ent-Verticilide B1 inhibits type 2 ryanodine receptor channels and is antiarrrhythmic in Casq2<sup>−/−</sup> mice

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Manuscript information:

Running title: Antiarrhythmic efficacy of an 18-membered cyclic depsipeptide.

Text pages: 19
Tables: 1
Number of figures: 5
Number of references: 26
Number of words in Abstract: 197
Number of words in Introduction: 537
Number of words in Discussion: 707

Abbreviations:

RyR2 – cardiac ryanodine receptor
Casq2 – cardiac calsequestrin
CPVT – catecholaminergic ventricular tachycardia
PVC – premature ventricular complex
SR – sarcoplasmic reticulum
VT – ventricular tachycardia
Abstract

Intracellular Ca\(^{2+}\) leak from cardiac ryanodine receptor (RyR2) is an established mechanism of sudden cardiac death (SCD), whereby dysregulated Ca\(^{2+}\) handling causes ventricular arrhythmias. We previously discovered the RyR2-selective inhibitor ent-(+)-verticilide (ent-1), a 24-membered cyclooligomeric depsipeptide that is the enantiomeric form of a natural product (nat-(−)-verticilide). Here, we examined its 18-membered ring-size oligomer (ent-verticilide B1; “ent-B1”) in RyR2 single channel and \[^{3}H\]ryanodine binding assays, and in Casq2\(^{-/-}\) cardiomyocytes and mice, a gene-targeted model of SCD. ent-B1 inhibited RyR2 single channels and RyR2-mediated spontaneous Ca\(^{2+}\) release in Casq2\(^{-/-}\) cardiomyocytes with sub-micromolar potency. ent-B1 was a partial RyR2 inhibitor, with maximal inhibitory efficacy of less than 50%. ent-B1 was stable in plasma, with a peak plasma concentration of 1460 ng/ml at 10 min and half-life of 45 min after intraperitoneal administration of 3 mg/kg in mice. *In vivo*, ent-B1 significantly reduced catecholamine-induced ventricular arrhythmias in Casq2\(^{-/-}\) mice in a dose-dependent manner. Hence, we have identified a novel chemical entity – ent-B1 – that preserves the mechanism of action of a hit compound and shows therapeutic efficacy. These findings strengthen RyR2 as an antiarrhythmic drug target and highlight the potential of investigating the mirror-image isomers of natural products to discover new therapeutics.
Significance statement

The cardiac ryanodine receptor (RyR2) is an untapped target in the stagnant field of antiarrhythmic drug development. We have confirmed RyR2 as an antiarrhythmic target in a mouse model of sudden cardiac death and shown the therapeutic efficacy of a second enantiomeric natural product.
Introduction

Patients with heart rhythm disorders have a major, unmet need for new treatments. Among all causes of mortality in the United States, sudden cardiac death (SCD) – a result of ventricular arrhythmias – is responsible for 15% of all deaths annually (Zheng et al., 2001). With the exception of beta-adrenergic receptor inhibitors, none of the agents marketed as antiarrhythmic drugs in the U.S. prevent SCD, with most of them increasing rates of mortality in patients with ischemic heart disease or heart failure (Cardiac Arrhythmia Suppression Trial, 1989; Kober et al., 2008; Waldo et al., 1996). The common mechanism of action shared by FDA-approved antiarrhythmic drugs is the modulation of ion channels or G-protein coupled receptors expressed in the cell membrane (Brunton and Knollmann, 2023). As such, better antiarrhythmic drugs are needed. Here we build on our previous work (Batiste et al., 2019; Kryshtal et al., 2021) to help establish a new class of antiarrhythmic drugs by further validating the intracellular target of cardiac ryanodine receptors (RyR2) for antiarrhythmic drug development.

RyR2s are Ca$^{2+}$ release channels located in the membrane of the sarcoplasmic reticulum (SR) (Terentyev et al., 2008). The mechanism of Ca$^{2+}$-induced Ca$^{2+}$ release to facilitate excitation-contraction (EC) coupling in cardiomyocytes is well-studied, with RyR2 serving as the release channel for SR Ca$^{2+}$ stores (Bers, 2002). Pathologic Ca$^{2+}$ release from RyR2 has been reported in both genetic and acquired arrhythmia disorders through gain of function mutations (Watanabe and Knollmann, 2011) or post-translational modifications to RyR2 (Marx et al., 2000; Respress et al., 2012; Terentyev et al., 2008) respectively. In either scenario, an increased open probability of RyR2
causes Ca$^{2+}$ to “leak” from the SR, which disrupts both the temporal and functional integrity of cardiac Ca$^{2+}$ handling. Specifically, increased \([\text{Ca}^{2+}]_{\text{cytosolic}}\) is pumped extracellularly through the electrogenic Na$^+/\text{Ca}^{2+}$ exchanger, leading to delayed afterdepolarizations driven by the untimely influx of Na$^+$ as Ca$^{2+}$ homeostasis is restored (Knollmann and Roden, 2008).

