A novel RyR1-selective inhibitor prevents and rescues sudden death in mouse models of malignant hyperthermia and heat stroke

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Mutations in the type 1 ryanodine receptor (RyR1), a Ca2+ 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 an oxolinic acid-derivative RyR1-selective inhibitor, 6,7-(methylenedioxy)-1-octyl-4-quinolone-3-carboxylic acid (Compound 1, Cpd1), effectively prevents and treats MH and heat stroke in several mouse models relevant to MH. Cpd1 reduces resting intracellular Ca2+, inhibits halothane- and isoflurane-induced Ca2+ release, suppresses caffeine-induced contracture in skeletal muscle, reduces sarcolemmal cation influx, and prevents or reverses the fulminant MH crisis induced by isoflurane anesthesia and rescues animals from heat stroke caused by environmental heat stress. Notably, Cpd1 has great advantages of better water solubility and rapid clearance in vivo over dantrolene. Cpd1 has the potential to be a promising candidate for effective treatment of patients carrying RyR1 mutations.
he 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. During excitation–contraction (E–C) coupling, RyR1 releases Ca^{2+} through physical association with the sarcoplasmic slow voltage gated Ca^{2+} channel (dihydropyridine receptor, DHPR) during depolarization of T-tubule membrane. This mechanism is referred to as depolarization-induced Ca^{2+} release (DICR). The RyR1 channel can also be directly activated by Ca^{2+}, which is referred to as Ca^{2+}–induced Ca^{2+} release (CICR), although its importance in the function is much lower than RyR2 and RyR3.

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

The only drug approved for ameliorating the symptoms of MH is dantrolene. Dantrolene was first developed in 1960s as a muscle relaxant and was later shown to prevent Ca^{2+} release by direct interaction with RyR1. However, dantrolene has several disadvantages for clinical use: the main disadvantage is its poor water solubility which makes rapid preparation difficult. This disadvantage has been developed over 40 years since the first discovery.

We have recently identified oxolinic acid as a RyR1-selective inhibitor with better water solubility using efficient high-throughput screening platform for RyR1 inhibitors. 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, Cpd1) with extremely high potency which is comparable to dantrolene in vitro study. In this study, we tested whether Cpd1 has the ability to reversibly prevent and reverse a 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 Cpd1 is rapidly metabolized in mice. Our results provide crucial evidence for Cpd1 as a RyR1 inhibitor that may prove to be clinically useful.

Results

Generation of 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 Methods, Fig. 1a). An additional XbaI site was introduced to screen the genotype by PCR-RFLP. The homozygous RYR1-p.R2509C mice died in late embryonic stage, but heterozygous RYR1-p.R2509C (R2509C) mice grew normally and were as fertile as wild type (WT) (Fig. 1b).

It has previously been shown when 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 lead to death. When anesthetized by isoflurane, nearly 80% (68 out of 83) heterozygous R2509C mice rapidly increased their rectal temperature to over 40°C (42.3 ± 1.5°C, n = 16) (Fig. 1c, d), exhibited muscle rigidity (Fig. 1c, inset) and died within 90 min (64 ± 30 min, n = 16) from start of exposure (Fig. 1e). In the remaining R2509C mice, exposure to isoflurane caused an increase in rectal temperature but it did not exceed 39°C. In contrast, WT mice showed no elevation in rectal temperature in response to isoflurane (Fig. 1c, d). Elevation of rectal temperature looks two-phase reaction; it rose slowly at first and then surged around 39°C (Fig. 1c). Interestingly, time from rectal temperature of 39°C to death was quite similar among all individual mice (16 ± 4 min, n = 16) (Fig. 1e). We further investigated sex difference in response to isoflurane. More male mice (90%) succumbed to isoflurane than females (65%) (Fig. 1f). Among the mice that succumbed, there was no significant sex difference in the rectal temperature (Fig. 1g) and time to death (Fig. 1h, i). These findings suggest that R2509C mice can reproduce human MH symptoms in response to exposure to volatile anesthetics and thus are a useful model for test of Cpd1.

