In silico prediction of drug therapy in catecholaminergic polymorphic ventricular tachycardia

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Key points

- The mechanism of therapeutic efficacy of flecainide for catecholaminergic polymorphic ventricular tachycardia (CPVT) is unclear.
- Model predictions suggest that Na⁺ channel effects are insufficient to explain flecainide efficacy in CPVT.
- This study represents a first step toward predicting therapeutic mechanisms of drug efficacy in the setting of CPVT and then using these mechanisms to guide modelling and simulation to predict alternative drug therapies.

Abstract

Catecholaminergic polymorphic ventricular tachycardia (CPVT) is an inherited arrhythmia syndrome characterized by fatal ventricular arrhythmias in structurally normal hearts during β-adrenergic stimulation. Current treatment strategies include β-blockade, flecainide and ICD implementation – none of which is fully effective and each comes with associated risk. Recently, flecainide has gained considerable interest in CPVT treatment, but its mechanism of action for therapeutic efficacy is unclear. In this study, we performed in silico mutagenesis to construct a CPVT model and then used a computational modelling and simulation approach to make predictions of drug mechanisms and efficacy in the setting of CPVT. Experiments were carried out to validate model results. Our simulations revealed that Na⁺ channel effects are insufficient to explain flecainide efficacy in CPVT. The pure Na⁺ channel blocker lidocaine and the antianginal ranolazine were additionally tested and also found to be ineffective. When we tested lower dose combination therapy with flecainide, β-blockade and CaMKII inhibition, our model predicted superior therapeutic efficacy than with flecainide monotherapy. Simulations indicate a polytherapeutic approach may mitigate side-effects and proarrhythmic potential plaguing CPVT pharmacological management today. Importantly, our prediction of a novel polytherapy for CPVT was confirmed experimentally. Our simulations suggest that flecainide therapeutic efficacy in CPVT is unlikely to derive from primary interactions with the Na⁺ channel, and benefit may be gained from an alternative multi-drug regimen.
Introduction

First-line pharmacotherapy for treating catecholaminergic polymorphic ventricular tachycardia (CPVT) patients utilizes β-blockade at maximally tolerated doses; however, 30% of patients continue to experience arrhythmic episodes and often require mechanical implantable cardioverter defibrillator (ICD) implantation (Liu et al. 2008). β-Blockade is associated with fatigue, diminished physical activity level and reduced quality of life, while ICD discharge is associated with significant pain and anxiety, which can induce additional episodes of electrical storm (Sears et al. 1999; Nakamura et al. 2007).

CPVT is an inherited arrhythmia syndrome arising from mutations in the ryanodine receptor (RyR2) as well as the calcium buffer and regulator of RyR2, calsequestrin (CASQ2). As its name suggests, CPVT sets up a pathological substrate for arrhythmogenesis under the influence of a catecholamine surge, which leads to bidirectional and polymorphic ventricular tachycardia. Sudden cardiac arrest and death are not uncommon for individuals harbouring CPVT mutations, and a lethal episode may present as the first manifestation of disease (Priori et al. 2002).

Flecainide, a class IC antiarrhythmic suppresses the emergence of CPVT in both mice harbouring the CASQ2(−/−) mutation and humans with either a CASQ2 or RyR2 (S412G) mutation (Watanabe et al. 2009; van der Werf et al. 2011). However, the mechanism and efficacy of flecainide remain disputed. For example, Katz et al. (2010) found little efficacy for CASQ2 subtypes, while Liu et al. suggested virtually no effect on Ca2+ handling properties, rather proposing that flecainide is effective due to its Na+ channel blocking properties (Watanabe et al. 2011; Liu et al. 2011a). Still other studies demonstrated flecainide interaction and blockade of RyR2 (Hilliard et al. 2010; Galimberti & Knollmann, 2011; Mehra et al. 2014).

Studies of other class I antiarrhythmics showed that only those that modulated RyR2 activity demonstrated efficacy in models of CPVT, including propafenone (Hwang et al. 2011). Moreover, the type of interaction with the RyR2 was found to be significant as well; tetracaine, an RyR2 closed state blocker, failed to regulate Ca2+ properties in a CASQ2(−/−) model of CPVT (Galimberti & Knollmann, 2011). The late Na+ channel blocker ranolazine has also been studied for treatment of CPVT; however, two studies reveal conflicting results: Parikh et al. (2012) demonstrated strong interaction between ranolazine and RyR2, whereas Galimberti & Knollmann (2011) failed to demonstrate such an interaction.

Discrepant experimental observations, likely to be due to different experimental set-ups and models of CPVT, make reconciling the aforementioned studies difficult. Thus, the focus of the present study is to use a computational modelling approach to understand the dominant mechanism(s) underlying antiarrhythmic drug success and failure. In particular, is the effect of flecainide to normalize aberrant Na+ channel activation sufficient to explain observed clinical efficacy? The latter would be particularly important, given the wealth of clinical (The Cardiac Arrhythmia Suppression Trial (CAST) Investigators, 1989; Nakamura et al. 2007) and experimental data (Moreno et al. 2011) that reveal flecainide-induced proarrhythmia. Finally, can we use the model as a therapeutic prediction tool to optimize a multidrug regimen in CPVT?

Methods

Ethics approval

Animal use was approved by the Institutional Animal Care and Use Committee, Baylor College of Medicine, and conformed to the Guide for the Care and Use of Laboratory Animals (National Research Council, 2011).

Experimental methods

Eight animals (six R176Q/+ and two wild-type (WT)) were studied with ECG telemetry as previously described (van Oort et al. 2010). Telemeters (Data Sciences International, St Paul, MN, USA) were implanted in the abdominal cavity with subcutaneous lead placement in a lead II configuration. Following telemetry implantation, mice were injected with subcutaneous 0.3 mg kg−1 buprenorphine. The isoflurane was discontinued and mice were placed on a warming pad with blow-by oxygen. They were observed continuously until awake with normal breathing pattern and spontaneous movement. The mice were observed continuously for the first 2 h

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Abbreviations

APD, action potential duration; BCL, Basic Cycle Length; CaMKII, Ca2+/calmodulin-dependent protein kinase; CASQ2, calsequestrin; CPVT, catecholaminergic polymorphic ventricular tachycardia; DAD, delayed afterdepolarizations; EAD, early afterdepolarizations; ICD, implantable cardioverter defibrillator; ISO, isoprenol; KO, knockout; PKA, cAMP-dependent protein kinase; PMF, potential of mean force; P_o, open probability; RyR2, ryanodine receptor; SCaR, spontaneous Ca2+ release event; T_m, mean closed time; T_o, mean open time; TB, tonic block; VT, ventricular tachycardia; WT, wild-type.
then daily for 7 days. Buprenophine, 0.2–0.3 mg kg\(^{-1}\) subcutaneous injection, was provided every 6–12 h for the first 24 h. Ambulatory telemetry recordings were obtained after all animals had at least 7 days’ recovery from surgery and a 24 h baseline recording. Repeat baseline recordings were obtained 15 min prior to all injections. Mice were injected with a single intraperitoneal (i.p.) dose of adrenaline (120 mg kg\(^{-1}\), Sigma-Aldrich, St Louise, MO, USA) and caffeine (120 mg kg\(^{-1}\), Avantor Performance Materials, Center Valley, PA, USA) in sterile saline followed by continuous 24 h telemetry recordings. After a 7 day wash-out period, all mice underwent repeated injections with pretreatment. Baseline ECG telemetry was recorded for 15 min. Separate i.p. injections were given of flecainide (12 mg kg\(^{-1}\), Sigma-Aldrich), metoprolol (10 mg kg\(^{-1}\), Sigma-Aldrich) and KN-93 (30 μmol kg\(^{-1}\), Calbiochem). All drugs were mixed in sterile water. After 30 min, mice were injected with a single i.p. dose of adrenaline (2 mg kg\(^{-1}\)) and caffeine (120 mg kg\(^{-1}\)). Following injection of adrenaline and caffeine, the mice were monitored by telemetry for a total of 8 h. Data collection was performed using Dataquest A.R.T. version 4.31 (Data Sciences International, St Paul, MN, USA). Off-line analysis was performed by ECG Auto analysis version 3.1 (EMKA technologies, Falls Church, VA, USA).