To address the need for a first-in-class RyR2-selective drug, we discovered a bioactive enantiomer of the fungal natural product verticilide (nat-(−)-verticilide) (Shiomi et al., 2010). This hit compound, called ent-(+)-verticilide (ent-1), is a cyclooligomeric depsipeptide (COD) that selectively inhibits RyR2 \textit{in vitro} (Batiste et al., 2019). Unlike nat-(−)-verticilide, ent-1 therapeutically inhibited RyR2 Ca$^{2+}$ leak in mice with catecholaminergic polymorphic ventricular tachycardia (CPVT), a genetic disease that can present as exercise- or stress-induced SCD in childhood (Leenhardt et al., 1995). While ent-verticilide has become a useful tool compound in exploring the potential of RyR2-targeted therapy in animal models of arrhythmia (Kim et al., 2023), a follow-up structure-activity study revealed that the oligomeric structure of ent-1 can be modified to an alternate ring-size analog that maintains an affinity for RyR2 (Smith et al., 2021). The 18-membered ring analog ent-verticilide B1 (ent-B1) (MW = 639.8 g/mol; ent-1 MW = 853.1 g/mol) retained RyR2 inhibitor activity in a Ca$^{2+}$ spark assay using permeabilized cardiomyocytes, an accepted measure of isolated RyR2 Ca$^{2+}$ release (Smith et al., 2021). ent-B1 is also the mirror image of a natural product, verticilide B1, that is not active against RyR2 or insect RyR (Ohshiro et al., 2012). Here, we studied the \textit{in vitro} pharmacology of ent-B1 and explored the potential of ent-B1 as a novel antiarrhythmic.
compound through *in vivo* experiments in an established CPVT mouse model (*Casq2*−/− mice).

**Material and Methods**

*Drugs, chemicals, and reagents* – All chemicals and reagents were purchased from Sigma-Aldrich unless otherwise stated. We tested one novel molecular entity, *ent*-B1, that is an 18-membered ring size analogue to the 24-membered cyclic depsipeptide *ent*-1 reported previously (Batiste et al., 2019); *ent*-B1 used here was synthesized in the Johnston laboratory using the methods for synthesis and identification as reported in (Smith et al., 2021).

*Single Channel Recording* – SR vesicles containing RyR2 were isolated from porcine hearts and incorporated in artificial bilayer membranes as previously described (Laver et al., 1995). Lipid bilayers were formed across an aperture with diameter 150-250 mm of a delrin cup using a lipid mixture of phosphatidylethanolamine, phosphatidylserine, and phosphatidylcholine (5:3:2 wt/wt, Avanti Polar Lipids, Alabaster, AL) in n-decane (50 mg/ml, ICN Biomedicals, Irvine, CA). During the SR vesicle fusion period, the cis (cytoplasmic) chamber contained 250 mM Cs⁺ (230 mM Cs(CH₃)₃O₃S, 20 mM CsCl) + 1.0 mM CaCl₂ and the trans (luminal) chamber contained 50 mM Cs⁺ (30 mM Cs(CH₃)₃O₃S, 20 mM CsCl) + 1 mM CaCl₂. When ion channels were detected in the bilayer, the trans Cs⁺ was raised to 250 mM by aliquot addition of 4 M Cs(CH₃)₃O₃S. During experiments, the cis solution was exchanged by a perfusion system (O’Neill et al., 2003) to one containing 250 mM Cs⁺ plus 2 mM ATP and free Ca²⁺ of 100 nM followed by exchange
with the same plus ent-B1. Thus, the perfusion system allowed repeated application and washout of ent-B1 within ~3 s.

All solutions were pH buffered using 10 mM TES (N-tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid; ICN Biomedicals) and titrated to pH 7.4 using CsOH (ICN Biomedicals). Free Ca$^{2+}$ of 100 nM was generated from 1 mM CaCl$_2$ and 4.5 mM BAPTA (1,2-bis(o-aminophenoxy)ethane-N,N',N''-tetraacetic acid; obtained from Invitrogen) and this was validated using a Ca$^{2+}$ electrode (Radiometer, Brea, CA). ATP was in the form of the di-sodium salt and obtained from Enzo Life Sciences (Farmingdale, NY) and Cs$^+$ salts were obtained from Sigma-Aldrich (St Louis, MO). CaCl$_2$ was obtained from BDH Chemicals (VWR, Radnor, PA). Cytoplasmic recording solutions were buffered to a redox potential of -232 mV with glutathione disulfide (GSSG; 0.2 mM) and glutathione (GSH; 4 mM; MP Biomedicals), and luminal solutions were buffered to a redox potential of -180 mV with GSSG (3 mM) and GSH (2 mM), both obtained from MP Biomedicals. ent-B1 was prepared as a stock solution in DMSO.