Effect of Cpd1 on intracellular Ca^{2+} homeostasis in the isolated single muscle cells. Cpd1 is a derivative of oxolinic acid with an octyl group at nitrogen atom (oxolinic acid has an ethyl group). This modification increased the potency for inhibiting the RyR1 channel nearly 70-fold in a HEK293 cell-based assay (IC_{50} values from 810 nM for oxolinic acid to 12 nM for Cpd1). Since Cpd1 is a free acid, it is virtually water insoluble. We therefore prepared its sodium salt, which neutralizes the carboxyl group and significantly increases water solubility (Fig. 2a). The Cpd1 sodium salt exhibited good solubility in normal saline (0.9% NaCl) as well as pure water and glucose solution (Table 1). In contrast, dantrolene sodium salt was soluble in pure water and glucose solution but hardly soluble in normal saline, which is in accord with previous studies.

We initially tested the effect of Cpd1 in in vitro experiments using enzymatically isolated single flexor digitorum brevis (FDB) muscle cells from the R2509C mice. Abnormal resting intracellular Ca^{2+} ([Ca^{2+}]_i) homeostasis in skeletal muscle cells is one of the most common features associated with MH in susceptible humans. We therefore examined the effects on muscle cells when exposed to CsCl. At 0.1 μM reduced resting [Ca^{2+}], [Ca^{2+}]_i was significantly lower in R2509C cells in a dose-dependent manner (Fig. 2b, c). Pretreatment of R2509C cells with Cpd1 (0.1μM) reduced resting [Ca^{2+}], [Ca^{2+}]_i (Supplementary Fig. 1a, b) and completely abolished halothane-induced increases in [Ca^{2+}], [Ca^{2+}]_i (Fig. 2b, c). We then used the ratiometric Ca^{2+} indicator fura-2 to estimate the unstimulated resting [Ca^{2+}], and whether or not it could be modified by Cpd1. At rest fura-2 F_{340}/F_{380} ratios were significantly higher in R2509C cells than in WT cells and Cpd1 reduced [Ca^{2+}]_i in R2509C cells in a dose-dependent manner but had no significant effect on WT (Fig. 2d). Similar to the effects of halothane, exposure to isoflurane increased [Ca^{2+}]_i in R2509C but not in WT cells and Cpd1 completely prevented this effect (Fig. 2e, f). It has been shown that sarcoclemmal cation influx is accelerated in skeletal muscle of several MH model mice compared with WT mice. This is due to enhanced sarcoclemmal Ca^{2+} entry in response to chronic reduced SR Ca^{2+} load. We therefore measured resting Mn^{2+} quench of fura-2 fluorescence. Unexpectedly, rate of Mn^{2+} quench in R2509C muscles was not different from WT and in this model Cpd1 had no effect (Supplementary Fig. 2a, b).
Effect of Cpd1 on contraction force in isolated muscles. The In Vitro Contracture Test (IVCT) using muscle biopsies is widely used for diagnosis of MH susceptibility in humans. A subject is considered MH positive if the muscle exhibited a greater contracture at a lower dose of caffeine or halothane. We therefore tested the effect of Cpd1 on caffeine-induced contracture in soleus muscles isolated from R2509C mice. WT muscles showed only minimal contracture in responses to caffeine up to 20 mM (Fig. 3a). In contrast, R2509C muscles exhibited dose-dependent contracture during exposure to caffeine and pretreatment with 3 µM Cpd1 decreased caffeine-induced contracture tension of R2509C muscles at 20 mM caffeine (Fig. 3b).

It has been reported that soleus muscles from MH model mice exhibit heat-induced increase in the basal force at temperatures of 35 °C or higher. Basal tension at 42 °C was significantly increased in R2509C solei compared to WT muscles (Fig. 3c, d) and pretreatment with 3 µM Cpd1 significantly reduced the heat-induced contracture in R2509C muscles.

Dantrolene is known to suppress twitch and tetanic contractions in both normal and MH muscles. We tested whether Cpd1 affects twitch and tetanic contractions and whether or not there was a difference in the Cpd1 effect between MH and WT. Untreated soleus muscles from both WT and R2509C mice showed similar twitch and tetanic contraction force and...
treatment with 3 μM Cpd1 significantly reduced both twitch and tetanic contraction force in both groups (Fig. 3e–g). The effect was stronger on twitch (72% inhibition) (Fig. 3f) than on tetanic (35% inhibition at 100 Hz) (Fig. 3g) contractions, which is similar to dantrolene41.

Effect of Cpd1 on in vivo isoflurane challenge. We next tested whether Cpd1 can prevent or treat MH episodes in R2509C mice in vivo. We first tested the preventive effect of Cpd1 on MH episodes in response to isoflurane exposure. We used male mice for the experiments, since male mice were more sensitive to isoflurane than female mice (see Fig. 1f). Doses of either 3 mg/kg or 10 mg/kg of Cpd1 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. 4a–c). 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, Cpd1 successfully prevented any rise in rectal temperature (Fig. 4a, b). All the treated mice survived 90 min after induction of anesthesia (Fig. 4c) and they behaved normally for at least 24 h after recovery from anesthesia.