### Molecular dynamics and free energy simulations

Force-field parameters for flecainide were developed using the general automated atomic model parameterization (GAAMP) server developed by Huang & Roux (2013). Briefly, all geometry optimization, charge fitting and torsional parameter fitting were done using B3LYP functional at the 6–31G* (2d,2p) level of theory. Umbrella sampling simulations for partitioning of the neutral and cationic forms of flecainide into model POPE bilayer (128 lipids) were performed using a protocol described previously by Li et al. (2008). Harmonic constraints of 5 kcal (mol Å\(^2\))\(^{-1}\) were applied to the drug’s centre of mass along the reaction coordinate spanning 62 Å from −31 Å to 31 Å normal to the membrane plane. We used five starting conformations for the drug and 124 windows spaced every 0.5 Å to describe drug partitioning into the bilayer. A flat-bottomed cylindrical restraint with \(R = 15\) Å was used to confine drug in the xy plane along the reaction coordinate. It allows for good sampling in a lateral plane. Each potential of mean force (PMF) window was equilibrated for 2.5 ns in the presence of restraining potential and then subjected to a 5 ns production run. The resulting 1D PMF was reconstructed with the weighted histogram analysis method (WHAM) (Roux, 1995). The WHAM convergence tolerance was set at 0.0001 kcal mol\(^{-1}\). We used symmetrized density to compute resulting PMFs. The statistical uncertainties were estimated by separating the data into seven blocks and were found to be within ±1.5 kcal mol\(^{-1}\) for neutral and charged forms of flecainide, respectively.

### Computational ventricular myocyte models

Briefly, we developed mouse and rabbit in silico models of catecholaminergic polymorphic ventricular tachycardia (CPVT) by 'knocking out' the SR Ca\(^{2+}\) buffer calsequestrin (Kornyeyev et al. 2012) in the Morotti–Grandi mouse cardiac cell model (Morotti et al. 2014) and in the Soltis–Saucerman rabbit cardiac cell model (Soltis & Saucerman, 2010), respectively. Flecainide interactions with the ryanodine receptor were modelled assuming open state associations (Hilliard et al. 2010). We also incorporated the model of the cardiac Na\(^{+}\) channel and I\(_{Kr}\) and their interaction with flecainide as previously (Moreno et al. 2011, 2013). We utilized the Morotti–Grandi computational model for mouse (Morotti et al. 2014) and the Soltis–Saucerman computational model for rabbit (Soltis & Saucerman, 2010), which included all the relevant components required for a detailed analysis, including accurate cellular electrophysiology, Ca\(^{2+}\) handling (Shannon et al. 2004) and the cAMP-dependent protein kinase (PKA) (Saucerman et al. 2003) and Ca\(^{2+}\)/calmodulin-dependent protein kinase (CaMKII) (Soltis & Saucerman, 2010) phosphorylation pathways. The computational model for flecainide interaction with the cardiac Na\(^{+}\) channel is from (Moreno et al. 2011), with adaptations designed to recreate the CPVT phenotype and flecainide’s interaction with the RyR2, informed and validated by experimental data (Hilliard et al. 2010; Hwang et al. 2011).

### Wild-type Na\(^{+}\) channel models and inclusion of drug binding

The wild-type drug-free Na\(^{+}\) channel model was used as previously described (Moreno et al. 2013). The Na\(^{+}\) drug-channel model parameters for the on- and off-rates of flecainide, ranolazine and lidocaine are taken from experiments where available. These include diffusion rates that indicate drug on-rates \(k_{\text{in}} = [\text{drug}] \times D\) (diffusion rate) and affinities \((K_d)\) to discrete conformations that determine drug off-rates \(k_{\text{off}} = K_d \times D\) (diffusion rate). Rates were also constrained by experimental data (described in detail below) and microscopic reversibility as in Colquhoun et al. (2004). The transition rates are given in Table 1.

**Flecainide.** The flecainide model was used as previously described (Moreno et al. 2011), but now includes a drug-bound bursting regime (drug binding to red states as indicated in Scheme 1; Top 8 states are ‘normal’ gating mode, while red states indicate burst mode). Rate constants in the upper (normal mode) states are from

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Table 1. Markov model transition rates (ms\(^{-1}\)) for Drug free WT Na\(^{+}\) channel

| States of Markov model | Rate constants |
|------------------------|----------------|
| I′\(_{C3}\) → I′\(_{C2}\), C\(_{3}\) → C\(_{2}\), BC\(_{3}\) → BC\(_{2}\) | α\(_{11}\) = 8.5539/(7.4392e-2*exp(-V/17.0)+2.0373e-1*exp(-V/150)) |
| I′\(_{C2}\) → I′\(_{C1}\), C\(_{2}\) → C\(_{1}\), BC\(_{2}\) → BC\(_{1}\) | α\(_{12}\) = 8.5539/(7.4392e-2*exp(-V/15.0)+2.0373e-1*exp(-V/150)) |
| C\(_{1}\) → O, BC\(_{1}\) → BO | α\(_{13}\) = 8.5539/(7.4392e-2*exp(-V/12.0)+2.0373e-1*exp(-V/150)) |
| I′\(_{C2}\) → I′\(_{C3}\), C\(_{2}\) → C\(_{3}\), BC\(_{2}\) → BC\(_{3}\) | β\(_{11}\) = 7.5215e-2*exp(-V/20.3) |
| IF → I′\(_{C2}\), C\(_{1}\) → C\(_{2}\), BC\(_{1}\) → BC\(_{2}\) | β\(_{12}\) = 2.7574*exp((-V/5)/20.3) |
| O → C\(_{1}\), BO → BC\(_{1}\) | β\(_{13}\) = 4.7755e-1*exp((-V/10)/20.3) |
| I′\(_{C3}\) → C\(_{3}\), C\(_{2}\) → C\(_{1}\), I′ \(\leftarrow\) C\(_{1}\) | α\(_{2}\) = 13.3705*exp(V/43.749) |
| O → BO | β\(_{2}\) = (α\(_{13}\) + α\(_{12}\) + α\(_{11}\))β\(_{3}\) |
| IS → O | α\(_{x}\) = 3.4229e-2α\(_{2}\) |
| BC\(_{3}\), BC\(_{2}\), BC\(_{1}\), BO | β\(_{x}\) = 1.7898e-2α\(_{3}\) |
| C\(_{3}\), C\(_{2}\), C\(_{1}\), O → BC\(_{3}\), BC\(_{2}\), BC\(_{1}\), BO | μ\(_{1}\) = 2.0462e-7 |
| BC\(_{3}\), BC\(_{2}\), BC\(_{1}\), BO → C\(_{3}\), C\(_{2}\), C\(_{1}\), O | μ\(_{2}\) = 8.9731e-4 |

Moreno et al. (2011). Bursting state affinity for the charged form of flecainide was initially set at the value found by assuming the affinity of tonic block of late I\(_{Na}\) was equal to K\(_{d}\) at −100 mV. The value for K\(_{d,0}\) was then calculated and used as an initial value in the optimization. For example, the affinity of tonic block (TB) I\(_{Na,L}\) for WT is ~44 μM (Nagatomo et al. 2000); if that value is assumed to equal K\(_{d,−100mV}\), K\(_{d,0,bursting}\) was initially set at 3.202 μM (e.g. the K\(_{d,0,bursting}\) that would give K\(_{d,−100mV,bursting}\) = 44 μM). The optimized K\(_{d,0,bursting}\) was 95.2165 μM. Full parameters are given in Table 2.

**Ranolazine.** The ranolazine–Na\(^{+}\) channel drug interaction model was as previously described (Moreno et al. 2013) and the transition rates are given in Table 3.

**Lidocaine.** The wild-type lidocaine model was as previously described (Moreno et al. 2011), but now includes a drug-bound bursting (lower 4 states) regime. Non-bursting rates were from (Moreno et al. 2011). Bursting state affinity for the charged form of lidocaine was initially set at the value found by assuming the affinity of tonic block of late I\(_{Na}\) was equal to K\(_{d}\) at −100 mV. K\(_{d,0}\) was then calculated and used as an initial value in the optimization. For example, the affinity of TB I\(_{Na,L}\) for WT is ~12 μM (Fedida et al. 2006); if that value is assumed to equal K\(_{d,−100mV}\), K\(_{d,0,bursting}\) was initially set at 0.80 μM (e.g. the K\(_{d,0,bursting}\) that would give K\(_{d,−100mV,bursting}\) = 12 μM). The optimized K\(_{d,0,bursting}\) was 0.9810 μM. The transition rates are given in Table 4.

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RyR2–flecainide drug-channel interaction model development

The Shannon–Bers Markov model formulation of the RyR2 (Shannon et al. 2004) was modified to include a drug bound state DO with transitions k\(_{on}\) = D × [drug] and k\(_{off}\) = D × IC\(_{50,drug}\) to and from the open state O, which represent the drug diffusing to the receptor and binding or dissociating from the channel, respectively, as done previously (Moreno et al. 2011). The diffusion rate of flecainide (Scheme 2) was estimated at 5500 M\(^{-1}\) ms\(^{-1}\) (Zhu et al. 2006).