**Single Channel Recording Analysis and Data Acquisition** – Experiments were carried out at room temperature (23 ± 2 °C). Electric potentials are expressed using standard physiological convention (i.e. cytoplasm relative to SR lumen at virtual ground). Control of the bilayer potential and recording of unitary currents was done using an Axopatch 200B amplifier (Axon Instruments/Molecular Devices, Sunnyvale, CA). Channel currents were digitized at 50 kHz and low pass filtered at 5 kHz. Before analysis the current signal was redigitized at 5 kHz and low pass-filtered at 1 kHz. Individual readings of open probability were derived from 30-60 s of RyR2 recording. Single channel open probability was measured using a threshold discriminator at 50% of channel amplitude.
[^3]H]ryanodine ligand binding assay – [^3]H]ryanodine, [9,21-3H(N)] (56 Ci/mmol) was obtained from PerkinElmer. Porcine cardiac SR vesicles were isolated as previously described (Fruen et al., 2000) and incubated with, 200 nM CaM binding peptide, 0.1 µM CaCl2, 20 mM PIPES, 150 mM KCl, 5mM GSH, 0.1 mg/mL BSA, 1 µg/mL aprotinin, 1µg/mL leupeptin, and 1 µM DTT for 30 min at 37 °C. Samples were centrifuged at 110,000 x g for 25 min at 4 °C and resuspended to at final concentration of 15 mg/mL in 20 mM PIPES, 150 mM KCl, 5mM GSH, 0.1 mg/mL BSA, 1 µg/mL aprotinin, 1µg/mL leupeptin, and 1 µM DTT. In 96-well plates, cardiac SR membranes (CSR, 3 mg/mL) were pre-incubated with 1% v/v ent-B1 (to yield the indicated drug concentrations) for 30 min, at 22 °C, in a solution containing 150 mM KCl, 5 mM GSH, 1 µg/mL Aprotinin/Leupeptin, 1 mM EGTA, and 23 µM or 1.62 mM CaCl2 (100 nM or 30 µM free Ca^{2+}, respectively as determined by MaxChelator), 0.1 mg/mL BSA, and 20 mM K-PIPES (pH 7.0). Non-specific [^3]H]ryanodine binding to SR was assessed by addition of 15 µM non-radioactive ryanodine. Maximal [^3]H]ryanodine binding was assessed by addition of 5 mM adenylyl-imidodiphosphate (AMP-PNP), supplemented with 20 mM caffeine. These control samples were each loaded over four wells per plate. Binding of [^3]H]ryanodine (7.5 nM) was determined following a 3 h incubation at 37 °C and filtration through grade GF/B glass microfiber filters (Brandel Inc., Gaithersburg, MD, US) using a M96T-Brandel Harvester. Filters were immersed in 4 mL of Ecolite scintillation cocktail and incubated 24 hours prior to [^3]H] counting in a Perkin-Elmer Tri-Carb 4810.

Intracellular Ca^{2+} measurements in intact cardiomyocytes – Ventricular cardiomyocytes were isolated from two male 10-week-old and one female 13-week-old Casq2^{−/−} mouse as described previously (Knollmann et al., 2003). Cardiomyocytes were pre-incubated
for 2 hours with DMSO or ent-B1. Myocytes were then loaded with Fura-2 acetoxymethyl ester (Fura-2 AM; Invitrogen) as described previously (Batiste et al., 2019). Briefly, isolated single ventricular myocytes were incubated with 2 μM Fura-2 AM for 7 minutes to load the indicator in the cytosol. Myocytes were then washed twice for 10 minutes with normal Tyrode (NT) solution containing 250 μM probenecid (all solutions also contained either vehicle [DMSO] or ent-B1). The composition of NT used for Fura-2 loading and washing was (in mM): 134 NaCl, 5.4 KCl, 1.2 CaCl2, 1 MgCl2, 10 glucose, and 10 HEPES, pH adjusted to 7.4 with NaOH. After Fura-2 loading, all experiments were conducted in NT solution containing 1 μM isoproterenol and 2 mM CaCl2. Fura2-loaded myocytes were electrically paced at 3 Hz field stimulation and Ca²⁺ transients were recorded for 20 seconds followed by no electrical stimulation for 40 seconds to record spontaneous Ca²⁺ release events. After that, myocytes were perfused with 10 mM caffeine in NT solution for 5 seconds to estimate total SR Ca²⁺ content. Fura-2 was measured using a dual-beam excitation fluorescence photometry setup (IonOptix Corp.) and analyzed using commercially available data analysis software (IonWizard, IonOptix, Milton, MA). All experiments were conducted at room temperature.

Action potential measurements in intact cardiomyocytes – Membrane potential was recorded in current-clamp mode with ruptured patch in isolated ventricular myocytes from one male 13-week-old and one female 11-week-old Casq2⁺/⁺ mouse. To match the drug incubation time of the intracellular Ca²⁺ measurements, myocytes were pre-incubated in vehicle (DMSO) or 0.3 μM ent-B1 for 2.5 hrs before AP measurement. Action potential (APs) were measured using pipette solutions containing (in mmol/L)
120 K-aspartate, 20 KCl, 5 NaCl, 5 MgATP, 0.1 EGTA and 10 HEPES adjusted to pH 7.2 with KOH. Whole-cell patch was established in perfused Tyrode solution containing (in mmol/L) 134 NaCl, 5.4 KCl, 1 MgCl₂, 2 CaCl₂, 10 glucose and 10 HEPES and pH adjusted to 7.4 with NaOH. All Tyrode solution contained DMSO or 0.3 µM ent-B1. APs were triggered by application of a 2 ms current injection at 20-30% above threshold with 1 Hz and obtained using Multiclamp 700B, Digidata 1550B. APDs were analyzed using pClamp 10.6 software (Axon Instruments, CA, USA). All experiments were conducted at room temperature.

