A novel saline-soluble, rapidly-metabolized RyR1 inhibitor rescues volatile anesthesia-induced death and environmental heat stroke in a mouse model relevant to malignant hyperthermia

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Running title: A novel RyR1 inhibitor rescues MH

Abbreviations: MH, malignant hyperthermia; RyR1, type 1 ryanodine receptor; Wt, wild-type; Het; heterozygous; Hom; homozygous

Abstract

Mutations in the type 1 ryanodine receptor (RyR1), a Ca^{2+} release channel in skeletal muscle, hyperactivate the channel to cause malignant hyperthermia (MH) and are implicated in severe heat stroke. Dantrolene, the only approved drug for MH, has the disadvantages of having very poor water solubility and long plasma half-life. We show here that a novel RyR1-selective inhibitor, 6,7-(methylenedioxy)-1-octyl-4-quinolone-3-carboxylic acid (Compound 1), effectively rescues MH and heat stroke in several mouse models relevant to MH. Compound 1 reduced resting intracellular Ca^{2+}, inhibited halothane-induced Ca^{2+} release, suppressed caffeine-induced contracture in skeletal muscle, reduced sarcolemmal cation influx, and prevented or reversed the fulminant MH crisis by isoflurane anesthesia and heat stroke by environmental heat stress. Notably, Compound 1 has great advantages of better water solubility and rapid clearance *in vivo* over dantrolene. Compound 1 has the potential to be a promising new candidate for effective treatment of patients carrying RyR1 mutations.

Introduction

The type 1 ryanodine receptor (*RYR1*; MIM# 180901) is a Ca^{2+}-release channel in the sarcoplasmic reticulum (SR) of skeletal muscle that plays a central role in muscle contraction[1,2]. During excitation-contraction (E-C) coupling, RyR1 releases Ca^{2+} through physical association with the sarcolemmal slow voltage gated Ca^{2+} channel (dihydropyridine receptor, DHPR) during depolarization of T-tubule membrane. This is referred to as depolarization-induced Ca^{2+} release (DICR) [3,4]. The RyR1 channel can also directly be activated by Ca^{2+}, which is referred to as Ca^{2+}-induced Ca^{2+} release (CICR) [5,6].

Genetic mutations in the *RYR1* gene are associated with malignant hyperthermia (MH; MIM# 145600) [7]. MH is a life-threatening disorder characterized by skeletal muscle rigidity and elevated body temperature in response to halogenated anesthetics such as halothane or isoflurane [8]. MH mutations are thought to hyperactivate CICR, which causes massive Ca^{2+} release by halogenated anesthetics under resting conditions [6,9,10]. Heat stroke is a medical emergency with a high body temperature and altered mental status, which is triggered by exercise or environmental heat stress [11,12]. It has been reported that MH mutations in the *RYR1* gene are also implicated in some heat stroke [13-15].

The only drug approved for ameliorating the symptoms of MH is dantrolene [16,17]. Dantrolene was first developed in 1960s as a muscle relaxant [18] and was later shown to prevent Ca^{2+} release by direct interaction with RyR1 [19,20]. However, dantrolene has several disadvantages for clinical use: the main disadvantage is its poor water solubility which makes rapid preparation difficult in emergency situations [21]. In addition, dantrolene has long plasma half-life ($t_{1/2}$ ~12 h), which causes long-lasting side effects such as muscle weakness [22]. To date, no alternative drugs improving these disadvantages have been developed over 50 years since the first discovery.

We have recently identified oxolinic acid as a novel RyR1-selective inhibitor with better water solubility using efficient high-throughput screening platform for RyR1 inhibitors [23,24]. We synthesized a series of oxolinic acid derivatives by structural development, and successfully identified 6,7-(methylenedioxy)-1-octyl-4-quinolone-3-carboxylic acid (Compound 1) with extremely high potency which is comparable to dantrolene in *in vitro* study [25]. In this study, we tested whether Compound 1 has therapeutic effects in treating the MH crisis by creating a novel MH mouse model carrying *RYR1*-p.R2509C, a corresponding mutation (p.R2508C) in humans [26,27]. Evaluations were also done with two other MH mouse models, *RYR1*-p.R163C [28] and *RYR1*-p.G2435R [29]. We found that Compound 1 effectively prevents and rescues mice from fulminant MH crisis triggered by isoflurane anesthesia. The drug also treats mice with heat stroke caused by environmental heat stress. A notable finding is that Compound 1 is rapidly metabolized in mice. Our results provide crucial evidence for Compound 1 as a RyR1 inhibitor that may prove to be clinically-useful.

Results

Generation of novel MH model mice carrying *RYR1*-p.R2509C mutation

A MH mouse model carrying p.R2509C mutation in the *RYR1* gene (*RYR1*-p.R2509C) was created using CRISPR/Cas9 system (see Materials and Methods, **Fig. 1a**). An additional *Xba I* site was introduced to screen the genotype by PCR-RFLP. The homozygous (Hom) *RYR1*-p.R2509C mice died in late embryonic stage, but heterozygous (Het) *RYR1*-p.R2509C mice grew normally and were as fertile as wild type (Wt) (**Fig. 1b**).

It has previously been shown when MH susceptible mice, swine or humans with mutations in the *RYR1* gene are exposed to volatile anesthetics, it causes a MH crisis and if untreated can cause death [28-33]. As with other animal models, when anesthetized by isoflurane,

RYR1-p.R2509C Het mice rapidly increased their rectal temperature to over 42°C (**Fig. 1c**), exhibited muscle rigidity (**Fig. 1d**) and died after 65±31 min (mean±SD, n=14) of exposure (**Fig. 1e**). In contrast, Wt mice showed no elevation in rectal temperature in response to isoflurane. Time from start of anesthesia to death varied largely among individual mice and took significantly longer in females (87±33 min, n=5) than males (53±24 min, n=9) ($p<0.05$) (**Fig. 1f**). Interestingly, time from rectal temperature of 39°C to death was similar among all individuals, and no sex difference was observed ($p=0.19$) (**Fig. 1g**). These findings suggest that *RYR1*-p.R2509C mice can reproduce human MH symptoms in response to exposure to volatile anesthetics and thus are a useful model for test of Compound 1.

Intracellular Ca^{2+} homeostasis in the isolated muscles

Structures of oxolinic acid and Compound 1 are shown in **Fig. 2a**. Compound 1 has longer alkyl chain at nitrogen atom compared to oxolinic acid. Addition of the moiety nearly 70-fold increased the potency in inhibiting the RyR1 channel (IC_{50} values from 810 nM for oxolinic acid to 12 nM for Compound 1) in HEK293 cell-based assay [25].

We initially tested the effect of Compound 1 on *in vitro* experiments using the isolated muscles. Abnormal resting intracellular Ca^{2+} ($[Ca^{2+}]_i$) homeostasis in skeletal muscle cells is one of the most common functional features associated with MH in humans [34], swine [30,31], and model mice [28,29,32,33]. We therefore tested whether Compound 1 could correct abnormal $[Ca^{2+}]_i$ homeostasis using enzymatically-isolated single flexor digitorum brevis (FDB) muscle fibers from the *RYR1*-p.R2509C mice. $[Ca^{2+}]_i$ was fluorometrically monitored with a Ca^{2+} indicator, Cal520. We applied halothane to muscle fibers, which stimulates RyR1 and is used for MH testing. Whereas halothane up to 0.1% (v/v) did not change $[Ca^{2+}]_i$ in Wt fibers, it caused increase in $[Ca^{2+}]_i$ in Het fibers in a dose-dependent manner (**Fig. 2b, 2c**). Pretreatment of Het fibers with Compound 1 (0.1 μM) completely abolished halothane

induced increase in $[Ca^{2+}]_i$. In addition, Compound 1 (0.1 μM) significantly reduced resting $[Ca^{2+}]_i$ in Het fibers but not Wt fibers (**Extended Data Fig. 1**).

Contraction of isolated muscles

The In Vitro Contracture Test (IVCT) using muscle biopsies is widely used for diagnosis of MH susceptibility in humans. A test is considered positive if the degree of contracture from exposure to caffeine or halothane is significantly enhanced (greater contracture at a lower dose of caffeine, or the presence of contracture after exposure to halothane) in MH susceptible patients [35]. We therefore tested the effect of Compound 1 on caffeine-induced contracture using soleus muscles isolated from *RYR1*-p.R2509C mice. Wt muscles showed only minimal contracture in responses to caffeine up to 20 mM (**Extended Data Fig. 2** and **Fig. 2d**). In contrast, Het muscles exhibited dose-dependent contracture during exposure to caffeine. Application of 3 μM Compound 1 decreased caffeine-induced contracture tension of Het muscles from 0.83 ± 0.27 to 0.55 ± 0.05 N/cm^2 (n = 9) at 20 mM caffeine.

Dantrolene is known to suppress twitch and tetanic contractions, which causes muscle weakness as a major side effect of the drug [36,37]. We therefore tested whether Compound 1 affects twitch and tetanic contractions. Untreated soleus muscles from Het *RYR1*-p.R2509C mice showed similar twitch and tetanic contraction force to that recorded from Wt soleus muscles (**Extended Data Fig. 2** and **Fig. 2e**). 3 μM Compound 1 reduced both twitch and tetanic contractions. The effect was stronger with twitch (72% inhibition) than with tetanic (35% inhibition at 100 Hz) contractions, which is similar to dantrolene [36].

***In vivo* isoflurane challenge**

We next tested whether Compound 1 can prevent or treat MH episodes in

RYR1-p.R2509C Het mice *in vivo*. Since Compound 1 is a free acid having carboxyl group (**Fig. 2a**), and is virtually water insoluble, we prepared its sodium salt, which neutralizes the carboxyl group and significantly increases water solubility. Compound 1 sodium salt also exhibited good solubility (849±58 μg/mL, n=4) in normal saline (0.9% NaCl) (Table 1). Dantrolene sodium salt, in contrast, was hardly soluble in normal saline (25.7±2.1 μg/mL, n=4) under the same conditions, which is in accord with the previous study [38] (**Extended Data Table 1**).

We first tested the preventive effect of Compound 1 on MH episodes in response to isoflurane exposure. Doses of either 3 mg/kg or 10 mg/kg of Compound 1 solubilized in normal saline were administered *i.p.* 10 min before isoflurane challenge. The 3 mg/kg dose did not prevent rise in rectal temperature and only 1 out of 6 mice survived (**Fig. 3a-3c**). Time to death for mice treated at 3 mg/kg (47±15 min, n=5) was not significantly different than that for control mice (53±24 min, n=9). However, with the 10 mg/kg dose, Compound 1 successfully prevented any rise in rectal temperature and all the mice survived 90 min after inducing anesthesia (**Fig. 3a-3c**).

We next tested whether Compound 1 could rescue mice from MH episodes. Het mice were anesthetized with isoflurane and the drug was administered *i.p.* when rectal temperature reached 39°C. The 3 mg/kg dose was able to stop the rise in rectal temperature (-0.03±0.81°C at 10 min after administration, compared to 1.79±0.82°C in controls), but rectal temperature started to rise again ~30 min after administration of the drug (**Fig. 3d, 3e**). The 10 mg/kg dose decreased body temperature (-0.64±0.29°C at 10 min after administration) and maintained it at that level until the experiment was terminated at 60 min. At that time 50% of the mice in the 3 mg/kg group and all the mice in the 10 mg/kg survived at the 60 min time point (**Fig. 3f**).

