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RESEARCH ARTICLE

Approved drugs ezetimibe and disulfiram enhance mitochondrial Ca^{2+} uptake and suppress cardiac arrhythmogenesis

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Background and Purpose: Treatment of cardiac arrhythmia remains challenging due to severe side effects of common anti-arrhythmic drugs. We previously demonstrated that mitochondrial Ca^{2+} uptake in cardiomyocytes represents a promising new candidate structure for safer drug therapy. However, druggable agonists of mitochondrial Ca^{2+} uptake suitable for preclinical and clinical studies are still missing.

Experimental Approach: Herewe screened 727 compounds with a history of use in human clinical trials in a three-step screening approach. As a primary screening platform we used a permeabilized HeLa cell-based mitochondrial Ca^{2+} uptake assay. Hits were validated in cultured HL-1 cardiomyocytes and finally tested for anti-arrhythmic efficacy in three translational models: a Ca^{2+} overload zebrafish model and cardiomyocytes of both a mouse model for catecholaminergic polymorphic ventricular tachycardia (CPVT) and induced pluripotent stem cell derived cardiomyocytes from a CPVT patient.

Key Results: We identifiedtwo candidate compounds, the clinically approved drugs ezetimibe and disulfiram, which stimulate SR-mitochondria Ca^{2+} transfer at nanomolar concentrations. This is significantly lower compared to the previously described mitochondrial Ca^{2+} uptake enhancers (MiCUps) efsevin, a gating modifier of the voltage-dependent anion channel 2, and kaempferol, an agonist of the mitochondrial Ca^{2+} uniporter. Both substances restored rhythmic cardiac contractions in a zebrafish cardiac arrhythmia model and significantly suppressed arrhythmogenesis in freshly isolated ventricular cardiomyocytes from a CPVT mouse model as well as induced pluripotent stem cell derived cardiomyocytes from a CPVT patient.

Abbreviations: AEQ_{mt}, mitochondrial matrix-targeted aequorin; CPVT, catecholaminergic polymorphic ventricular tachycardia; MiCUp, mitochondrial Ca^{2+} uptake enhancer; NCC, NIH Clinical Collection; RyR, ryanodine receptor; SR, sarcoplasmic reticulum; *tre*, *tremblor*; VDAC2, voltage-dependent anion channel 2.

Conclusion and Implications: Taken together we identified ezetimibe and disulfiram as novel MiCUps and efficient suppressors of arrhythmogenesis and as such as, promising candidates for future preclinical and clinical studies.

KEYWORDS
anti-arrhythmic, arrhythmia, CPVT, MCU, mitochondrial Ca^{2+} uptake pathway, mitochondrial Ca^{2+} uptake enhancers, mitochondria

1 | INTRODUCTION

While mortality and morbidity rates related to cardiovascular diseases are generally declining, arrhythmia-related incidents are still on the rise (Benjamin et al., 2018). This is in part related to limited effectiveness and major side effects of common anti-arrhythmic drugs. Anti-arrhythmic drugs of Vaughan Williams class I, III and IV act by targeting plasma membrane ion channels and suppressing propagation of ectopic signals. However, due to their effects on the cardiac action potential and thus cardiac conduction speed, they are prone to proarrhythmic side effects. Therefore, novel therapeutic strategies that suppress the initiation of arrhythmogenic signals inside cardiomyocytes are currently in focus of the search for novel and safer anti-arrhythmic therapies.

We have recently demonstrated a critical role of mitochondrial Ca^{2+} uptake for the regulation of cardiac rhythmicity (Shimizu et al., 2015). The cardiac contraction cycle is initiated by influx of extracellular Ca^{2+} into cardiomyocytes and subsequent Ca^{2+} release predominantly from the sarcoplasmic reticulum (SR) to initiate muscle contraction (Bers, 2002, 2008). Mitochondria are located in close proximity to the SR (Rog-Zielinska et al., 2016) and can rapidly take up Ca^{2+} on a beat-to-beat interval (Robert et al., 2001) through a selective mitochondrial Ca^{2+} uptake pathway consisting of pore proteins in both mitochondrial membranes and several positive and negative regulators (Baughman et al., 2011; De Stefani et al., 2011; Kirichok et al., 2004; Waldeck-Weiermair et al., 2013). In cardiomyocytes, the mitochondrial Ca^{2+} uptake pathway is directly tethered to the Ca^{2+} release sites of the SR. Within the Ca^{2+} microdomain around the Ca^{2+} release sites of the SR, the local cytosolic Ca^{2+} concentration reaches values high enough to activate the mitochondrial Ca^{2+} uptake pathway allowing for rapid and direct shuttling of Ca^{2+} from the SR into mitochondria (De la Fuente et al., 2016; De la Fuente & Sheu, 2019). Under pathological conditions, erratic Ca^{2+} release of single Ca^{2+} release sites in form of Ca^{2+} sparks followed by Ca^{2+} waves during diastole leads to spontaneous contractions and arrhythmia (Allen et al., 1984). Pharmacological activation of mitochondrial-Ca^{2+} uptake locally buffers these events and thereby suppresses arrhythmogenic signals in cardiomyocytes, while systolic events remain unaltered (Shimizu et al., 2015). Indeed, treatment with agonists of mitochondrial-Ca^{2+} uptake suppressed episodes of arrhythmia in a murine model for catecholaminergic polymorphic ventricular tachycardia (CPVT) (Schweitzer et al., 2017). Interestingly, despite enhancing mitochondrial Ca^{2+} uptake over several days, no signs of severe adverse effects, for example, apoptosis, were observed neither in cellular nor in animal models (Schweitzer et al., 2017; Shimizu et al., 2015). Modulation of mitochondrial-Ca^{2+} uptake could thus serve as novel pharmacological strategy for the treatment of human cardiac arrhythmias.

This can be accomplished by agonists of either the voltage-dependent anion channel 2 (VDAC2) in the outer mitochondrial membrane or the mitochondrial Ca^{2+} uniporter in the inner mitochondrial membrane. Two such compounds, the VDAC2 gating modulator **efsevin** (Shimizu et al., 2015; Wilting et al., 2020) and the mitochondrial Ca^{2+} uniporter agonist **kaempferol** (Montero et al., 2004), were found to be effective in reducing arrhythmia, thus providing a proof of concept that mitochondrial Ca^{2+} uptake enhancers (MiCUps) represent, in this respect, a pharmacologically highly relevant class of molecules. However, both drugs are so far only used experimentally and are currently too poorly characterized to have clinical potential.

What is already known

- Cardiac arrhythmia results from imbalances in cellular Ca^{2+} homeostasis.
- Activation of mitochondrial Ca^{2+} uptake suppresses arrhythmogenic Ca^{2+} signals during diastole.

What does this study add

- The two clinically approved drugs, ezetimibe and disulfiram activate mitochondrial Ca^{2+} uptake.
- Both efficiently suppress arrhythmogenesis in translational models in a nanomolar range.

What is the clinical significance

- The results strengthen mitochondrial Ca^{2+} uptake as a pharmacological target.
- The identification of clinically approved drugs allows for repurposing studies.

Furthermore, both are effective at concentrations around 10–20 μM at the *in vitro* target site, making them poor candidates for clinical use.

Here, we applied a three-step protocol to identify novel MiCUps. First, we screened a chemical library consisting of 727 compounds with a history of use in human clinical trials for novel mitochondrial Ca^{2+} uptake enhancers using an established screening platform for mitochondrial Ca^{2+} uptake modifiers (Arduino et al., 2017). We identified three hits, the FDA- and EMA-approved drugs, **ezetimibe** and **disulfiram**, and the natural compound **honokiol**, which significantly increased mitochondrial-Ca^{2+} uptake in permeabilized HeLa cells. To transfer these results to a cardiac system, we measured SR-mitochondria Ca^{2+} transfer in a standardized cultured cardiomyocyte assay (Schweitzer et al., 2017; Wilting et al., 2020) and found that two of them, ezetimibe and disulfiram, enhanced SR-mitochondria Ca^{2+} transfer at significantly lower concentrations than efsevin and kaempferol. Finally, we performed efficacy testing in translational arrhythmia models (Schweitzer et al., 2017; Shimizu et al., 2015) and found that both substances suppress cardiac fibrillation in zebrafish embryos and arrhythmogenic Ca^{2+} signals in murine and human cardiomyocytes carrying mutations associated with CPVT. Altogether, we identified two highly interesting candidates for further preclinical and clinical testing of MiCUp-based anti-arrhythmic therapy.