In vivo pharmacokinetic study – The pharmacokinetic study was carried out by a contract research organization, Pharmaron, Inc. Mice were housed with free access to food and water. All protocols and procedures were compliant with Animal care and Use Application approved by the Institution Animal Care and Use Committee of Pharmaron, Inc., (protocol # PH-DMPK-VUMC-22-003) following the guidance of the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC). Based on our previous study with ent-1 (Blackwell et al., 2023), an ent-B1 dose of 3 mg/kg (drug/body weight) was used. Three male CD1 mice, age 6-8 weeks, were selected for the study. Mice were injected intraperitoneally with ent-B1 dissolved in solution containing 10% Tween 20, 10% DMSO, 40% water, and 40% PEG-400 (v/v, 5 mL/kg), with a final concentration of 0.6 mg/mL. 30 µL of blood was collected from each animal at 10, 20, 30, 60, 180, and 480 minutes following drug administration and centrifuged at 5000 x g, 4 °C for 5 minutes to obtain plasma. The samples were stored at -75 ± 15 °C until analysis. Clinical observation showed no abnormality during the entire experiment.
**LC-MS/MS analysis of ent-B1** – LC-MS/MS with electrospray ionization in the positive ion mode setting was used to detect ent-B1 followed by multiple reaction monitoring of precursor and product ions as follows: ent-B1 (mass-to-charge ratio [m/z] 640.18 to 214.00). Mouse plasma was quantified using nine standards (0.5 – 1000 ng/ml) and four quality control levels (1, 2, 50, 800 ng/ml) prepared independently of those used for the standard curve. 20 µL of standards, quality control samples, and unknown samples (10 µL plasma and 10 µL blank solution) were added to 200 µL acetonitrile containing internal standard (dexamethasone; [m/z] 393.40 to 373.30) for protein precipitation. The samples were vortexed for 30 seconds, centrifuged for 15 minutes at 4000 rpm and 4 °C, and the supernatant was diluted five times with water. Samples were then loaded into SIL-30AC autosampler and 10 µL was injected into a Shimadzu LC-30AD Series HPLC coupled to an AB Sciex Triple Quad 5500 mass spectrometer. Analytes were separated on a Raptor Biphenyl column (50 x 2.1 mm, 2.7 µm) using a 95:5 (v/v) mobile phase mixture of 0.1% formic acid in water (mobile phase A) and 0.1% formic acid in acetonitrile (mobile phase B) at the flow rate of 0.6 ml/min. The gradient for mobile phase B was increased to 95% over 1.4 minutes with a total run time of 2.5 minutes for each sample. All quality controls and standards met the following acceptance criteria: 1) standard curve of at least five standards are within 15% of their nominal concentrations and at least 50% at each quality control level (low, medium, and high) were within 15% of their nominal concentrations.

**Pharmacokinetic analysis** – Plasma concentrations of ent-B1 at each time point were imported to Phoenix WinNonlin® 8.0 software (Certara USA, Inc., Princeton, NJ). Plasma concentration time profiles for individual animals were analyzed by
noncompartmental analysis using model 200 (Plasma; Single Extravascular Dose; Linear Log Trapezoidal Method) to approximate the elimination rate constant (ke), half-life ($T_{1/2}$), maximum observed plasma concentration ($C_{\text{max}}$), time to maximum observed plasma concentration ($T_{\text{max}}$), the area under the plasma concentration-time curve from zero to infinity (AUC$_{\text{inf}}$). Dose was normalized to 3 mg/kg for each animal and used to derive estimates of extravascular clearance (Cl/F) and extravascular volume of distribution (Vz/F) by noncompartmental analysis.

**Plasma stability experiments**- Drug-free mouse plasma was preincubated in a 96-well polypropylene plate for 30 minutes on a microplate shaker at 37 °C prior to the addition of ent-B1 (final concentration 1900 ng/mL). Drug was added to individual wells (n=3 per compound), mixed thoroughly, and 50 µL of plasma was removed at 0, 15, 30, 45 60, 90, and 120 minutes to evaluate compound stability in mouse plasma at 37 °C. Samples removed at each time point were transferred to a 96-well polypropylene plate containing 300 µL of ice-cold acetonitrile containing internal standard (ent-verticilide-$d_{12}$; 100 ng/mL). Samples were mixed multiple times with a pipette to ensure precipitation of plasma proteins and the plate was covered with an adhesive film to prevent solvent evaporation. Samples remained on ice throughout the experiment until the last sample collection time point. The plate was centrifuged at 4800 rpm for 20 minutes and 300 µL of supernatant was transferred to a new 96 deep well polypropylene deep well plate, evaporated under a stream of nitrogen gas, and reconstituted with 200 µL of mobile phase prior to LCMS analysis. Peak area ratios of the analyte and internal standard for each sample were analyzed by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) and normalized to the area ratio for time point 0 (baseline) to
calculate the percent of ent-B1 remaining at each time point. Criteria for establishing compound stability was set at 90% and 80% remaining at 60 and 120 minutes, respectively.

*Electrocardiogram (ECG) recording* – Nine male and thirteen female Casq2+/− mice 17-20 weeks old were randomly assigned to a three-by-three crossover design such that every treatment sequence was sampled. Mice were pretreated with intraperitoneal injection of vehicle (DMSO), 3 mg/kg ent-B1, or 30 mg/kg of ent-B1 fifteen minutes prior to baseline ECG recording. Mice were then injected with 3 mg/kg isoproterenol intraperitoneally. Recordings were continued for 5 minutes or for 1 minute after cessation of ventricular ectopy. A washout period of one week was used between treatments. The Vanderbilt Institutional Animal Care and Use Committee (IACUC) approved the use of mice in these studies (protocol # M1900057-01). Animals had free access to food and water.