We next tested whether Cpd1 could rescue mice from MH episodes. R2509C mice (both males and females) 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 reduce rectal temperature by −0.03 ± 0.81 °C at 10 min after administration, compared to an increase of 1.79 ± 0.82 °C in controls, but rectal temperature started to rise again ~30 min after administration of the drug in some mice (Fig. 4d, e). The 10 mg/kg dose decreased body temperature by −0.64 ± 0.29 °C at 10 min after administration and maintained it at that level until the experiment was terminated 60 min after administration of the drug. At that time 60% of the mice in the 3 mg/kg group and 100% of the mice in the 10 mg/kg group survived (Fig. 4f) and all the survivors behaved normally for at least 24 h after experiments.

Effect of Cpd1 on in vivo environmental heat stress challenge. It has been shown that environmental heat stress causes an increase in body temperature and death in other MH model mice29,30,33,34. We therefore tested whether R2509C 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 at a relatively constant level around 37 °C (37.4 ± 0.8 °C, n = 7), the rectal temperature of R2509C mice steadily increased to 41.4 ± 1.4 °C (n = 13) (Supplementary Fig. 3a). Almost all the mice responded to heat and died (Supplementary Fig. 3b). Although the time from start of heat stress to death largely varied among individual mice, the variation in time to death after reaching body temperatures of 38 °C (36 ± 13 min, n = 13) or 39 °C (22 ± 8 min, n = 12) became smaller (Supplementary Fig. 3c).

Using this model, we initially tested the preventive effect of Cpd1 on the heat stroke. Pretreatment of mice with Cpd1 (10 mg/kg) slowed the rate of temperature rise (Fig. 5a), but maximum rectal temperature did not change (Fig. 5b) and none of the treated mice (0 out of 4) survived 120 min of heat stress (Fig. 5c). 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. 5d).

We next tested whether Cpd1 could rescue mice from environmental heat stroke. The R2509C mice were incubated in the test chamber and the drug was administered i.p. when their rectal temperature reached 39 °C. All the untreated mice died within 30 min of the time of injection (time to death, 22 ± 8 min, n = 13). The 3 mg/kg Cpd1 dose slowed rise in the body temperature post injection (Fig. 5e, f) and 3 out of 5 mice survived 60 min after administration (Fig. 5g). The 10 mg/kg Cpd1 dose showed more pronounced effect: it suppressed rise in the body temperature and 10 out of 11 mice survived for 60 min after administration. After removing from the heat stress environment, the survivors behaved normally for at least 24 h after being and regaining consciousness. When the drug was administered at body temperature of 38 °C, after which most untreated mice died within 60 min (time to death, 36 ± 13 min, n = 13), both the 3 and 10 mg/kg Cpd1 doses strongly suppressed the continued rise in the body temperature (Supplementary Fig. 4a, 4b) and almost all mice survived 60 min after its administration (Supplementary Fig. 4c).

Pharmacokinetics of Cpd1. We observed transient effects with the 3 mg/kg dose of Cpd1 in rescue experiments in both in vivo isoflurane and heat stress challenges with R2509C mice (see Figs. 4d and 5e). This implies fast metabolism or excretion of the drug in mice. We therefore measured plasma concentration of Cpd1 after i.p. administration. Cpd1 rapidly declined in mouse blood following first order kinetics with t1/2 of ~8 min for both the 3 mg/kg and 10 mg/kg dose (Fig. 6a). There was no sex difference in the kinetics of Cpd1 (Supplementary Fig. 5a, b). Since Cpd1 has hydrophobic moiety of octyl group (see Fig. 2), it can be metabolized in the liver. In vitro drug metabolism assay using
mouse liver microsomes demonstrated that Cpd1 was reduced to 20% and 5% of the original amount at 10 min and 60 min, respectively (Fig. 6b). Cpd1 was metabolized more slowly by human liver microsomes; it was reduced to 20% at 60 min.

The duration of the effect of Cpd1 in mice was also evaluated using measurements of in vivo muscle strength, since we showed that the drug inhibited twitch and tetanic contractions in 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 Cpd1, and that this deficit was almost completely recovered at 60 min, suggesting short duration of the medicinal effect in vivo (Fig. 6c). No sex nor genotype differences were observed in the effect of Cpd1 on in vivo muscle contraction strength (Supplementary Fig. 6a–c).