![Scheme 2](image)

Isoproterenol-stimulated Ca\(_{2+}\) waves in CASQ2 knockout (KO) CASQ2(−/−) mice were inhibited by flecainide with an IC\(_{50}\) of 2.0 ± 0.2 μM (Hwang et al. 2011), while other experimental preparations measured an IC\(_{50}\) range from 2 to 17 μM (Brunton et al. 2010; Hilliard et al. 2010; Hwang et al. 2011; Mehra et al. 2014). Drug binding was modelled as open-state block, using experimental data for drug affinity to the open state to determine off and on rates, respectively. We also predicted cases for variable flecainide IC\(_{50}\) = 3, 4, and 5 μM shown in Fig. 1. We examined the cases of 3, 4 and 5 μM of IC\(_{50}\) acting on each of the targets under the influence of 1 μM isoproterenol, over a range of clinically relevant pacing frequencies, from 0.5 to 3.0 Hz (30–180 beat min\(^{-1}\); Leenhardt et al. 1995), at 0.01 Hz increments.

We assumed rapid partitioning of flecainide into its charged (98% at pH 7.4, pK\(_{a}\) = 9.3) and neutral (2%)
fractions (Liu et al. 2003), diffusion of the neutral component across the membrane, and equilibration on the cytosolic side, resulting in equimolar concentrations of flecainide inside and outside of the cell, consistent with experimental observations and the mechanism of flecainide’s interaction with the Na⁺ channel (Liu et al. 2003). The kinetics of the state transitions for the channel are adjusted for temperature by a $Q_{10}$ factor of 1.5 (Sitsapesan et al. 1991). The presence of a 20% RyR sub-conductance state in response to flecainide binding of the channel (Hilliard et al. 2010) is modelled at the whole cell level as a reduction to 20% maximal conductance of the channel.

In single channel simulations mimicking the experimental conditions tested, the IC$_{50}$ of 16 μM was used to validate the model. It is important, however, to justify the use of the 2 μM IC$_{50}$ value used in cellular and tissue level simulations. The 16 μM IC$_{50}$ value corresponds to single channel conditions (Hilliard et al. 2010), which does not account for the influence of coupling between adjacent ryanodine receptors on the kinetics of the channel (Marx et al. 2001). Similarly, the IC$_{50}$ value of 16 μM
for spark inhibition in permeabilized myocytes cannot be used, due to the constant maintenance of $[\text{Ca}^{2+}]$ in those experiments – the high concentration of Ca$^{2+}$ used to elicit consistent Ca$^{2+}$ sparks in experiments is static and is an unrealistic representation of paced myocytes (Galimberti & Knollmann, 2011). The high external Ca$^{2+}$ concentration used would cause a higher IC$_{50}$ than that seen using a lower Ca$^{2+}$ concentration in the intact myocyte used to generate Ca$^{2+}$ waves during diastole, due to a persistently overactive ryanodine receptor by constitutive cytosolic activation.

Thus the measured IC$_{50}$ for wave inhibition in the isoproterenol-stimulated Ca$^{2+}$ waves in CASQ2(--/--) mice (IC$_{50}$ = 2.0 ± 0.2 μM; Hwang et al. 2011), was used, since it reflects realistic conditions for our model system. Its weakness is that the IC$_{50}$ does not separate out external contributions to the IC$_{50}$, such as lowered junctional Ca$^{2+}$ by $I_{\text{NCX}}$ activity via lowered [Na$^+$]. Experiments in paced rat myocytes indicate that this contribution may significantly influence flecainide’s ability to inhibit spontaneous Ca$^{2+}$ wave formation, and thus future work will need to evaluate whether the contribution of $I_{\text{NCX}}$ regulation of Na$^+$ is indeed significant in influencing flecainide’s efficacy (Sikkel et al. 2013).

$$k_{\text{Cas}SR} = \text{Max}_{SR} - \frac{\text{Max}_{SR} - \text{Min}_{SR}}{1 + \left( \frac{[\text{Ca}^{2+}]_{SR}}{[\text{Ca}^{2+}]_{SR}^{50}} \right)^{H/2}}$$
Table 4. Markov model transition rates (ms⁻¹) for Lidocaine

| Parameter Name | Value |
|----------------|-------|
| kon = kclosed, on | D⁺ to Diffusion |
| koff = kclosed, off | kᵣopen * Diffusion; (kdᵣopen = 318e⁻₆ * exp(-0.7 * V/Fᵣ * T)) |
| kbursting, on = kclosed bursting, on | [D⁺] * Diffusion; (kdᵣbursting, Open = 0.981e⁻₆ * exp(-0.7 * V/Fᵣ * T)) |
| kbursting, off = kclosed bursting, off | | |
| kneutral, on | | |
| kneutral, off | | |
| kneutral, inactivated, on | | |
| kneutral, inactivated, off | | |
| kneutral, closed, on | 3.4e⁻²⁶ Diffusion |
| kneutral, closed, off | 900e⁻⁶ Diffusion |
| Diffusion | 500 M⁻¹ ms⁻¹ |

| States of Markov model | Parameters |
|------------------------|------------|
| D⁺IC3 → D⁺IC2, D⁺C3 → D⁺C2, | α₁₁ |
| DIC3 → DIC2, DC3 → DC2 | | |
| D⁺IC2 → D⁺IC1, D⁺C2 → D⁺C1, | α₁₂ |
| DIC2 → DIC1, DC2 → DC1 | | |
| D⁺IC2 → D⁺IC3, D⁺C2 → D⁺C3, | β₁₁ |
| DIC2 → DIC3, DC2 → DC3 | | |
| D⁺IF → D⁺IC2, D⁺C1 → D⁺C2, | β₁₂ |
| DIC2 → DIC1, DC → DC2 | | |
| D⁺O → D⁺IS | αₓ₁ = 6.3992e⁻⁷ ❍ x |
| D⁺IS → D⁺O | βₓ₁ = 1.3511e⁻⁰ * βₓ |
| D⁻C1 → D⁻O | α₁₃C = 5.6974e⁻³ ❍ α₁₃ |
| DC1 → D₀ | α₁₃n = 8.4559e⁺¹ ❍ α₁₃ |
| D⁻O → D⁻C1 | β₁₃C = (β₁₃C * kᵣcon * kᵣoff * α₁₃C)/(kon * kᵣcon * α₁₃C) |
| DO → DC1 | β₁₃n = (β₁₃n * kᵣcon * α₁₃n * kᵣoff)/(kᵣcon * α₁₃n) |
| Dᵣ → DO | βₓ₂ = (βₓ₁ * kᵣcon * αₓ₁ * kᵣoff)/(αₓ₁ * kᵣcon * kᵣoff) |
| D⁻O → D⁻IF | α₂₂ = 6.7067e⁻⁶ ❍ α₂ |
| DO → Dᵣ | α₂₂ = 1.7084e⁻⁵ ❍ α₂ |
| D⁻IF → D⁻O | β₂₂ = (α₁₃C * α₂₂ * α₃C)/(β₁₃C * β₃₃) |
| Dᵣ → Dᵣ | β₂₂ = (α₃₃ * α₁₃n * α₂₂)/(β₃₃ * β₁₃n) |
| D⁻C₃ → D⁻IC3, D⁻C₂ → D⁻IC₂, D⁻C₁ → D⁻IF | β₃₃ = 1.9698e⁻⁵ ❍ β₃ |
| DC₃ → DIC₃, DC₂ → DIC₂, DC₁ → Dᵣ | β₃₃ = 4.8477e⁺⁰ ❍ β₃ |
| D⁻IC₃ → D⁻IC₂, D⁻IC₂ → D⁻IC₁, D⁻IF → D⁻C₁ | α₃₃ = 3.2976e⁺⁰ ❍ α₃ |
| DIC₃ → DIC₂, DIC₂ → DIC₁, DIC₁ → DIC₀ | α₃₃ = (kᵣcon * α₃C * kᵣoff * β₃₃)/(kᵣcon * kᵣcon * kᵣoff) |

RI = (kᵣcon I - kᵣSRCA [Ca]ᵣ/R) - (kᵣcon I - kᵣSRCA [Ca]ᵣ/R) - (kᵣcon I - kᵣSRCA [Ca]ᵣ/R)

JᵣSR Cor = kᵣ (0.2 * DC) × ([Ca]ᵣSR - [Ca]ᵣ)

JᵣLeak = kᵣLeak ([Ca]ᵣSR - [Ca]ᵣ)

The parameters are presented in Table 5.