2 | METHODS

2.1 | Primary drug screen in permeabilized HeLa cells

Drug screening was performed as described previously (Arduino et al., 2017). Briefly, HeLa cells (ATCC, RRID:CVCL_0030) stably expressing a mitochondrial matrix-targeted aequorin (AEQ_{mt}) were grown in Dulbecco's modified Eagle's medium with high-glucose, 10% FBS and 100 $\mu g \cdot mL^{-1}$ geneticin (Life Technologies). On the day of the experiment, active aequorin was reconstituted from apoaequorin in the mitochondrial matrix by incubating cells with 3-μM coelenterazine derivative *n* (Biotium) for 3 h at room temperature. Then, Ca^{2+} was depleted from intracellular stores by incubation with 200-nM **thapsigargin** (VWR) for 20 min, and cells were permeabilized with 60-μM digitonin for 5 min. Experiments were performed in an intracellular-like buffer containing (in mM) 140 KCl, 1 KH_2PO_4/K_2HPO_4, 1 $MgCl_2$, 20 HEPES, 1 Na^+-pyruvate, 1 ATP/$MgCl_2$, 2 Na^+-succinate and 0.1 EGTA as a Ca^{2+} chelator (pH 7.2 with KOH) at a density of ~70,000 cells/well in a white 96-well compound plate. The NIH Clinical Collection (NCC) library, consisting of 727 compounds (10 μM, in 0.1% [v/v] DMSO) was screened in biological duplicates. DMSO (0.1%) was used as a vehicle control and Ru360 (10 μM, VWR), a classical inhibitor of mitochondrial Ca^{2+} uniporter, was used as an experimental control to prove that aequorin luminescence is selective for mitochondrial Ca^{2+} uniporter (MCU)-mediated mitochondrial Ca^{2+} uptake. Permeabilized cells were incubated in the presence of compounds for 5 min at room temperature and Ca^{2+}-stimulated light signals upon a bolus application of 4-μM free Ca^{2+} was recorded at 469 nm every 0.1 s with a luminescence counter (MicroBeta^2 LumiJET Microplate Counter, PerkinElmer). Drug screens were analysed as described previously (Arduino et al., 2017). Briefly, after smoothing the dynamics of mitochondrial-Ca^{2+}-dependent luminescence obtained for each compound using a cubic spline function as described previously (Arduino et al., 2017), both peak (maximal amplitude of the luminescence signal) and uptake rate (left slope) were automatically determined. Based on these parameters a score (S_{drug}) was assigned to each compound as shown in Figure 1.

2.2 | Mitochondrial Na^+/Ca^{2+} exchanger-mediated Ca^{2+} extrusion assay

HeLa cells were harvested in an external medium containing (in mM): 145 NaCl, 5 KCl, 1 $MgCl_2$, 10 glucose, 10 HEPES and 0,5 EGTA (pH 7.4/NaOH), treated with 200-nM thapsigargin (VWR) for 10 min and resuspended in an intracellular-like buffer containing (in mM) 140 KCl, 3 KH_2PO_4, 2,5 $MgCl_2$, 10 HEPES, 0.05 EGTA, 5 Malate, 5 Pyruvate, 5 Succinate, pH 7.4 with KOH. After permeabilization with 60-μM digitonin for 3 min, cells were resuspended in intracellular-like buffer with 0.1-μM calcium green-5N (Thermo Fischer), seeded into a black 96-well plate at a density of ~1 million cells/well and incubated for 5 min with compounds or vehicle (0.1% DMSO) before measurements. Calcium Green-5N fluorescence was monitored at Ex506/Em531 every 8 s at room temperature using a CLARIOstar microplate reader (BMG Labtech Perkin-Elmer Envision) and injected with of $CaCl_2$ (100 μM). After 3 min, the mitochondrial Ca^{2+} uniporter inhibitor Ru360 (10 μM, VWR) was added to each well together with either vehicle or the mitochondrial Na^+/Ca^{2+} exchanger inhibitor CGP-37157 (10 μM, VWR). Afterwards, 60-mM NaCl was injected and mitochondrial Na^+/Ca^{2+} exchanger-dependent Ca^{2+} efflux was recorded.

2.3 | SR-mitochondria Ca^{2+} transfer

Ca^{2+} transfer from the SR into mitochondria was measured as described in Schweitzer et al. (2017). In brief, HL-1 cardiomyocytes (RRID:CVCL_0303, received as a gift from Dr. William Claycomb, Lousiana State University) (Claycomb et al., 1998) were plated in a 96-well plate randomly assigned to experimental groups of equal group size, loaded with 4-μM Rhod-2, AM (Thermo Fisher) and permeabilized with 25-μM digitonin in internal solution (in mM: 1 BAPTA (VWR), 20 HEPES, 100 L-aspartic acid potassium salt, 40 KCl, 0.5 $MgCl_2$, 2 maleic acid, 2 glutamic acid, 5 pyruvic acid, 0.5 KH_2PO_4, 5 MgATP and 0.47 $CaCl_2$, [pH = 7.2 with Trizma base]). Fluorescence at Ex = 540 ± 9 nm and Em = 580 ± 20 nm was recorded on an Infinite® 200 PRO multimode reader (Tecan, Maennedorf, Switzerland). After 22 s, caffeine was injected to a final concentration of 10 mM. To exclude signals that are not related to Ca^{2+} mobilizations, all experiments included a control well with **ruthenium red**, as a blocker

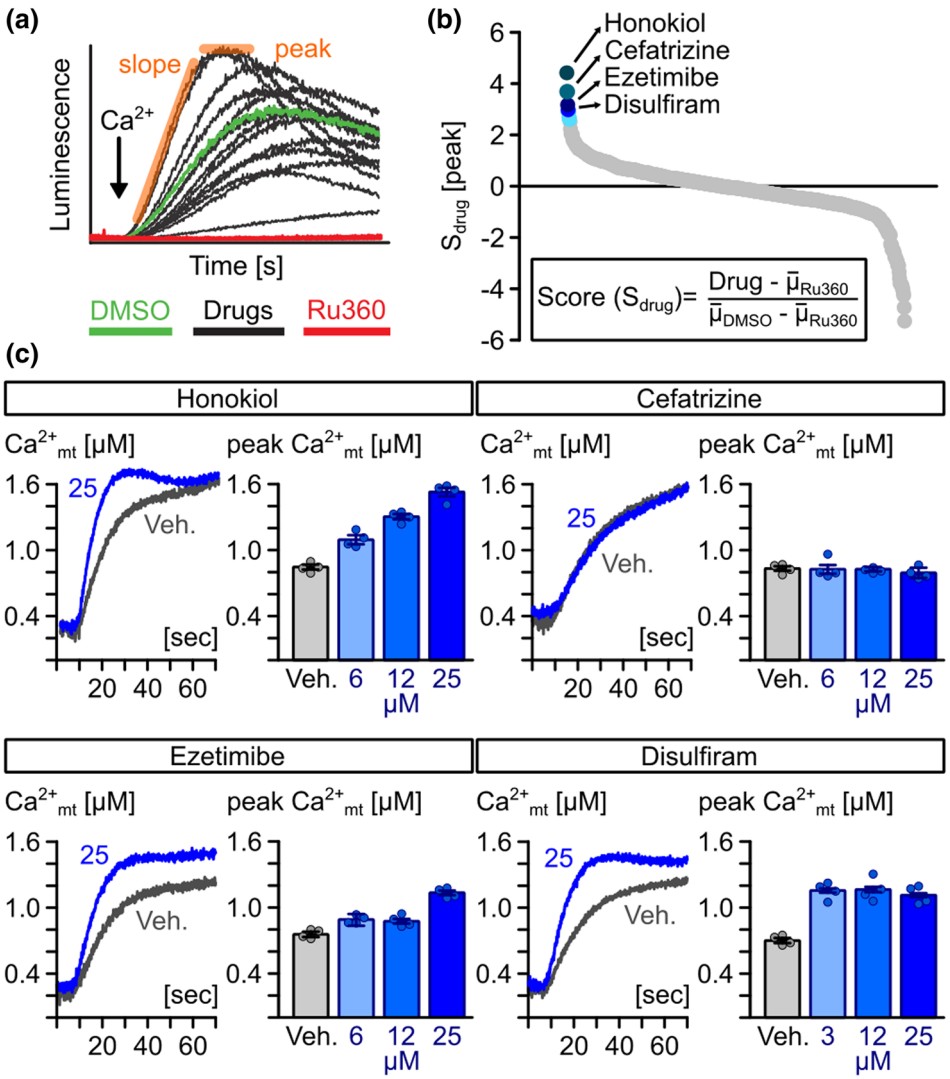

FIGURE 1 Identification of novel MiCUps by chemical screening. (a) Example of Ca^{2+}-dependent, mitochondrial aequorin kinetics in digitonin-permeabilized HeLa cells upon the addition of 4-μM free Ca^{2+} (black arrow) in the presence of candidate drugs (10 μM), experimental (10-μM Ru360) and vehicle controls (0.1% [v/v] DMSO). (b) Ranking of compounds based on S_{drug} scores. (c) Representative traces of averaged mitochondrial Ca^{2+} kinetics and quantification of mitochondrial (mt)-Ca^{2+} uptake rate in mitochondria of permeabilized HeLa cells treated with different concentrations of honokiol, cefatrizine, ezetimibe and disulfiram and 0.1% (v/v) DMSO (vehicle; Veh.) ($n = 4$)

for SR-mitochondria Ca^{2+} transfer (ryanodine receptor/RyR, VDAC and mitochondrial Ca^{2+} uniporter), and only experiments in which SR-mitochondria Ca^{2+} transfer was blocked by ruthenium red were included in the analysis. Cells were visually inspected after measurement and only recordings from wells where cells were still attached and morphologically intact were used for analysis. EC50 values were calculated using Quest Graph™ EC50 Calculator (AAT Bioquest, Inc.) at https://www.aatbio.com/tools/ec50-calculator.