Environmental heat stress

It has been shown that environmental heat stress causes an increase in body temperature and death of existing MH model mice [28,29,32,33]. We therefore tested whether *RYR1*-p.R2509C Het mice exhibit heat stroke triggered by environmental heat stress. Mice were anesthetized using intravenous anesthetics and transferred to a 35 °C test chamber. Whereas the rectal temperature of Wt mice was maintained a relatively constant level around 37°C (37.4±0.8°C, n=7), the rectal temperature of Het mice steadily increased (41.4±1.4°C, n=13) and all the mice died (**Extended Data Fig. 3a, 3b**). The time from start of heat stress to death largely varied among individual mice (74±21 min, n=13), but the variation became smaller for time to death after reaching body temperatures of 38°C (36±13 min, n=13) or 39°C (22±8 min, n=13) (**Extended Data Fig. 3c**).

Using this model, we initially tested preventive effect of Compound 1 on the heat stroke. Pretreatment of mice with Compound 1 (10 mg/kg) slowed the rate of temperature rise (**Fig. 4a**), but none of the treated mice (0 out of 4) survived 120 min of heat stress (**Fig. 4b**). However, time to death for the treated mice (98±17 min, n=4) was significantly prolonged compared to the control mice (74±21 min, n=13) (**Fig. 4c**).

We next tested whether Compound 1 could rescue mice from environmental heat stroke. The mice were incubated at the test chamber and the drug was administered *i.p.* when rectal temperature reached 38°C. All the untreated mice died within 60 min (time to death, 36±13 min, n=13). Compound 1 at the 3 mg/kg dose suppressed rise in the body temperature (**Fig. 4d, 4e**) and 5 out of 6 mice survived 60 min after administration (**Fig. 4f**). Compound 1 at the 10 mg/kg dose showed more pronounced effect: it transiently dropped the body temperature and all the mice (6 out of 6) survived for 60 min after administration. The rescue effect of the drug was also observed when the drug was administered when body temperature reached 39°C, after which most untreated mice died within 30 min (time to death, 22±8 min,

n=13). When given at this time, Compound 1 (10 mg/kg) strongly suppressed the continued rise in the body temperature and 5 out of 6 mice survived 60 min after its administration (**Extended Data Fig. 4**).

In vivo $[Ca^{2+}]_i$ homeostasis in skeletal muscle at rest and during MH episodes

We demonstrated that Compound 1 prevented halothane-induced $[Ca^{2+}]_i$ elevation and reduced resting $[Ca^{2+}]_i$ in the isolated skeletal muscle fibers (see **Fig. 1** and **Extended Data Fig. 1**). To test whether this is also the case *in vivo*, $[Ca^{2+}]_i$ in skeletal muscle was measured with Ca^{2+}-selective microelectrodes [39] using MH model mice carrying the *RYR1*-p.R163C mutation[28]. At rest, $[Ca^{2+}]_i$ was threefold higher in *RYR1*-p.R163C Het muscle (330 ± 43 nM, n = 17) compared to Wt muscle (122 ± 3 nM, n = 20) (**Fig. 5a**). Administration of Compound 1 *i.p.* effectively reduced $[Ca^{2+}]_i$ in Het muscle in a dose-dependent manner. Compound 1 also reduced $[Ca^{2+}]_i$ in Wt muscles, but the effect was significantly smaller. When mice were exposed to 1.5% (v/v) isoflurane, $[Ca^{2+}]_i$ was greatly increased in untreated Het *RYR1*-p.R163C muscles (1226 ± 154 nM, n = 19), indicating a MH episode. Administration of isoflurane had no effect on $[Ca^{2+}]_i$ in Wt muscles (121 ± 4 nM, n = 18) (**Fig. 5b**). Pretreatment with Compound 1 greatly reduced $[Ca^{2+}]_i$ in Het *RYR1*-p.R163C muscle in a dose-dependent manner and prevented the MH episode at doses of 5 and 10mg/kg *i.p.*. To validate *in vivo* experiments, we tested the effect of Compound 1 in the isolated *RYR1*-p.R163C FDB muscle fibers (**Extended Data Fig. 5**). Similar to the *in vivo* findings, $[Ca^{2+}]_i$ was significantly higher in quiescent Het fibers (326 ± 27 nM, n = 16) compared to Wt fibers (121 ± 3 nM, n = 20). Compound 1 reduced $[Ca^{2+}]_i$ in Het fibers in a dose-dependent manner and $[Ca^{2+}]_i$ at 2.5 μM or higher was close to the level of treated Wt fibers.

Sarcolemmal cation influx in skeletal muscle

It has been shown that sarcolemmal cation influx is accelerated in skeletal muscle of MH model mice compared with Wt mice [39,40]. This is due to enhanced sarcolemmal Ca^{2+} entry in response to chronic reduced SR Ca^{2+} load. Thus, sarcolemmal cation influx is a useful index for the RyR1 channel activity. To test the effect of Compound 1 on sarcolemmal cation influx, we measured resting Mn^{2+} quench of Fura-2 fluorescence using skeletal muscle fibers from MH model mice carrying the *RYR1*-p.G2435R mutation [29]. Rate of Mn^{2+} quench in fibers from homozygous (Hom) *RYR1*-p.G2435R mice was twice that from Wt mice (**Fig. 5c, 5d**). Compound 1 reduced Mn^{2+} quench in Hom *RYR1*-p.G2435R fibers in a dose-dependent manner and the Mn^{2+} quench rate at the 10 μM dose was the same as untreated Wt fibers.

Pharmacokinetics

In *in vivo* experiments, we observed transient effects of Compound 1 in our isoflurane challenge and heat challenge experiments (see **Fig. 3, 4**). This implies fast metabolism or excretion of the drug in mice. We therefore measured plasma concentration of Compound 1 after *i.p.* administration. Compound 1 rapidly declined from mouse blood following first order kinetics with $t_{1/2}$ of ~10 min at both the 3 mg/kg and 10 mg/kg dose (**Fig. 6a**). Since Compound 1 has hydrophobic moiety of long alkyl chain of 8 carbons (see **Fig. 2**), it can be metabolized in the liver. *In vitro* drug metabolism assay using liver microsomes demonstrated that Compound 1 was reduced to 20% and 5% of the original amount at 10 min and 60 min, respectively, after incubation of mouse liver microsomes (**Fig. 6b**). Compound 1 was metabolized more slowly by human liver microsomes; it was reduced to 20% at 60 min.

Duration of the effect of Compound 1 in mice was also evaluated measuring *in vivo* muscle strength, since the drug inhibited twitch and tetanic contractions of the isolated skeletal muscle (see **Fig. 2**). Grip strength tests demonstrated that muscle strength of Wt mice was reduced by 15% 10 min after administration of 10 mg/kg Compound 1, and that this

deficit was almost completely recovered at 60 min, suggesting short duration of the medicinal effect *in vivo* (**Fig. 6c**).

The above results indicate that transient effects of Compound 1 in the isoflurane challenge and heat challenge experiments is due to rapid metabolism of the drug in mice. We therefore tested whether additional administrations of Compound 1 could prevent or treat mice from the crisis. Repeated administrations of the 3 mg/kg dose successfully reduced the rectal temperature and prevented death during isoflurane-induced MH crisis (**Extended Data Fig. 6**).

Discussion

MH is a life-threatening disorder in response to halogenated anesthetics such as halothane or isoflurane [8]. Here we examined the therapeutic effects of Compound 1, a novel RyR1-selective inhibitor with high potency [25], on anesthesia-induced MH crisis. Compound 1 showed good solubility in normal saline. Using three different model mice, including a novel *RYR1*-p.R2509C MH model created for this study, we demonstrated that Compound 1: (1) prevents increase in $[Ca^{2+}]_i$ by inhalational anesthetics (halothane and isoflurane) and decreases resting $[Ca^{2+}]_i$ in skeletal muscle *in vitro* and *in vivo*, (2) inhibits sarcolemmal cation entry caused by chronic store Ca^{2+} depletion, (3) inhibits caffeine-induced contracture in isolated skeletal muscle, (4) effectively prevents and treats isoflurane-induced fulminant MH crisis, and (5) treats severe heat stroke caused by environmental heat stress. These results provide crucial evidence that Compound 1 is capable of effectively preventing and treating the MH crisis by inhibiting the RyR1 channel.

Several strains of MH mouse models carrying mutations in the *RYR1* gene have been reported to date [28,29,32,33]. We created a novel mouse model carrying p.R2509C mutation in the *RYR1* gene, which corresponds to MH mutation (p.R2508C) in human *RYR1* gene [26]. In

a HEK293 expression system, the p.R2508C mutant exhibited the highest CICR activity among mutants in the central region tested [27]. Whereas Hom mice were embryonic lethal, Het mice grew normally and were as fertile as Wt (**Fig. 1**). Het mice showed a halothane-induced increase in $[Ca^{2+}]_i$ and caffeine-induced contracture in skeletal muscle (**Fig. 2**). In addition, *RYR1*-p.R2509C mice exhibited a MH crisis when exposed to isoflurane anesthesia (**Fig. 3**) and heat stroke when exposed to environmental heat stress (**Fig. 4**). These properties are common to those seen in the other model mice carrying severe MH mutations in the *RYR1* gene, e.g., p.R163C [28] and p.Y524S [32] and p.G2435R [29]. Thus, the *RYR1*-p.R2509C mice are a useful model for MH research.

A major problem of dantrolene in clinical use is its poor aqueous solubility [21]. First, dantrolene must be solubilized with sterile water and injected or infused by the intravenous route, since it is virtually insoluble in normal saline. Second, dantrolene is prepared at a diluted concentration (0.33 mg/mL) due to its low water solubility, which makes rapid preparation difficult in emergency situations. The second problem has recently been solved by nanocrystalline suspension of dantrolene (Ryanodex®) which can be solubilized at 50 mg/mL [41]. We found that Compound 1 exhibits good solubility (~1 mg/mL) in normal saline, which is threefold higher compared to dantrolene in water (**Extended Data Table 1**). This enables continuous administration of the drug by infusion using the intravenous route and expands usage of the drug before and after surgery for prevention of MH and postoperative MH [42].

Another drawback of dantrolene in clinical use is its long plasma half-life (10-12 h in humans after intravenous injection) [43]. Therefore, it is difficult to control the appropriate plasma concentration and reduce side effects such as muscle weakness [21]. Pharmacokinetic analysis revealed that Compound 1 is rapidly metabolized in mice with plasma half-life of ~10 min, probably by metabolism by the liver (**Fig. 6**). Indeed, because of this a single dose of Compound 1 showed only a transient effect during MH episodes (**Fig. 3**), and

environmental heat stress (**Fig. 4**) and the muscle weakness was transient (**Fig. 6**). Thus, Compound 1 may be beneficial for a temporary treatment of MH episodes. The slower metabolism of the drug with human liver microsomes (**Fig. 6**) suggests longer plasma half-life in humans compared to mice. Pharmacokinetic analysis in humans awaits completion.

Heat stroke is a life-threatening condition clinically diagnosed as a severe elevation in body temperature with central nervous system dysfunction [11,12]. It has been reported that MH mutations in the *RYR1* gene is implicated in some heat stroke [13-15]. Indeed, dantrolene has therapeutic effects on some patients with severe exertional heat stroke [44]. Compound 1 effectively prevents and treats fulminant heat stroke caused by environmental heat stress in *RYR1*-p.R2509C model mice (**Fig. 4**). Since Compound 1 is soluble in normal saline, it has the potential to be administered by continuous infusion which could be necessary for treatment of severe heat stroke.

Dantrolene is also used for the treatment of neuroleptic malignant syndrome, a life-threatening neurologic emergency associated with the use of antipsychotic agents [45] as well as overdose of 2,4-dinitrophenol (a prohibited weight loss agent that interrupts ATP synthesis and causes hyperthermia) [46]. Since Compound 1 has similar effects in preventing and treating MH and heat stroke, it might also be a potential candidate for treatment of these emergency situations.