Simulation of IKr blockade

To simulate the effects of flecainide or ranolazine on IKr current, we decreased the peak conductance, GᵣKr, in a concentration dependent fashion using a
A
Flecainide 2 μM on both targets

IC50 = 3 μM

IC50 = 4 μM

IC50 = 5 μM

B
Flecainide 2 μM on I_{Na}

C
Flecainide acting on only RyRs

Figure 1. Testing IC_{50} of flecainide in the cell model for I_{Na}, RyR2 and both targets in combination
Voltage dynamics is shown for each IC_{50} case, and the voltage and Ca^{2+} transient maxima are binned for each pacing frequency between 0.5 and 3 Hz during the second half of a 2 min simulation time course. A, CASQ2(−/−) myocytes with 3, 4 and 5 μM IC_{50} effects on both Na^{+} channels and RyRs. B, flecainide interaction with just Na^{+} channels. C, flecainide concentrations acting on only RyRs.
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| Parameter Name | Value / Units |
|----------------|---------------|
| $k_{on}$       | [D+]$^+$Diffusion |
| $k_{off}$      | 0.011         |
| Diffusion      | 5500 M$^{-1}$ms$^{-1}$ |
| $k_0$          | 25 ms$^{-1}$  |
| $k_{Ca}$       | 10 mM$^{-2}$ms$^{-1}$ |
| $k_{m}$        | 0.06 ms$^{-1}$ |
| $k_{Ca}$       | 0.5 mM$^{-1}$ms$^{-1}$ |
| $k_{m}$        | 0.005 ms$^{-1}$ |
| $E_{Ca}$       | 0.45 mM       |
| $M_{x}$        | 15            |
| $M_{SR}$       | 1             |
| $H_{SR}$       | 2.5           |
| $K_{SR}$       | 5.348e-6 ms$^{-1}$ |

Table 5. Parameters for SR Ca$^{2+}$ release

concentration–response relationship with a Hill coefficient of 1 ($n = 1$) as follows:

$$G_{1Kr} = G_{1Kr, max} \times \frac{1}{1 + ([\text{Drug}]/IC_{50})^n}$$

where $G_{1Kr,max}$ is the nominal conductance value from the given rabbit ventricular myocyte model (Soltis & Saucerman, 2010) and $IC_{50}$ is the concentration of drug that produces a 50% inhibition of $I_{Kr}$ current. The $IC_{50}$ values for the drug to inhibit $I_{Kr}$ current are for flecainide 1.5 μM (Belardinelli et al. 2013) and for ranolazine 12 μM (Rajamani et al. 2008).

Simulation of effect of metoprolol on β-adrenergic activity and CaMKII blocking effects of KN-62

We modelled the $\beta_1$ and CaMKII blocking effects of metoprolol ($IC_{50} = 105$ nM) and KN-62 ($IC_{50} = 468$ nM) as a competitive inhibitor of β-agonist and limiter of free endogenous total CaMKII, respectively. Dose–response curves (Fig. 2) for the effect of metoprolol on β-adrenergic activity were extracted from an $E_{max}$ experimental model (Abrahamsson et al. 1990), where the percentage inhibition of $E_{max}$ represents the fraction of ISO inhibition. In preliminary tests of the computational model involving myocyte pacing, 50 nM ISO achieved 98% of the maximal response to agonist; ISO inhibition was therefore modelled as a percentage of 50 nM applied ISO. Thus, in Fig. 2A, percentage $\beta_1$-receptor blockade refers to the percentage of $\beta_1$-receptors that are competitively blocked by metoprolol. KN-62 was modelled as an inhibitor of endogenously available CaMKII (Tokumitsu et al. 1990). Dose–response data were obtained from Davies et al. (2000) and fitted to a percentage block of CaMKII for given doses of KN-62; similarly, percentage CaMKII inhibition in Fig. 2B refers to the percentage of endogenous CaMKII that is inhibited by KN-62. Data are from Abrahamsson et al. (1990) (metoprolol) and Tokumitsu et al. (1990) (KN-62). We applied the $E_{max}$ model from Abrahamsson et al. (1990) to determine our dose–response relation as follows:

$$\% \beta - \text{Blockade or } % \text{CaMKII inhibition} = \frac{E_{max} \times C}{IC_{50} + C}$$

where $E_{max} = 100\%$, and $C$ is the metoprolol or KN-62 concentration.

CPVT CASQ2(−/−) model development

The CPVT phenotype was modelled via a computational CASQ2(−/−) (Knollmann et al. 2006) by modifying the Soltis–Saucerman model (Soltis & Saucerman, 2010). We set the calsequestrin buffer concentration and the time derivative of the calsequestrin buffer concentration to 0. While this has no direct effect on regulating RyR2 function, it was expected to still demonstrate a functional effect on RyR2 activity because of alterations to intracellular SR Ca$^{2+}$. Equations for the regulation of Na$^+/K^+$-ATPase activity by PKA as mediated by phospholemman were incorporated from the Yang–Saucerman model (Yang & Saucerman, 2012). Our previously validated Na$^+$ channel (Moreno et al. 2011) and modified RyR2 channel were inserted into the cellular model (Soltis & Saucerman, 2010).
Stochastic single channel RyR2 simulations

Individual RyR2 channels were modelled stochastically in Monte Carlo simulations using Gillespie’s algorithm (Gillespie, 1977). Parameters were constrained by fixed concentrations of flecainide (0 and 50 μM), luminal Ca\(^{2+}\) (1 mM), and junctional Ca\(^{2+}\) (0.1 μM), mimicking experimental conditions (Hwang et al. 2011). Stochastic error diminishes over a large number of simulations, approaching the expected mean open time (\(T_o\)), mean closed time (\(T_c\)), and open probability (\(P_o\)) as derived by the methods of Colquhoun & Hawkes (1981).

Cellular and tissue simulations

**Mouse ventricular myocyte model.** The \(I_{Na}\) channel was replaced in the Morotti–Grandi mouse cardiac cell model (Morotti et al. 2014) with our published Markov model (Moreno et al. 2013). We then adjusted three transition rates for the mouse model to simulate the mouse \(I_{Na}\) kinetics as follows: O→C1, \(\beta_1\) \(=\) 0.45; O→IF, \(\alpha_2\) \(=\) 0.45; O→IS, \(\alpha_3\) \(=\) 0.45. Virtual myocytes were paced using a \(-9.5\) pA pF\(^{-1}\) current stimulus for 5 ms in single cells for 150 s at 2 Hz pacing frequency in the presence and absence of drug and 0.5 μM isoproterenol (ISO).

**Rabbit ventricular myocyte model.** Virtual whole cells and tissues (both WT and KO) were allowed to ‘rest’ without external stimuli for 10 min to establish initial conditions. Cells were then virtually paced (using a \(-80\) pA pF\(^{-1}\) current stimulus for 0.5 ms in single cells and \(-500\) pA pF\(^{-1}\) stimulus for 2.0 ms in tissues) for 5 min at specific pacing frequencies in the absence of drug or agonist. Cells were finally paced for an additional 2 min in the presence and absence of drug and 1 μM ISO. Parameters including upstroke velocity, action potential duration (APD) and the number of early and delayed afterdepolarizations (EADs and DADs) were tracked over the course of each simulation.

One-dimensional (1D) tissue was simulated as a fibre of 165 cells (1.65 cm) (Glukhov et al. 2010) with reflective boundary conditions. Transmural heterogeneity was incorporated into the tissue by a linear decrease to 25% maximal \(I_{Io}\) conductance (Myles et al. 2010), corresponding to a linear transition from epicardial to endocardial tissue (Fedida & Giles, 1991) and an APD gradient of 205–224 ms. The diffusion coefficient \(D_x\) was set to 0.002 cm\(^2\) ms\(^{-1}\) to establish a conduction velocity of 61–73 cm s\(^{-1}\) (epicardium–endocardium in WT conditions) (Brugada et al. 1990).

Code for simulations and analysis was written in C++ and MATLAB 2014a (The Math Works, Inc., Natick, MA, USA). Code was run on an Apple Mac Pro machine with two 2.93 GHz 6-Core Intel Xeon processors, and an HP ProLiant DL585 G7 server with a 2.3 GHz 48-core AMD Opteron processor. Code was compiled with the Intel ICC compiler 2013. Numerical results were visualized using MATLAB R2014b. All source code used in this paper is available online or can be obtained by emailing ceclancy@ucdavis.edu.

Results

We modelled the interaction between flecainide and RyR2 by modifying the Shannon–Bers model of the cardiac RyR2 channel (Shannon et al. 2004) (see Methods) that reproduces gain and fractional SR Ca\(^{2+}\) release, and includes regulation by both luminal and junctional Ca\(^{2+}\) fractions. Based on experimental data, drug binding was modelled as voltage-independent open-state block that results in a \(\sim 20\%\) subconductance state (Hi1liard et al. 2010; Hwang et al. 2011; Mehra et al. 2014). A different recent study suggested that flecainide does not block the RyR (Bannister et al. 2015). These effects are also included in the cellular level simulations as no RyR block by flecainide.

This assumption resulted in predictions of drug effects that were consistent with experiments in single channel lipid bilayer studies (Hi1liard et al. 2010). Stochastic Monte Carlo simulations reveal intermittent closures that interrupt channel openings, corresponding to flecainide binding and unbinding the open-state (Fig. 3A) (Hwang...
et al. 2011). Figure 3B shows model predictions (lines) closely match experimental measurements (symbols) of drug concentration-dependent effects on channel open probability (left) and channel mean open time (middle) (Hwang et al. 2011). Mean closed time displays no dose dependence in this binding scheme, as in experimental findings (Fig. 3B, right). We modelled flecainide interactions with the Na\(^+\) and hERG K\(^+\) channel as described previously (Moreno et al. 2011) (Moreno et al. 2013).