The above results indicate that transient effects of Cpd1 in the isoflurane challenge and heat challenge experiments are due to rapid metabolism of the drug in mice. In fact, second and third administrations of the 3 mg/kg dose when rectal temperature reached 39 °C successfully decreased the rectal temperature (Supplementary Fig. 7). We therefore tested whether repeated administrations of Cpd1 could prevent mice from the crisis. Eight
administrations of 1 mg/kg Cpd1 at 10 min intervals successfully prevented rise in the rectal temperature by exposure either to isoflurane (Fig. 6d) or to heat stress (Fig. 6e) and all the mice survived the full 90 min after initiating either challenge (Fig. 6f). Pharmacokinetic analysis of Cpd1 after repeated administrations revealed that Cpd1 rapidly declined from mouse blood as was the case of single administration (Fig. 6g). Interestingly, $t_{1/2}$ was slightly longer in female (14.8 ± 2.1 min, $n = 3$) than in male (9.6 ± 2.7 min, $n = 3$), whereas $C_{\text{max}}$ was significantly higher in males (0.67 ± 0.11 µg/mL, $n = 3$) than in females (0.15 ± 0.11 µg/mL,
Fig. 3 Contractile function in soleus muscles from WT and R2509C mice. a Typical traces of basal stress after exposure to caffeine. Caffeine at indicated concentrations was applied for 2 min. Soleus muscles from R2509C mice exhibit much greater increase in basal stress after exposure to caffeine than those from WT mice. Pretreatment of muscles with Cpd1 (3 μM) effectively reduces the increase in basal stress caused by caffeine in R2509C muscles. b Dose dependence of the increase in basal stress in response to caffeine exposure. Data are shown as means with individual data points (n = 5 for each group) and were analyzed by one-way ANOVA with Tukey’s test. c Typical traces of basal stress with increased temperature. d The average values of basal stress for 30 s after exposure to 42 °C. Data are shown as means with individual data points (n = 5 for each group) and were analyzed by one-way ANOVA with Tukey’s test.

n = 3). Muscle weakness was transiently observed after the repeated administrations in both male and female, but it returned to normal by 60 min (Fig. 6h).

Effect of Cpd1 on RYRI-p.R163C MH model mice. We demonstrated that Cpd1 prevented and reversed MH and heat stroke in R2509C mice. To test whether this is also the case with other MH model mice carrying different mutations, we examined the effects of Cpd1 on heterozygous RYRI-p.R163C (R163C) mice using in vivo measurements of [Ca²⁺]i with Ca²⁺-selective microelectrodes. Resting [Ca²⁺]i was threefold higher in R163C muscle (330 ± 43 nM, n = 17) compared to WT muscle (122 ± 3 nM, n = 20) (Fig. 7a). Administration of Cpd1 i.p. effectively reduced [Ca²⁺]i in R163C
muscle in a dose-dependent manner. Cpd1 also reduced \([Ca^{2+}]_i\) in WT muscles, but the effect was significantly smaller. When mice were exposed to 1.5% (v/v) isoflurane, \([Ca^{2+}]_i\) rapidly increased in untreated R163C muscles (1226 ± 154 nM, \(n = 19\)), indicating a MH episode (Fig. 7b, c). Administration of isoflurane had no effect on \([Ca^{2+}]_i\) in WT muscles (121 ± 4 nM, \(n = 18\)) (Fig. 7c). Pretreatment with Cpd1 suppressed increase in \([Ca^{2+}]_i\) in R163C muscle in a dose-dependent manner (Fig. 7b, c) and effectively prevented the MH episode and death at doses of 5 and 10 mg/kg (Fig. 7d). When Cpd1 at 10 mg/kg was administered to R163C mice 15 min after induction of isoflurane anesthesia, it was able to abort the MH crisis and rapidly decreased \([Ca^{2+}]_i\) to the levels measured in WT muscle (Fig. 7e, f).

To validate in vivo experiments, we tested the effect of Cpd1 in FDB muscle fibers isolated from the R163C mice. Similar to the in vivo findings, \([Ca^{2+}]_i\) was significantly higher in quiescent R163C fibers (326 ± 27 nM, \(n = 16\)) compared to WT fibers (121 ± 3 nM, \(n = 20\)) and Cpd1 reduced \([Ca^{2+}]_i\) in R163C fibers in a dose-dependent manner to the level of WT fibers. (Supplementary Fig. 8).

