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# Fropofol prevents disease progression in mice with hypertrophic cardiomyopathy

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**Aims** Increased myofilament contractility is recognized as a crucial factor in the pathogenesis of hypertrophic cardiomyopathy (HCM). Direct myofilament desensitization might be beneficial in preventing HCM disease progression. Here, we tested whether the small molecule fropofol prevents HCM phenotype expression and disease progression by directly depressing myofilament force development.

**Methods and results** Force, intracellular $Ca^{2+}$, and steady-state activation were determined in isolated trabecular muscles from wild-type (WT) and transgenic HCM mice with heterozygous human α-myosin heavy chain R403Q mutation (αMHC 403/+). αMHC 403/+ HCM mice were treated continuously with fropofol by intraperitoneal infusion for 12 weeks. Heart tissue was analysed with histology and real-time PCR of prohypertrophic and profibrotic genes. Fropofol decreased force in a concentration-dependent manner without significantly altering $[Ca^{2+}]_i$ in isolated muscles from both WT and αMHC 403/+ HCM mouse hearts. Fropofol also depressed maximal $Ca^{2+}$-activated force and increased the $[Ca^{2+}]_i$ required for 50% activation during steady-state activation. In whole-animal studies, chronic intra-abdominal administration of fropofol prevented hypertrophy development and diastolic dysfunction. Chronic fropofol treatment also led to attenuation of prohypertrophic and profibrotic gene expression, reductions in cell size, and decreases in tissue fibrosis.

**Conclusions** Direct inhibition of myofilament contraction by fropofol prevents HCM disease phenotypic expression and progression, suggesting that increased myofilament contractile force is the primary trigger for hypertrophy development and HCM disease progression.

**Keywords** Hypertrophic cardiomyopathy • Myofilament • Fropofol • Calcium

## 1. Introduction

Hypertrophic cardiomyopathy (HCM) is one of the most common genetic heart disorders, with a prevalence of 1 in 200 (0.6% of the general population).[1,2] Hallmarks of the disease process include a hypercontractile state resulting from increased ATPase sensitivity to $Ca^{2+}$,[3,4] faster cross-bridge sliding velocity,[5,6] and higher force production by individual cross-bridges.[7,8] Thus, β-adrenergic receptor blockers and $Ca^{2+}$ channel blockers, which inhibit contraction primarily by decreasing $Ca^{2+}$ availability, often are not very effective.[9] The ineffectiveness of these drugs underscores the fact that the increased contractility stems from mutations in the myofilament proteins themselves. Importantly, increased power output was recently shown to be the primary stimulus for disease progression, and the small myosin ATPase inhibitive molecule MYK-461, which targets the force-generating cross-bridges, proved to be preventive.[10] This

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breakthrough study used a small molecule to bind the same protein that carries a single missense mutation to oppose increases in contractility. However, the dramatic decrease in force at higher concentrations of the drug could limit the margin of safety and cause heart failure if overdosed. Thus, we are still searching for safe small molecule myofilament desensitizers/inhibitors.

One potential candidate is fropofol, a small molecule derivative of propofol (a well-known anaesthetic). It shares many chemical and physical properties with propofol but lacks anaesthetic efficacy.[11] Recently, we characterized the effect of fropofol in normal rat cardiac muscles and those with elevated contractility.[12] Fropofol decreased force development by targeting directly to force-generating cross-bridges and myofilament proteins. Moreover, the cardiac force development was depressed by 30% at maximum effect. These unique features of fropofol motivated us to test its ability to prevent HCM disease development and progression.

# 2. Methods

## 2.1 Animal care

We used a well-known mouse model of transgenic HCM,[13] which has a heterozygous arginine (R)-to-glutamine (Q) mutation in α-myosin at position 403 (αMHC 403/+ HCM mice). These mice recapture the key features of human HCM. Animal care and experimental protocols were approved by the Animal Care and Use Committee of The Johns Hopkins University School of Medicine. All studies complied with the NIH guidelines or the guidelines from Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes.