We generated a virtual calsequestrin knockout mouse cardiac myocyte (CASQ2(-/-)) by setting the concentration of CASQ to zero in the Morotti–Grandi model of the mouse ventricular myocyte, which includes the β-adrenergic signalling and CaMKII pathways (Morotti et al. 2014). We then predicted the effects of the virtual CASQ2(-/-) on electrical and Ca\(^{2+}\) dynamics. Figure 4A–D shows action potentials (APs) (top) and Ca\(^{2+}\) transients (bottom) of cells paced at a frequency of 2 Hz. As expected, the Ca\(^{2+}\) transient amplitude increases upon application of 0.5 μM isoproterenol (ISO) (Fig. 4B). In the absence of ISO, CASQ2(-/-) myocytes demonstrate reduced but intact Ca\(^{2+}\) transients and APs compared with control (compare Fig. 4C to Fig. 4A) consistent with experimental observations in CSQN mutant and CSQN underexpression mouse myocytes (Viatchenko-Karpinski et al. 2004; Terentyev et al. 2008; Gyorke & Terentyev, 2008). Consistent with multiple experimental observations, application of 0.5 μM ISO induces spontaneous Ca\(^{2+}\) release events (red arrows Fig. 4D, bottom), which drive afterdepolarizations (Knollmann et al. 2006; Gyorke & Terentyev, 2008; Hilliard et al. 2010; Liu et al. 2013). Following development of the virtual mouse model of CPVT, which is validated by many published experimental data, we generated a virtual calsequestrin rabbit knockout (CASQ2(-/-)). This approach allows for cross species testing, e.g. to predict how larger animals (more ‘human-like’) would respond, and to also look for model independence of our findings.
The rabbit model was constructed by setting the concentration of CASQ to zero in the Soltis–Saucerman model of the rabbit ventricular myocyte, which contains the β-adrenergic signalling and CaMKII pathways. Figure 4E–H shows simulated rabbit APs (top) and Ca\(^{2+}\) transients (bottom) of control cells paced at a frequency of 1 Hz. As observed in the mouse model, the Ca\(^{2+}\) transient amplitude increases upon application of 1 μM ISO (Fig. 4F). In the absence of ISO, CASQ\(^{-/-}\) myocytes demonstrate morphologically similar Ca\(^{2+}\) transients and APs to control (compare Fig. 4G to Fig. 4E). No differences were found with other pacing frequencies. Consistent with experimental observations (Kornyeyev et al. 2012), application of 1 μM ISO induces spontaneous Ca\(^{2+}\) release events (red arrows Fig. 4H, bottom), which drive depolarizations that trigger APs (red arrows in Fig. 4H, top).

Recent publications have suggested that flecainide may be insufficiently concentrated inside the cell to affect the RyR directly (Liu et al. 2011a, 2012). Thus, we employed a novel atomic scale modelling approach to compute free energy profiles for flecainide transport in the extracellular, membrane and intracellular compartments (Fig. 5A).

With a pK\(_a\) value of 9.8, 97% of flecainide will exist in the protonated form at physiological pH. However, recent studies suggest that the hydrophobic environment of the lipid bilayers promotes stabilization of the neutral form, allowing for flecainide entry upon deprotonation. To assess feasibility of this mechanism, we determined

![Figure 5. Atomic scale prediction of flecainide transport across the membrane](image)
the underlying thermodynamics governing flecainide movement on the surface and in the hydrophobic core of the membrane and the barriers associated with transport of cationic or neutral forms.

**Force (PMF) or Potential mean force free energies ($\Delta G_A$) for partitioning of charged and neutral flecainide**

Both forms of flecainide display preference for interfacial partitioning with stabilization of about 8 and 11 $k_B T$ for charged and neutral flecainide, respectively. The simulations show that concentration of neutral flecainide will increase substantially on the surface as compared to the bulk phase and will be predominant in the hydrophobic core of the bilayer (site 2). There is a substantial penalty for protonated drug to cross a hydrophobic core, although it will act as a sink for a neutral flecainide with a very small crossing barrier for the drug ($\sim 2k_B T$ not shown).

The difference between the potentials of mean force ($\Delta$PMF ($Z$)) across the bilayer, and position-dependent free energies for charged and neutral flecainide ($\Delta$G$_{A}$) at the membrane interface (site 1) and hydrophobic core of the bilayer (site 2) are shown in Fig. 5B. We calculated the $pK_z$ shift due to drug deprotonation upon partitioning into lipid bilayers from the difference between the position-dependent free energies for charged and neutral flecainide ($\Delta$G$_{A}^{\text{deprot}}$).

$$\Delta G_{\text{deprot}} = W_{\text{Flec}^0}(z) - W_{\text{Flec}^+}(z)$$

$$\Delta pK_A = \frac{\Delta \Delta G_{\text{deprot}}}{2.3 k_B T}$$

$\Delta pK_A$ values are also indicated as relative fractions of neutral to charged drug ($Fq^-/Fq^+$) in the aqueous solution (red), site 1 (blue) and site 2 (black) in Fig. 5B. The $pK_z$ shift is close to zero in the bulk (as expected) and then sharply drops at site 1. This indicates that flecainide will readily lose its proton in the membrane environment.

Is diffusion of neutral flecainide sufficient for effective accumulation? We estimated diffusion coefficients from local friction factors along the reaction coordinate in free energy simulations (from fluctuations in the constraining force) (Boggara & Krishnamoorti, 2010). The diffusion coefficient for flecainide was calculated as $\sim 4.9 \times 10^{-7}$ cm$^2$ s$^{-1}$ in the bilayer, about an order of magnitude lower than bulk water similar to previously reported values for various drugs (Boggara & Krishnamoorti, 2010). Flecainide is predicted to undergo extremely rapid equilibration.

We next predicted the effects of flecainide on control and CASQ2(--/-) rabbit ventricular myocyte models for clinically relevant frequencies at a high therapeutic dose of flecainide (2 $\mu$M). In order to map the action potential (AP) dynamics–frequency relationship for the CPVT CASQ2(--/-) with flecainide, the maximum values of the voltage following the upstroke of the AP were collected during the second half of a 2 min simulation and binned vertically for each constant pacing frequency; for clarity, only steady state behaviour is shown ($t = 60$–$120$ s).

In the control simulation there was a stimulus to AP ratio of 1:1 (Fig. 6A). The time course of AP at 2 Hz (grey line) is shown on the left (Fig. 6A, C, E, G and J). The peak of the calcium transient was similarly monitored (control – Fig. 6B), with the time course of the calcium transient at 2 Hz (grey line) in the left of each of the right panels (Fig. 6B, D, F, H and J).

In the absence of drug, CASQ2(--/-) myocytes displayed triggered APs at frequencies greater than 0.95 Hz (notice the horizontal line in the plot at frequencies slower than 0.95 Hz, corresponding to a stimulus to AP ratio of 1:1), when challenged by 1 $\mu$M ISO (Fig. 6C). Pathological electrical activity was preceded by spontaneous Ca$^{2+}$ release events from the RyR2 (Fig. 6D), which, when resulting in a sufficient release of Ca$^{2+}$, can integrate in time to induce electrogenic forward-mode sodium–calcium exchange current (3 inward Na$^+$/1 outward Ca$^{2+}$) and depolarize the cell, ultimately activating $I_{Na}$.

Application of a high therapeutic dose flecainide (2 $\mu$M), acting on the rabbit RyR2, $I_{Ks}$ and $I_{Na}$ rectified the electrical activity at all frequencies tested (Fig. 6E). The electrical stability coincided with suppression of diastolic Ca$^{2+}$ release, but was also accompanied by a markedly reduced Ca$^{2+}$ transient (Fig. 6F). The therapeutic range of plasma concentrations for flecainide is 0.5–2.0 $\mu$M (Brunton et al. 2010). We examined the cases of 0.5, 1.0 and 1.5 $\mu$M flecainide acting on each of the targets under the influence of 1 $\mu$M isoproterenol, over a range of clinically relevant pacing frequencies, from 0.5 to 3.0 Hz (30–180 beats min$^{-1}$; Leenhardt et al. 1995), at 0.01 Hz increments (Fig. 7). Low dose flecainide (0.5 $\mu$M) failed to suppress Ca$^{2+}$ alternans, a marker of proarrhythmia (Chudin et al. 1999), which emerged at frequencies greater than 1.6 Hz and suggests incomplete efficacy in the lower range of therapeutic dosing (Fig. 7A).