2.4 | Cytosolic Ca^{2+} measurements on HL-1 cardiomyocytes

For the measurement of Ca^{2+} release, HL-1 cells were plated in a 96-well plate loaded with 5-μM fura-2, AM (Thermo Fisher) or 1 h at 37°C, before washing with external solution (in mM: 140 NaCl, 6 Kcl, 2 $CaCl_2$, 1 $MgCl_2$, 10 glucose, 20 HEPES, pH = 7.4 with NaOH). After 30 min incubation to allow for total de-esterification of the dye, cells were washed, randomly assigned to experimental groups, and measured in an Infinite® 200 PRO multimode reader (Tecan, Maennedorf, Switzerland) at Ex = 340 ± 9 nm and 380 ± 9 nm and Em = 510 ± 20 nm. Test compounds were added 5 min prior to the experiment. Caffeine to a final concentration of 10 mM was added 22 s after start of the recording.

2.5 | Isolation of ventricular cardiomyocytes from $RyR2^{R4496C/WT}$ mice

Mice from strain $Ryr2^{tm1Sgp}$ (RRID:MGI:3653876) mimic the human CPVT phenotype (Cerrone et al., 2005) and were used as models for human cardiac arrhythmia. Mice were bred in the animal facility of the Walther Straub Institute of Pharmacology and Toxicology at LMU Munich, which is approved by the Bavarian animal welfare committee. Mice were kept in groups of 5–6 animals in ventilated plastic cages with wood shavings (Blue Line, Tecniplast, Buguggiate, Italy). Ventricular cardiomyocytes of 8–16 weeks old male and female mice were isolated by retrograde perfusion through the aorta as described previously (O'Connell et al., 2007; Schweitzer et al., 2017). After cervical dislocation, the heart was excised and placed on a Langendorff mode perfusion system. Tissue was digested by perfusion with Liberase™ TM at a final concentration of 0.075 $ml \cdot ml^{-1}$ for 6 min. After

mechanical separation of the ventricles by trituration, cardiomyocytes were consecutively transferred into Tyrode's solution with 0.1, 0.5 and finally 1-mM $CaCl_2$ to reintroduce physiological Ca^{2+} concentrations. Only excitable, rod-shaped, quiescent cells were used for experiments. All animal procedures had local approval and conformed to the guidelines from Directive 2010/63/EU on the protection of animals used for scientific purposes.

2.6 | Human iPSC cardiomyocytes

Human iPSCs from a 60-year-old male donor presenting with a severe form of CPVT were generated as described previously (Moretti et al., 2010). Spontaneously beating areas were explanted after 2–5 months and enzymatically dissociated into single cardiomyocytes. Single cells were plated on fibronectin-coated glass bottom dishes (MatTek, Ashland, MA, USA) and used for experiments at approximately 7 days after dissociation (Schweitzer et al., 2017). Individual dishes were randomly assigned to experimental groups. Human iPSC generation was performed under a human research subject protocol approved by the institutional review boards and the ethic committee of the Klinikum rechts der Isar, Technical University of Munich, which strictly complied with the Helsinki Declaration regarding donor source. Written informed consent was obtained from the affected patient and healthy volunteer.

2.7 | Confocal Ca^{2+} imaging

Ca^{2+} waves were analysed by confocal microscopy as described previously (Schweitzer et al., 2017). In brief, cardiomyocytes were loaded with 1-μM Fluo-4, AM (Thermo Fisher), and Ca^{2+} transients were elicited by electric field stimulation using a S48 square pulse Stimulator (Grass Technologies, Warwick, RI, USA) on an inverted confocal microscope (Leica TCS SP5 or Zeiss LSM 880). Line scan series were generated along the long axis of a myocyte. After reaching steady state pulsing was stopped and cells were analysed for the occurrence of spontaneous diastolic Ca^{2+} waves. Every experiment contained a group with isoprenaline and only preparations that were sensitive to stimulation with isoprenaline were used for drug testing. Depending to the quality of the preparation and the yield of cells, the remaining cells were randomly attributed to experimental groups. All groups were measured in random order to avoid the influence of a decrease in the quality of cells over time and were stopped when cell quality decreased as indicated by a hyperexcitability or insensitivity to external stimuli.

2.8 | Zebrafish husbandry and phenotype rescue experiments

Zebrafish of the mutant line Tg (myl7:eGFP)/*tremblor* (*tre*^{tc318d}, RRID:ZFIN_ZDB-GENO-150505-3) were used as a model for Ca^{2+}-induced cardiac arrhythmia (Shimizu et al., 2015) and were maintained and bred in the animal facility of the Walther Straub Institute of Pharmacology and Toxicology at LMU Munich, which is approved by the Bavarian animal welfare committee. Fish are kept in groups of approximately 10 adult fish in modified mouse cages in a stand-alone zebrafish rack (PP Module, Aqua Schwarz, Göttingen, Germany) under constant surveillance of water quality. Embryos collected from heterozygous *tre* crosses were kept in E3 buffer (in mM: 5 NaCl, 0.33 $MgSO_4$, 0.17 KCl, 0.33 $CaCl_2$) and randomly distributed into equal groups of 50–20 embryos exposed to test substances starting at 6 h post fertilization (hpf). Depending on the size of clutches 2–4 groups could be tested in one experiment. Embryos were dechorionated with 30 $\mu g \cdot mL^{-1}$ protease from *Streptomyces griseus* at 26 hpf and cardiac function was analysed at 30 hpf by visual examination of the embryonic hearts under a fluorescence stereomicroscope (Euromex DZ5040). Only groups with homogeneous, synchronized development of all embryos were analysed. All animal procedures had local approval and conformed to the guidelines from Directive 2010/63/EU on the protection of animals used for scientific purposes.

2.9 | Data and Statistical analysis

Data and statistical analysis complied with the recommendations of the *British Journal of Pharmacology* on experimental design and analysis in pharmacology (Curtis et al., 2018). Because effects and effective concentrations of compounds were not predictable and, in an effort, to reduce animal use, a sample size estimation was not performed and experiments were evaluated after each single experiment. Data are presented as mean ± SEM without outlier removal. *N* values represent biological replicates. Normality of data was determined by Shapiro–Wilk test. Tests for statistical significance were conducted as indicated and were ANOVA for comparison of groups with normal distribution of data and Kruskal–Wallis test with Dunn's post hoc test for multigroup comparison. ($^*P < 0.05$).

2.10 | Materials

Test substances ezetimibe and disulfiram were purchased from Molekula (Germany). All other laboratory chemicals were purchased from Sigma-Aldrich (Germany) or Carl Roth GmbH (Germany) unless noted otherwise in the methods section. Laboratory devices are specified in the respective methods sections.

2.11 | Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in the IUPHAR/BPS Guide to PHARMACOLOGY http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Harding et al., 2018).

3 | RESULTS

3.1 | Screening for novel mitochondrial Ca^{2+} uptake enhancers

Since pharmacological enhancement of mitochondrial-Ca^{2+} uptake can prevent arrhythmogenesis in cardiomyocytes (Schweitzer et al., 2017; Shimizu et al., 2015), but agonists of mitochondrial-Ca^{2+} uptake are still scarce and experimental, we set out screen for novel, potent MiCUps for preclinical and clinical testing. As a first step, we took advantage of a previously established and validated mitochondrial-Ca^{2+} uptake assay. In a previous study, we have developed a permeabilized HeLa cell-based screening approach for the identification of small molecule inhibitors of mt-Ca^{2+} uptake and have successfully identified specific mitochondrial Ca^{2+} uniporter blockers (Arduino et al., 2017). Here, we applied this screening approach for the identification of enhancers and screened the NCC consisting of 727 compounds with a history of use in human clinical trials. Drug effects on luminescence-based mitochondrial-Ca^{2+} dynamics were quantified by scoring each drug at 10 μM (S_{drug}) based on its effect on the aequorin-luminescence peak when compared to vehicle (DMSO) and experimental (Ru360) controls (Figure 1a). Compounds were then ranked by their score and the highest scores were selected as MiCUps (Figure 1b, Supporting Information). The top four hits were further investigated: those included the bioactive biphenolic phytochemical honokiol, the antimicrobial cephalosporine cefatrizine, the cholesterol uptake inhibitor ezetimibe and disulfiram, a drug used to treat chronic alcoholism. Their effect was further validated by analysis of the dose dependency of the mitochondrial-Ca^{2+} uptake over a wider range of concentrations (Figure 1c). This revealed dose-dependent enhancement for honokiol, ezetimibe and disulfiram starting at 3–6 μM but not for cefatrizine, which was thus considered a false positive hit from the primary screen. We next confirmed that hits from our screen are indeed enhancers of mitochondrial Ca^{2+} uptake rather than blockers of mitochondrial Ca^{2+} extrusion, which would likewise lead to enhanced mitochondrial Ca^{2+} accumulation. Therefore, we measured mitochondrial Na^+/Ca^{2+} exchanger-mediated mitochondrial Ca^{2+} extrusion in permeabilized HeLa cells: unlike the established **NCLX** blocker **CGP-37157**, neither of our three candidate drugs affected mitochondrial Na^+/Ca^{2+} exchanger activity (Figure 2).