## 2.2 Intraperitoneal implantation of mini-osmotic pumps

Forty male αMHC 403/+ HCM mice and 10 male wild-type (WT) mice were used in the study. Of the 40 αMHC 403/+ HCM mice, 20 were randomly selected to receive chronic fropofol treatment and 20 were untreated. The 10 WT mice were also divided into fropofol and untreated groups. We implanted mini-osmotic pumps (Alzet, Cupertino, CA, USA) into the abdomen of all mice when they were 14 weeks old, when hypertrophy began to develop. Mice were anaesthetized with 3% isoflurane and continuously monitored for heart rate, breathing, and temperature. An area of ∼1–2 $cm^2$ in the lower abdomen (either side) was de-haired and sterilized with alcohol. A 3–5 mm incision was made into the abdominal cavity and an Alzet osmotic pump filled with fropofol (100 μL, rate: 0.11 μL/h) was implanted into the cavity. The incision was closed with sutures and then dressed with sterile tape. The animals were observed for approximately 1 h and placed back into their cages if no abnormalities were noted. Pain was controlled with ketamine (1 mg/kg) if needed. Each implantation lasted ∼4 weeks and the animals were subjected to two more re-implantations of fropofol-loaded pumps for a total of 12 weeks. Mice tolerated the implantation and re-implantation surgeries well in our preclinical trials. The pump allowed a maximal concentration of 0.11 mg/h delivery based on the special gravity ratio of 0.93. At this rate, the (free) blood concentration of fropofol was ∼3 μM after 1 week and was maintained thereafter for the remainder of the trial, as determined by HPLC. After a week of recovery from the surgery, all mice in the trial were subjected to 90 min swimming twice a day to simulate strenuous exercise. The inclusion of strenuous exercise was intended to promote pathologic remodelling, including hypertrophy and fibrosis, in the HCM mice, as we found in our preliminary studies and as was shown in recent animal studies.[14,15] To prevent drowning, the mice were trained to swim for 1 week before the trial. The training consisted of incremental 15 min periods of swimming from day 1 to 7. All mice tolerated swimming training well. At the end of 12 weeks treatment, all mice were sacrificed for *in vitro* experiments.

To verify that the 3 μM concentration of fropofol would be effective *in vivo* with coronary perfusion, we assessed the effect of fropofol on left ventricular (LV) pressure development in Langendorff perfused mouse hearts at body temperature (Supplementary material online, *Figure S1*). In brief, mice were euthanized with an anaesthetic overdose of pentobarbital (100 mg/kg) injected intraperitoneally (i.p.). After deep anaesthesia was achieved, the hearts were excised, mounted, and perfused in a Langendorff setup. Over 50% of the maximal depression in LV pressure development was achieved at 3 μM fropofol (i.e. $EC_{50}$ was 2.6 μM).

## 2.3 Transthoracic echocardiograms

Mice were also monitored with serial transthoracic echocardiograms (TTEs) every 2 weeks after the beginning of the study period until death or the end of the trial (i.e. 12 weeks). Mice were lightly sedated with isoflurane (1–3%) and monitored for EKG and temperature. LV function was assessed by echocardiography (Agilent Sonos 5500, Santa Clara, CA, USA, 15 MHz probe, 3 cm depth). LV wall thickness, chamber dimensions, fractional contraction, and fractional wall thickening were determined as previously described.[16,17] Diastolic function was evaluated from transmitral Doppler flow patterns.

## 2.4 Trabecular/small papillary muscles

At the end of the 12-week study, the mice were euthanized with an anaesthetic overdose of pentobarbital (100 mg/kg, i.p.). After deep anaesthesia was achieved, the hearts were excised via sternotomy and perfused in a retrograde fashion in a dissection dish. Trabeculae and small papillary muscles were dissected from the right ventricle of hearts from both WT and αMHC 403/+ HCM mice, and from selected mice that were chronically treated with fropofol in the study. Force, sarcomere length, and intracellular $Ca^{2+}$ ($[Ca^{2+}]_i$) were measured simultaneously as described in our previous studies. Steady-state force-$[Ca^{2+}]$ relationship was determined in the same preparations before and after chemical skinning.[17–19] See Supplementary Methods for details. The experiments were performed at room temperature (20–22°C).

