Suppression of Arrhythmia by Enhancing Mitochondrial Ca\textsuperscript{2+} Uptake in Catecholaminergic Ventricular Tachycardia Models

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HIGHLIGHTS

- Fast transfer of Ca\textsuperscript{2+} from the sarcoplasmic reticulum into mitochondria in cardiomyocytes can be enhanced by the MiCUps efsevin, targeting the VDAC2, and kaempferol, targeting the MCU.
- Enhancing sarcoplasmic reticulum-to-mitochondria Ca\textsuperscript{2+} transfer with MiCUps suppresses arrhythmogenic Ca\textsuperscript{2+} events and spontaneous action potentials in cardiomyocytes from a mouse model of CPVT.
- In vivo treatment of CPVT mice with MiCUps reduces episodes of ventricular tachycardia after adrenergic stimulation.
- In induced pluripotent stem cell-derived cardiomyocytes from a CPVT patient, both MiCUps reduce arrhythmogenic Ca\textsuperscript{2+} events.
- Our data establish fast mitochondrial Ca\textsuperscript{2+} uptake as a promising candidate structure for pharmacological treatment of human cardiac arrhythmia.
SUMMARY

Cardiovascular disease-related deaths frequently arise from arrhythmias, but treatment options are limited due to perilous side effects of commonly used antiarrhythmic drugs. Cardiac rhythmicity strongly depends on cardiomyocyte Ca\(^{2+}\) handling and prevalent cardiac diseases are causally associated with perturbations in intracellular Ca\(^{2+}\) handling. Therefore, intracellular Ca\(^{2+}\) transporters are lead candidate structures for novel and safer antiarrhythmic therapies. Mitochondria and mitochondrial Ca\(^{2+}\) transport proteins are important regulators of cardiac Ca\(^{2+}\) handling. Here, the authors evaluated the potential of pharmacological activation of mitochondrial Ca\(^{2+}\) uptake for the treatment of cardiac arrhythmia. To this aim, the authors tested substances that enhance mitochondrial Ca\(^{2+}\) uptake for their ability to suppress arrhythmia in a murine model for ryanodine receptor 2 (RyR2)-mediated catecholaminergic polymorphic ventricular tachycardia (CPVT) in vitro and in vivo and in induced pluripotent stem cell-derived cardiomyocytes from a CPVT patient. In freshly isolated cardiomyocytes of RyR2R4496C/WT mice, efsevin, a synthetic agonist of the voltage-dependent anion channel 2 (VDAC2) in the outer mitochondrial membrane, prevented the formation of diastolic Ca\(^{2+}\) waves and spontaneous action potentials. The antiarrhythmic effect of efsevin was abolished by blockade of the mitochondrial Ca\(^{2+}\) uniporter (MCU), but could be reproduced using the natural MCU activator kaempferol. Both mitochondrial Ca\(^{2+}\) uptake enhancers (MiCUps) and safer antiarrhythmic therapies.

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**METHODS**

**ISOLATION OF CARDIOMYOCYTES.** Isolation of ventricular cardiomyocytes from heterozygous knock-in RyR2\(^{-4496C/WT}\) mice (24) was performed using a Langendorff perfusion-based enzymatic digestion protocol (25) with minor modifications. Only excitable, rod-shaped, quiescent cells were used for experiments.

**HUMAN iPSC-BASED MODEL.** iPSCs from a skin biopsy from a CPVT patient carrying the RyR2\(^{S406L/WT}\) mutation and from a healthy donor were differentiated into spontaneously beating explants (26,27) and enzymatically dissociated into single cardiomyocytes.

**Ca\(^{2+}\) IMAGING.** Ca\(^{2+}\) transients, spontaneous diastolic Ca\(^{2+}\) waves, and Ca\(^{2+}\) sparks were measured in cardiomyocytes loaded with Fluo-4 acetoxyethyl ester (AM) (Thermo Fisher Scientific, Darmstadt, Germany), using confocal microscopy in line scan mode. Cells were paced by extracellular electrodes at 0.5 Hz.