Materials and Methods

Materials

Compound 1 was synthesized as described previously [25]. Sodium salt of Compound 1 was prepared as follows. Compound 1 (100 mg, 0.29 mmol) was dissolved in 3 mL of

tetrahydrofuran. Then, 44 μL of NaOH aqueous solution (25%, 0.28 mmol) was added to the solution, and the suspension was sonicated for 10 min to afford white precipitate. After 24 h at room temperature, the colorless precipitates were collected by filtration, and washed with cold tetrahydrofuran. The residual material was dried under reduced pressure to give the sodium salt of Compound 1 as colorless powder.

Determination of thermodynamic solubility

Test compound (2 mg) was suspended with 1 mL of saline or 5% glucose aqueous solution in glass tube, and the suspension was shaken at 150 rpm for 48 h at 25 °C. An aliquot was filtered by PVDF filter unit (Mini-Uni Prep™, GE Healthcare), and the filtrate was diluted four times with pure water. The diluted sample solution was injected into HPLC system (column (Mightysil RP-18, Kanto Chemical Co. Inc.), UV/vis detector (UV-2077, JASCO), pump (PU-2089, JASCO), and column oven (CO-965, JASCO)) and the peak area was recorded at 254 nm. The concentration of solution was calculated with a calibration curve. Examinations of all conditions were performed four times.

Animals

All animal-related procedures were in accordance with the guidelines of the Jikei University School of Medicine, Juntendo University School of Medicine, the National Center of Neurology and Psychiatry (NCNP), Mount Sinai Hospital and Leeds University. Mice were housed in isolator cages, fed with food and water ad libitum, and kept under controlled lighting conditions (12 h-light/12 h-dark) in specific pathogen-free conditions in the Jikei University, Juntendo University, National Institute of Neuroscience, NCNP, Mount Sinai Hospital and the Leeds Institute of Medicine animal facilities.

Generation of mutant mouse by gene editing using CRISPR-Cas9 system

Mouse genomic sequence within upstream- and downstream-50 bps each to that corresponding to human mutation site was searched by CRISPR design tool (http://crispr.mit/edu) to select PAM and its consequent guide sequence with high specificity. CAATGCCATACACTCGGTCC**AGG** (bold: PAM sequence) was selected for crRNA synthesis. A single-stranded donor oligonucleotide carrying Xba I site (underlined), CCGAAGATGTCTGCATCCTTCGTGCCTGACCAGGCATCCATGGTGCTTTTTCTAGA CTGCGTGTATGGCATTGAGAACCAGGACTTCTTGCTGCATGTGCTGGATGTGGG was chemically synthesized for homologous recombination (**Fig. 1**). A *RYR1*-genomic fragment containing PAM and the consequent guide sequence were amplified by PCR from wild-type mouse genomic DNA. RNP complex formed by mixing of tracrRNA and crRNA with recombinant Cas9 protein, was added to the PCR fragment and incubated for 1 hour. Cleavage of PCR fragment into two fragments was confirmed by agarose electrophoresis. RNP complex and single-stranded donor oligonucleotide were introduced to mouse one-cell-stage zygotes by electroporation. The electroporated zygotes were transferred to the oviducts of pseudopregnant females. Genomic DNA was prepared from newborns' tail and knock-in mice were screened by PCR-RFLP method using a pair of primers (R2509C_Forward: GCGTCAGGAATGGTTAGAATAGAT; R2509C_Reverse: TTGTCTGATGCTAGGTAGAAGGTG) and Xba I digestion (**Fig. 1**). Crossing obtained knock-in founders with wild-type mice to get F1 generations were done by standard protocol.

Preparation of single flexor digitorum brevis (FDB) fibers

Mice were deeply anesthetized with intraperitoneal (*i.p.*) injection of pentobarbital (300 mg/kg) before euthanasia. Flexor digitorum brevis (FDB) muscles were dissected and incubated with 2 mg/mL collagenase (Worthington Biochemical Co., NJ, USA) in the

HEPES-Krebs solution containing 2 mg/mL bovine serum albumin for 2-3 hours at 37°C. Following incubation, single fibers were separated by gentle trituration in collagenase-free HEPES-Krebs solution. Isolated single FDB fibers were seeded on a cover slip of the 35 mm glass-bottom dish for Ca^{2+} imaging or on 96 well plate coated with Cultrex basement membrane matrix (Trevigen, Minneapolis, USA) for Mn^{2+} quench assay.

Ca^{2+} imaging of isolated single FDB cells

Isolated single FDB fibers were incubated with 4 µM Cal520-AM (AAT Bioquest, CA, USA) in the HEPES-Krebs solution for 30 min at 37°C. The fibers were then washed three times with the HEPES-Krebs solution to remove excess Cal520AM, and after 30 min of de-estrification, fluorescence images were obtained with a 20× (NA=0.75) objective lens of an inverted microscope (Nikon TE2000-E Japan) equipped with the sCMOS camera (Zyla, Andor, Belfast, Northern Ireland). Cal520 was excited at 480±15 nm, and fluorescence was measured at wavelengths of 525±25 nm. Imaging experiments were carried out at 26°C by superfusing a HEPES-Krebs solution (140 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 11 mM glucose, 5 mM HEPES, pH 7.4) with or without halothane and/or Compound 1.

Measurement of muscle force *in vitro*

Muscle contraction measurement was assessed using intact soleus muscle as described previously [47]. Briefly, mice were anesthetized with an *i.p.* injection of sodium pentobarbital (70 mg/kg body weight) and the soleus muscles were dissected. The isolated muscles were mounted between a force transducer (UL-100; Minebea Co., Tokyo, Japan) and a fixed hook in a chamber containing Krebs solution (140 mmol/L NaCl, 5 mmol/L KCl, 2 mmol/L $CaCl_2$, 1 mmol/L $MgCl_2$, 1 mmol/L NaH_2PO_4, 25 mmol/L $NaHCO_3$, and 11 mmol/L glucose) bubbled with 95% O_2 and 5% CO_2 at 25°C. The muscles were stretched to determine the

length which produced maximum twitch contraction in response to field stimulation for 1 msec at supramaximal voltage. For caffeine contracture studies, muscles were exposed for 2 min to Krebs solution containing 10, 15, or 20 mM caffeine. For twitch and tetanic contraction measurements, muscles were stimulated at 1 min intervals with 20 trains of 1, 2, 5, 10, 20, 50, 100, and 200 Hz pulses. Absolute contractile force was normalized to the maximum diameter of the muscle.

In vivo isoflurane challenge

Mice were weighed and then placed into an anesthetic chamber. Anesthesia was induced with 2.0-3.0 vol% isoflurane in air using a precision vaporizer (TK-7w BioMachinery isoflurane system, Japan) until there was no detectable response to tweezer pinches (within 60 s). Then, mice were rapidly placed atop a bed prewarmed at 37°C and anesthesia was maintained with isoflurane, 1.5 -2.0 vol% in air via a nose cone attachment. Rectal temperature was continuously monitored with a rectal probe (RET-3; Physitemp Instruments, LLC, NJ, USA) using Powerlab 26T (AdInstruments, Dunedin, New Zealand). Data were recorded every 10 s for up to 90 min or until the animal died of an MH crisis. Compound 1 was administered *i.p.* either 10 min before the start of isoflurane anesthesia for test of the preventive effect or when the rectal temperature was achieved 39°C for treatment of MH episodes.

In vivo heat stress challenge

To investigate heat stress responses, mice were anesthetized (*i.p.*) with an anesthetic mixture (0.75 mg/kg medetomidine, 4 mg/kg midazolam and 5 mg/kg butorphanol) and transferred to a test chamber prewarmed at 35°C. Rectal temperature was measured continuously during the heat stress challenge (up to 90 min) or until the time of fulminant heat stroke. Compound 1 was administered (*i.p.*) either 10 min before the start of heat stress challenge for test of the

preventive effect or when the rectal temperature was achieved 38 or 39°C for treatment of heat stroke.

Ca^{2+} determinations in intact muscle fibers using ion-specific microelectrodes

Ca^{2+} ion specific microelectrode recordings were performed as described previously [39,48]. For *in vivo* measurements, Wt and *RYR1*-p.R163C Het mice were anesthetized with 100 mg/kg ketamine and 5 mg/kg xylazine (*i.p.*) and placed on a 37°C heating pad and their temperature was monitored. The *vastus lateralis* was exposed surgically after which its muscle fibers were impaled with double-barreled Ca^{2+}-selective microelectrode, and the potentials were recorded via a high-impedance amplifier (WPI Duo 773 electrometer; WPI, Sarasota, FL, USA). The potential from the 3 M KCl microelectrode (V_m) was subtracted electronically from the potential of the Ca^{2+} electrode (V_{CaE}) to produce a differential Ca^{2+}-specific potential (V_{Ca}) that represents the $[Ca^{2+}]_i$. V_m and V_{Ca} were filtered (30–50 kHz) to improve the signal-to-noise ratio and stored in a computer for further analysis. Determinations were carried out at resting condition and then 5-10 min after commencing exposure to 1.5% isoflurane in the inspired air. Compound 1 (0-10 mg/kg) was administrated *i.p*. before exposure to isoflurane and measurements made every 5 min. For *in vitro* measurements, single flexor digitorum brevis (FDB) muscle fibers were enzymatically isolated as described above and placed in mammalian Ringer solution of following composition (in mM): 140 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 5 glucose, and 10 HEPES, pH 7.4. Muscle fibers were impaled with the microelectrodes as above and $[Ca^{2+}]_i$ was determined in the presence of varying (0-5 µM) concentrations of Compound 1.

Mn^{2+} quench assay

Assays were carried out as described previously [39]. Briefly, 5µM of Fura2-AM dye (Thermo Fisher Scientific) dissolved in mammalian Ringer solution (133 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 25 mM HEPES, 5.5 mM glucose, 2 mM $CaCl_2$, pH 7.4) was loaded onto the dissociated Wt and *RYR1*-p.G2435R Hom FDB fibers in 96 well plates for 30 mins at 37°C, 5% CO2. Fibers were then washed twice with Ringer solution and then incubated with the desired concentration of Compound 1 dissolved in Ringer solution for the final wash in the drug treated samples. After washing fibers were maintained at 37°C for 20 mins for de-esterification of Fura2-AM and in the presence of the tested concentration of Compound 1. The fibers were then transferred to the stage of a Nikon TE2000 inverted microscope (Nikon, Tokyo, Japan). Fura-2 was illuminated at its isosbestic wavelength (360nm) with the X-Cite 120 metal halide light source (Exfo, Ontario, Canada) and fluorescence emission at 510 nm was recorded using a 10 x 0.3na objective. The fibers were perfused with Ringer solution for 30 sec to obtain the baseline signal and then perfused with manganese quench buffer (133 mM NaCl, 5 mM KCl, 0.5 mM $MnCl_2$, 25 mM HEPES, 5.5 mM glucose, pH 7.4) for 180 s followed by reperfusion with Ringer solution for 30 sec to wash away the manganese quench buffer. Data were collected using an intensified 10-bit digital intensified CCD at 30 fps (Stanford Photonics, Stanford, CA) from regions of interest on 3-6 individual fibers per well and analyzed using Piper software (Stanford Photonics, Stanford, CA). The fluorescence signals were plotted as arbitrary fluorescence units over time. GraphPad Prism 7 was used to fit the baseline signal and quench signal into linear regression models ($y = mx + c$). The fluorescence quench rate was calculated by subtracting the slope of the baseline signal from that of the quench signal to obtain the net slope for both drug-treated and untreated samples.