*ECG analysis* – LabChart (AD Instruments, Inc) was used to analyze ECG recordings by a reviewer blinded to treatment dose. ECG records were examined to quantify premature ventricular contractions (PVCs), duration of ventricular tachycardia, heart rate, and baseline ECG parameters (PR, QRS and QT interval). Arrhythmias were scored on a five point ordinal scale based on the number of PVCs with the following criteria: 1) zero point for no PVCs; 2) one point for isolated PVCs; 3) two points for bigeminy (alternating sinus beats and PVCs); 4) three points for couplets (two consecutive PVCs); and 5) four points for three or more consecutive PVCs (ventricular tachycardia).
**Statistical Analysis** – Statistics were carried out in GraphPad Prism (v9.5.0) or Matlab as indicated in the figure legends. Notably, given the known *in vitro* and *in vivo* efficacy of *ent*-1 – a ring size analogue to *ent*-B1 – we conducted exploratory studies of *ent*-B1 without a prespecified null hypothesis. Hence, all p-values should be interpreted as descriptive and not hypothesis-testing. All tests were two-sided and a p-value cutoff of <0.05 was used to denote significance. Concentration-response curves for RyR2 single-channel, \(^{3}\text{H}\)ryanodine ligand binding and intact myocyte assays were generated using non-linear regression with the equation: \(Y = \text{Bottom} + \frac{(\text{Top} - \text{Bottom})}{(1+(\text{IC}_{50}/X)^{\text{HillSlope}})}\) using least square regression, no weighting, and no constrains on the parameters. The distributions of relative RyR2 open probability at each [*ent*-B1] were normalized by taking the log of each sample. For the 3 x 3 crossover *in vivo* ECG study, a pre-test was conducted using a mixed model with fixed effects of sequence and period; mice were treated as random effects. When warranted, a post-hoc test was conducted and used, as reported in the pertaining figure legends, to test the null hypothesis that the 3 mg/kg or 30 mg/kg doses do not deviate from treatment with vehicle.

**Results**

*ent*-B1 inhibits RyR2 single channels and \(^{3}\text{H}\)ryanodine binding in SR vesicles

To determine the effect of *ent*-B1 on RyR2 channel activity, we studied isolated SR vesicles derived from porcine cardiac muscle. RyR2 channels were incorporated into artificial lipid bilayers and exposed to *ent*-B1 via perfusion (Fig. 1). We tested the effect of seven concentrations of *ent*-B1 (0.03, 0.1, 0.3, 1, 3, 10, and 30 µM) on RyR2
channel open probability. Cytosolic Ca\(^{2+}\) concentration was kept at 100 nM to match physiological concentrations during diastole. ent-B1 inhibited RyR2 channels in a concentration-dependent manner, with an estimated IC\(_{50}\) of ~0.24 µM [95% confidence interval (CI): 0.08 – 1.00] and incomplete maximal channel inhibition (Fig. 1C). There was a corresponding shift in the current amplitude histogram toward I=0 in the presence of ent-B1, with no detectable substates (Fig. 1D). To establish a more detailed concentration-response relationship, we performed a \(^{3}\)Hryanodine binding assay using SR samples from porcine cardiac muscle (Terentyev et al., 2008). In this assay of RyR2 channel activity, the presence of an inhibitor reduces the amount of \(^{3}\)Hryanodine bound to the samples. When compared to vehicle (DMSO), ent-B1 reduced \(^{3}\)Hryanodine binding with a low micromolar potency (IC\(_{50}\) = 1.9 µM) and incomplete maximal inhibition of approximately 30% (Fig. 2). Taken together, these results demonstrate that ent-B1 directly inhibits RyR2 channels.

**ent-B1 reduces spontaneous Ca\(^{2+}\) release in intact mouse CPVT cardiomyocytes**

Direct pharmacological block of RyR2 necessitates a compound permeate the cell membrane (sarcolemma). Single channel and \(^{3}\)Hryanodine assays do not require cell membrane permeability or transport. Hence, we next examined the activity of ent-B1 in cardiomyocytes isolated from Casq2\(^{-/-}\) mice to determine whether ent-B1 blocks RyR2 in intact cells. To quantify drug efficacy, we measured the rate of spontaneous RyR2-mediated spontaneous SR Ca\(^{2+}\) release events (Chopra et al., 2007) following a 3 Hz pacing protocol (Fig. 3A). Compared to vehicle (DMSO), ent-B1 reduced the rate of SCRs in a concentration-dependent manner (IC\(_{50}\) = 0.23 ± 0.1 µM) with ~60% efficacy.
at the highest concentration tested (Fig. 3B). This indicates that after a 2.5 hour incubation, ent-B1 significantly inhibits pathologic Ca\(^{2+}\) release in intact cardiomyocytes with similar potency to the RyR2 single channel assay (0.24 µM, Fig. 1). Only the highest concentration of ent-B1 tested (3 µM) reduced diastolic Ca\(^{2+}\) levels, the amplitude of the systolic Ca\(^{2+}\) transient, and the amplitude of the caffeine-induced Ca\(^{2+}\) transient (Fig. S1), which is consistent with the mechanism of action of a partial RyR2 inhibitor. ent-B1 had no effect on other measures of SR Ca\(^{2+}\) release such as time-to-peak of Ca\(^{2+}\) transients or Ca\(^{2+}\) decay kinetics (Fig. S1C, D), the latter being an indicator of SERCA2 SR Ca\(^{2+}\) uptake rate. We also saw no effect of ent-B1 on the decay rate of the caffeine-induced SR Ca\(^{2+}\) transient (Fig. S1F), which is a standard measure of the Na\(^{+}\)-Ca\(^{2+}\) exchanger activity. To quantify any potential off-target effects on ion channels or ion transporters in the plasma membrane, we recorded action potentials from current-clamped intact Casq2\(^{-/-}\) cardiomyocytes (Fig S2). ent-B1 (0.3 µM) had no significant effects on the ventricular action potential (Fig. S2), suggesting that at therapeutic concentrations that block RyR2 channels ent-B1 does not inhibit sarcolemmal ion channels that contribute to the cardiac action potential.