3.2 | Ezetimibe and disulfiram enhance SR-mitochondria Ca^{2+} transfer in cardiomyocytes

Significant differences were described for mitochondrial Ca^{2+} uptake between non-excitable cells like HeLa cells and cardiomyocytes (Chen et al., 2011; Mammucari et al., 2018). In particular, in cardiomyocytes a specialized mechanism of SR-mitochondria Ca^{2+} transfer was recently described involving a tight coupling between the ryanodine receptor 2 (**RyR2**), VDAC2 and mitochondrial Ca^{2+} uniporter to allow for rapid transfer of Ca^{2+} from the SR into mitochondria (De la Fuente et al., 2016; Min et al., 2012). Because this direct SR-mitochondria Ca^{2+} transfer was proposed to be the

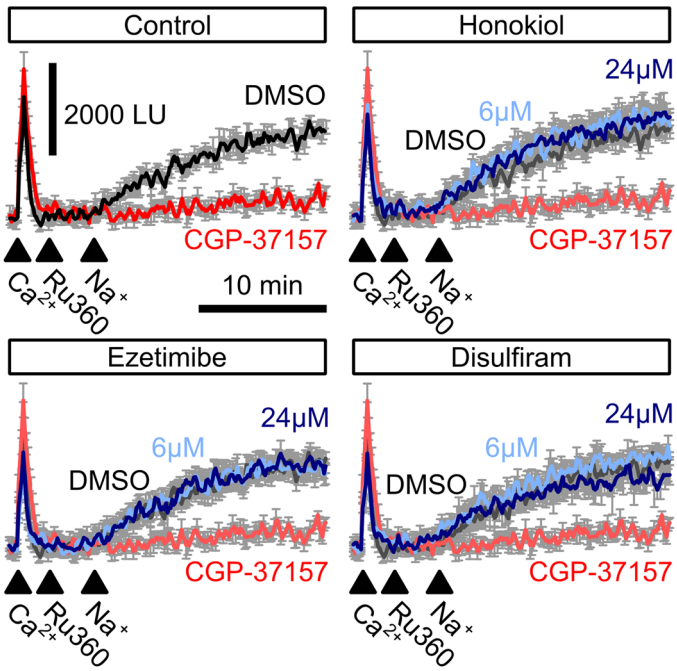

FIGURE 2 Mitochondrial Na^+/Ca^{2+} exchanger-mediated mitochondrial Ca^{2+} extrusion. Free Ca^{2+} in the supernatant of permeabilized HeLa cells was recorded using Calcium Green-5N before and after injection of 100-μM Ca^{2+} (first arrowhead) followed by a block of mitochondrial Ca^{2+} uniporter using Ru360 (second arrowhead). Addition of 60-mM Na^+ (third arrowhead) induced mitochondrial Na^+/Ca^{2+} exchanger-mediated Ca^{2+} extrusion (control, black trace) which could be blocked by the mitochondrial Na^+/Ca^{2+} exchanger blocker CGP-37157 (control, red trace). Neither ezetimibe, nor disulfiram or honokiol affected NCLX activity ($n = 4$)

molecular prerequisite for the anti-arrhythmic effect of MiCUps (Schweitzer et al., 2017; Shimizu et al., 2015; Wilting et al., 2020), we tested the potential of the newly identified MiCUps ezetimibe, disulfiram and honokiol to specifically enhance SR-mitochondria Ca^{2+} transfer in cardiomyocytes. To this aim, we used a plate-reader-based assay on permeabilized HL-1 cardiomyocytes. In contrast to the HeLa-based assay, in this assay Ca^{2+} mobilization is triggered by the addition of caffeine in the presence of the fast chelator BAPTA to limit cytosolic Ca^{2+} diffusion to the low micrometre range. This assay allows for standardized comparison of multiple conditions (Schweitzer et al., 2017; Wilting et al., 2020) to generate dose–response relationships and was previously successfully used to investigate the role of VDAC2 in promoting SR-mitochondria Ca^{2+} transfer (Min et al., 2012; Wilting et al., 2020). Rapid mitochondrial uptake of Ca^{2+} released from the SR by a **caffeine** pulse was significantly enhanced by ezetimibe and disulfiram, while no significant effect of honokiol could be observed (Figure 3a,b). Strikingly, analysis of dose–response curves revealed that the two active substances, ezetimibe and disulfiram, enhanced mitochondrial-Ca^{2+} uptake at markedly lower concentrations compared to the established MiCUps, the mitochondrial Ca^{2+} uniporter activator kaempferol (Montero et al., 2004) and the VDAC2 modifier efsevin (Wilting et al., 2020). To exclude a putative false-positive result due to a potential effect of ezetimibe and/or disulfiram on SR Ca^{2+} release, we next measured

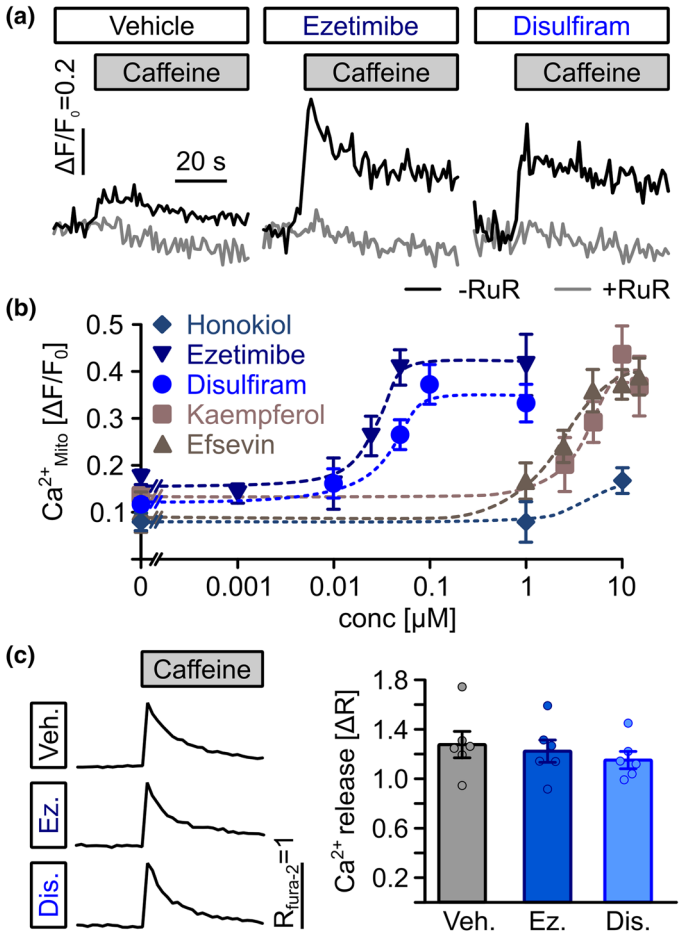

FIGURE 3 Direct transfer of Ca^{2+} from the sarcoplasmic reticulum into mitochondria in cardiomyocytes. (a) Representative recordings of mitochondrial (mt)-Ca^{2+} uptake (black line) in permeabilized HL-1 cardiomyocytes. Superfusion with 10-mM caffeine induced uptake of Ca^{2+} released from the SR into mitochondria, which was enhanced by 1-μM ezetimibe and 1-μM disulfiram. Ruthenium red as a blocker of RyR, VDAC and mitochondrial Ca^{2+} uniporter was used to block SR-mitochondria Ca^{2+} transfer as a negative control. (b) Ezetimibe and disulfiram enhanced SR-mitochondria (Mito) Ca^{2+} transfer dose dependently from 0.17 ± 0.01 ($n = 26$ biological replicates from nine experiments) in vehicle-treated control cells to maximum values of $\Delta F/F_0 = 0.41 \pm 0.04$ at 50 nM for ezetimibe ($n = 19$ replicates from seven experiments) and from 0.15 ± 0.01 ($n = 20$ replicates from seven experiments) to 0.37 ± 0.04 at 100 nM for disulfiram ($n = 9$ replicates from nine individual experiments) and at significantly lower concentrations then the established MiCUps kaempferol and efsevin (ezetimibe: $EC_{50} = 25.5$ nM, disulfiram: $EC_{50} = 36.8$ nM, kaempferol: $EC_{50} = 3.7$ μM, efsevin: $EC_{50} = 2.9$ μM), while no significant effect of honokiol could be observed. (c) Ezetimibe (Ez) and disulfiram (Dis) did not alter SR Ca^{2+} release as assessed by fura-2 fluorescence in intact HL-1 cardiomyocytes after addition of 10-mM caffeine. The baseline fura-2 fluorescence ratio ($R_{340nm/380nm}$) was 0.75 ± 0.03 for vehicle-treated cells, 0.72 ± 0.03 for cells treated with 1-μM ezetimibe and 0.79 ± 0.03 for cells treated with 1-μM disulfiram. Release of Ca^{2+} from the SR induced a change in fluorescence (ΔR) of 1.07 ± 0.09 for vehicle-treated cells, 1.02 ± 0.08 for cells treated with 1-μM ezetimibe and 0.96 ± 0.06 for cells treated with 1-μM disulfiram ($n = 34$ replicates from six experiments, ANOVA)