**ELECTROPHYSIOLOGY.** Action potentials were recorded in current clamp mode, using the perforated patch-clamp technique. Cells were paced by repetitive, depolarizing intracellular current injections at 0.5 Hz, followed by a 60-s pause to detect potentially proarrhythmic events during this diastolic phase.

**cAMP ACCUMULATION ASSAY.** For evaluation of intracellular cAMP levels, cardiomyocytes were labeled with \(^{3}\)H-labeled adenine to measure accumulation of [\(^{3}\)H]cAMP over 15 min.

**IN VIVO ARRHYTHMIA TESTING.** Drugs were administered to RyR2\(^{R4496C/WT}\) mice 8 to 12 weeks of age through osmotic minipumps, and subsequently, electrocardiography recordings were performed under light isoflurane anesthesia, to monitor ventricular tachycardia after bolus injection of epinephrine/caffeine (epi/caff). All animal procedures were performed in accordance with national and European ethical regulations (directive 2010/63/EU) and approved by the responsible government agency (BMWFUW-66.010/0012-WF/V/3b/2015).

**MITOCHONDRIAL Ca\(^{2+}\) UPTAKE.** Mitochondrial Ca\(^{2+}\) uptake in response to 10 mM caffeine to open RyR2 was measured in Rhod-2 AM-loaded permeabilized HL-1 cardiomyocytes (28) on a fluorescence 96-well plate reader. The Ca\(^{2+}\) chelator 1,2-bis(o-aminophenoxy) ethane-N,N,N',N'-tetraacetic acid (BAPTA) (1 mM) was used to restrict Ca\(^{2+}\) to the low micrometer range around RyR clusters (8,29).

**STATISTICAL ANALYSIS.** Data are mean ± standard error of the mean. Normality of data was determined by Shapiro-Wilk test, and respective tests for statistical significance were conducted as indicated. Post hoc tests were Dunn’s test for Kruskal-Wallis tests and Tukey’s multiple comparisons test for ANOVA. Significance for contingency tables was calculated using Fisher’s exact test.
Please see the detailed Methods in the Supplemental Appendix.

RESULTS

EFSEVIN REDUCES ARRHYTHMOGENIC Ca²⁺ WAVES IN RyR2<sup>R4496C/WT</sup> CARDIOMYOCYTES. The triggers for arrhythmia originating from imbalanced cellular Ca²⁺ homeostasis, such as CPVT, are intracellular Ca²⁺ waves during diastole, which arise from an increased SR Ca²⁺ leak through RyR2 (30). We therefore recorded diastolic Ca²⁺ waves in freshly isolated ventricular cardiomyocytes from RyR2<sup>R4496C/WT</sup> mice and their wild-type (WT) littermates. Under control conditions (vehicle), neither WT cardiomyocytes nor RyR2<sup>R4496C/WT</sup> cells showed spontaneous Ca²⁺ waves within 90 s after preceding electrical stimulation at 0.5 Hz. However, unlike WT cells, RyR2<sup>R4496C/WT</sup> cardiomyocytes displayed pronounced spontaneous, diastolic Ca²⁺ waves after stimulation with the catecholamine isoproterenol (ISO) (Figures 1A to 1C).

Strikingly, application of 15 μM efsevin significantly reduced the number of cells displaying such Ca²⁺ waves and the average number of Ca²⁺ waves per minute.
Subsequent treatment with 15 mM (Supplemental Figure 1). –RyR2R4496C/WT cardiomyocytes. We observed a significant increase in the frequency of spontaneous action potentials (31), which can propagate spontaneously in patch-clamped unstimulated RyR2R4496C/WT cells and WT cells.

RyR2R4496C/WT CARDIOMYOCYTES. EFSEVIN REDUCES SPONTANEOUS ACTION POTENTIALS IN 1 minute in RyR2R4496C/WT cardiomyocytes to levels of unstimulated RyR2R4496C/WT cells and WT cells. Notably, in contrast to previous data from unstimulated cells (15), efsevin did not exert any significant effects on the amplitude and kinetics of electrically evoked Ca^{2+} transients under ISO stimulation (Supplemental Figure 1).