## 2.5 Histology, myocyte size, and fibrosis quantification

HCM mice that were treated chronically with fropofol ($n = 3$) or untreated ($n = 3$) and WT mice ($n = 3$) were euthanized as described above, and their hearts were excised. The hearts were washed in 23°C phosphate-buffered saline (PBS) and then fixed with 4% paraformaldehyde at 4°C overnight. Paraffin-embedded hearts were sectioned at a thickness of 5 μm to obtain short-axis, two-chamber views for three levels from apex to base. Sections were stained with haematoxylin–eosin for histopathology. To determine the myocyte cross-sectional area, we stained sections with wheat germ agglutinin. For fibrosis quantification, sections were stained with Masson's trichrome. Stained slides were scanned by an Aperio VERSA Pathology Scanner (LEICA Biosystems Imaging, Inc., Vista, CA, USA). We selected 15 histological fields at random at a magnification of ×40 on slides from the same level of each heart. Image J (Java 1.8.0_112, National Institutes of Health) was used for quantification of blue-stained collagen to highlight fibrosis within the red-stained myocardium unaffected by fibrosis. We processed images with Corel Draw (Ottawa, Canada) to remove pixels of background and pink cardiomyocyte tissue and to leave blue-stained areas in the images. We measured the

integrated density of these processed images and excluded pixel areas less than 3 to minimize noise. All images were adjusted to the same threshold. The blue- and red-stained areas were quantified and the ratio of the blue-stained pixel area to total pixel area was determined. Images of wheat germ agglutinin-stained sections (×40) were captured on an IX73 microscope system (Olympus, Inc., Waltham, MA, USA). Myocyte cross-sectional area and fibrosis area fraction were determined with Image-Pro 6 software (Media Cybernetics, Inc., Silver Spring, MD, USA).

## 2.6 Real-time polymerase chain reaction

Study mice were euthanized as described above. The hearts were isolated rapidly, washed in 23°C PBS to evacuate blood, and then immersed in RNALater (Qiagen, Germantown, MD, USA) at room temperature. Total RNA from the left ventricle was extracted with the RNeasy Plus kit (Qiagen) according to the manufacturer's protocol. The yield and purity of RNA were estimated spectrophotometrically by using the A260/A280 ratio. We reverse transcribed the RNA (5 μg) into cDNA using a High Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, Waltham, MA, USA). Fluorescent quantification of relative genes was carried out on an ABI 7500 fast real-time PCR system (Applied Biosystems, Foster City, CA, USA) with a 20 μL reaction system that included the following: cDNA, 1 μL; forward and reverse primers, 2 μL; Fast SYBRGreen Master Mix, 10 μL (Thermo Fisher Scientific); and nuclease-free water, 7 μL. The primers used in this process are listed in Supplementary material online, *Table S1*. We obtained the primers from Integrated DNA Technologies (Coralville, IA, USA). The relative mRNA levels were normalized to glyceraldehyde 3-phosphate dehydrogenase mRNA with the $2^{-\Delta\Delta CT}$ method.

## 2.7 Statistical analysis

Paired Student's *t*-tests, Student's *t*-tests, and ANOVAs (one-way and two-way) were used for statistical analysis of the data. A *P*-value of <0.05 was considered to indicate significant differences between groups. Unless otherwise indicated, pooled data are expressed as means ± S.E.M.

# 3. Results

## 3.1 Effect of fropofol on force development and steady-state force-$[Ca^{2+}]_i$ relationships in cardiac muscles from HCM mice

To demonstrate the effect of fropofol in HCM cardiac muscles *in vitro*, we first studied trabecular and small papillary muscles from the right hearts of αMHC 403/+ HCM mice. Fropofol concentration-dependently decreased force development in HCM muscles after 10 min of exposure (*Figure 1*). Significant depression occurred at a fropofol concentration of 50 μM ($EC_{50}$ was estimated to be 37 μM). Importantly, the maximum depression achieved at the highest fropofol concentration (200 μM) was 25% from baseline. Diastolic force was also significantly depressed in a concentration-dependent manner with no change in diastolic $Ca^{2+}$ (*Figure 1A–D*). Fropofol did not affect the dynamics of contraction or intracellular $Ca^{2+}$ transients (*Figure 1E* and *F*). The effect of fropofol on contraction in WT muscles was similar, except that diastolic force was unchanged (Supplementary material online, *Figure S2A and B*). Myofilament ATPase activity was also inhibited by fropofol (Supplementary material online, *Figure S2C*). Notably, fropofol had no effect on force development in mouse skeletal muscle (Supplementary material online, *Figure S2D*).