**Effect of Cpd1 on **\(RYR1\)-p.G2435R MH model mice.** We also tested the effect of Cpd1 on a third MH model using homozygous \(RYR1\)-p.G2435R (G2435R) mice. G2435R muscles have also been shown to have enhanced cation influx compared to WT which is thought to be in response to chronic reduced SR Ca\(^{2+}\) load. To test the effect of Cpd1 on sarcolemmal cation influx, we measured resting Mn\(^{2+}\) quench of fura-2 fluorescence in isolated G2435R and WT skeletal muscle fibers. The rate of Mn\(^{2+}\) quench in G2435R fibers was twice that in WT fibers (Fig. 8c, d). Cpd1 reduced Mn\(^{2+}\) quench in G2435R fibers in a dose-dependent manner and the Mn\(^{2+}\) quench rate at the 10 µM dose was the same as that in untreated WT fibers.

**Discussion**

Here we examined the therapeutic effects of Cpd1, an oxolinic acid-derivative RyR1-selective inhibitor with high potency, on the volatile anesthetic and increased environmental temperature-induced MH crisis. Cpd1 showed good solubility in normal saline (Table 1). Using three different model mice, we demonstrated...
that Cpd1: (1) prevents increase in \([\text{Ca}^{2+}]_i\) by volatile anesthetics (halothane and isoflurane) and decreases resting \([\text{Ca}^{2+}]_i\) in skeletal muscle in vitro and in vivo (Figs. 2 and 7), (2) inhibits sarcolemmal cation entry caused by chronic store \([\text{Ca}^{2+}]_i\) depletion (Fig. 8), (3) inhibits caffeine- and heat-induced contracture in isolated skeletal muscle (Fig. 3), (4) effectively prevents and treats isoflurane-induced fulminant MH crisis (Figs. 4 and 7), and (5) treats severe heat stroke caused by environmental heat stress (Figs. 5 and 8). These results provide crucial evidence that Cpd1 is capable of effectively preventing and treating the MH crisis and heat stroke by inhibiting the RyR1 channel.

Several strains of MH mouse models carrying mutations in the \(\text{RYR1}\) gene have been reported to date.\(^29,30,33,34\) We created a mouse model carrying the \(\text{RYR1}-\text{p.R2509C}\) mutation, which corresponds to...
n = mutations in the common to those seen in the other model mice carrying severe MH microelectrode. Iso after exposure to iso normally and were as fertile as WT (Fig. 1). R2509C mice showed a milder phenotype than R163C mice.

Although dantrolene is the only compound that is available and used to treat and prevent MH, a major problem of dantrolene in clinical use is its poor aqueous solubility. 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 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.

Another drawback of dantrolene in clinical use is its long plasma half-life (10–12 h in humans after intravenous
solution. Fura-2 fluorescence quenching in G2435R fibers almost overlapped with that for WT. D Slope of the decline in fura-2 fluorescence (f.a.u./sec) after switching to 1 mM Mn$^{2+}$ containing external solution. G2435R fibers show faster fluorescence quenching than WT fibers, an indicative of enhanced divalent cation entry. Cpd1 reduces the rate of fluorescence quenching in G2435R fibers in a dose-dependent manner to the level seen in WT fibers. Data are shown as box-whisker plot, with the median for all subjects shown as the center line, the box representing the 25–75 percentile, and the lines showing the range of the data (WT: n = 73; G2435R, 0 μM: n = 73; G2435R, 1 μM: n = 39; G2435R, 10 μM: n = 60) and were analyzed by one-way ANOVA with Tukey’s test. Source data are provided as a Source Data file.

Fig. 8 Effect of Cpd1 on homozygous RYR1-p.G2435R (G2435R) mice. 