cytosolic Ca^{2+} levels in intact ezetimibe- and disulfiram-treated HL-1 cardiomyocytes using fura-2 and analysed basal Ca^{2+} levels and Ca^{2+} release after superfusion with 10-mM caffeine (Figure 3c). We observed no significant change for either baseline fura-2 fluorescence ratio ($R_{340nm/380nm}$) or release of Ca^{2+} from the SR (ΔR) (Figure 3c). In conclusion, the above data indicates that both, ezetimibe and disulfiram, specifically enhance mitochondrial-Ca^{2+} uptake in HL-1 cardiomyocytes without affecting baseline cytosolic Ca^{2+} levels or SR Ca^{2+} release and have significantly lower EC_{50} values then the established MiCUps kaempferol and efsevin.

3.3 | Ezetimibe and disulfiram suppress arrhythmia in a zebrafish model of Ca^{2+}-overload induced arrhythmia

Consequently, we set out to evaluate the potency of the newly identified MiCUps identified in our screening approach to suppress arrhythmia in translational models. Prior to application of MiCUps to these models, we confirmed that both substances, ezetimibe and disulfiram, effectively enhance mitochondrial Ca^{2+} uptake also in intact HeLa cells at concentrations comparable to those effective in permeabilized cells (Figure S1). While we found enhanced mitochondrial-Ca^{2+}

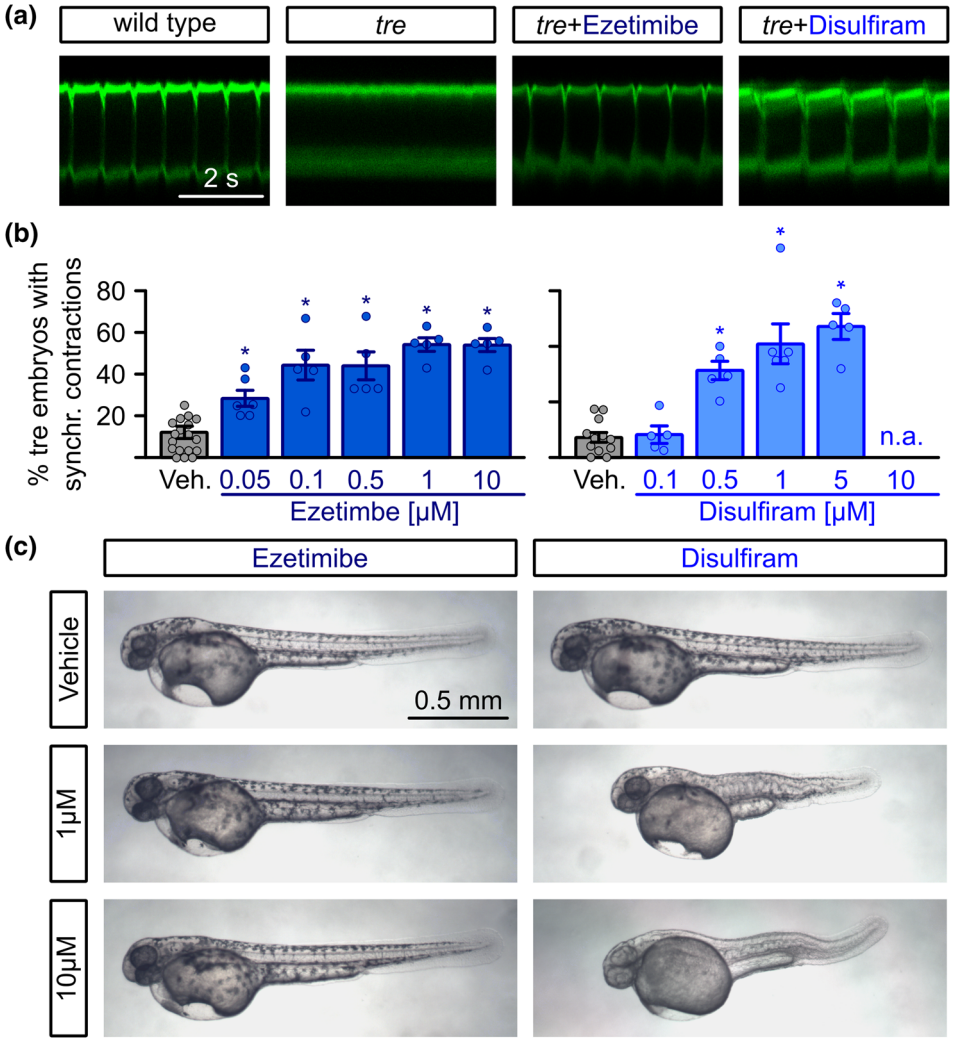

FIGURE 4 Restoration of rhythmic cardiac contractions in the zebrafish arrhythmia model *tremblor*. (a) Representative confocal linescan recordings through atria of beating GFP-labelled hearts from living zebrafish embryos (TG (myl7:eGFP)) at 1 day after fertilization. Rhythmic cardiac contractions can be observed in wild-type but not *tremblor* (*tre*) embryos. Treatment of *tre* embryos with 1 µM ezetimibe of 1 µM disulfiram restored rhythmic cardiac contractions. (b) Quantification of the percentage of *tre* embryos with synchronized contractions revealed a rescue of the *tre* phenotype from 11.1 ± 2.04% embryos with synchronized contractions in the vehicle control ($n = 1289$ embryos in 15 individual experiments) to 28.39 ± 3.9% of *tre* embryos at 0.05 µM ($n = 155$ embryos in six individual experiments), 44.07 ± 7.21% ($n = 448$ embryos in five individual experiments) after addition of 0.1 µM and a maximum of 53.85 ± 3.38% at 1 µM ezetimibe ($n = 354$ embryos in five individual experiments). While 0.1 µM disulfiram did not induce a significant effect ($n = 507$ embryos in five individual experiments), 1 µM disulfiram enhanced the percentage of embryos with synchronized contractions from 9.66 ± 2.31% ($n = 1133$ embryos from 12 individual experiments) to 54.27 ± 9.48% at 1 µM ($n = 371$ embryos from five individual experiments) and to 62.83 ± 5.6% at 5 µM ($n = 418$ embryos from five individual experiments) (Kruskal–Wallis test). (c) Treatment of zebrafish embryos with ezetimibe did not alter gross morphology of embryos, while disulfiram induced severe malformations of the body and a lack of pigmentation

uptake for both substances at 3–6 μM, higher concentrations of disulfiram showed a decrease in mitochondrial-Ca^{2+} uptake indicating cellular toxicity, while higher concentrations of ezetimibe further enhanced mitochondrial-Ca^{2+} uptake. We then took advantage of a previously established *in vivo* arrhythmia model in which we recently demonstrated that zebrafish embryos of the transgenic line *tremblor* (*tre*^{tc318}), lacking a cardiac isoform of the Na^+/Ca^{2+} exchanger, display Ca^{2+} overload-induced cardiac arrhythmia (Langenbacher et al., 2005; Shimizu et al., 2015). In contrast to wild-type embryos, which consistently show rhythmic cardiac contractions at day 1 of development. Homozygous *tre* embryos display a hypercontracted heart that shows only chaotic contractions within the myocardium (Langenbacher et al., 2005). This phenotype can be rescued by treatment with efsevin (Shimizu et al., 2015). We therefore tested the newly identified MiCUps ezetimibe and disulfiram for their potential to restore rhythmic cardiac contractions in this model (Figure 4a). Addition of 0.05-μM ezetimibe restored rhythmic cardiac contractions in roughly one quarter of *tre* embryos and approximately 60% of *tre* embryos at concentrations above 1 μM (Figure 4b), while only a tenth of control embryos treated with vehicle showed synchronized contractions. In agreement with the dose–response curves in HL-1 cardiomyocytes slightly higher concentrations of disulfiram were needed to obtain a similar phenotype rescue in *tre* embryos, where 0.1 μM disulfiram did not induce a significant effect but again approximately one half to 60% of all *tre* embryos were rescued at concentrations above 0.5 μM. Although not all embryos treated with ezetimibe or disulfiram showed rhythmic contractions, this rescue efficiency is comparable to the rescue efficiency initially described with 10 μM efsevin (Shimizu et al., 2015). Interestingly however, zebrafish embryos treated with disulfiram showed signs of intoxication such as a lack of pigmentation