**EFSEVIN REDUCES SPONTANEOUS ACTION POTENTIALS IN RyR2R4496C/WT CARDIOMYOCYTES.** Cardiac arrhythmia is triggered when Ca^{2+} waves activate the sarcolemmal sodium–calcium exchanger, leading to a transient depolarizing sodium inward current and finally spontaneous action potentials (31), which can propagate along the myocardium. Hence, we recorded spontaneous action potentials in patch-clamped RyR2R4496C/WT cardiomyocytes. We observed a significant increase in the frequency of spontaneous action potentials after cells were superfused with 1 μM ISO. Subsequent treatment with 15 μM efsevin effectively reduced these spontaneous depolarizations to baseline levels (Figures 1D and 1E). Notably, efsevin did not exert any effects on the resting membrane potential and amplitude of electrically evoked action potentials under ISO stimulation but caused a significant change of the repolarization phase (Supplemental Figure 1), namely a prolongation of the action potential duration at 50% repolarization (APD50) but not at 90% repolarization (APD90). To evaluate whether this effect seen on the fast, inactivating mouse action potential (32) could lead to QT prolongation in human cells, we recorded action potentials from human iPSC-derived cardiomyocytes in the presence of efsevin and found no changes in APD50 and APD90 compared to vehicle-treated cells (Supplemental Figures 2A and 2B). Furthermore, efsevin did not inhibit hERG channel activity in a heterologous expression system at relevant concentrations (Supplemental Figure 2C).

**THE ANTIARRHYTHMIC EFFECT OF EFSEVIN IS MEDIATED BY MITOCHONDRIAL Ca^{2+} UPTAKE.** We next investigated the mechanism of efsevin’s antiarrhythmic effect. To exclude the possibility that efsevin directly blocks catecholaminergic stimulation by ISO, we measured cAMP accumulation in efsevin-treated RyR2R4496C/WT cardiomyocytes. Stimulation by ISO induced a significant increase in cellular cAMP, which was blocked by the beta-adrenoreceptor blocker propranolol. Addition of efsevin alone did not increase or decrease cellular cAMP concentrations, and addition of efsevin to stimulated cells had no effect on the ISO-induced cAMP increase, indicating that efsevin does not influence beta-adrenergic signaling in cardiomyocytes (Figure 2).

Efsevin significantly enhanced transfer of Ca^{2+} from the SR into mitochondria in a Ca^{2+} uptake assay in permeabilized cultured HL-1 cardiomyocytes. Mitochondrial Ca^{2+} was measured after addition of 10 mM caffeine to open RyRs in the presence of the Ca^{2+} chelator BAPTA to restrict Ca^{2+} released from RyRs to the low micrometer range around RyR clusters (8,29) (Figure 3A). The efsevin-sensitive Ca^{2+} transfer between the SR and mitochondria was blocked by addition of the MCU blocker ruthenium red. To test whether the enhanced SR-mitochondria Ca^{2+} transfer was directly responsible for the reduction of diastolic Ca^{2+} wave frequency in RyR2R4496C/WT cardiomyocytes, we assessed whether blocking of mitochondrial Ca^{2+} uptake abolished the antiarrhythmic effect of efsevin. We measured catecholamine-induced Ca^{2+} waves in RyR2R4496C/WT myocytes under simultaneous blockade of mitochondrial Ca^{2+} uptake, using the MCU inhibitor Ru360 (Figure 3B). We observed a moderately higher Ca^{2+} wave frequency under all conditions, consistent with the idea that mitochondrial Ca^{2+} uptake prevents Ca^{2+} wave formation. Most strikingly, the ability of efsevin to suppress the ISO-induced increase in Ca^{2+} wave frequency was abolished in the presence of Ru360, indicating that the suppression of diastolic Ca^{2+} waves by efsevin is solely mediated by enhanced mitochondrial Ca^{2+} uptake.
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We next tested if enhancing mitochondrial Ca$^{2+}$ uptake was a general pharmacological approach to suppress arrhythmogenic events or if it is limited to a specific effect of efsevin on VDAC2. To this purpose, we used another activator of mitochondrial Ca$^{2+}$ uptake, the natural plant flavonoid kaempferol, which was reported to directly activate MCU in the inner mitochondrial membrane (33,34). Indeed, kaempferol increased SR–mitochondria Ca$^{2+}$ transfer, comparable to efsevin (Figure 3D). To evaluate the antiarrhythmic potential of kaempferol, we measured diastolic Ca$^{2+}$ waves in kaempferol-treated RyR2$^{R4496C/WT}$ cardiomyocytes under ISO to induce catecholaminergic stimulation. Strikingly, 10 μM kaempferol completely eliminated ISO-induced arrhythmogenic Ca$^{2+}$ waves in RyR2$^{R4496C/WT}$ cardiomyocytes (Figure 3E).