Pharmacokinetics

Compound 1 (3 mg/kg and 10 mg/kg) dissolved in normal saline was administered by *i.p.*

injection to male C57BL/6J mice. Serial tail bled blood samples (~20 μL) were collected using heparinized tip at 5, 10, 30, and 60 min after administration. Aliquots (5μL) of plasma samples obtained from each blood were treated with 50 μL of acetonitrile and organic layer were injected onto LC-MS/MS system (Waters Corp., Milford, MA, USA). The pharmacokinetic parameters included maximum concentration (*C*max), elimination half-life (*T*½).

Metabolic stability in liver microsomes.

Compound 1 (final concentration was 0.5 μM) was incubated with human or mouse hepatic microsomes (XenoTech Ltd, Kansas City, KS) with nicotinamide adenine dinucleotide phosphate (NADPH) for 10 and 60 min at 37°C. Each reaction was stopped by acetonitrile, and the value of unchanged compound 1 was determined by quantitating using LC-MS/MS, and the metabolic stability was evaluated as a remaining rate (%).

Measurement of muscle force *in vivo*

Muscle force was measured *in vivo* by grip-force test. The tests were performed before and 10 and 60 min after *i.p*. injection of saline or Compound 1 (10 mg/kg). Mice (8- to 12-week old) were gently held by the tails and allowed them to grasp the horizontally positioned metal bar of the Grip Strength Meter (MK308M, Muromachi Kikai, Tokyo, Japan) with their forelimbs and hindlimbs (4-grips test). Each mouse performed four trials for force measurement at every time points. The highest force value applied to the metal bar was recorded and normalized by body weight [49].

Statistics

Data are presented as the mean ± SD. Statistical analysis was performed using Prism 8 (GraphPad Software, Inc., La Jolla, USA). Student's *t*-test was used for comparisons between two groups. One-way analysis of variance (ANOVA), followed by Dunnett's test, was performed to compare multiple groups. Statistical significance was defined as $p < 0.05$.

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Conflict of Interest

The authors declare that they have no conflict of interest.

Author contributions

T.Y., T.K., N.K. and T.M. concepted and designed the work. T.Y., T.K., N.K., M.K., S.Noguchi., T.I., Y.U.I., S.M., H.I., N.M., A.U., J.A., J.R.L, X.L., S.K., K.I., B.L., K.N. and T.M. acquired and analyzed data. T.Y., T.K., N.K., M.K., S.Noguchi, T.I., Y.U.I., I.N., S.M., H.I., N.M., H.K., A.U., J.A., J.R.L., X.L., P.D.A., S.K., K.I., B.L., K.N., S.Nakagawa, T.S. and T.M. interpreted data. T.Y., T.K., N.K., S.K., J.R.L, P.D.A. and T.M. wrote and revised the manuscript.

Figure legends

Fig. 1. Generation and characterization of *RYR1*-p.R2509C mice. *RYR1*-p.R2509C mice were generated by CRISPR/Cas9 system using single-stranded donor oligonucleotide carrying *Xba I* restriction site. **a.** Sequences of an amplified DNA fragment from a tail of Wt, Het, and Hom offspring. Sequencing confirms the presence of both the R2509C mutation and a Xba I site on both alleles in Hom and on one allele in Het. **b.** Gross morphology and allele-specific PCR-RFLP analysis of Wt, Het, and Hom embryos at E19 day. *Top*, Het embryos showed no apparent differences from Wt embryos, but Hom embryos were dead. *Bottom*, presence of a single 688 bp band denotes Wt mice, 366 bp and 326 bp bands (not well separated in this gel) denote Hom mice, and all three bands denote Het mice. **c.** Rectal temperature of mice after isoflurane challenge. Het mice but not WT mice exhibited elevation in rectal temperature and died by fulminant MH crisis (†). **d.** Het mice responded with fully body contractions as reflected in arching of their backs and extension of their legs. **e.** Maximum rectal temperature of Wt and Het mice during isoflurane challenge. *$p < 0.05$ compared to Wt. All the Het mice died (n=14). **f.** Time to death from isoflurane challenge. Female mice appeared to take longer time to death compared to male mice. *$p < 0.05$ compared to male mice. **g.** Time to death from body temperature of 39°C by isoflurane challenge. Variations were smaller than time to death and there was no difference between male and female mice.

Fig. 2. Intracellular Ca^{2+} signals in isolated FDB muscle cells from Wt and *RYR1*-p.R2509C Het mice. a. Structure of oxolinic acid and Compound 1. **b.** Representative Ca^{2+} signals in the isolated FDB muscle fibers from Wt and Het mice at various concentrations of halothane in the absence (Wt, Het) and presence of 0.1 μM Compound 1 (Het, Compound 1). Fluorescence Ca^{2+} signals (F) were normalized to the initial value (F_0). **c.**

Average resting Ca^{2+} signals at various concentrations of halothane and effects of 0.1 μM Compound 1. Data are means ± SD (Wt: n=4, Het: n=7, Het, compound 1: n=5). *p <0.05 compared to Wt. #p <0.05 compared to Het. **d, e.** Contractile function in soleus muscles from Wt and *RYR1*-p.R2509C Het mice. **d.** Caffeine contracture. Soleus muscles from Het mice exhibit much greater caffeine contracture than those from Wt mice. Compound 1 (3 μM) effectively reduces caffeine contracture of Het muscles. Data are given as box and whisker plots (Wt: n=6, Het: n=10, Het, compound 1: n=9). *p <0.05 compared to Wt. #p <0.05 compared to Het. **e.** Stress-frequency relationship in soleus muscles. No statistically significant difference is observed between Wt and Het muscles. Compound 1 (3 μM) significantly reduces twitch and tetanic tension. Data are means ± SD (Wt: n=13, Het: n=9, Het, compound 1: n=9). *p <0.05 compared to Wt. #p <0.05 compared to Het.

Fig. 3. *In vivo* isoflurane challenge in Wt and *RYR1*-p.R2509C Het mice. A-C. Preventive effects of Compound 1. **a.** Rectal temperature in mice after anesthesia by isoflurane. Compound 1 (0, 3, 10 mg/kg) was administered *i.p.* to Het mice 10 min before anesthesia. †, death by fulminant MH crisis. **b.** Maximum rectal temperature. Data are means ± SD (Wt: n=6; Het, 0 mg/kg: n=9; Het, 3 mg/kg: n=6; Het, 10 mg/kg: n=5). *p <0.05 compared to Wt. #p <0.05 compared to Het, 0 mg/kg. **c.** Survival rate of mice 90 min after anesthesia. Administration of Compound 1 at 10 mg/kg but not 3 mg/kg prevented Het mice from fulminant MH crisis. **d-f.** Rescue effects of Compound 1 on fulminant MH crisis in Het mice. **d.** Rectal temperature in mice after administration of Compound 1 during anesthesia. Mice were anesthetized by isoflurane and Compound 1 (0, 3, 10 mg/kg) was administered *i.p.* when their body temperature reached 39°C (arrow). †, death by fulminant MH crisis. **e.** Change in rectal temperature 10 min after administration of Compound 1. Data are means ± SD (0

mg/kg: n=14; Het, 3 mg/kg: n=10; Het, 10 mg/kg: n=8). #p <0.05 compared to 0 mg/kg. **f.** Survival rate of mice 60 min after administration of Compound 1.

Fig. 4. *In vivo* heat stress challenge in Wt and R2509C Het mice. Anesthetized mice were placed in a test chamber prewarmed at 35°C. Compound 1 (10 mg/kg) was administered 10 min before heat stress challenge. **a.** Rectal temperature in mice. **b.** Survival rate of mice 120 min after heat stress challenge. **c.** Time to death from beginning of heat stress challenge. Data are means±SD (0 mg/kg: n=13; 10 mg/kg: n=4). #p <0.05 compared to 0 mg/kg. **d-f.** Rescue effects of Compound 1 on heat stroke in Het mice. **d.** Rectal temperature in mice after administration of Compound 1 during heat stress challenge. Compound 1 (0, 3, 10 mg/kg) was administered *i.p.* when their body temperature reached 38°C (arrow). †, death by heat stroke. **e.** Change in the rectal temperature from 38°C at the endpoint. Data are means ± SD (0 mg/kg: n=13; 3 mg/kg: n=6; 10 mg/kg: n=5). #p <0.05 compared to 0 mg/kg. **f.** Survival rate of mice 60 min after administration of Compound 1.

Fig. 5. *In vivo* $[Ca^{2+}]_i$ homeostasis and divalent cation entry in skeletal muscle of MH model mice. a, b. $[Ca^{2+}]_i$ in *vastus lateralis* muscle from Wt and *RYR1*-p.R163C Het mice was determined *in vivo* by Ca^{2+} selective microelectrode. Compound 1 (0, 2.5, 5, 10 mg/kg) was administered *i.p.* 5 min before measurements. **a.** $[Ca^{2+}]_i$ at rest without anesthesia. **b.** $[Ca^{2+}]_i$ at rest during 1.5% isoflurane anesthesia. Compound 1 dose-dependently reduced $[Ca^{2+}]_i$ in Het mice to a level in Wt mice. Data are mean±SD (n=15-21). *p <0.05 compared to Wt. #p <0.05 compared to 0 mg/kg Compound 1. **c** and **d.** Divalent cation entry was measured by Mn^{2+} quench assay with the isolated FDB muscle fibers from Wt and *RYR1*-p.G2435R Hom mice. **c.** Change in fura-2 fluorescence by Mn^{2+} in the external solution. Trace for Hom with 10 μM Compound 1 was almost overlapped with that for Wt. **d.** Slope of

the decline in fura-2 fluorescence (f.a.u./s) after switching to Mn^{2+} containing external solution. Hom fibers show faster Mn^{2+} entry than Wt fibers, an indicative of enhanced divalent cation entry. Compound 1 reduces the rate of Mn^{2+} entry in Het fiber in a dose-dependent manner to a level in Wt fibers. Data are mean±SE (WT: n=4; Hom, 0 μM: n=73; Hom, 1 μM: n=39; Hom, 10 μM: n=78). *p <0.05 compared to Wt. #p <0.05 compared to Het, 0 mg/kg Compound 1.

Fig. 6. Pharmacokinetics of Compound 1 in mice. a. Averaged plasma concentration-time profiles of Compound 1 following *i.p.* injection of 3 mg/kg and 10 mg/kg. Data are mean±SD (n=3 per time point). **b.** Metabolic stability of Compound 1 in the presence of mouse (white) or human (black) liver microsomes. Residual rates of Compound 1 at 0 min were considered 100%. Data are mean±SD (n=4). **c.** Effect of Compound 1 on muscle force *in vivo*. Muscle force was measured *in vivo* by grip force test (4-grips test). The tests were performed before (pre) and 10 and 60 min after *i.p.* injection of saline or Compound 1. The force values were normalized by body weight (BW). Data are mean±SD (n=10). #*p*<0.05, compared to the value of saline-injected control.

Extended Data Table 1. Solubilities of Compound 1 sodium salt and dantrolene sodium salt in normal saline (0.9% NaCl) and 5% glucose aqueous solution.

	Normal saline	5% Glucose
Compound 1 (μg/mL)	849 ± 58	351 ± 14
Dantrolene (μg/mL)	25.7± 2.1	744 ± 22

*Values are means±SD (n=4).

Extended Data Fig. 1. Effect of Compound 1 on resting Ca^{2+} in skeletal muscle fibers. a. Representative effects of 100 nM Compound 1 (blue bar) on resting Ca^{2+} signals in FDB fibers from Wt and *RYR1*-p.R2509C Het mice. **b**. Resting Ca^{2+} levels before and during application of 0.1 μM Compound 1. Data are means ± SD (Wt: n=5, Het: n=6). *p <0.05 compared to Wt.