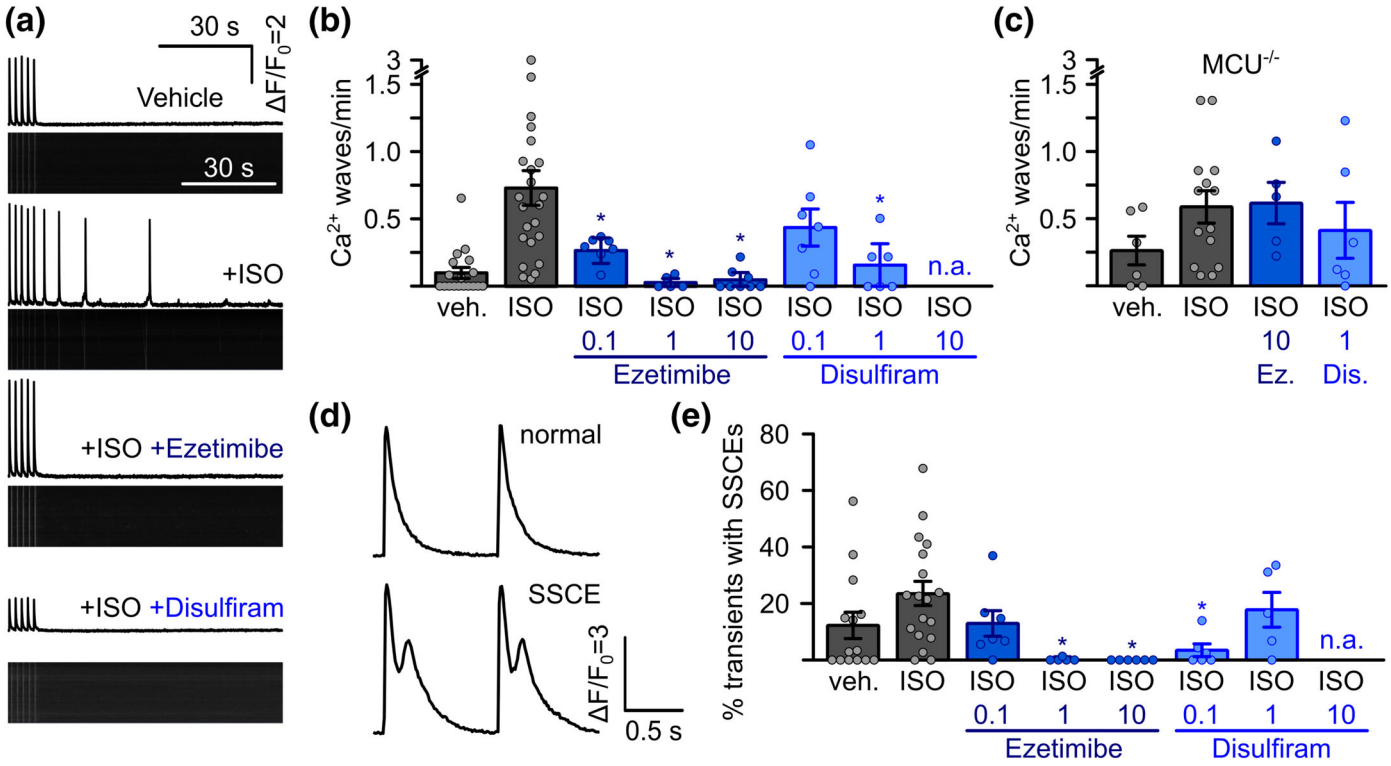

FIGURE 5 Suppression of arrhythmogenesis in freshly isolated murine RyR2$^{C4496R/WT}$ cardiomyocytes by ezetimibe and disulfiram. (a) Representative confocal linescan recordings of intracellular Ca^{2+} in freshly isolated ventricular cardiomyocytes from RyR2$^{C4496R/WT}$ mice. Cells were continuously pulsed at 0.5 Hz and spontaneous Ca^{2+} waves were analysed during 1.5 min after pulsing was stopped. Addition of isoprenaline (ISO) induced spontaneous Ca^{2+} waves which could be blocked by the addition of ezetimibe or disulfiram. (b) Quantitative analysis of the experiments in (a). Addition of ISO raised the propensity for spontaneous Ca^{2+} waves from 0.10 ± 0.04 waves per minute (n = 143 cells from 18 mice) to 0.73 ± 0.13 (n = 222 cells from 24 mice) in RyR2$^{C4496R/WT}$ mice. Addition of MiCups lowered waves to 0.27 ± 0.06 under 0.1 μM ezetimibe (n = 64 cells from seven mice), 0.032 ± 0.02 under 1 μM ezetimibe (n = 35 cells from five mice) and, finally, 0.05 ± 0.03 under 10 μM ezetimibe (n = 80 cells from eight mice) and to 0.44 ± 0.14 under 0.1 (n = 49 cells from seven mice) and 0.15 ± 0.08 under 1 (n = 35 cells from six mice) for disulfiram (Kruskal–Wallis test). (c) Disruption of mitochondrial Ca^{2+} uptake by genetic ablation of mitochondrial Ca^{2+} uniporter (MCU) in *MCU*$^{-/-}$ /RyR2$^{C4496R/WT}$ mice abolished the effect of ezetimibe (Ez) and disulfiram (Dis) indicated by comparable values of 0.61 ± 0.15 (n = 42 cells from five mice, P > 0.05 Kruskal–Wallis-test) for ISO + 10 μM ezetimibe and 0.39 ± 0.20 (n = 47 cells from six mice, P > 0.05, Kruskal–Wallis-test) for ISO + 1 μM disulfiram compared to 0.59 ± 0.12 (n = 107 from 14 mice) under ISO alone. (d) Representative recording from electrically induced systolic Ca^{2+} transients showing normal transients (upper trace) and transients with secondary systolic Ca^{2+} elevations (SSCEs). (e) SSCEs were observed in 12.4 ± 4.7% (n = 121 cells from 14 mice) of all transients in the vehicle control and rose to 23.3 ± 4.4% after addition of ISO (n = 134 cells from 18 mice). Addition of ezetimibe dose-dependently suppressed SSCEs to a minimum of 0.0% under 10 μM ezetimibe (n = 53 cells from six mice). Disulfiram decreased SSCEs to 3.4 ± 2.2% at 0.1 μM (n = 42 cells from six mice), while cells treated with 1 μM showed SSCEs in 17.5 ± 6.5% of all transients (n = 32 cells from five mice, Kruskal–Wallis test). n.a. = not analysable

and a disturbed morphology, which was not observed in ezetimibe-treated embryos (Figure 4c). Taken together, low micromolar concentrations of both drugs can efficiently restore rhythmic cardiac contractions in *tre* embryos but disulfiram appears to have toxic effects at effective concentrations above 1 μM.

3.4 | Ezetimibe and disulfiram suppress arrhythmogenesis in cardiomyocytes of a murine tachycardia model

We next tested both compounds for their anti-arrhythmic potential in freshly isolated cardiomyocytes of a murine model for catecholaminergic polymorphic ventricular tachycardia (CPVT) (Cerrone et al., 2005). Consistent with the phenotype of CPVT, cardiomyocytes from RyR2$^{R4496C/WT}$ mice develop spontaneous Ca^{2+} waves during diastole when stimulated with catecholamines (Figure 5a) (Schweitzer et al., 2017; Sedej et al., 2010). This effect excels RyR2$^{R4496C/WT}$ cardiomyocytes as a valuable model for *ex vivo* testing, since a putative reduction of these waves induced by test compounds can not only be directly compared to the disease state (i.e. after catecholamine stimulation) but also to the pretrigger control in the same set of cells. Ca^{2+} waves originate from the spontaneous release of Ca^{2+} through mutated RyR2s and represent the origin of ectopic cardiac excitations that cause arrhythmia (Allen et al., 1984). Both MiCUps dose-dependently reduced the number of isoprenaline (ISO)-induced Ca^{2+} waves to levels comparable to unstimulated control cells (Figure 5b). Interestingly, and comparable to our zebrafish data, disulfiram displayed toxic effects at higher concentrations (10 μM) reflected by an elevation of basal cytosolic Ca^{2+} levels. Cells treated with 10-μM disulfiram showed very high fluo-4 fluorescence (51.5 ± 4.8FU compared to 15.3 ± 0.5FU under isoprenaline alone) and extensive spontaneous activity, which prevented further analysis (Figure S2).

To confirm that this striking effect in CPVT cardiomyocytes is solely attributable to the enhanced mitochondrial-Ca^{2+} uptake induced by the two MiCUps, we crossbred RyR2^{R4496C} mice with *MCU*$^{-/-}$ mice, which lack the central pore forming subunit of the mitochondrial Ca^{2+} uniporter complex (Pan et al., 2013). In freshly isolated RyR2$^{R4496C/WT}$/*MCU*$^{-/-}$ cardiomyocytes, we again observed an induction of diastolic Ca^{2+} waves upon isoprenaline treatment compared to untreated control cells. Strikingly, application of the highest effective doses, 10 μM ezetimibe and 1 μM disulfiram, failed to reduce Ca^{2+} waves in both cases (Figure 5c), indicating that mitochondrial Ca^{2+} uptake is the effective target of ezetimibe and disulfiram.