**ent-B1 is stable in plasma and has druggable pharmacokinetic properties**

Once we established that ent-B1 could directly bind to RyR2 and permeate the sarcolemma to inhibit pathologic Ca\(^{2+}\) release, we wanted to test its therapeutic efficacy in an *in vivo* arrhythmia study. To guide dose selection, we first determined ent-B1’s pharmacokinetic properties in mice. Plasma samples were collected and ent-B1 concentrations measured after a 3 mg/kg ent-B1 intraperitoneal (i.p.) injection.
Substantial plasma concentrations were readily achieved, indicating favorable systemic exposure after i.p. administration. The mean peak plasma concentration (C_{max}) was 1460 ng/mL (2.3 µM) at 10 min after i.p. injection and exhibited a biphasic decline with a mean elimination half-life (t_{1/2}) of 45.4 minutes (Fig. 4A, Table 1). The half-life for ent-B1 was substantially shorter than the half-life of its larger ring size analogue, ent-1, which was 6.9 and 6.4 hours for the 3 mg/kg and 30 mg/kg doses, respectively (Blackwell et al., 2023). To determine whether the shorter half-life of ent-B1 was due to instability of the drug in plasma, we used LC-MS/MS to measure ent-B1 incubated in murine plasma. ent-B1 was stable over the course of two hours, with ~95% of the parent compound remaining (Fig. 4B).

*ent-B1 reduces ventricular arrhythmia burden in a mouse model of CPVT*

*Casq2^{−/−}* mice are a validated model for preclinical testing of antiarrhythmic drugs for CPVT (Batiste et al., 2019; Watanabe et al., 2009). Our hit compound ent-1 had antiarrhythmic efficacy *in vivo* in a single dose study at 3 and 30 mg/kg i.p. in *Casq2^{−/−}* mice (Blackwell et al., 2023). We tested the same doses of ent-B1 in a triple-crossover design with each mouse receiving vehicle (DMSO), 3 mg/kg, and 30 mg/kg with a one-week washout period between experiments. Based on the pharmacokinetic properties of ent-B1 (Table 1), we chose an *in vivo* arrhythmia challenge protocol with the data collection occurring 15 minutes after ent-B1 i.p. administration. Mice were anesthetized with isoflurane and a baseline electrocardiogram (ECG) was established. Ventricular arrhythmias were elicited with a catecholamine challenge using the β-adrenergic agonist isoproterenol (3 mg/kg i.p.), a well-established drug challenge for antiarrhythmic efficacy.
testing in this model (Watanabe et al., 2009). ECGs were analyzed in blinded fashion and ventricular arrhythmias quantified. **Fig. 5A** gives examples of ventricular arrhythmias induced by the catecholamine challenge. ent-B1 caused a dose-dependent reduction in the number of total ectopic beats and incidence of VT (**Fig. 5B, C**). We also scored the mice based on the ventricular arrhythmia severity, i.e., premature ventricular complexes (PVCs) < bigeminy < couplets < ventricular tachycardia (Blackwell et al., 2022). The lower arrhythmia risk scores in mice receiving ent-B1 indicate therapeutic efficacy (**Fig. 5D**). Consistent with ent-B1’s lack of effect on the cardiac action potential *in vitro* (**Fig. S2**), no significant changes in heart rate and in baseline ECG parameters (PR, QRS and QT interval) were observed (**Figs. S3, S4**).

**DISCUSSION**

Our experiments highlight the potential of leveraging the unique chemical biology of natural product enantiomers to design novel molecular entities with therapeutic efficacy. While the promise of traditional drug candidates rested on maintaining a low molecular weight (<500 Da), limited O and N atoms and H-bond donors as well as hydrophilic properties, the clinical use of natural product compounds and their derivatives is growing despite typically defying one or more of these rules (Caron et al., 2021; Lipinski, 2004; Veber et al., 2002). Within the natural product space, cyclic peptides offer large templates for *de novo* drug design. For example, we have previously shown that modification of ring size and *N*-methylation of units of ent-1 preserve biochemical properties (Smith et al., 2021). Here we show that, in the case of ring size modification, this preserves selectivity and translates to therapeutic efficacy.
This confirms our hypothesis that the complete structure of the cyclic peptide ent-1 is not required for modulation of its target, RyR2.