Extended Data Fig. 2. Typical traces for contractile function in soleus muscles from Wt and *RYR1*-p.R2509C Het mice. a. Caffeine contracture. Caffeine at indicated concentrations was applied for 2 min. Soleus muscles from Het mice exhibit much greater caffeine contracture than those from Wt mice. Pretreatment of muscles with Compound 1 (3 μM) effectively reduces caffeine contracture of Het muscles. **b.** Stress-frequency relationship in soleus muscles. Muscles were stimulated at 1 min intervals with 5 trains of 0.5 Hz pulse and subsequent 20 trains of 1, 2, 5, 10, 20, 50, 100, and 200 Hz pulses. Absolute contractile force was normalized to the maximum diameter of the muscle.

Extended Data Fig. 3. ***In vivo* heat stress challenge of Wt and *RYR1*-p.R2509C mice**. Mice were placed in a test chamber at 35°C. **a.** Rectal temperature of mice. Het mice but not WT mice exhibited rise in rectal temperature and died by fulminant heat stroke (†). **b.** Maximum rectal temperature of Wt and Het mice during heat stress challenge. Data are means ± SD (Wt: n=7; Het: n=13). *p <0.05 compared to Wt. **c-e.** Time to death from beginning of heat stress challenge (**c**), 38°C (**d**) or 39°C (**e**)**.** Data are means ± SD (beginning: n=13; 38°C: n=13; 39°C: n=12).

Extended Data Fig. 4. Rescue effect of Compound 1 on heat stress challenge in *RYR1*-p.R2509C Het mice. a. Rectal temperature in mice after administration of Compound

1 during heat stress challenge. Compound 1 (0 or 10 mg/kg) was administered *i.p.* when their body temperature reached 39°C (arrow). †, death by heat stroke. **b.** Change in the rectal temperature 10 min after administration of Compound 1 and the maximum values during measurement. Data are means±SD (0 mg/kg: n=8; 10 mg/kg: n=6). #p <0.05 compared to 0 mg/kg. **c.** Survival rate of mice 60 min after administration of Compound 1.

Extended Data Fig. 5. *In vitro* $[Ca^{2+}]_i$ homeostasis of skeletal muscles isolated from Wt and *RYR1*-p.R163C mice. FDB muscle fibers were isolated and $[Ca^{2+}]_i$ was determined using Ca^{2+} selective microelectrodes. $[Ca^{2+}]_i$ was significantly higher in quiescent Het fibers (326 ± 27 nM, n = 16) compared to Wt fibers (121 ± 3 nM, n = 20). Compound 1 reduced $[Ca^{2+}]_i$ in Het fibers in a dose-dependent manner. Data are means±SD (n=10-20). *p<0.05 compared to Wt. #p <0.05 compared to the value without Compound 1.

Extended Data Fig. 6. Effect of repeated application of Compound 1 on *in vivo* isoflurane challenge. Rectal temperature in *RYR1*-p.R2509C Het mouse was measured after anesthesia by isoflurane. Compound 1 (3 mg/kg) was administered *i.p.* when the body temperature reached 39°C (arrow). †, death by fulminant MH crisis.

Fig. 1

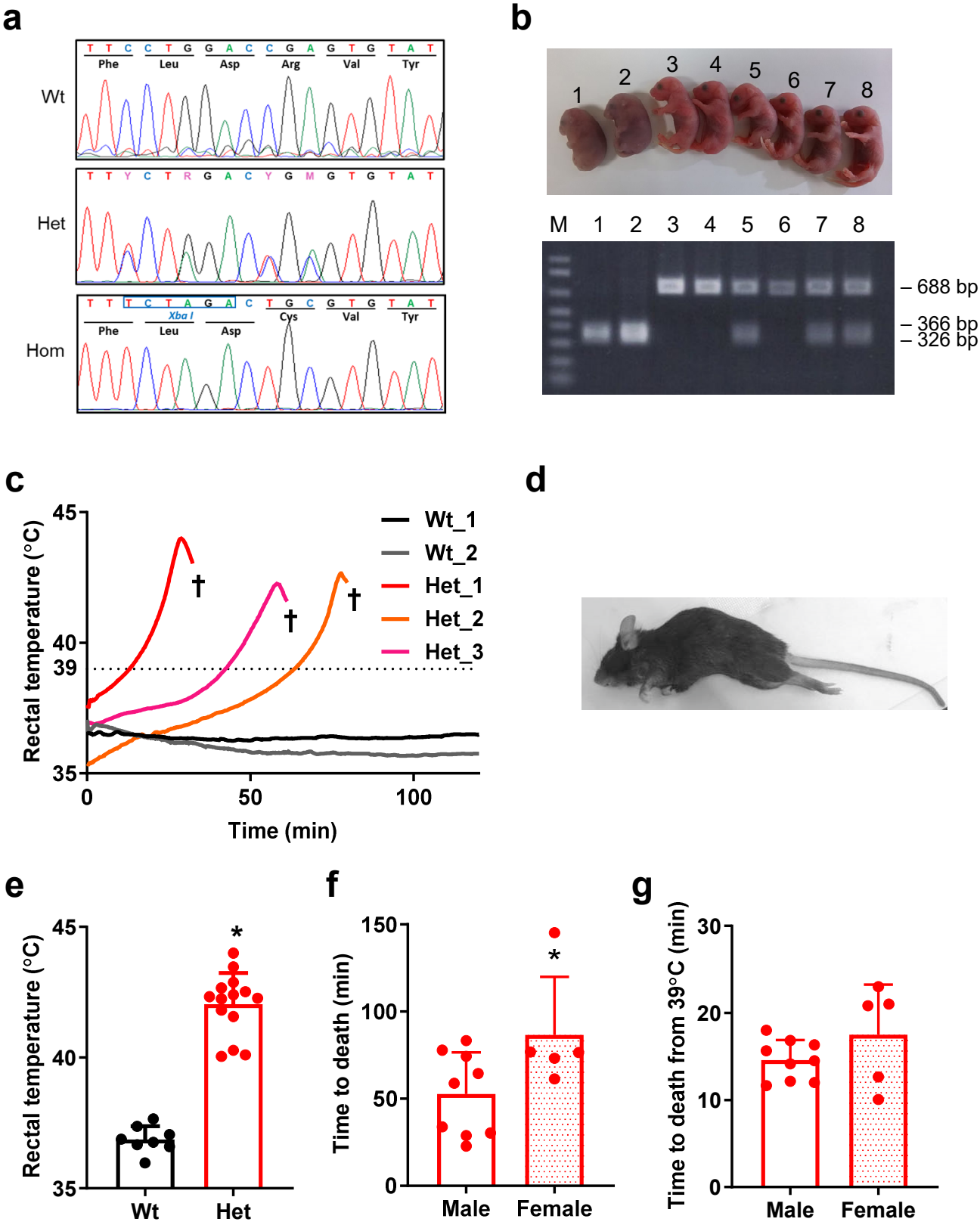

a
Wt
Phe
Leu
Asp
Arg
Val
Tyr
Het
Hom
Xba I
Cys
b
1
2
3
4
5
6
7
8
M
– 688 bp
– 366 bp
– 326 bp
c
Rectal temperature (°C)
Time (min)
Wt_1
Wt_2
Het_1
Het_2
Het_3
d
e
Rectal temperature (°C)
Wt
Het
f
Time to death (min)
Male
Female
g
Time to death from 39°C (min)
Male
Female

Fig. 2

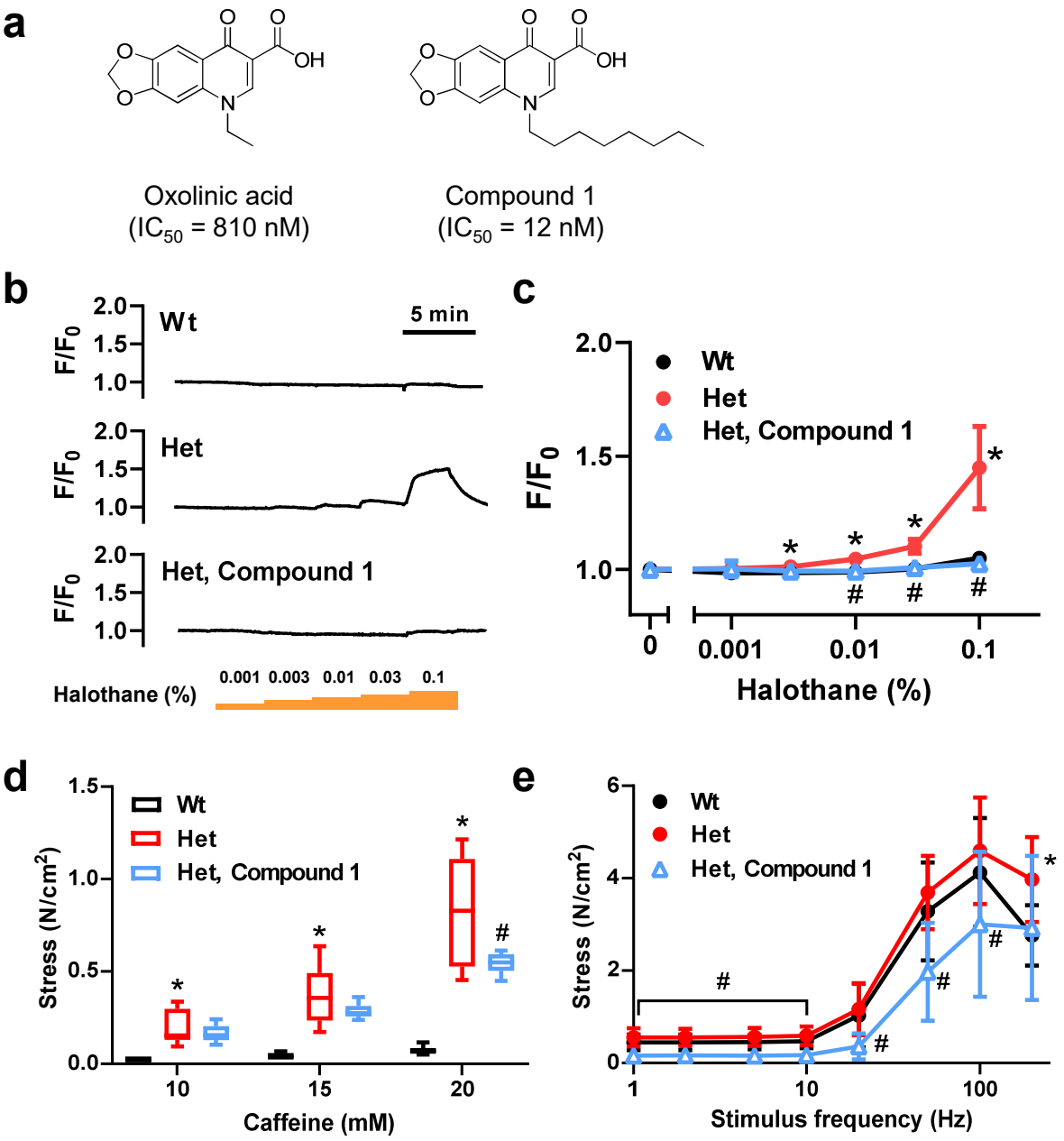

a
Oxolinic acid
(IC50 = 810 nM)
Compound 1
(IC50 = 12 nM)
b
Wt
Het
Het, Compound 1
5 min
F/F0
Halothane (%)
0.001 0.003 0.01 0.03 0.1
c
Wt
Het
Het, Compound 1
F/F0
Halothane (%)
d
Wt
Het
Het, Compound 1
Stress (N/cm2)
Caffeine (mM)
e
Wt
Het
Het, Compound 1
Stress (N/cm2)
Stimulus frequency (Hz)