Apart from diastolic Ca^{2+} waves, systolic Ca^{2+} activity was suggested as a potential trigger for arrhythmia in CPVT (Němec et al., 2010; Němec et al., 2016). We therefore also analysed systolic Ca^{2+} transients of our recordings for the occurrence of secondary systolic Ca^{2+}-elevations (SSCEs) and found that a significantly higher portion of transients recorded from cells treated with isoprenaline showed secondary systolic Ca^{2+}-elevations then transients recorded

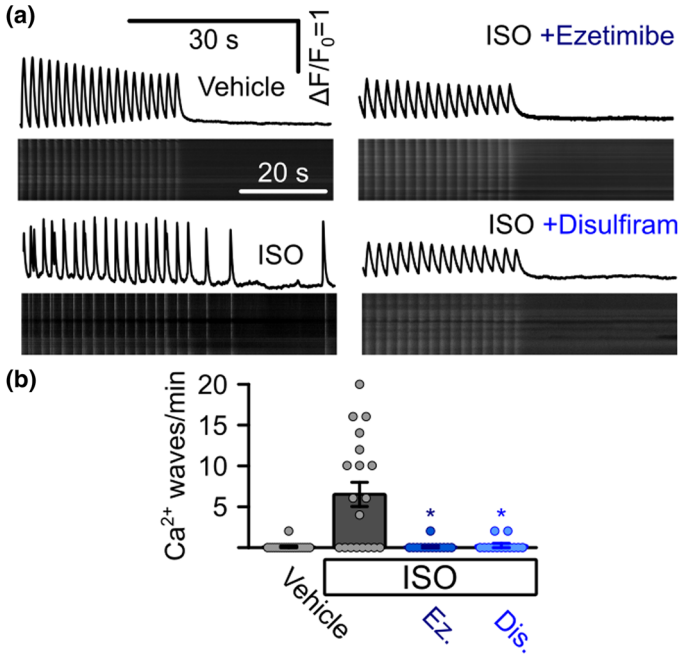

FIGURE 6 Suppression of arrhythmogenic Ca^{2+} signals in iPSC-derived cardiomyocytes from a catecholaminergic polymorphic ventricular tachycardia (CPVT) patient. (a) Representative confocal linescan recordings of intracellular Ca^{2+} in iPSC-derived cardiomyocytes from a CPVT patient. Cells were continuously pulsed at 0.5 Hz and spontaneous Ca^{2+} waves were analysed after pulsing was stopped. Addition of isoprenaline (ISO) induced spontaneous Ca^{2+} waves which could be blocked by the addition of ezetimibe or disulfiram. (b) Quantitative analysis of the experiments in (a). Addition of ISO induced the occurrence of waves in CPVT cells (0 waves per minute for vehicle control, $n = 11$ cells, 5.87 ± 1.80 under ISO, $n = 16$, Kruskal–Wallis test), which could be suppressed by the addition of ezetimibe (Ez) or disulfiram (Dis) (0.10 ± 0.07 waves per minute for ezetimibe, $n = 13$ cells, 0.05 ± 0.05 waves per minute for disulfiram, $n = 13$, Kruskal–Wallis test, data from four independent enzymatic dissociations of 19 individual beating cell clusters)

from vehicle-treated control cells. Strikingly, both, ezetimibe and disulfiram, significantly reduced secondary systolic Ca^{2+}-elevations (Figure 5d,e). A clear dose-dependence was observed for ezetimibe; however, higher concentrations of disulfiram induced higher spontaneous Ca^{2+} activity, which might again be attributed to the enhanced baseline Ca^{2+} observed under disulfiram (Figure S2).

3.5 | Ezetimibe and disulfiram suppress arrhythmogenesis in human iPSC-derived CPVT cardiomyocytes

Finally, we tested both substances for their ability to suppress arrhythmogenic Ca^{2+} waves in human cells to estimate the translatability of our data and the potential of ezetimibe and disulfiram to serve as candidates for a human therapy (Figure 6). To this aim, we used iPSC-derived cardiomyocytes from a CPVT patient. Comparable to murine CPVT cardiomyocytes and in line with the CPVT phenotype, these cells did not display spontaneous diastolic Ca^{2+} waves under unstimulated control conditions while addition of isoprenaline (ISO) induced prominent Ca^{2+} waves (Figure 6a,b). Strikingly, addition of ezetimibe and disulfiram reduced Ca^{2+} waves to control conditions.

4 | DISCUSSION

4.1 | Mitochondrial Ca^{2+} uptake enhancer screen

We have previously demonstrated that the VDAC2 agonist efsevin as well as the mitochondrial Ca^{2+} uniporter agonist kaempferol potently suppress arrhythmia in murine and human models of CPVT (Schweitzer et al., 2017; Shimizu et al., 2015). However, in regard of a clinical application of these substances, it is of note that both enhance SR-mitochondria Ca^{2+} transfer with an EC_{50} of around 5 μM, a concentration that is likely to favour off-target effects. Furthermore, data concerning efsevin's bioavailability, pharmacodynamics, stability and toxicity are largely missing. Though kaempferol was used in clinical studies before, it was only used at a low dose as a nutritional supplement and was shown to bind multiple targets including the NF-κB (Kadioglu et al., 2015), the fibroblast growth factor (Lee et al., 2018) and other signalling pathways (Kim et al., 2015; Wu et al., 2017; Yao et al., 2014). Thus, though our previous findings established the mitochondrial Ca^{2+} uptake pathway as a promising candidate target for future preclinical and clinical development of a human anti-arrhythmic therapy, efsevin and kaempferol represent poor candidates for this purpose and novel substances for further testing are needed. While several studies have already identified effective blockers of the mitochondrial Ca^{2+} uniporter complex (Arduino & Perocchi, 2018; Di Marco et al., 2020; Kon et al., 2017; Nathan et al., 2017; Woods & Wilson, 2020), agonists still remain scarce.

We therefore set up a three-step protocol to identify novel MiCUps with anti-arrhythmic effects: primary screening was performed in a previously validated HeLa-cell based assay that allows screening of large compound libraries in a system that is cost efficient and automatable. Further target validation was performed in HL-1 cells allowing for testing of multiple candidate substances at multiple concentrations on cardiac myocytes. Finally, only candidates passing steps one and two were further evaluated for their anti-arrhythmic potential in translational animal models thereby reducing animal usage.

Using this approach, we screened the NCC consisting of 727 compounds with a history of use in human clinical trials to identify novel specific enhancers of mitochondrial-Ca^{2+} uptake. Three compounds, honokiol, disulfiram and ezetimibe, were selected as hits based on their stimulatory effect on mt-Ca^{2+} uptake without blocking mitochondrial Ca^{2+} extrusion in primary drug screens as well as dose-dependent measurements in permeabilized HeLa cells. We then further tested these compounds for their ability to selectively enhance the transfer of Ca^{2+} from the SR into mitochondria in cardiomyocytes as the mechanism proposed to be the molecular prerequisite for the anti-arrhythmic effect of MiCUps (Schweitzer et al., 2017; Shimizu et al., 2015; Wilting et al., 2020). Interestingly, honokiol, which displayed the most pronounced effects in HeLa cells, was inactive in this system, while ezetimibe and disulfiram consistently enhanced mitochondrial-Ca^{2+} uptake. This might be attributable to differences in the mitochondrial Ca^{2+} uptake pathway between non-excitable and excitable cells and highlights the importance of re-evaluation of hits from our primary screening platform in cardiac cells. After passing both assays, ezetimibe and disulfiram were then further tested for their anti-arrhythmogenic potential in translational models and both were shown to indeed suppress arrhythmogenesis.

Taken together, we successfully identified two novel, potent MiCUps by applying a combination of a HeLa-based chemical screening followed by cardiomyocyte-specific target validation and final testing in arrhythmia models. With this protocol, we identified ezetimibe and disulfiram as novel candidates for the use in further preclinical and eventually clinical tests on the efficacy of MiCUps for the treatment of cardiac arrhythmia, but also as valuable compounds for further basic research on the mitochondrial Ca^{2+} uptake pathway and preclinical research for other indications. In this respect enhancing mitochondrial Ca^{2+} uptake was recently suggested to be beneficial to facilitate cerebral blood flow after traumatic brain injury (Murugan et al., 2016) and to promote metabolism/secretion coupling in type 2 diabetes (Bermont et al., 2020).