To assess the effect of fropofol on myofilament properties, we assessed the steady-state force-$[Ca^{2+}]_i$ relationship in intact muscles (*Figure 1G*). Fropofol (100 μM) decreased the maximal $Ca^{2+}$-activated force ($F_{max}$; 100.7 ± 2.7 vs. 79.7 ± 2.4 mN/mm$^2$, $P < 0.001$, ANOVA) and increased the $[Ca^{2+}]_i$ required for 50% activation ($Ca_{50}$; 0.653 ± 0.07 vs. 1.04 ± 0.09, $P < 0.01$, ANOVA). To confirm that these changes were indeed from alterations in the myofilaments themselves, we chemically skinned the same muscles (so that all cellular soluble regulatory mechanisms were removed and only the myofilaments remained functional[20]) and exposed them to varied $Ca^{2+}$ concentrations in the presence of fropofol. The fropofol-induced changes in $F_{max}$ and $Ca_{50}$ observed in intact muscles were reproduced in the same skinned muscles ($F_{max}$: 89.8 ± 1.1 vs. 67.5 ± 0.7 mN/mm$^2$, $P < 0.01$; $Ca_{50}$: 0.72 ± 0.1 vs. 1.10 ± 0.13 μM, $P < 0.05$; *Figure 1H*). The consistency of changes in the steady-state force-$[Ca^{2+}]$ relationship induced by fropofol *before* and *after* skinning indicates a *direct* myofilament modification by fropofol.

## 3.2 Fropofol treatment prevents pathological remodelling in HCM mice

Having confirmed that fropofol depresses force development by directly targeting myofilaments themselves, we proceeded to conduct trials in αMHC 403/+ HCM mice to test the hypothesis that circumventing cross-bridge force/power generation prevents the pathological remodelling and phenotypical expression of HCM disease. Mice were subjected to strenuous swimming for 90 min twice a day and monitored by TTE (*Figure 2*). Ejection fraction (EF) and fractional shortening (FS) of the LV wall decreased by approximately 10% at the end of the 12-week study period (*Figure 2A*). Development of hypertrophy was significantly attenuated by fropofol, as LV wall thickness, determined from the mid short-axis view at the level of papillary muscles, had changed little in αMHC 403/+ HCM mice at the end of the 12-week treatment (*Figure 2B–D*). On the other hand, hypertrophy in untreated αMHC 403/+ HCM mice progressed significantly. Diastolic function, as indicated by the ratio of early diastolic transmitral peak Doppler flow (the E wave) to the late diastolic peak flow by atrial contraction (the A wave), known as the E/A ratio, remained unchanged in the fropofol-treated group (*Figure 2E*). We also followed changes in LV wall thickness, EF, FS, E/A ratio, and other systolic and diastolic functions by performing serial TTEs at 2-week intervals from 4 weeks of treatment to the end of study (*Figure 3* and *Table 1*). LV EF remained at baseline level (52.2%) in the fropofol group (50%) but continued to rise in the untreated group (from 52.3% to 60%, $P < 0.0001$; *Figure 3A*). By 4 weeks of treatment, LV EF was 55.3% in the untreated group and 49% in the fropofol group, a 6% *decrease*. Similarly, FS remained largely unchanged at around 23.2–24.8% throughout the course of fropofol treatment ($P = 0.47$) but increased from 23.5% to 30% during the same period in the untreated group ($P < 0.0001$; *Figure 3B*). The increase in LV mass was also insignificant in the fropofol-treated group (*Table 1*). Fropofol prevented a decrease in the E/A ratio (*Figure 3C*) and prevented increases in the interventricular septum and LV anterior wall thickness (*Figure 3D* and *E*). Fropofol treatment also improved other parameters of diastolic function (*Table 1*). These echocardiographic results indicate that chronic fropofol treatment moderated the levels of LV hypertrophy and diastolic dysfunction in αMHC 403/+ HCM mice. Fropofol treatment did not affect these parameters in WT mice (*Figure 3*).