It was previously reported that enhanced mitochondrial Ca$^{2+}$ uptake in cardiomyocytes restricts diffusion of Ca$^{2+}$ inside the cytosol (13) and thereby prevents propagation of cytosolic Ca$^{2+}$ signals under conditions of Ca$^{2+}$ overload (15). Because an enhanced RyR-mediated Ca$^{2+}$ leak was reported to be the mechanism responsible for arrhythmogenesis in CPVT (35-37), we recorded Ca$^{2+}$ sparks from RyR2$^{R4496C/WT}$ cardiomyocytes (Figure 4). We observed an increase in Ca$^{2+}$ spark frequency and amplitude after treatment with ISO, consistent with previous work (36), thus explaining the enhanced Ca$^{2+}$ wave frequency under catecholaminergic stimulation. Strikingly, the SR leak in cells treated with ISO together with efsevin was reduced compared to that in cells treated with ISO alone, as indicated by a decrease in Ca$^{2+}$ spark frequency and amplitude. Also, we found removal of cytosolic Ca$^{2+}$ was accelerated under efsevin stimulation, leading to a reduction of full width and full duration of Ca$^{2+}$ sparks. Together, these effects explain the suppressive effect of efsevin on propagating Ca$^{2+}$ waves.

**MICups REDUCE EPISODES OF STRESS-INDUCED VENTRICULAR TACHYCARDIA IN VIVO.** To assess the potency of both of the mitochondrial Ca$^{2+}$ uptake enhancers (MiCups), efsevin and kaempferol, to suppress arrhythmia in vivo, we administered efsevin and kaempferol to RyR2$^{R4496C/WT}$ mice, using implantable osmotic minipumps. All mice recovered well from surgery, and their behavior was grossly normal. Treated mice showed no signs of discomfort, stress, or abnormal behavior. After 3 days, efsevin and kaempferol showed no effect on electrocardiography (ECG) parameters such as the interval between atrial and ventricular depolarization (PR), the interval between ventricular depolarization and subsequent repolarization (QT), or conduction of ventricular
depolarization (QRS) and basal heart rate (Figures 5A and 5B). To activate their adrenergic response, we injected mice with a bolus of 2 mg/kg epinephrine and 120 mg/kg caffeine (epi/caff). The epi/caff injection induced a significant increase in heart rate in all 3 groups, but no differences were observed between vehicle-treated mice and mice treated with MiCUps. Most strikingly, both of the MiCUps significantly reduced episodes of bidirectional ventricular tachycardia under catecholaminergic stimulation. Injection of epi/caff provoked bidirectional ventricular tachycardia in all vehicle-treated control animals (n = 11) but only in 6 of 10 mice treated with efsevin and 6 of 11 mice treated with kaempferol (Figures 5D and 5E). Comparable results were obtained from mice treated with efsevin for 8 days (Supplemental Figure 3).

**MiCUps REDUCE ARRHYTHMOGENIC Ca^{2+} WAVES IN iPSC-DERIVED CARDIOMYOCYTES FROM A CPVT PATIENT.** In order to evaluate the translational potential of MiCUps for the treatment of CPVT, we used a human cell-based arrhythmia model for CPVT. Human iPSC-derived cardiomyocytes from a CPVT patient heterozygous for the RyR2R4496C mutation associated with CPVT (26) were used to record arrhythmogenic Ca^{2+} waves, whereas cells obtained from a healthy 32-year-old female without history of cardiac disease served as control. In accordance with the CPVT phenotype, where patients show a normal ECG pattern under baseline conditions, only few untreated cells displayed Ca^{2+} waves (Figure 6), whereas beta-adrenergic stimulation induced by ISO led to a significant increase in diastolic Ca^{2+} waves in RyR2R4496C/WT cells but did not induce Ca^{2+} waves in control cells. Treatment of RyR2R4496C cells with either efsevin or kaempferol significantly reduced the number of cells displaying Ca^{2+} waves and the average frequency of Ca^{2+} waves per minute to baseline levels.