Fig. 3

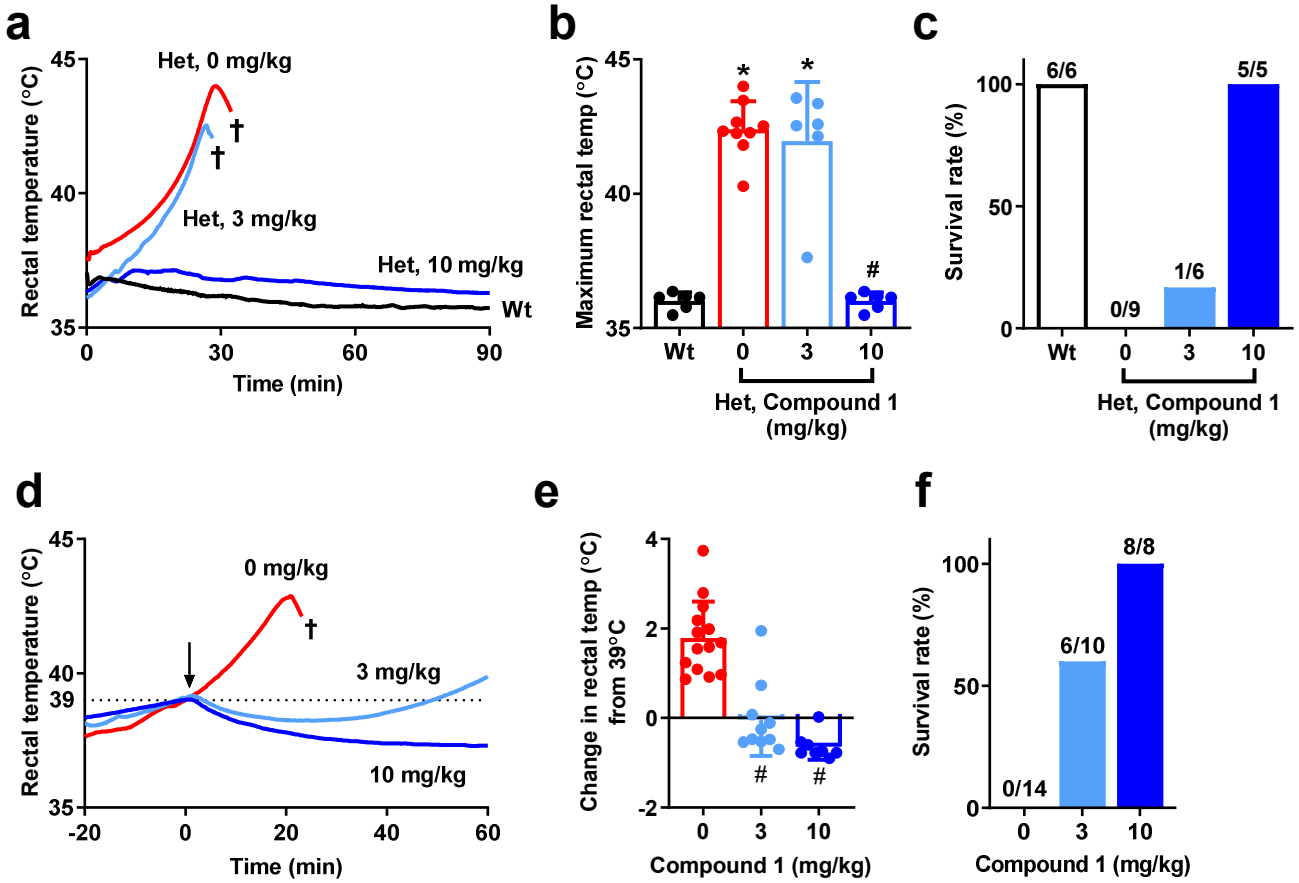

a
Rectal temperature (°C)
45
40
35
Het, 0 mg/kg
†
†
Het, 3 mg/kg
Het, 10 mg/kg
Wt
0
30
60
90
Time (min)
b
Maximum rectal temp (°C)
45
40
35
*
*
#
Wt
0
3
10
Het, Compound 1 (mg/kg)
c
Survival rate (%)
100
50
0
6/6
0/9
1/6
5/5
Wt
0
3
10
Het, Compound 1 (mg/kg)
d
Rectal temperature (°C)
45
40
39
35
0 mg/kg
†
3 mg/kg
10 mg/kg
-20
0
20
40
60
Time (min)
e
Change in rectal temp (°C) from 39°C
4
2
0
-2
#
#
0
3
10
Compound 1 (mg/kg)
f
Survival rate (%)
100
50
0
0/14
6/10
8/8
0
3
10
Compound 1 (mg/kg)

Fig. 4

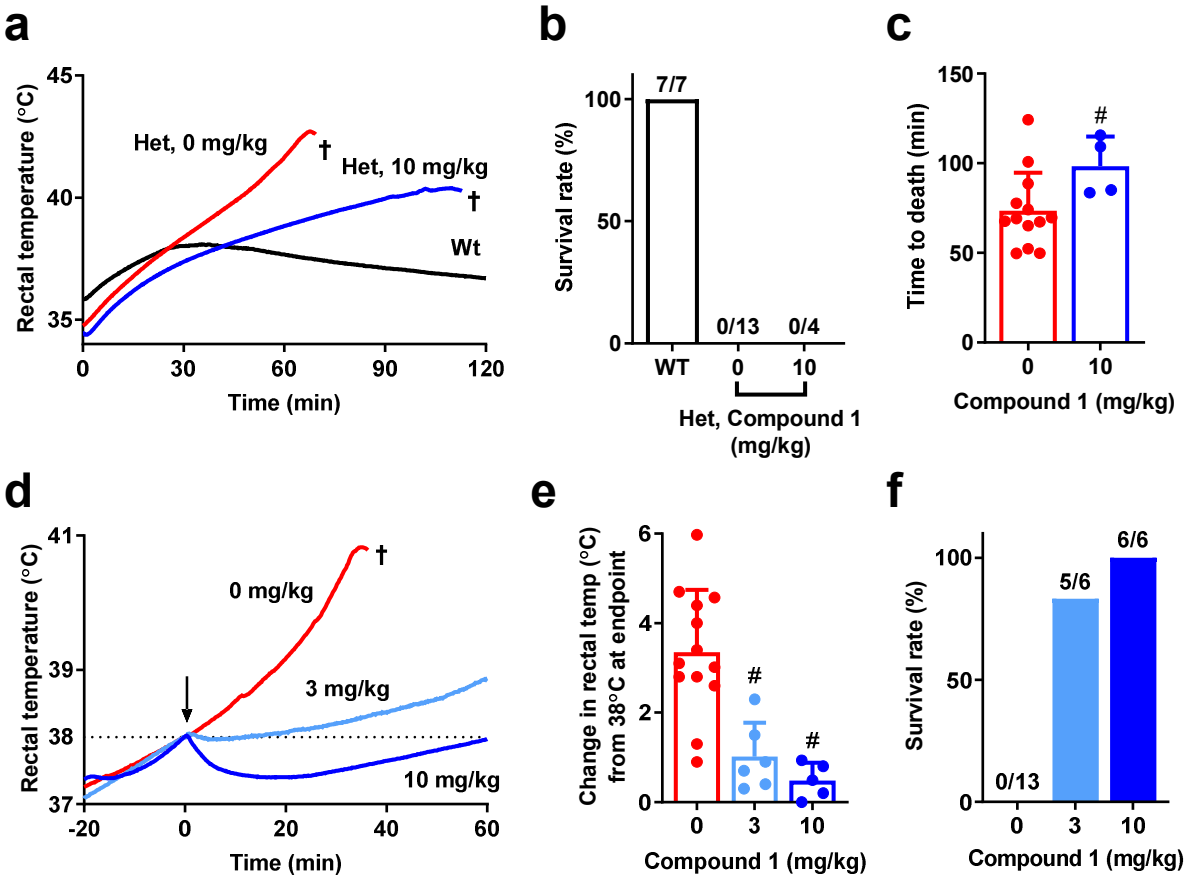

a
Rectal temperature (°C)
45
40
35
Het, 0 mg/kg
†
Het, 10 mg/kg
†
Wt
0
30
60
90
120
Time (min)
b
Survival rate (%)
100
50
0
7/7
0/13
0/4
WT
0
10
Het, Compound 1 (mg/kg)
c
Time to death (min)
150
100
50
0
#
0
10
Compound 1 (mg/kg)
d
Rectal temperature (°C)
41
39
38
37
0 mg/kg
†
3 mg/kg
10 mg/kg
-20
0
20
40
60
Time (min)
e
Change in rectal temp (°C) from 38°C at endpoint
6
4
2
0
#
#
0
3
10
Compound 1 (mg/kg)
f
Survival rate (%)
100
50
0
0/13
5/6
6/6
0
3
10
Compound 1 (mg/kg)