Although not directly compared here, our in vitro experiments suggest that ent-B1 has a slightly lower potency than the larger ring-size analogue ent-1. For example, the IC\textsubscript{50} for ent-1 in the ryanodine binding assay was 0.1 µM (Batiste et al., 2019), whereas ent-B1 was 1.3 µM (Fig. 2). In the intact cardiomyocyte assay, the difference in potency was less, with an IC\textsubscript{50} of 0.09 µM for ent-1 (Batiste et al., 2019), and 0.23 µM for ent-B1 (Fig. 3). Importantly, the ent-B1 potency in the most direct assay, the RyR2 single channel assay, was 0.24 µM (Fig. 1), like the intact myocyte assay (Fig. 3). However, the in vivo potency of ent-B1 on ventricular arrhythmia suppression was comparable to that of ent-1, with both compounds dosed at 30 mg/kg producing a 70% reduction in ventricular ectopy burden using the same arrhythmia induction protocol (see Fig. 5B and (Batiste et al., 2019)). As reported for ent-1 (Blackwell et al., 2023), we also observe a clear dose-dependent effect of ent-B1 in vivo (Fig. 5). At the same time, ventricular ectopy was not completely suppressed even though peak plasma concentrations in vivo (Table 1) were more than 10-fold higher than the IC\textsubscript{50} in cardiomyocytes. We attribute this result to the incomplete inhibition of RyR2 observed in vitro, although high plasma protein binding and/or low membrane permeability could be contributory. It should be noted that a statistically significant suppression of ventricular tachycardia, the most clinically-relevant arrhythmia parameter, was observed only at the higher dose tested (30 mg/kg, Fig. 5). Although further lead optimization would likely be required to optimize drug efficacy in vivo, ent-B1 holds promise as a preclinical lead
given its antiarrhythmic efficacy in the most physiologically relevant assay, the in vivo arrhythmia challenge.

Our in vitro assays suggest that, like ent-1, ent-B1 directly binds to RyR2 and produces incomplete inhibition, unlike recently identified RyR2 inhibitory compounds (chloroxylenol, methyl orsellinate, and riluzole) which produce complete RyR2 block (Takenaka et al., 2023). ent-B1 likely acts as a negative allosteric modulator of channel function, similar to ent-1, for which we recently identified a putative binding site (Seflova et al., 2023). ent-B1’s combination of sub-micromolar potency and partial channel inhibition is ideal for our target, as a stronger inhibition of RyR2 could be fatal given its central role in physiologic excitation-contraction coupling. Although peak plasma concentration was comparable to ent-1, our pharmacokinetic study revealed a more rapid systemic clearance of ent-B1 ($t_{1/2} = 45$ min, table 1) relative to ent-1 (415 min) in mice (Blackwell et al., 2023). Given the short half-life, ent-B1 would only be useful in acute clinical scenarios unless the drug formulation is modified to produce a sustained-release profile. Assuming this property is preserved in large animal species and humans, ent-B1 could be developed for treatment of ventricular tachycardia storm (“VT storm”), an acute, life-threatening arrhythmia disorder consisting of sequential episodes of sustained VT in a 24-hour window. One limitation of our study with regards to this purpose for ent-B1 is that the intraperitoneal route of administration likely produces a markedly different pharmacokinetic profile than that of intravenous administration. Taken together, our data with ent-B1 further validate RyR2 as an antiarrhythmic drug target and introduce a second preclinical stage compound whose mirror image is a fungal natural product.
**Authorship contributions**

Participated in research design: Gochman, Do, Kim, Schwarz, Blackwell, Akers, Cornea, Laver, Knollmann

Conducted experiments: Gochman, Do, Kim, Schwarz

Contributed new reagents or analytic tools: Thorpe, Smith, Johnston, Rebbeck

Performed data analysis: Gochman, Do, Kim, Ritschel, Schwarz, Blackwell

Wrote or contributed to the writing of the manuscript: Gochman, Do, Blackwell, Akers, Rebbeck, Laver, Cornea, Johnston, Knollmann

**Acknowledgment**

None

**Data Availability Statement**

All data/datasets are contained in the paper.
References


Footnotes

This research was supported in part by the National Institutes of Health National Heart, Lung, and Blood Institute [R35 HL144980 (to B.C.K.), R01 HL151223 (to J.N.J., B.C.K., R.L.C.), R01 HL139065 and HL138539 (to R.L.C.), F30 HL168829 (to A.G); the PhRMA Foundation Postdoctoral Fellowship (to D.J.B.); the American Heart Association Arrhythmia and Sudden Death Strategically Focused Research Network grant [19SFRN34830019 (to B.C.K.)]. No author has an actual or perceived conflict of interest with the contents of this article.

An earlier version of this MS has been published as a preprint on BioRxiv.

URL. https://www.biorxiv.org/content/10.1101/2023.07.03.547578v2
Legends for Figures

**Figure 1:** RyR2 single channel recording in artificial lipid bilayers. A) Chemical structure of 24-membered cyclic depsipeptide ent-1 and 18-membered cyclic depsipeptide ent-B1. B) Representative trace of RyR2 single channel recording before and after application of ent-B1 at +40 mV, with 2 mM ATP and 100 nM Ca\(^{2+}\) (cis) and 1 mM Ca\(^{2+}\) (trans). Channel openings are in the upward direction (c, closed; o, open). C) Open probability of RyR2 in the presence of ent-B1 relative to vehicle (DMSO). Fitting values using non-linear regression to a Hill-function (line) yielded an IC\(_{50}\) of 0.24 µM [95% CI: 0.08 – 1.00] and a maximal inhibitory effect I\(_{\text{max}}\) of 61% (95% CI: 52% - 72%). D) Probability distributions of current amplitude of 30-second segments of recording from (B) before and after addition of 30 µM ent-B1.