4.2 | Cardiac selectivity of novel mitochondrial Ca^{2+} uptake enhancers

In this and previous studies, we successfully applied MiCUps on different models from cell cultures to *in vivo* systems, but never observed gross effects, for example, apoptosis, on other tissues than the heart (Schweitzer et al., 2017; Shimizu et al., 2015). Though these experiments never exceeded treatment times of several days to weeks, these data suggest MiCUps as promising candidates for the treatment of cardiac diseases. It has been proposed that, although most components of the mitochondrial Ca^{2+} uptake pathway are

ubiquitously expressed throughout the organism, tissue selectivity of mitochondrial Ca^{2+} uptake is determined by composition and subcellular localization of individual components and channel subunits. Here, we observed differences in drug effects and effective doses between cardiomyocytes and non-excitable HeLa cells, which might be at least one explanation for their specificity. Honokiol was active in HeLa cells but did not show activity in HL-1 cardiomyocytes. Furthermore, the concentrations of ezetimibe and disulfiram needed to activate mitochondrial-Ca^{2+} uptake significantly vary between the two systems. A possible explanation could be differences in tissue-specific mitochondrial Ca^{2+} uniporter activity (Fieni et al., 2012), mitochondrial Ca^{2+} uptake pathway composition (Patron et al., 2019; Raffaello et al., 2013), or physiological adaption (Lambert et al., 2019). Since the target protein of the identified MiCUps within the mitochondrial Ca^{2+} uptake pathway remains elusive, it is conceivable that the mitochondrial Ca^{2+} uptake pathway in cardiac cells lacks the component required for honokiol binding or that this component is only present at a very low level. Vice versa, it is possible that the specific mitochondrial Ca^{2+} uptake pathway composition that is present in cardiomyocytes is a better target for ezetimibe and disulfiram. Alternatively, different subcellular localizations of mitochondrial Ca^{2+} uptake pathway components in the two cell types might account for the observed differences. By using caffeine as a trigger and BAPTA in the intracellular solution, we specifically analyse local transfer of Ca^{2+} from the SR into mitochondria in HL-1 cardiomyocytes. In contrast, global addition of 4-μM Ca^{2+} was used as a trigger in HeLa cells. Since both channels of the mitochondrial Ca^{2+} uptake pathway, VDAC2 and the mitochondrial Ca^{2+} uniporter preferentially accumulate in areas of the mitochondrion which interact with the SR (De la Fuente et al., 2016, 2018; Min et al., 2012; Sander et al., 2021) and the concentration of Ca^{2+} released from the SR reaches concentrations of up to 1 mM in close vicinity to the Ca^{2+} release sites, this could explain why lower concentrations of ezetimibe and disulfiram are active in cardiomyocytes. Finally, the existence of two different mitochondrial Ca^{2+} uniporter subpopulations with distinct subunit composition and Ca^{2+} sensitivity, one in the SR-mitochondria interface and one outside these junctions could account for the system-dependent activities. Such subpopulations could be established to differentiate between local and global Ca^{2+} signals. In this scenario, honokiol might only target the latter population, while ezetimibe and disulfiram are more selective for the SR-associated population.

The discussed differences in composition and localization of the different mitochondrial Ca^{2+} uptake pathway components could thus explain the observed cardio-selectivity of MiCUps. Therefore, MiCUps could act as specific enhancers of mitochondrial-Ca^{2+} uptake in the heart, while being relatively inert in other tissues. However, future studies are needed to evaluate this hypothesis and to specifically investigate effects of MiCUps on other tissues. It is of note that we used MiCUps for acute treatment and it is thus feasible that changes in mitochondrial-Ca^{2+} uptake have an immediate effect on cardiomyocytes but not in other cell types. Still, even subtle differences in mitochondrial-Ca^{2+} uptake might interfere with cellular Ca^{2+} homeostasis when they persist chronically and might induce long-term effects in other organs. Thus, additional investigations on other cell types and in particular long-term studies are needed. The identified substances from this study might serve as tools to address these questions.

4.3 | Novel mitochondrial Ca^{2+} uptake enhancers for preclinical and clinical testing

We identified two compounds, ezetimibe and disulfiram, which enhance mitochondrial-Ca^{2+} uptake at significantly lower concentrations than efsevin and kaempferol and could thus serve as candidate drugs for preclinical and eventually clinical trials on the efficacy of MiCUps for the treatment of cardiac arrhythmia. In contrast to common anti-arrhythmic drugs, which target ion channels in the cell membrane and thereby modulate the cardiac action potential to suppress propagation of ectopic signals, MiCUps act intracellularly by inhibiting the generation of arrhythmogenic triggers such as early and late afterdepolarizations. Due to this mechanism of action, which does not directly interfere with ionic currents of the systolic cardiac action potential, MiCUps are expected to be less prone to proarrhythmic side effects than commonly used anti-arrhythmic drugs of Vaughan Williams class I and III, which retard and prolong systolic cardiac action potentials. Both, ezetimibe and disulfiram, are FDA and EMA approved and currently in use. Ezetimibe is used as a blocker of the **Niemann-Pick C1-like intracellular cholesterol transporter 1 (NPC1L1/SLC65A2)** (Garcia-Calvo et al., 2005) protein in epithelial cells of the small intestine to reduce uptake of cholesterol. It is used for the prevention and treatment of cardiovascular disease. Although ezetimibe is well tolerated, it is however not first-line therapy, due to a lower efficiency compared to the commonly used statins. Ezetimibe is membrane permeable (Alhayali et al., 2018) and orally bioavailable, and plasma concentrations reach a maximum approximately 2 h after intake. It is enterohepatically metabolized (Kosoglou et al., 2005). However, approximately 80%–90% of ezetimibe is rapidly metabolized into ezetimibe-glucoronide (Kosoglou et al., 2005). Though both forms were shown to be active inhibitors of NPC1L1, we found that ezetimibe glucoronide is less effective to reduce Ca^{2+} waves in $RyR2^{R4496C/WT}$ cardiomyocytes (Figure S3). Thus, although plasma concentrations of approximately 300 nm for both forms were described after a 10 mg per 10 days oral intake (Ezzet et al., 2001), which would be well in the effective range in our studies, further experiments in preclinical models need to evaluate effective *in vivo* doses.

Disulfiram is an inhibitor of acetaldehyde dehydrogenase and is used for the treatment of alcohol abuse, but serious side effects limit the use of the drug. Also in our experiments, disulfiram induced severe malformations in zebrafish and showed signs of cellular toxicity in intact HeLa cells and cardiomyocytes at higher concentrations. It is of note however that disulfiram is administered to zebrafish embryos at a very sensitive step of development while it is envisioned to serve as an anti-arrhythmic drug for adult subjects. Furthermore, the disulfiram concentration needed for efficient rescue in zebrafish was significantly higher compared to concentrations applied in cellular models which

might be explained by differences in the differentiation state of cardiomyocytes in the distinct models or a limited uptake into zebrafish embryos through the embryonic skin. However, also in cardiomyocytes, we observed enhanced Ca^{2+} activity during diastole upon treatment with higher doses disulfiram. This might at least in part be explained by a direct destabilizing effect of disulfiram on the RyR2 during diastole, which would be in agreement with a recently identified modulatory effect of disulfiram on the skeletal muscle isoform RyR1 (Rebbeck et al., 2017). Furthermore, disulfiram was shown to influence the permeability of the inner mitochondrial membrane and to release Ca^{2+} from mitochondria at higher concentrations (Balakirev & Zimmer, 2001; Chávez et al., 1989) which is in line with our results from intact cells. Although these results speak against the use of disulfiram as an antiarrhythmic agent in clinical use, on the other hand and consistent with our findings, previous preclinical studies already demonstrated an antiarrhythmic potential of disulfiram in different animal models (Fossa et al., 1982; Fossa & Carlson, 1983). These effects might now be explained by activation of mitochondrial-Ca^{2+} uptake. It is of note however that these reports also observed a negative inotropic effect of disulfiram at higher concentrations which might be explained by the unspecific binding to RyR2 or other unspecific effects as the ones outlined above. Taken together, further investigations on drug efficacy and safety are definitely needed for the use of disulfiram.

Summarizing our results, we have identified two novel mitochondrial Ca^{2+} uptake enhancers that are already in clinical use. However, before clinical tests for the treatment of human arrhythmia can be performed with these substances, follow-up studies comprising *in vivo* studies with different dosing and administration regimes are needed to determine effective doses and time points of administration to evaluate the potential of MiCUps to be used as preventive therapy to reduce the risk for arrhythmias or to stop an acute episode of arrhythmia, respectively.

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AUTHOR CONTRIBUTIONS

P.S., F.P. and J.S. conceptualized the study. P.S., M.F., M.K.S., F.W., S.M.G., D.M.A., S.F. and L.D., performed experiments. P.S., M.F., M. K.S., F.W., S.M.G., D.M.A., S.F., F.P. and J.S. analysed data. A.M., T. G., F.P. and J.S. provided funding and infrastructure. P.S. and J.S. wrote the manuscript. All authors commented on the manuscript.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

DECLARATION OF TRANSPARENCY AND SCIENTIFIC RIGOUR

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research as stated in the *British Pharmacological Journal* guidelines for Design and Analysis and Animal Experimentation, and as recommended by funding agencies, publishers and other organizations engaged with supporting research.

DATA AVAILABILITY STATEMENT

The data underlying this article are available in the article and in its supporting information. Raw data are available upon request from the corresponding author.

ETHICS APPROVAL STATEMENT

All human and animal studies have been approved by the appropriate ethics committee and have therefore been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments.

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