a–b In vivo heat challenge of G2435R mice. Mice were placed in a 38 °C test chamber. Cpd1 (0 or 30 mg/kg) was administered i.p. 10 min before heat challenge. a Time course of rectal temperature of mice. The steepest and least steep representative rectal temperature changes from 0 mg/kg and 30 mg/kg Cpd1 groups are shown. b Slope of temperature change from onset to endpoint at 40 °C. Data are means ± SD (0 mg/kg: n = 7; 30 mg/kg: n = 5) and were analyzed by two-tailed unpaired t-test. c–d Divalent cation entry was measured by Mn$^{2+}$ quench assay with isolated FDB muscle fibers. c Change in fura-2 fluorescence by switching to 1 mM Mn$^{2+}$ containing external solution. Fura-2 fluorescence was expressed as fluorescence arbitrary units (f.a.u.). The trace for G2435R with 10 μM Cpd1 almost overlapped with that for WT. d Slope of the decline in fura-2 fluorescence (f.a.u./sec) after switching to 1 mM Mn$^{2+}$ containing external solution. G2435R fibers show faster fluorescence quenching than WT fibers, an indicative of enhanced divalent cation entry. Cpd1 reduces the rate of fluorescence quenching in G2435R fibers in a dose-dependent manner to the level seen in WT fibers. Data are shown as box-whisker plot, with the median for all subjects shown as the center line, the box representing the 25–75 percentile, and the lines showing the range of the data (WT: n = 78; G2435R, 0 μM: n = 73; G2435R, 1 μM: n = 39; G2435R, 10 μM: n = 60) and were analyzed by one-way ANOVA with Tukey’s test. Source data are provided as a Source Data file.

injection)$. Therefore, it is difficult to control the appropriate plasma concentration and reduce side effects such as muscle weakness. Pharmacokinetic analysis revealed that Cpd1 is rapidly metabolized in mice with plasma half-life of ~8 min, probably by metabolism by the liver (Fig. 6). Muscle weakness was completely recovered within 60 min (Fig. 6). Rapid metabolism of Cpd1 was also true even after repeated administration of 1 mg/kg Cpd1 at 10 min intervals, which successfully prevented rise in the rectal temperature by exposure either to isoflurane or heat stress (Fig. 6). Thus, Cpd1 may be beneficial for a treatment of MH episodes without the concern of residual weakness.

Heat stroke is a life-threatening condition clinically diagnosed as a severe elevation in body temperature with central nervous system dysfunction. It has been reported that MH mutations in the RYR1 gene is implicated in some heat stroke as well as overdose of 2,4-dinitrophenol (a prohibited weight loss agent that interrupts ATP synthesis and causes hyperthermia). Since Cpd1 has similar effects in preventing and treating MH and heat stroke, it might also be a potential candidate for treatment of these emergency situations.

**Methods**

**Materials.** Cpd1 was synthesized according to our reported procedure. Briefly, a solution of benzol[de][1,3]dioxol-5-amine and diethyl ethoxymethyleneimaleonate in ethanol was heated at 90 °C for 18 h. After the evaporation, the residue was extracted with ethyl acetate and water. The organic layer was washed with brine, dried over sodium sulfate and concentrated under reduced pressure. The residue was purified by silica gel column chromatography to give diethyl 2-((benzo[d][1,3]dioxol-5-ylamino)methylene)malonate. A solution of 2-((benzo[d][1,3]dioxol-5-ylamino)methylene)malonate in diphenylether was heated at 250 °C for 10 h. After cooling, precipitates were washed with ether, and dried under reduced pressure to give ethyl 8-oxo-5,8-dihydro-1,3[dioxol]-4,5-[g]quinoline-7-carboxylate. A solution of ethyl 8-oxo-5,8-dihydro[1,3]dioxol[4,5-g]quinoline-7-carboxylate in dry DMF was added to a suspension of sodium hydride in dry dimethylformamide at 0 °C. After stirring for 10 min at room temperature, a solution of o-nitryl bromide was added to the mixture. The reaction mixture was...
heated at 55 °C for 9 h. After removal of the solvent in vacuo, the residue was extracted with ethyl acetate and water. The organic layer was washed with brine, dried over anhydrous sodium sulfate, and evaporated. The residue was purified by silica gel column chromatography to give a mixture of ethyl and n-octyl ester of 5-octyl-8-oxo-5,8-dihydo[1,3]dioxolo[4,5-c]quinoline-7-carboxylic acid, which was used in the next hydrolysis step without further separation. 2 M NaOH was added to a solution of a mixture of ethyl and n-octyl ester of 5-octyl-8-oxo-5,8-dihydo[1,3]dioxolo[4,5-c]quinoline-7-carboxylic acid in MeOH, and the mixture was stirred at room temperature for 8 h. The reaction mixture was quenched with ice-cold 2 M hydrochloric acid and stirred for 1 h to form colorless precipitates. The precipitates were collected, washed with water, and recrystallized to give Cpd1. Sodium salt of Cpd1 was dissolved in 3 mL of ethyl acetate and evaporated. The residue was puriﬁed by silica gel column chromatography to give a mixture of ethyl and n-octyl ester of 5-octyl-8-oxo-5,8-dihydo[1,3]dioxolo[4,5-c]quinoline-7-carboxylic acid, which was used in the next hydrolysis step without further separation. The residue was washed with 1 mL of normal saline, pure water, or 5% glucose aqueous solution and dried under reduced pressure to give the sodium salt of Cpd1 as colorless powder.