At the end of the 12-week study period, we performed histochemical histoimmunological analysis of heart tissues from the mice. Hearts from the untreated HCM mice were larger than those of the fropofol-treated HCM mice. Additionally, the heart weight-to-tibia length ratio and the

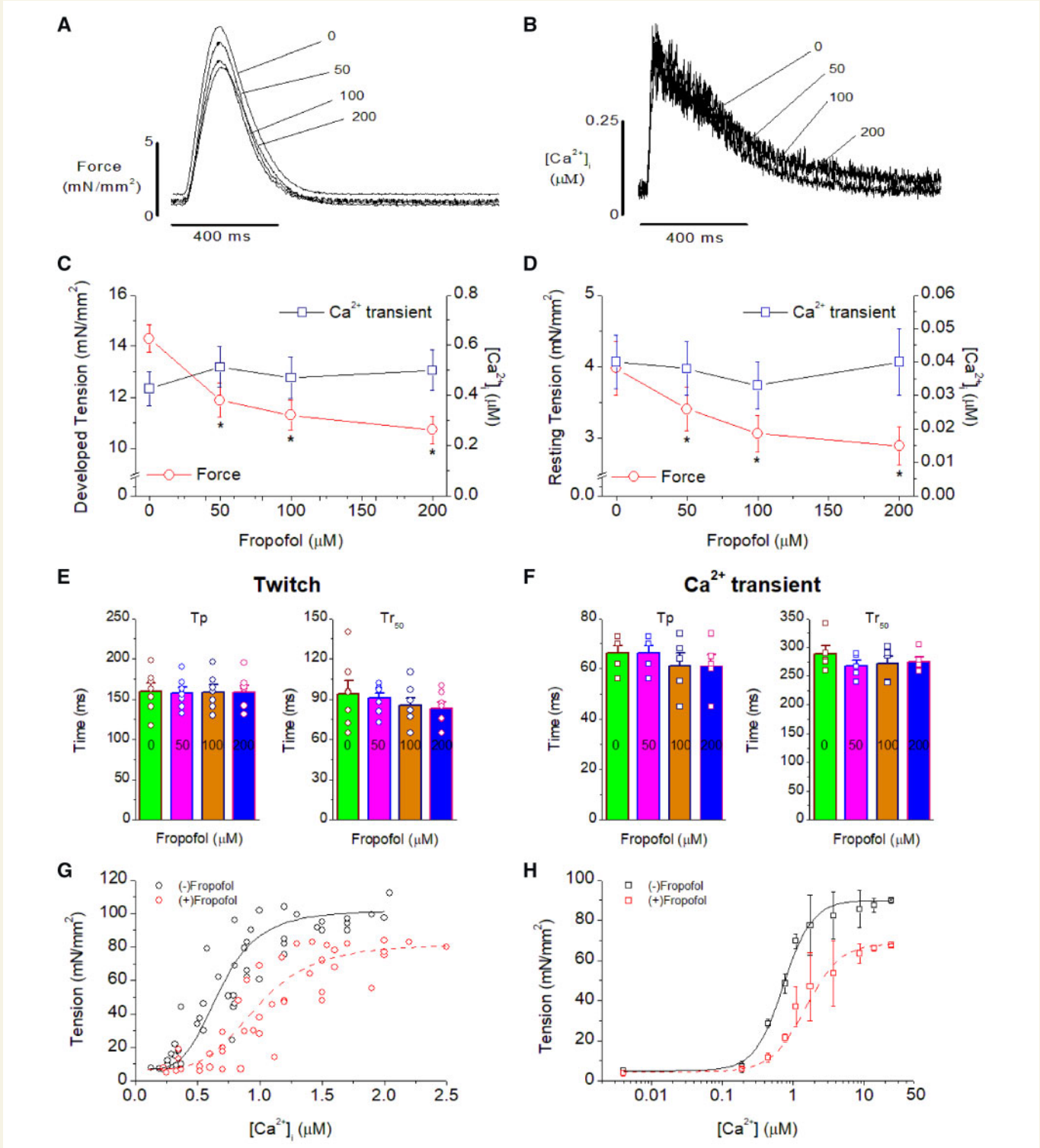


**Figure 1** Fropofol decreases twitch force development in HCM muscle. (*A*) Raw recordings of force development in a trabecular muscle from HCM heart in the presence of fropofol. The numbers indicate concentrations of fropofol in μM. (*B*) Raw recordings of $Ca^{2+}$ transients in the same trabecular muscle from *A* in the presence of fropofol. (*C*) Pooled data of systolic force development and amplitudes of intracellular $Ca^{2+}$ transients in the presence of different concentrations of fropofol. Systolic force became significantly less than that at baseline at concentrations greater than 50 μM. *P*-values from paired Student's *t*-test: $^*P < 0.01$ vs. baseline, $n = 7$. (*D*) Effects of fropofol on diastolic force and intracellular $Ca^{2+}$ levels. (*E*) Effects of fropofol on twitch dynamics, measured as time to peak force (Tp) and time from peak to half relaxation ($Tr_{50}$) ($n = 7$). (*F*) Effects of fropofol on the time course of intracellular $Ca^{2+}$ transients, measured as time to peak $[Ca^{2+}]_i$ and time to half $[Ca^{2+}]_i$ during relaxation ($n = 5$). (*G*) Effects of fropofol (100 μM) on the steady-state force and intracellular $Ca^{2+}$ relation in intact HCM muscles ($n = 9$). (*H*) The same muscles in which steady-state relations were first obtained were chemically skinned and activated with various $Ca^{2+}$ concentrations in the absence and presence of fropofol. Temperature = 22°C; external $Ca^{2+}$ = 2.0 mM; stimulation rate = 0.5 Hz.