Fig. 5

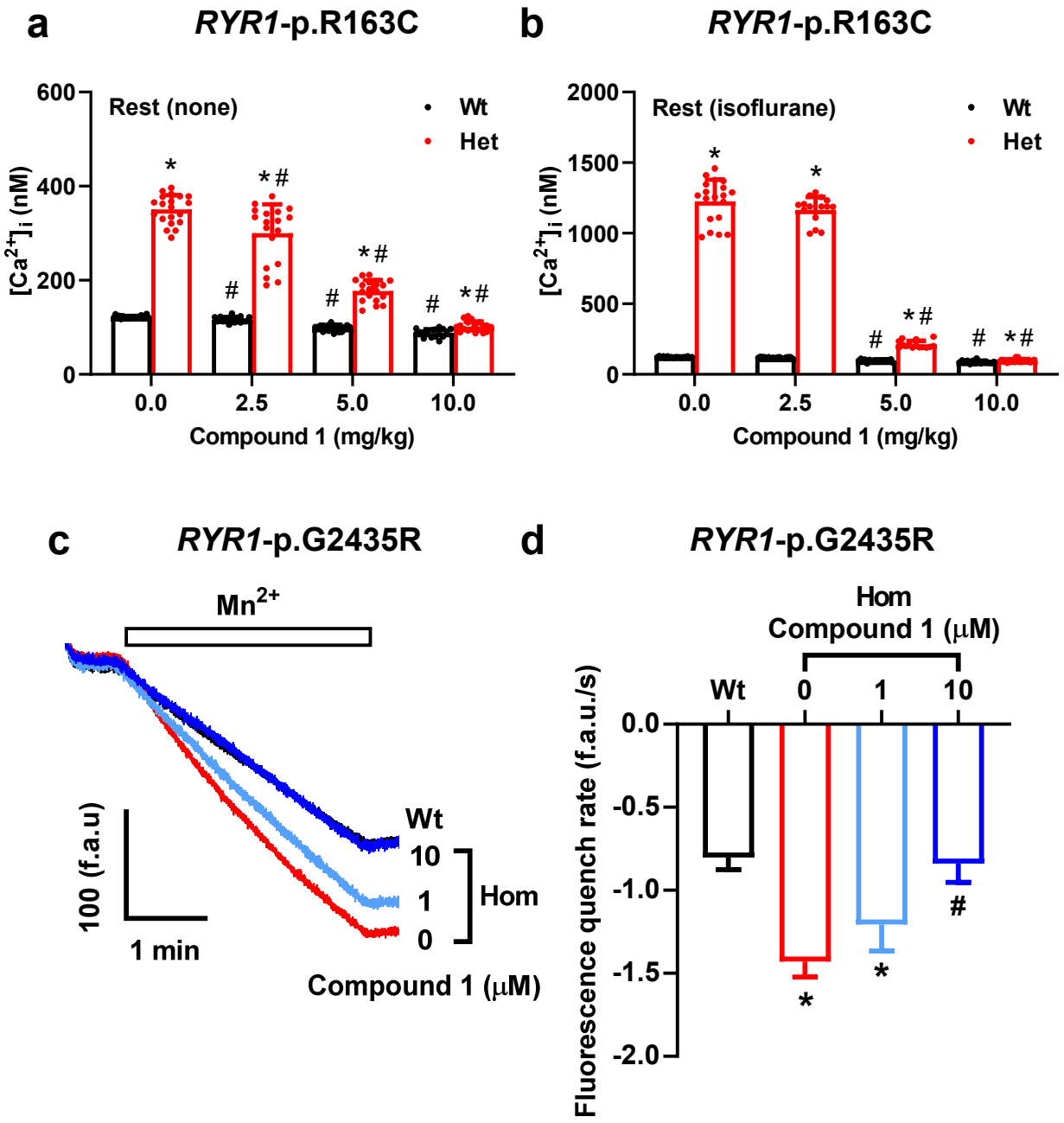
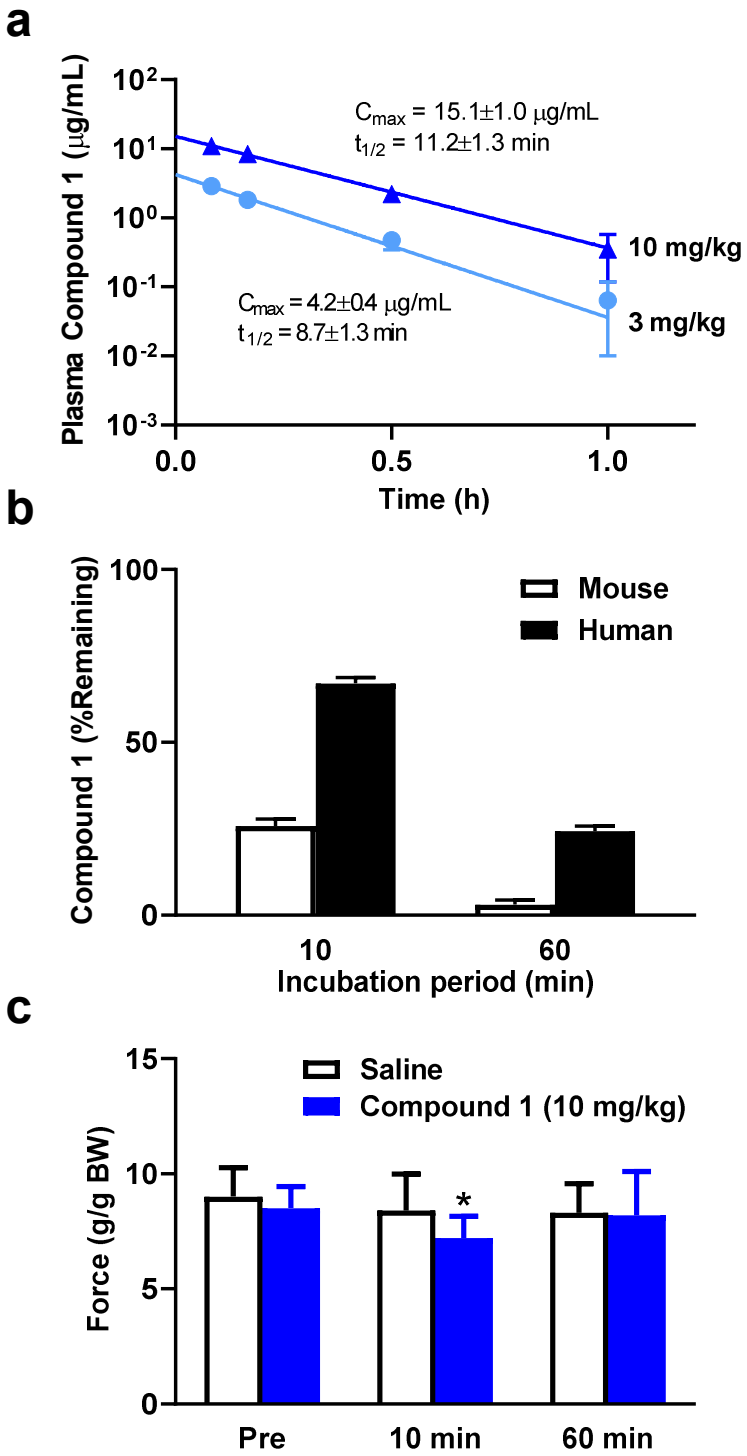
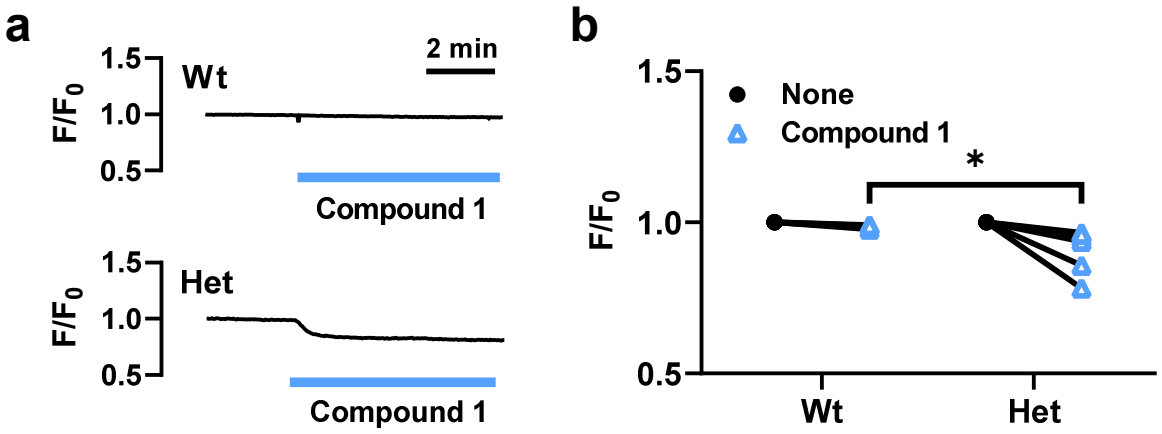
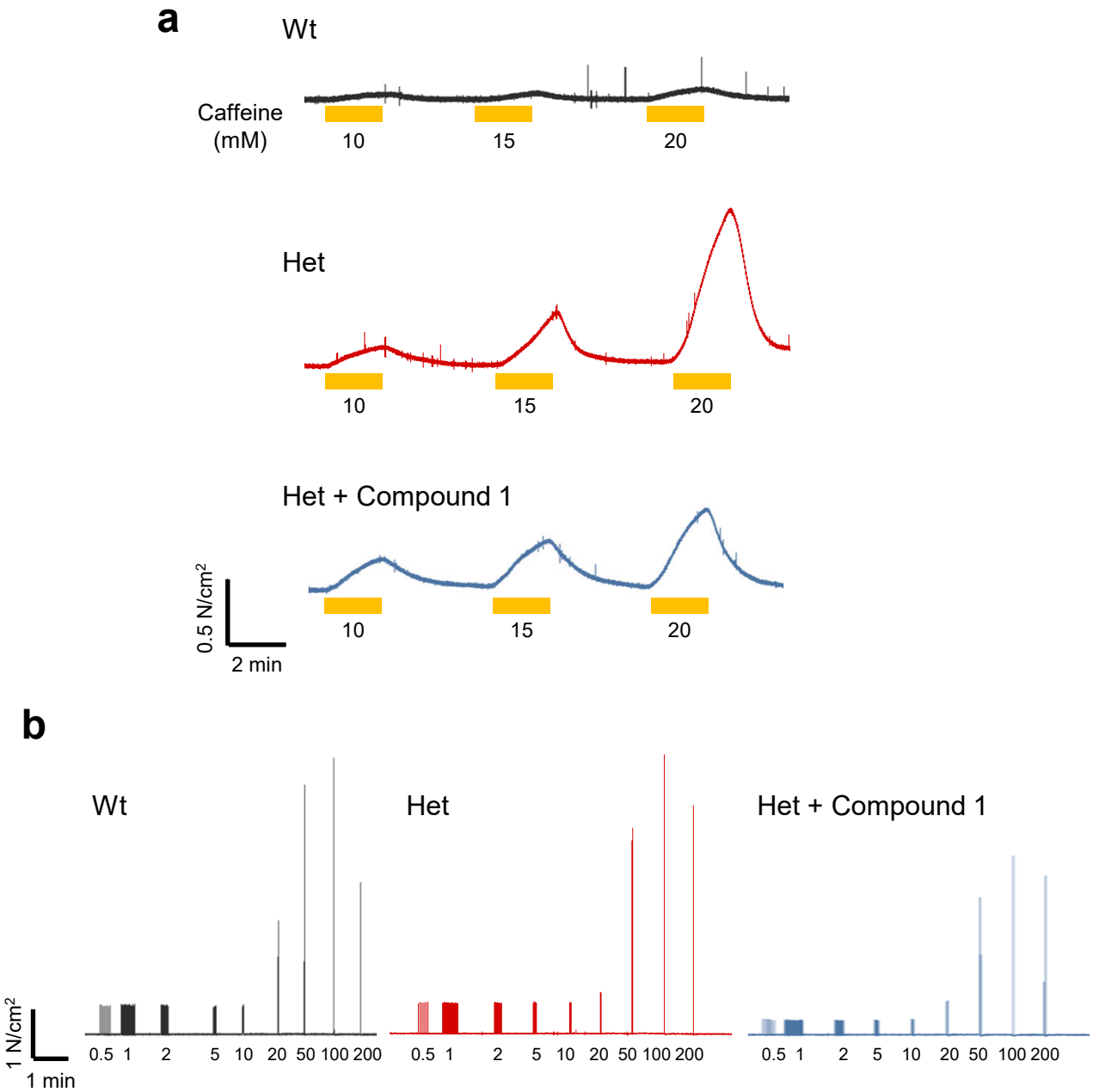

a
RYR1-p.R163C
Rest (none)
Wt
Het
[Ca2+]i (nM)
600
400
200
0
0.0
2.5
5.0
10.0
Compound 1 (mg/kg)
b
RYR1-p.R163C
Rest (isoflurane)
Wt
Het
[Ca2+]i (nM)
2000
1500
1000
500
0
0.0
2.5
5.0
10.0
Compound 1 (mg/kg)
c
RYR1-p.G2435R
Mn2+
100 (f.a.u)
1 min
Wt
10
1
0
Hom
Compound 1 (μM)
d
RYR1-p.G2435R
Hom
Compound 1 (μM)
Wt
0
1
10
Fluorescence quench rate (f.a.u./s)
0.0
-0.5
-1.0
-1.5
-2.0