**DISCUSSION**

**MITOCHONDRIA AS DRUG TARGETS.** Mitochondria occupy approximately 30% of the cardiomyocyte volume (6,7). Although their crucial roles in ATP synthesis, regulation of respiratory rate, and apoptosis are well understood, mitochondrial contributions to cardiac Ca^{2+} handling are still under debate. While it is generally accepted that a gradual and moderate rise in mitochondrial Ca^{2+} enhances energy production (18), the role of a low-affinity/high-conductance, fast mitochondrial Ca^{2+} uptake remains unclear. Different experimental approaches suggest an immediate role for this uptake in the regulation of cardiomyocyte bioenergetics (38), contraction (39), and rhythmicity (15,21), but the potential of this rapid mitochondrial Ca^{2+} uptake mechanism to serve as a drug target in cardiovascular disease has not been sufficiently evaluated. It was proposed that inhibition of MCU by Ru360 ameliorates myocardial damage after ischemia reperfusion injury in rats, presumably by inhibiting depolarization of mitochondria and following opening of the mitochondrial permeability transition pore (40).
However, in healthy myocardium, the role of fast mitochondrial Ca\(^{2+}\) uptake remains elusive. Regarding arrhythmia, protective (22) as well as pro-arrhythmic (41) effects of activated mitochondria were discussed. We have recently identified the novel compound efsevin, which binds to the outer mitochondrial membrane VDAC2, enhances mitochondrial Ca\(^{2+}\) uptake, and restores rhythmic cardiac contractions in a zebrafish model for Ca\(^{2+}\)-induced cardiac arrhythmia (15). Here we show that enhancing mitochondrial Ca\(^{2+}\) uptake by using MiCUs efficiently suppresses arrhythmia in both cellular models and a human model of CPVT in vitro and in vivo. Our results establish pharmacological activation of rapid mitochondrial Ca\(^{2+}\) uptake as a novel preventive and therapeutic strategy against CPVT. We have previously shown that efsevin suppresses arrhythmia in a murine model of CPVT and in a human model of CPVT (15). Thus, due to their potent role of suppressing arrhythmogenic Ca\(^{2+}\) waves in both Ca\(^{2+}\) overload (15) and CPVT, it is conceivable that MiCUs may also be applied in other more common forms of Ca\(^{2+}\)-induced cardiac arrhythmias. These include arrhythmias in the setting of heart failure, which are triggered by cellular Ca\(^{2+}\) overload or atrial fibrillation, also linked to imbalances in cardiomyocyte Ca\(^{2+}\) handling (5). This study serves as a proof-of-principle, holding great promise for additional indications.

**OPTIMIZED MiCUs.** Our work shows that enhancing mitochondrial Ca\(^{2+}\) uptake efficiently reduces arrhythmia in experimental models of CPVT. In order to develop MiCUs toward human therapeutics, several steps of optimization must be taken. Candidate compounds need to be optimized to achieve a high affinity MiCu with low side effects and suitable pharmacokinetics for application in human subjects. We show that the antiarrhythmic effect can be achieved by activation of at least 2 distinct target proteins within the mitochondrial Ca\(^{2+}\) uptake complex: efsevin, targeting VDAC2 in the outer mitochondrial membrane, and kaempferol, targeting MCU in the...
inner membrane. Suppression of arrhythmia is thus attributable to enhanced mitochondrial Ca\(^{2+}\) uptake and is independent of the molecular target protein within the fast mitochondrial Ca\(^{2+}\) uptake complex. Our work thus establishes the entire protein complex as a pharmacological target structure and allows for future optimization of this therapeutic concept through novel compounds and targets. Apart from VDAC2 and MCU, the auxiliary MCU regulators MICU1 and -2, MCUb, EMRE, and MCUR1\(^{16,17}\) may serve as future candidate targets.