We next simulated drug action on the Na$^+$ current or RyR alone to determine the contributions of each target to drug efficacy. The rabbit model predicted that neither the high dose of flecainide (2 $\mu$M in Fig. 6G and H), nor any lower concentrations, when tested on Na$^+$ current alone, eliminated pathological activity at all pacing frequencies (Fig. 7B). As expected from flecainide’s use-dependent blocking behaviour of $I_{Na}$, higher concentrations and faster pacing frequencies eliminated some arrhythmogenic behaviour, but DADs and triggered action potentials persisted. Derangement of Ca$^{2+}$ activity also persisted, and was largely unaffected by drug effects on the Na$^+$ current (Fig. 6H).
Selective application of flecainide on RyR2 yielded results comparable to the case flecainide acting on both targets (compare Fig. 6I and J (selective RyR2) with Fig. 6E and F (both targets)). Mid- and high-dose flecainide acting on the RyR2 alone resolved pathological behaviour at the cellular level, though Ca\(^{2+}\) alternans and DADs still emerged at lower doses (Fig. 7C).

We next modelled the physiological effects of acute application of isoproterenol on heart rate, mimicking acute sympathetic stimulation. In Fig. 8, rabbit cells were paced to steady-state (for 500 s) at 1 Hz and then subjected to isoproterenol and an increase in pacing frequency (cycle length = 600 ms). Compared with control virtual cells that responded uneventfully to sympathetic challenge (Fig. 8A), the virtual CASQ2(−/−) CPVT mutation generated multiple spontaneous APs, observed in the histogram of peak voltages in time (left side of left panels) and in the time course of the membrane potential (right of left panels during the time indicated by the boxed region in the left panels). As observed for steady-state pacing, simulated 2 \(\mu\)M flecainide application on both the Na\(^+\) current and RyR (Fig. 8C) normalized aberrant behaviour, while flecainide effects on the Na\(^+\) channel alone were insufficient (Fig. 8D). A dose of 2 \(\mu\)M flecainide acting on the RyR alone normalized the membrane potential and prevented spontaneous Ca\(^{2+}\) release (Fig. 8E). We also tested different pacing cycle lengths from 300 ms to 500 ms, and found similar results (data not shown).

We next predicted the effects of flecainide in a rabbit one-dimensional (1D) transmural heterogeneous tissue. Stimulation was applied to the endocardial site of the CASQ2(−/−) rabbit tissue at 2 Hz (120 beats min\(^{-1}\)) with 1 \(\mu\)M ISO, in the presence or absence of flecainide. Spontaneous Ca\(^{2+}\) releases from the RyR2 often resulted in sufficient calcium loading to drive inward \(I_{\text{NCX}}\), causing DADs that were occasionally sufficient in amplitude to activate Na\(^+\) channels for a triggered AP (red arrows – Fig. 9A).

Flecainide at 2 \(\mu\)M acting on both the Na\(^+\) channels and the RyRs suppressed propagated arrhythmogenic activity by regulating underlying SR Ca\(^{2+}\) release (Fig. 9B). Also consistent, 2 \(\mu\)M flecainide acting on \(I_{\text{Na}}\) alone did not prevent spontaneous Ca\(^{2+}\) release and DADs (Fig. 9C). However, 2 \(\mu\)M flecainide acting on the

![Figure 6. Model prediction of flecainide mechanism](image_url)

The time course of action potentials is in left columns and calcium transients (CaTs) in right columns at 2 Hz. Voltage and Ca\(^{2+}\) transient dynamics over a range of physiological frequencies is shown to the right of each simulated time course at 2 Hz: the voltage maxima are binned for each pacing frequency between 0.5 and 3 Hz during the second half of a 2 min simulation time course. A and B, control APs, CaTs. C and D, CASQ2(−/−) APs, CaTs. E and F, CASQ2(−/−) myocyte APs and CaTs with high clinical dose (2 \(\mu\)M) flecainide effects on both Na\(^+\) channels and RyRs. G and H, high dose flecainide acting only on Na\(^+\) channels. I and J, high dose flecainide (2 \(\mu\)M) on only RyRs.
**Figure 7.** Full parameter space of flecainide efficacy for $I_{\text{Na}}$, RyR2 and both targets in combination

Voltage dynamics is shown for each flecainide case, and the voltage and Ca$^{2+}$ transient maxima are binned for each pacing frequency between 0.5 and 3 Hz during the second half of a 2 min simulation time course. A, CASQ2(−/−) myocytes with 0.5, 1.0 and 1.5 μM flecainide effects on both Na$^{+}$ channels and RyRs. B, flecainide concentrations acting on only Na$^{+}$ channels. C, flecainide interaction with just RyRs.
RyR2 alone was sufficient for therapeutic suppression of DADs and triggered APs (Fig. 9D). Notably, 2 μM flecainide acting on $I_{\text{Na}}$ (e.g. Fig. 9B and C) slowed tissue conduction velocity from 66 to 44 cm s$^{-1}$ as compared with 2 μM flecainide acting on RyR2 alone, consistent with well-documented detrimental conduction velocity in coupled tissue (Starmer et al. 1991; Moreno et al. 2011).

We further probed concentration dependence of our RyR2-specific effects of flecainide, and found strong dose dependence. Subthreshold DADs arising from spatially discordant Ca$^{2+}$ alternans emerged in the presence of 0.5 μM and 0.65 μM flecainide acting on RyR2 (Fig. 10), alone and in combination with $I_{\text{Na}}$ block (not shown). Figure 10B and D show the time course of the Ca$^{2+}$ mediated alternans development for 0.5 μM and 0.65 μM flecainide, respectively, acting on RyR2 alone.

Given the therapeutic potential of flecainide to normalize Ca$^{2+}$ anomalies in the CPVT-linked CASQ2$^{-/-}$, we set out to predict whether other Na$^+$ channel blockers might suppress emergent-triggered activity in rabbit ventricular myocyte models. We simulated the pure Na$^+$ channel blocker lidocaine (20 μM) and the late Na$^+$ current/hERG channel blocker ranolazine (5 μM) (Moreno et al. 2013). Lidocaine and ranolazine
have both failed to demonstrate efficacy in the suppression of Ca\(^{2+}\) wave formation (Galimberti & Knollmann, 2011). For both lidocaine and ranolazine devoid of RyR2 interaction, our results mimicked selective \(I_{\text{Na}}\) inhibition by flecainide. Lidocaine (Fig. 11A) and ranolazine (Fig. 11B) did not prevent spontaneous depolarizations at all frequencies. Protocol is as in Fig. 6.

Low dose flecainide is predicted to be insufficient for normalizing the CASQ2\((-/-\)) CPVT mutation-induced proarrhythmia in simulated cells and tissues. The extension of these results is that high doses may be required for clinical therapy, which may paradoxically promote arrhythmia. \(\beta\)-Blockade is associated with suboptimal management and pronounced side effects. Thus, we set out to utilize modelling and simulation to guide a novel form of virtual pharmacopoeia in an attempt to identify alternative CPVT therapy. We considered combinations of on-market or preclinical drugs that would cumulatively regulate the RyR2 at lower concentrations, and minimize risk associated with off-target interactions of each drug.

We used the computational model to test the hypothesis that a multidrug combination of flecainide, \(\beta\)-blockade, and CaMKII inhibition would demonstrate efficacy in CPVT. Although little is known about CaMKII inhibition in humans, the signalling molecule regulates many targets and is highly distributed in the brain; thus it is expected that high doses of CaMKII inhibition should be avoided (Ledoux et al. 1999; Kaurstad et al. 2012). However, both \(\beta\)-blockers and CaMKII inhibitors are classes of drugs that functionally modulate RyR2 activity (Fig. 12A), and are suggested to provide efficacy in the management of CPVT (Pott et al. 2011; Liu et al. 2011b, Di Pasquale et al. 2013).

We modelled the \(\beta_1\)-blocking effects of metoprolol (IC\(_{50} = 105\) nM) as a competitive inhibitor of \(\beta\)-agonist, and CaMKII inhibition by KN-62 (IC\(_{50} = 468\) nM) as a limiter of free endogenous total CaMKII (see Fig. 2; individual drug efficacy of \(\beta\)-blockade and CaMKII).