Fig. 6

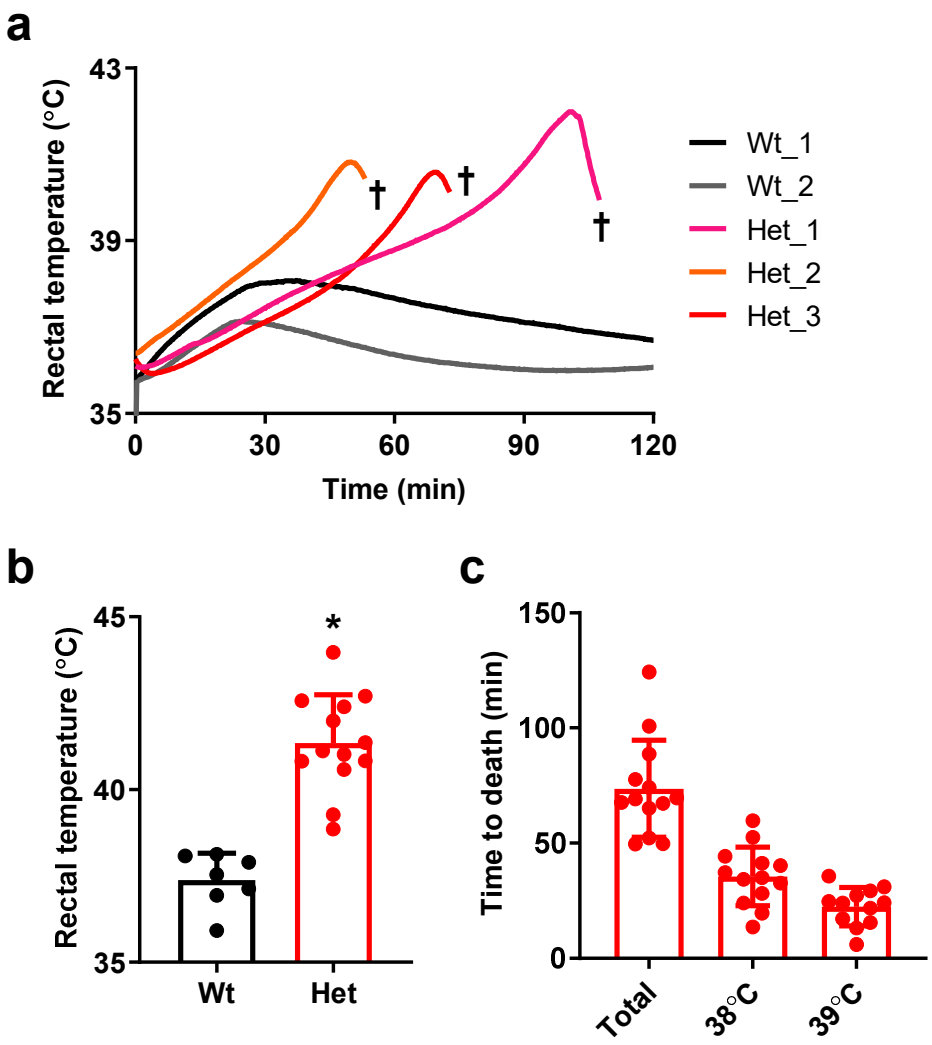

a
Plasma Compound 1 (μg/mL)
10^2
10^1
10^0
10^{-1}
10^{-2}
10^{-3}
C_{max} = 15.1±1.0 μg/mL
$t_{1/2}$ = 11.2±1.3 min
10 mg/kg
C_{max} = 4.2±0.4 μg/mL
$t_{1/2}$ = 8.7±1.3 min
3 mg/kg
0.0
0.5
1.0
Time (h)
b
Compound 1 (%Remaining)
100
50
0
Mouse
Human
10
60
Incubation period (min)
c
Force (g/g BW)
15
10
5
0
Saline
Compound 1 (10 mg/kg)
*
Pre
10 min
60 min



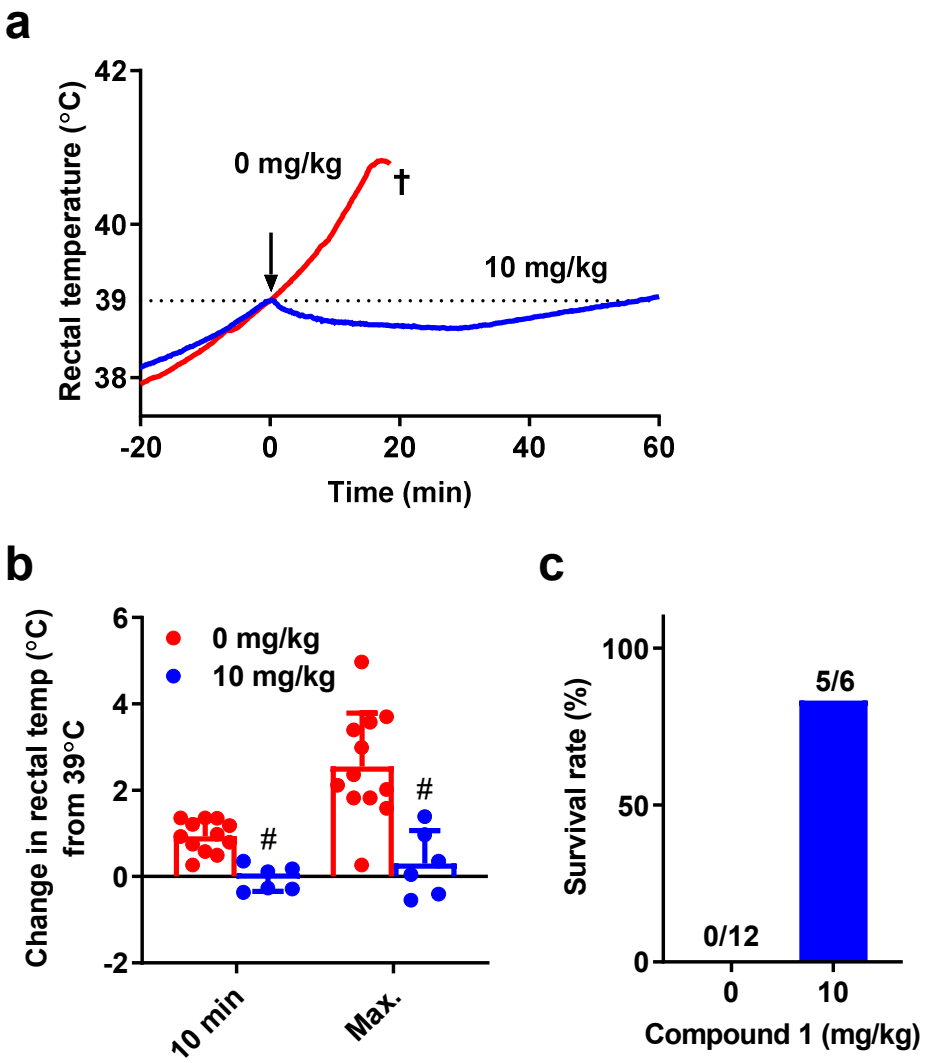
Extended Data Fig. 1. Effect of Compound 1 on resting Ca^{2+} in skeletal muscle fibers. a. Representative effects of 100 nM Compound 1 (blue bar) on resting Ca^{2+} signals in FDB fibers from Wt and *RYR1*-p.R2509C Het mice. **b**. Resting Ca^{2+} levels before and during application of 0.1 µM Compound 1. Data are means ± SD (Wt: n=5, Het: n=6). *p <0.05 compared to Wt.



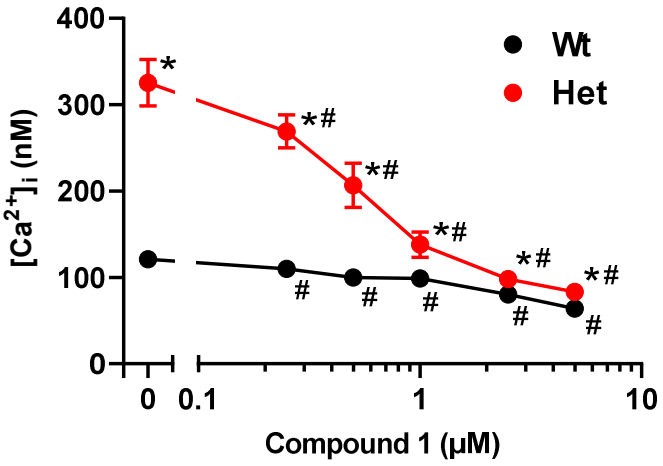
Extended Data Fig. 2. Typical traces for contractile function in soleus muscles from Wt and *RYR1*-p.R2509C Het mice. a. Caffeine contracture. Caffeine at indicated concentrations was applied for 2 min. Soleus muscles from Het mice exhibit much greater caffeine contracture than those from Wt mice. Pretreatment of muscles with Compound 1 (3 μM) effectively reduces caffeine contracture of Het muscles. **b.** Stress-frequency relationship in soleus muscles. Muscles were stimulated at 1 min intervals with 5 trains of 0.5 Hz pulse and subsequent 20 trains of 1, 2, 5, 10, 20, 50, 100, and 200 Hz pulses. Absolute contractile force was normalized to the maximum diameter of the muscle.



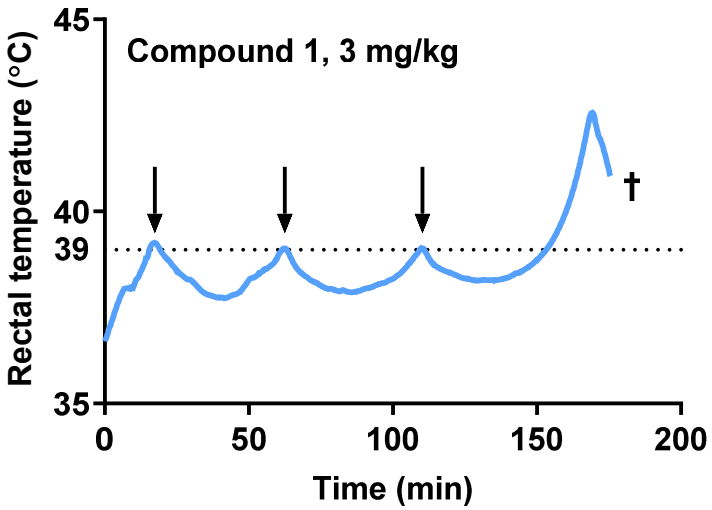
Extended Data Fig. 3. ***In vivo* heat stress challenge of Wt and *RYR1*-p.R2509C mice**. Mice were placed in a test chamber at 35°C. **a.** Rectal temperature of mice. Het mice but not WT mice exhibited rise in rectal temperature and died by fulminant heat stroke (†). **b.** Maximum rectal temperature of Wt and Het mice during heat stress challenge. Data are means ± SD (Wt: n=7; Het: n=13). *p <0.05 compared to Wt. **c-e.** Time to death from beginning of heat stress challenge (**c**), 38°C (**d**) or 39°C (**e**). Data are means ± SD (beginning: n=13; 38°C: n=13; 39°C: n=12).



Extended Data Fig. 4. Rescue effect of Compound 1 on heat stress challenge in *RYR1*-p.R2509C Het mice. a. Rectal temperature in mice after administration of Compound 1 during heat stress challenge. Compound 1 (0 or 10 mg/kg) was administered *i.p.* when their body temperature reached 39°C (arrow). †, death by heat stroke. **b.** Change in the rectal temperature 10 min after administration of Compound 1 and the maximum values during measurement. Data are means±SD (0 mg/kg: n=8; 10 mg/kg: n=6). #p <0.05 compared to 0 mg/kg. **c.** Survival rate of mice 60 min after administration of Compound 1.



Extended Data Fig. 5. *In vitro* $[Ca^{2+}]_i$ homeostasis of skeletal muscles isolated from Wt and *RYR1*-p.R163C mice. FDB muscle fibers were isolated and $[Ca^{2+}]_i$ was determined using Ca^{2+} selective microelectrodes. $[Ca^{2+}]_i$ was significantly higher in quiescent Het fibers (326 ± 27 nM, n = 16) compared to Wt fibers (121 ± 3 nM, n = 20). Compound 1 reduced $[Ca^{2+}]_i$ in Het fibers in a dose-dependent manner. Data are means ± SD (n=10-20). *$p<0.05$ compared to Wt. #$p<0.05$ compared to the value without Compound 1.



Extended Data Fig. 6. Effect of repeated application of Compound 1 on *in vivo* isoflurane challenge. Rectal temperature in *RYR1*-p.R2509C Het mouse was measured after anesthesia by isoflurane. Compound 1 (3 mg/kg) was administered *i.p.* when the body temperature reached 39°C (arrow). †, death by fulminant MH crisis.