**Figure 2:** Effect of ent-B1 on RyR2 activity in porcine SR vesicles. RyR2 activity was quantified by the fraction of [\(^3\)H]ryanodine bound to RyR2. The concentration-response curve shows results for ent-B1 relative to vehicle (DMSO). ent-B1 IC\(_{50}\) = 1.93 µM (95% CI 0.74-4.62 µM), I\(_{\text{max}}\) = 29%. Data are shown as mean ± SD (n = 6).

**Figure 3:** Spontaneous Ca\(^{2+}\) release in intact mouse Casq2\(^{-/-}\) cardiomyocytes. A) Representative fluorescence recordings from ventricular myocytes isolated from Casq2\(^{-/-}\) mice. Cells were field stimulated at 3 Hz for 20 s before recording. Blue marks indicate final stimulations of pacing train, arrows SCR events. Recording duration was 40 s before 10 mM caffeine application to assess total SR Ca\(^{2+}\) content. B) SCR frequency concentration-response curve for ent-B1 following 20 s pacing protocol. ent-B1 data normalized to vehicle (DMSO). Data are shown as mean ± SD. Fitting values using non-linear regression to a Hill-function yielded an IC\(_{50}\) of 0.23 µM [95% CI: 0.099 – 0.63] and...
a maximal inhibitory effect $I_{\text{max}}$ of 62% (95% CI: 53% - 89%) $N = 26, 30, 30, 30, \text{ and } 31$ cells for 0, 0.03, 0.1, 0.3, and 1 μM ent-B1 respectively.

**Figure 4: In vivo ent-B1 pharmacokinetics in CD1 mice.** A) Plasma concentrations of ent-B1 after intraperitoneal administration of 3 mg/kg dose. $N = 3$ mice. Plasma collected serially at 0.167, 0.333, 0.5, 1, 3, and 8 hours post-administration. B) Quantification of ent-B1 incubated ex vivo in murine plasma for 2 hours at 37 deg C. Individual data points (grey) shown alongside mean values with connecting lines.

**Figure 5: ent-B1 antiarrhythmic efficacy in Casq2−/− mice.** Triple crossover study design for in vivo arrhythmia challenge. $N = 22$ Casq2−/− mice were randomly assigned to vehicle (DMSO), 3 mg/kg, or 30 mg/kg ent-B1 and crossed over twice with one-week washouts between treatment. Mice underwent catecholamine-induced arrhythmia challenge with isoproterenol 15 min after intraperitoneal drug administration. A) Sample ECG traces of normal rhythm (top) and arrhythmias including PVCs and couplets (middle), and bigeminy and ventricular tachycardia (bottom). Ectopic beats are denoted by p (PVC), c (couplet), and vt (ventricular tachycardia). Normal sinus beats are not marked. B) Total number of ventricular ectopic beats per mouse. Individual data are shown with median and interquartile range. Bonferroni-adjusted $P$ values by pairwise Wilcoxon matched-pairs signed rank test. *$P = 0.020$ vs Veh; # $P = 0.039$ vs Veh. C) Incidence of ventricular tachycardia. # $P = 0.034$ vs Vehicle by Fisher’s exact test. D) Arrhythmia risk scores were based on an ordinal scale of: 4 = ventricular tachycardia, 3 = couplet, 2 = bigeminy, 1 = isolated PVC, 0 = no PVCs. Bonferroni-adjusted $P$ values by pairwise Wilcoxon matched-pairs signed rank test. # $P = 0.0058$ vs Vehicle.
Tables

Table 1: Noncompartmental pharmacokinetic (PK) parameter estimates of ent-B1 following 3 mg/kg intraperitoneal administration in CD1 mice

<table>
<thead>
<tr>
<th>PK parameter</th>
<th>3 mg/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>ke (h⁻¹)</td>
<td>0.932 ± 0.139</td>
</tr>
<tr>
<td>T_{1/2} (h)</td>
<td>0.756 ± 0.123</td>
</tr>
<tr>
<td>T_{max} (h)</td>
<td>0.167 ± 0.0</td>
</tr>
<tr>
<td>C_{max} (ng/ml)</td>
<td>1460 ± 90</td>
</tr>
<tr>
<td>AUC_{inf} (h x ng/ml)</td>
<td>1020 ± 184</td>
</tr>
<tr>
<td>Vz/F (L/kg)</td>
<td>3.32 ± 1.00</td>
</tr>
<tr>
<td>Cl/F (L/h/kg)</td>
<td>3.01 ± 0.50</td>
</tr>
</tbody>
</table>

ke: elimination rate constant; T_{1/2}: elimination half-life; T_{max}: time to maximum concentration; C_{max}: maximum concentration; AUC_{inf}: area under the plasma concentration curve from zero to infinity time; Vz/F: extravascular volume of distribution; Cl/F: extravascular clearance. Data reported as mean ± standard deviation. N = 3 mice.
Figure 1
Figure 2
Figure 3
Figure 4

A: Concentration-time profile of entB1 in plasma. The graph shows the mean and individual values of entB1 concentration over time (in hours).

B: Area ratio (% of baseline) of entB1 over time. The graph displays the mean and individual values of the area ratio over time (in hours).
Figure 5