We tested in a full factorial combination: the range of relevant concentration combinations in single cells at 2 Hz: 0–1 \(\mu\)M flecainide (0.1 \(\mu\)M increments), 0–420 nM metoprolol (corresponding to 0–80% block of \(\beta\) receptors, at 8% increments), and 0–468 nM KN-62 (corresponding to 0–50% CaMKII inhibition at 5% increments). Among the combinations tested, a combined dose of 0.5 \(\mu\)M flecainide, 70 nM metoprolol and 312 nM KN-62 resulted in controlled Ca\(^{2+}\) and voltage activity over the duration of single cell pacing at 2 Hz (Fig. 12B). Parameter space maps were generated for the three-drug combination indicating ranges of dose to prevent DAD formation and spontaneous Ca\(^{2+}\) release events. Among the 1331 combinations tested, the surfaces shown in Fig. 12C represent the efficacy of these combinations on voltage (top row) and spontaneous Ca\(^{2+}\) releases (bottom row). When the surface is dark blue, no DADs occurred (voltage), while the dark blue surface in the bottom panels of Fig. 12C indicates that no spontaneous Ca\(^{2+}\) release events occurred. Even for some drug combinations that remediated DADs, spontaneous Ca\(^{2+}\) releases still occurred (indicated by larger blue area in the DAD row than the spontaneous Ca\(^{2+}\) release row). The effect of flecainide alone is contained within these graphs where the concentration of the other drugs is zero. The red stars in Fig. 12C, right, show one predicted optimal therapeutic combination, a combined dose of 0.5 \(\mu\)M flecainide, 70 nM metoprolol and 312 nM KN-62, resulted in just one effective combination that remediated Ca\(^{2+}\) and voltage activity (indicated by a star). This is within a parameter space of local minima, indicating eradication of single cell proarrhythmia event markers (e.g. DADs and spontaneous Ca\(^{2+}\) release (SCaR) events).

Finally, we simulated our drug combination of 0.5 \(\mu\)M flecainide, 70 nM metoprolol and 312 nM KN-62 in CASQ2\((-/-\)) in 1D tissue, and compared the results with a simulated tissue pretreated with 0.5 \(\mu\)M flecainide (Fig. 12D). Compared with flecainide alone, combination
therapy was superior by (i) requiring a lower dose of flecainide (and thus maintaining conduction velocity 41 cm s$^{-1}$ for flecainide vs. 59 cm s$^{-1}$ for combination therapy); (ii) fully suppressing all DADs and triggered APs; (iii) reducing APD$_{90}$; (iv) normalizing $[\text{Ca}]_i$; and (v) preventing the occurrence of spatially discordant $\text{Ca}^{2+}$ alternans (see Table 6).

We next tested if the rabbit model prediction for flecainide, $\beta$-blockade and CaMKII polytherapy would hold true in the computational mouse model, as a test of the model independence of our findings. Just as we observed for the rabbit model predictions, high dose (2 $\mu$M) flecainide effects on both $I_{\text{Na}}$ and the RyR normalized the CASQ2(−/−) CPVT phenotype as shown in Fig. 13A. Application of flecainide in the mouse model did not affect the amplitude of the $\text{Ca}^{2+}$ transients, consistent with experimental observations in the mouse genetic models (Hilliard et al. 2010). Also consistent, the effects of high dose (2 $\mu$M) flecainide on $I_{\text{Na}}$ alone was predicted to be insufficient for normalizing the CASQ2(−/−) CPVT mutation-induced afterdepolarizations and spontaneous $\text{Ca}^{2+}$ release events as shown in Fig. 13B. However, 2 $\mu$M flecainide acting on the RyR2 alone was sufficient for therapeutic suppression of DADs (Fig. 13C). Low dose 0.5 $\mu$M flecainide acting on $I_{\text{Na}}$ and the RyR2 did not ablate spontaneous $\text{Ca}^{2+}$ release events as shown in Fig. 13D. We also tested if one of the predicted polytherapy combinations that was found to be effective in the rabbit model simulations was also effective in the mouse. The result, shown in Fig. 13E, shows that the drug combination of 0.5 $\mu$M flecainide, 70 nM metoprolol and 312 nM KN-62 in the mouse CASQ2(−/−) virtual myocyte completely normalized the cellular phenotype.

We next set out to experimentally test if the model prediction for flecainide, $\beta$-blockade and CaMKII polytherapy would be effective in the R176Q/+ CPVT experimental mouse model, an alternate CPVT genotype. The results are shown in Fig. 14. A knock-in R176Q/+ CPVT mouse model was used (six R176Q/+ , two WT).

**Figure 10. Simulated effects of low clinical dose flecainide effect on RyR only in CPVT one-dimensional tissue**

$\text{Ca}^{2+}$ alternans developed slowly over time, originating from the epicardial end (red arrows) and continued to the endocardium (blue arrows). A, simulated effects of low clinically relevant dose of 0.5 $\mu$M flecainide acting on the RyR alone on action potentials (top) and $\text{Ca}^{2+}$ transients (bottom). B, spatially discordant $\text{Ca}^{2+}$ alternans emerge over time. Red arrows correspond to epicardial (Epi) $\text{Ca}^{2+}$ alternans, while blue arrows correspond to endocardial (Endo) $\text{Ca}^{2+}$ alternans. Cell no. 160 (Epi) is shown from the time 53 s (bottom), and Cell no. 1 (Endo) is shown from the time 63 s (top). C, a modest increase in flecainide (0.65 $\mu$M) decreased the transient amplitude difference between beats and prolonged the onset of alternans. D, extended time course of $\text{Ca}^{2+}$ alternans development for 0.65 $\mu$M flecainide on the RyR alone. Cell no. 160 (Epi) is shown from the time 70 s (bottom), and Cell no. 1 (Endo) is shown from the time 80 s (top). In panels B and D, arrows mark the first instance of alternans, which persist for the duration of the simulation.
R176/+ mice develop bidirectional ventricular tachycardia (VT) with catecholamine provocation. At baseline, neither the WT nor the R176Q/+ mice demonstrated ventricular arrhythmias and average heart rate was 462 beats min$^{-1}$ (R176Q/) and 574 beats min$^{-1}$ (WT) (Table 7). After injection of adrenaline (2 mg kg$^{-1}$ i.p.) and caffeine (120 mg kg$^{-1}$ i.p.), six of six R176Q/+ mice developed bidirectional VT as shown in Fig. 14A and C. Ventricular tachycardia initiated within 30 s to 6 min and lasted between 1 and 51 min in length (average 21 min) with an average ventricular rate of 807 beats min$^{-1}$. Both WT and R176Q/+ mice demonstrated T-wave alternans lasting several hours after injection of adrenaline and caffeine. After a 7 day wash-out, the mice were injected with adrenaline and caffeine (2 mg kg$^{-1}$ i.p., respectively); however, 30 min prior, all mice were given pre-treatment with i.p. injections of flecainide (12 mg kg$^{-1}$, Sigma-Aldrich), metoprolol (10 mg kg$^{-1}$, Sigma-Aldrich) and KN-93 (30 μmol kg$^{-1}$, Calbiochem). All mice demonstrated statistically significant PR, QRS and QTc prolongation 30 min after administration of flecainide, metoprolol and KN-93 (Table 8). Some mice exhibited ST segment changes (data not shown). Heart rates were lower after drug administration but not significantly (Table 8). Adrenaline and caffeine following pre-treatment with flecainide, metoprolol and KN-93 failed to induce ventricular tachycardia in all R176Q/+ mice. Mice demonstrated T-wave alternans (one WT and five R176Q/+); however, higher-grade ectopy and the typical bidirectional VT were not seen as shown in Fig. 14B and C.

**Discussion**

The optimal pharmacological management of CPVT remains a clinical challenge. The current mainstay of pharmacotherapy often involves maximally tolerated β-blockade despite high (~30–50%) failure rates (Priori et al. 2002; Liu et al. 2008). In conjunction with pharmacotherapy, mechanical ICD implantation (Miyake et al. 2013) and left cardiac sympathetic denervation (Wilde et al. 2008) have been used clinically, but carry inherent morbidity and fail to target the mechanistic underpinnings of the CPVT arrhythmia triggers.

While alternative class I antiarrhythmics have been suggested (Galimberti & Knollmann, 2011; Hwang et al. 2011), only flecainide has shown moderate clinical success. Its well-studied mechanism of action is inhibition of the inward sodium current (I_Na), but data suggest that flecainide interacts with I_Kr and RyR2 (Hilliard et al. 2010; Galimberti & Knollmann, 2011). Flecainide has demonstrated lethal proarrhythmic effects in other patient populations (Echt et al. 1991), and previous clinical failures were largely the result of an incomplete understanding of the emergent proarrhythmic risk.