**SIDE EFFECTS.** Mitochondrial Ca\(^{2+}\) uptake proteins such as VDAC2 and MCU are ubiquitously expressed. Regarding a therapeutic application, it is thus important to evaluate potential side effects of a MiCUp-based therapy. It is important to note that we did not observe any adverse effects of MiCUs in mice treated with efsevin or kaempferol for 3 to 8 consecutive days. Furthermore, kaempferol was previously used in animal experiments, and no adverse effects, even after up to 1 year of treatment or at high doses, were observed\(^{42-44}\). However, further long-term experiments and large animal studies are needed to further evaluate safety of a MiCUp-based therapy. Although we did not observe changes in cytosolic Ca\(^{2+}\) in our experiments, long-term effects of enhanced mitochondrial Ca\(^{2+}\) uptake on a potential redistribution of cellular Ca\(^{2+}\) in the heart and other organs must be evaluated. Furthermore, because an enhanced mitochondrial Ca\(^{2+}\) uptake was observed to activate mitochondrial metabolism and reactive oxygen species production, special focus should be directed toward side effects related to changes in cellular bioenergetics.

Common side effects of actual antiarythmic drugs like Na\(^{+}\), K\(^{+}\), and Ca\(^{2+}\) channel blockers include...
changes in cardiac electrophysiology like deceleration of cardiac de- or repolarization, the latter often expressed as a prolonged QT interval. We observed an effect of efsevin on the repolarization phase of the action potential in mice (Supplemental Figure 1), namely a prolongation of APD50 but not APD90. However, whereas repolarization in mice is carried mainly by fast potassium currents (Ito, IK, slow), the human action potential displays a pronounced plateau phase, and phase 3 repolarization is carried predominantly by the delayed K+ currents Ikr and If (32). To rule out the possibility that the observed prolongation of APD50 by efsevin in mice could be relevant for human therapy, we showed that efsevin does not influence action potential duration in human iPSC-derived cardiomyocytes and does not block hERG activity. It is thus conceivable that efsevin has a direct impact on the fast repolarizing currents in mice but does not influence the human action potential. Most importantly, however, we did not observe effects of MiCUp administration on ECG parameters like PR, QT, and QRS interval and heart rate in mice treated with MiCUs. Because MiCUs target intracellular structures to suppress the generation of ectopic depolarizations and do not influence the cardiac action potential, they might be less prone to severe side effects like, for example, the typical pro-arrhythmic effects observed with class I or III antiarrhythmic drugs. However, the murine repertoire of ion channels governing the cardiac action potential of mice varies from the human one, and additional experiments in other mammalian species are needed to solve this issue.

CONCLUSIONS

Common antiarrhythmic drugs aim at inhibiting expansion of ectopic activity and display perilous side effects. Because major arrhythmias are often associated with imbalanced intracellular Ca2+ homeostasis (3–5), intracellular Ca2+ transporters are attractive candidates for newer and safer therapies. Here we show that enhancing mitochondrial Ca2+ uptake by pharmacological agonists of the mitochondrial Ca2+ uptake proteins VDAC2 and MCU efficiently suppresses arrhythmia in a murine and a human model for CPVT. Our data establish MiCUs as attractive compounds for a novel preventive and therapeutic strategy to treat Ca2+-triggered cardiac arrhythmias.

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PERSPECTIVES

COMPETENCY IN MEDICAL KNOWLEDGE: Mitochondria regulate cardiac rhythmicity. Pharmacological activation of mitochondrial Ca2+ uptake by MiCUs suppresses arrhythmogenesis in murine and human iPSC-based models for catecholaminergic polymorphic ventricular tachycardia.

TRANSLATIONAL OUTLOOK 1: Optimization of compounds and a careful investigation on pharmacokinetics and drug metabolism are needed to develop a potential MiCUp-based human therapy.

TRANSLATIONAL OUTLOOK 2: Additional experiments using models of other Ca2+-triggered arrhythmias could further expand the application range of MiCUs.

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