**Determination of Cpd1 thermodynamic solubility.** Test compound (2 mg) was suspended with 1 mL of normal saline, pure water, or 5% glucose aqueous solution and sonicated for 10 min to afford white precipitates. Test compound (2 mg) was dissolved in 3 mL of ethyl acetate and evaporated. The residue was puriﬁed by silica gel column chromatography to give a mixture of ethyl and n-octyl ester of 5-octyl-8-oxo-5,8-dihydo[1,3]dioxolo[4,5-c]quinoline-7-carboxylic acid, which was used in the next hydrolysis step without further separation.

**Animal experiments.** All animal-handling procedures were in accordance with the guidelines and approval of the ethics committee of the Institute of Medical Science of the University of Tokyo. Animals were housed in isolator cages, fed with food and water ad libitum, and kept under controlled environment with 12:12 light/dark cycles, 23–25 °C temperature, and 50–60% relative humidity in specific pathogen-free conditions in the Jikei University, Juntendo University, National Institute of Neuroscience, MCNP, Mount Sinai Hospital and UK Home Of

**Measurement of muscle force in vivo.** Muscle contraction measurement was assessed using intact soleus muscle as follows. WT and R2509C mice (8- to 20-week old) 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 on a force transducer (UX-100; Minebea Co., Tokyo, Japan) and a force transducer in a Krebs chamber containing (in mM) NaCl 5 mM, KCl 1 mM, MgCl2 11 mM glucose, 2 mM CaCl2, 10 mM HEPES, pH 7.4) to obtain the baseline signal and then perfused with Mn2+ quenching Krebs solution (140 mM NaCl, 5 mM KCl, 1 mM CaCl2, 11 mM glucose, 0.5 mM MnCl2, 10 mM HEPES, pH 7.4) for 180 s followed by reperfusion with basal Krebs solution for 120 s.

**In vivo isoﬂurane challenge.** WT and R2509C mice (8- to 20-week old) were weighed and then placed into an anesthetic chamber. Anesthesia was induced with 2.0–1.0% isoﬂurane in air using a precision vaporizer (TR-7W BioMachinery, Japan). 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 heat-induced contracture studies, muscles were exposed to Krebs solution warnned at 27, 35, 40, and 42 °C until basal force peaked. For twitch and tetanic contraction measurements, muscles were stimulated with basal Krebs solution containing 10, 20, 50, 100, and 200 Hz pulses. For Cpd1 treatment, muscles were incubated with Krebs solution containing 3 μM Cpd1 min prior to measurements. Absolute contractile force was normalized to the maximum diameter of the muscle.

**Ca2+ determinations in intact muscle fibers using ion-specific microelectrodes.** C2+- ion specific microelectrode recordings were performed as follows. For in vivo measurements, WT and heterozygous R163C mice (12- to 16-week old) were anesthetized by i.p. injection of 100 mg/kg ketamine and 5 mg/kg xylazine 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 Ca2+-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 (Vag) was subtracted electronically from the potential of the Ca2+-selective electrode (VCa) to produce a differential Ca2+-specific potential (VCa) that represents the [Ca2+]i. Vag and VCa 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 supramaximal voltage. When the rectal temperature was achieved 39 °C for treatment of MH episodes, the temperature was increased to 40 °C the experiment was ended and the animal was euthanized by cervical dislocation.

**In awake restrained G2435R mice: in this model to investigate the heat stress response, G2435R mice (12- to 16-week old) were administered Cpd1 i.p., placed into a mouse restrainer (Kent Scientific, CT, USA), and placed in an environmental isolarure anesthesia (Japan) system (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 supramaximal voltage. When the rectal temperature was achieved 39 °C for treatment of MH episodes, the temperature was increased to 40 °C the experiment was ended and the animal was euthanized by cervical dislocation.
digitorium brevis (FDB) muscle fibers were enzymatically isolated as described above and placed in mammalian Ringer solution of following composition (in mM): 140 NaCl, 5 mM KCl, 2 CaCl₂, 1 MgCl₂, 5 glucose, and 10 HEPES, pH 7.4. Muscle fibers were impaled with the microelectrodes as above and [Ca²⁺], was determined in the presence of varying (0–5 µM) concentrations of Cpd1.