Recent studies have suggested that because flecainide is 97–99% ionized at physiological pH, it is insufficiently concentrated inside the cell to act on the RyR (Liu et al. 2011a, 2012), though the study cited as evidence showed the opposite result (Liu et al. 2003). The authors made permanently charged flecainide, which when applied intracellularly (via a pipette) led to the same effect as when the same concentration of unadulterated flecainide was applied externally (in the bath). Permanently charged
Figure 12. Predicted low-dose polytherapy for CPVT
A, schematic diagram showing the primary targets of flecainide, metoprolol, and KN-62. B, prediction of low-dose combined therapy on action potentials (top) and calcium transients (bottom) at 2 Hz. C, 3 drug combination parameter space simulated in single cells tracking the number of DADs (top) and spontaneous Ca\(^{2+}\) releases (bottom). Red stars denote the location of the case in B of 0.5 μM flecainide, 70 nM metoprolol and 312 nM KN-62. D, simulated tissue effects of low dose flecainide. E, predicted tissue effects of combined simulated therapy.
flecainide had no effect when applied externally, showing that the drug action cannot be via an extracellular path. Here, we utilized a physics-based molecular dynamics approach to simulate the partitioning of flecainide by computing free energy profiles for flecainide transport in the extracellular, membrane and intracellular compartments (Fig. 5). The simulations suggest that flecainide accumulates on the membrane surface in

| Drug concentration                                         | CV (cm s⁻¹) | APD₉₀ (ms) | [Ca]ᵢ (μM) |
|------------------------------------------------------------|-------------|------------|------------|
| Drug free                                                  | 60          | Triggered APs | 0.6       |
| Flecainide 2 μM                                            | 41          | 192.5      | 0.285      |
| Flecainide 0.5 μM + 70 nM Metoprolol + 312 nM KN-62       | 59          | 177.8      | 0.55       |

Table 6. CASQ2(−/−) with ISO 1 μM at 2 Hz (rabbit)

Figure 13. Simulated drug effects in mouse ventricular myocytes
In each panel simulated membrane voltage (left) and intracellular Ca²⁺ transients (right) are shown during 2 Hz pacing. A, CASQ2(−/−) myocyte APs and CaTs with high clinical dose (2 μM) flecainide effects on both Na⁺ channels and RyRs. B, high dose flecainide acting only on Na⁺ channels. C, high dose flecainide (2 μM) on only RyRs. D, low dose flecainide 0.5 μM on both Na⁺ channels and RyRs. E, simulated test of predicted polytherapy in mouse ventricular myocytes.
cationic and neutral forms after which deprotonation occurs at the surface binding site and promotes membrane crossing down the concentration gradient. The drug then reacquires a proton once it is in the intracellular milieu. This process is extremely rapid, occurring with a diffusion coefficient of $\sim 10^{-7} \text{ cm s}^{-1}$, only an order of magnitude lower than in the bulk water.

Our predictions support the existing experimental data suggesting the following cell entry model for flecainide transport: (i) drug accumulates on the membrane surface in cationic and neutral forms; (ii) deprotonation occurs at the surface binding site (site 1) and promotes membrane crossing down the concentration gradient; and (c) drug acquires a proton once it is in the intracellular milieu.

As demonstrated in Fig. 4, virtual mouse and rabbit CASQ2(−/−) cells with β-agonist display increased Ca$^{2+}$ transients and spontaneous Ca$^{2+}$ release events, which are sufficient to drive triggered cellular electrical activity, and reproduce clinical behaviour of the CVPT phenotype (Liu et al. 2011a). The absence of calsequestrin as a buffer speeds up the dynamics of SR refilling, providing more interaction time for SR Ca$^{2+}$ to activate the RyR2, and results in functionally upregulated RyR2 activity. The resulting spontaneous Ca$^{2+}$ release events and depolarizations of the membrane persist after cessation of pacing, owing to cyclic refilling of the SR, spontaneous release of Ca$^{2+}$ into the cytoplasm via the RyR2, activation of $I_{Na}$ by inward $I_{NCX}$, influx of Ca$^{2+}$ via activation of $I_{CaL}$ and reuptake of Ca$^{2+}$ into the SR via SERCA. It is important to note that diastolic intervals in the rabbit are longer than in the mouse, and the model predicted that they could allow sufficient time to promote spontaneous diastolic release and triggered activity. Our model predicted that high dose flecainide prevents aberrant spontaneous Ca$^{2+}$ releases with resultant resolution of triggered AP activity in single virtual myocytes of mouse and rabbit and in rabbit transmural 1D virtual cardiac tissue (see Figs 6, 8 and 9), consistent with experimental data (Hilliard et al. 2010; Galimberti & Knollmann, 2011).

There is considerable disagreement in the literature as to whether the antiarrhythmic effects seen with flecainide are predominately the result of Na$^{+}$ channel blockade, RyR2 blockade, or both. Studies by Watanabe et al. (2009) and others (Hilliard et al. 2010; Galimberti & Knollmann, 2011; van der Werf et al. 2011) have documented a substantial interaction between flecainide and RyR2. They proposed that flecainide acts as a RyR2 open state blocker, with resultant increase in Ca$^{2+}$ spark rate, a decrease in Ca$^{2+}$ spark mass and disruption of Ca$^{2+}$ wave propagation.

Other studies have failed to document any effects of flecainide on RyR or Ca$^{2+}$ handling, and suggested flecainide’s efficacy is almost entirely due to its Na$^{+}$ channel blocking effects (Liu et al. 2011a; Bannister et al. 2015). There are a few potential explanations for these disparate findings, including a lower than expected flecainide concentration within the cell in Liu’s study, given rapid diffusion of neutral flecainide through the membrane and repartitioning within the bath solution, and potential mutation-induced lowered affinity of flecainide for RyR2.

When we decoupled flecainide’s presumed RyR2 activity from its well-known Na$^{+}$ channel blockade, we were surprised to find that the model predicted that Na$^{+}$ channel blockade – even at the high clinical doses – was not sufficient to explain the antiarrhythmic effects seen clinically with flecainide. As seen in Fig. 6 and 8 (single cells) and Fig. 9 (1D tissue), pure Na$^{+}$ channel
blockade failed to inhibit spontaneous Ca\(^{2+}\) release, and subsequent triggered activity, whereas the RyR2-specific flecainide interaction was predicted to yield results similar to flecainide acting on both targets.

Interestingly, our simulations with lidocaine (Moreno et al. 2011) as an additional control of pure Na\(^{+}\) channel blockade resulted in good concordance with experimental results (Katz et al. 2010; Galimberti & Knollmann, 2011; Hwang et al. 2011), namely that as a monotherapeutic approach, Na\(^{+}\) channel blockade is insufficient to resolve CPVT arrhythmia triggers. Consistent with experimental evidence in support of this mechanism of action, flecainide's interaction with the Na\(^{+}\) channel did resolve arrhythmia triggers in higher dimensions, but proarrhythmic DADs persisted.

Lastly, because the drugs used to treat CPVT act on different targets, we hypothesized that their actions might prove synergistic when used as a combination therapy. We tested the full parameter space of clinically relevant combination therapy with \(\beta\)-blockade, flecainide and CaMKII inhibition (Fig. 12), and found that all three drugs achieved better efficacy when used in combination with either or both of the other drugs tested. The potential to achieve therapeutic efficacy at lower drug concentrations potentially limits the danger of off-target and drug-related side-effects and are consistent with clinical reports of improved therapy with combined \(\beta\)-blockade and flecainide (Pott et al. 2011; Pflaumer & Davis, 2012).

Importantly, our polypharmacy simulations go beyond confirmation that a multidrug regimen is superior; for the first time (to our knowledge), our simulations are a proof-of-concept for prediction of a dosing strategy, and simulate a virtual phase 1–2 clinical trial of dose escalations with several thousand combinations of clinically relevant dosages.

We then utilized an experimental protocol to validate the virtual trial in R176Q/+ CPVT mice (Fig. 14). The CPVT R176/+ mice developed bidirectional ventricular tachycardia (VT) with catecholamine provocation. But, as predicted by the computer model in both rabbit (Fig. 12) and mouse (Fig. 13) virtual myocytes, pre-treatment with flecainide, metoprolol and KN-93 prevented catecholaminergically induced ventricular tachycardia in all R176Q/+ mice.

Our study is not without limitations. Most importantly, different CPVT mutations demonstrate differences in ultrastructure (Liu et al. 2013), protein expression (Song et al. 2007), RyR2 structural conformation and functionality (Cerrone et al. 2005; Goddard et al. 2008), and Ca\(^{2+}\) dynamics (Viatchenko-Karpinski et al. 2004; Terentyev et al. 2006). As flecainide and \(\beta\)-blockade have been used clinically, we know that the adverse effect profiles are concentration dependent. Little is known about the clinical consequences of CaMKII inhibition, and our predictions will obviously require careful testing and validation prior to clinical use.

Another important limitation of our study is that the models that we have utilized do not explicitly represent subcellular Ca\(^{2+}\) dynamics. For this reason, we are not able to simulate subcellular Ca\(^{2+}\) waves and the effects of the drugs on them. This will be an important subject of a future study. In addition, a full experimental
follow-up study to further test and hone the therapeutic efficacy of alternate polytherapy approaches needs to be conducted.

In conclusion, we utilized a computational modelling approach, informed by, and validated with experimental and clinical data, to make therapeutic predictions of drug mechanism and efficacy in the setting of CPVT. Our study sought to answer three fundamental questions of current pharmacotherapy for CPVT. (i) Is flecainide interaction with the Na\(^+\) channel sufficient for therapy? (ii) Can we predict if other Na\(^+\) channel blockers will be equally efficacious? (iii) Can a multi-targeted pharmacologic approach synergize to yield greater success than with individual therapeutics?

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