Mn²⁺–quench assay. Assays were carried out as follows: 5 µM of fura-2-AM dye (Thermo Fisher Scientific) dissolved in mammalian Ringer solution (133 mM NaCl, 5 mM KCl, 1 mM MgSO₄, 25 mM HEPES, 5.5 mM glucose, 2 mM CaCl₂, pH 7.4) was loaded into dissociated WT and homozygous G2435R FDB fibers in 96 well plates for 30 min at 37 °C, 5% CO₂. Fibers were then washed twice with Ringer solution and then incubated with the desired concentration of Cpd1 dissolved in Ringer solution for the final wash in the drug-treated samples. After washing fibers were maintained at 37 °C for 20 min for de-esterification of fura2-AM and in the presence of the tested concentration of Cpd1. 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 (360 nm) with the X-Cite 120 metal halide light source (Exfo, Ontario, Canada) and fluorescence emission at 510 nm was recorded using a 10 ×0.3na objective. The fibers were perfused with Ringer solution for 30 s to obtain the baseline signal and then perfused with manganese quench buffer (133 mM NaCl, 5 mM KCl, 0.5 mM MnCl₂, 25 mM HEPES, 5.5 mM glucose, pH 7.4) for 180 s followed by reperfusion with Ringer solution for 30 s 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 v1.3.04 (Stanford Photonics, Stanford, CA). The fluorescence signals were plotted as fluorescence arbitrary units (f.a.u.) over time. Prism v7 (GraphPad Software, Inc., La Jolla, USA) was used to fit the baseline signal and quench signal into linear regression models. 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. Cpd1 sodium salt (3 mg/kg and 10 mg/kg) dissolved in normal saline was administered i.p. to WT C57BL/6 J mice (10 week-old). Serial tail bled samples (~20 µL) were collected using heparinized tip at 5, 10, 30, and 60 min after administration. Aliquots (5 µL) of plasma samples obtained from each blood sample were enzymatically isolated as described above and [Ca²⁺]c, was determined by quantitating using LC-MS/MS system (Waters Corp., Milford, MA, USA) and pharmacokinetic parameters included maximum concentration (Cmax), elimination half-life (T1/2).

Metabolic stability in liver microsomes. Cpd1 (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 organic layer were injected onto LC-MS/MS system (Waters Corp., Milford, MA, USA) the pharmacokinetic

Measurement of muscle force in vivo. Muscle force was measured in vivo by grip-force test. The tests were performed as blinded, so the experimenter did not know which kind of solution (vehicle or Cpd1) was injected to each mouse. The force was measured before and 10 and 60 min after i.p. injection of saline or Cpd1 (10 mg/kg). Mice (8–12 week-old) were gently held by the tails and allowed them to grasp the horizontally positioned metal bar of the Grip Strength Meter (MK300M, Muromachi Kikai, Tokyo, Japan) with their forelimbs and hindlimbs (4-grips test). Each mouse performed four trials for force measurement at every time point. The highest force value applied to the metal bar was recorded and normalized by body weight[1].

Statistics. Data are presented as means ± SD. Unpaired Student’s t test was used for comparisons between two groups. One-way or two-way analysis of variance (ANOVA) followed by Tukey’s test was performed to compare three or more groups. Two-tailed tests were used for all analyses. Statistical analysis was performed using Prism v7 and v8 (GraphPad Software, Inc., La Jolla, USA).

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability. All data generated or analyzed in this study are available within the article and its Supplementary Information. All raw data supporting the findings from this study are available from the corresponding author upon request. Source data are provided with this paper.

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Author contributions
T.Y., T.K., N.K., and T.M. conceived and designed the work. T.Y., T.K., N.K., M.K., S.Noguchi, T.L., Y.U.I., S.M., H.I., N.M., A.U., J.A., J.R.I., X.L., C.D., S.K., K.I., B.L., Y.I., Y.N., and T.M. acquired and analyzed data. T.Y., T.K., N.K., M.K., S.Noguchi, T.L., Y.U.I., LN., S.M., H.I., N.M., H.K., A.U., J.A., J.R.I., X.L., C.D., P.D.A., S.K., K.I., B.L., Y.I., Y.N., S.N., 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. All authors reviewed and approved the revised manuscript.

Competing interests
The authors declare no competing interests.

Additional information
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