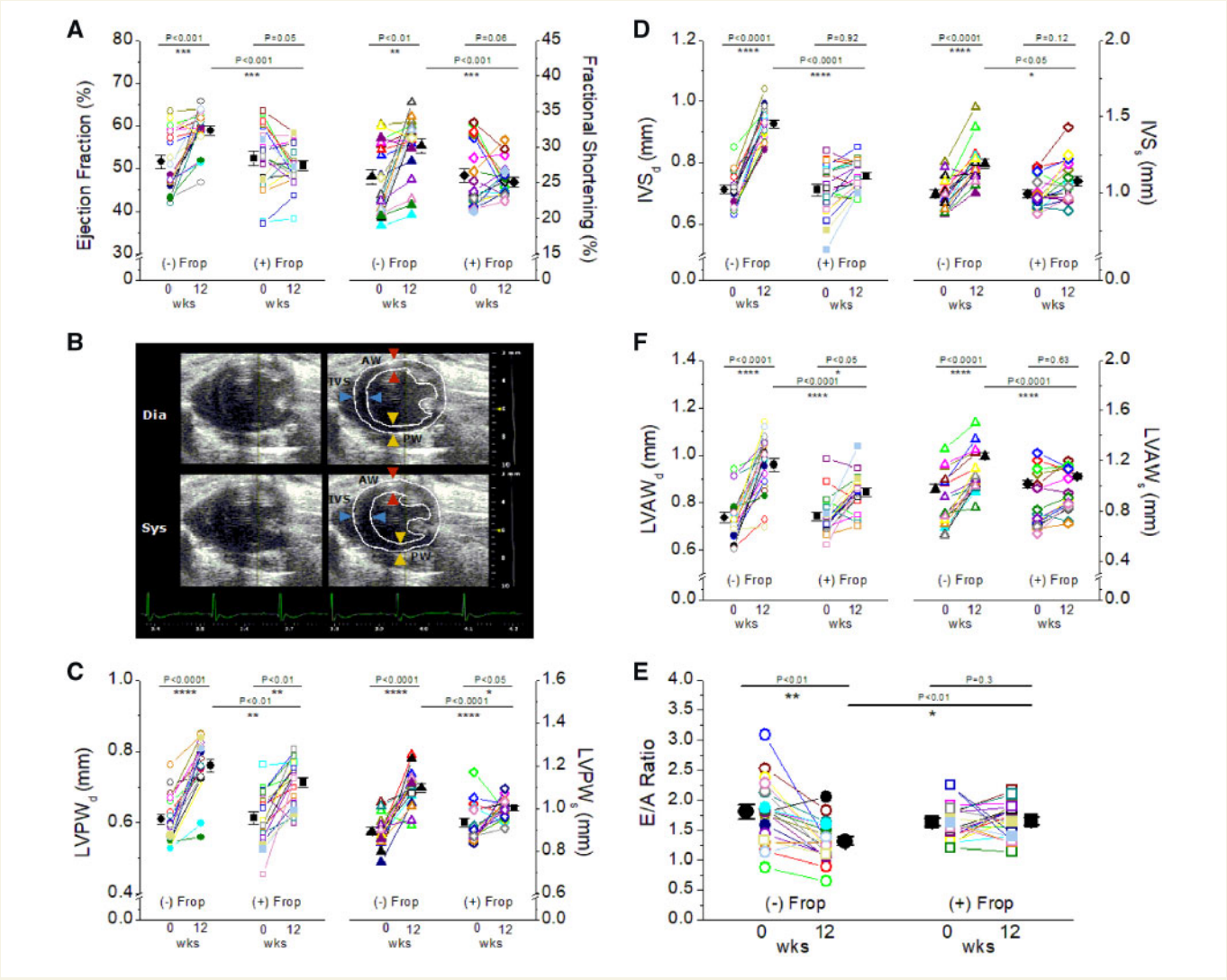


**Figure 2** Echocardiography of HCM mice before (0 week) and after (12 weeks) fropofol treatment. For all study mice, black-filled symbols indicate means for each group (i.e. fropofol and untreated). Colour-filled symbols represent data from animals that died during the trial between 6 and 10 weeks. (*A*) Pooled data for changes in ejection fraction and fractional shortening after 12 weeks. (*B–D*) Changes in left ventricular (LV) posterior wall (LVPW), interventricular septal wall (IVS), and LV anterior wall (LVAW) thickness during diastole ($_d$) and systole ($_s$). Wall thickness was measured in mid-LV short-axis view at the level of papillary muscles (B). The borders of LV walls were manually traced during both systole and diastole, and measurements were made at positions indicated by the arrowheads. (*E*) Changes in mitral E/A ratio. *P*-values from paired Student's *t*-test when compared to baseline within the group and from ANOVA when compared between fropofol-treated and untreated groups: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, $****P < 0.0001$, $n = 20$ in each group.

heart weight-to-body weight ratio were significantly higher in untreated mice than in WT and fropofol-treated mice (*Figure 4A* and *Table 2*). Hearts from untreated mice also exhibited key features of HCM, including increased cell size, myocyte disarray, and fibrosis (*Figure 4B–D*). On the other hand, fropofol-treated HCM hearts had a normal histological appearance, similar to that of WT hearts. These data indicate that inhibiting force generation prevented histopathological changes in HCM, further supporting the premise that increased force is the primary stimulus for the phenotypical expression of HCM. The prevention of fibrosis with fropofol treatment is also consistent with a previous finding that inhibiting force development early in the disease effectively prevents fibrotic response.[10] We also examined nine representative prohypertrophic and profibrotic genes (both myocyte- and fibroblast-expressed) that are significantly elevated in HCM.[10] The expression of these genes was significantly elevated in untreated HCM hearts as compared to that in WT hearts (*Figure 5*). However, the degree of elevation was reduced for eight of these genes, six of them significantly, after fropofol treatment. This finding indicates that inhibition of force development by fropofol attenuates hypertrophy and fibrosis by depressing prohypertrophic and profibrotic gene transcription.

## 3.3 Twitch dynamics and steady-state force-[$Ca^{2+}$] relationships in cardiac muscles from HCM hearts after fropofol treatment

One key question is whether fropofol treatment prevents abnormal contraction at the cellular level. We addressed this question by

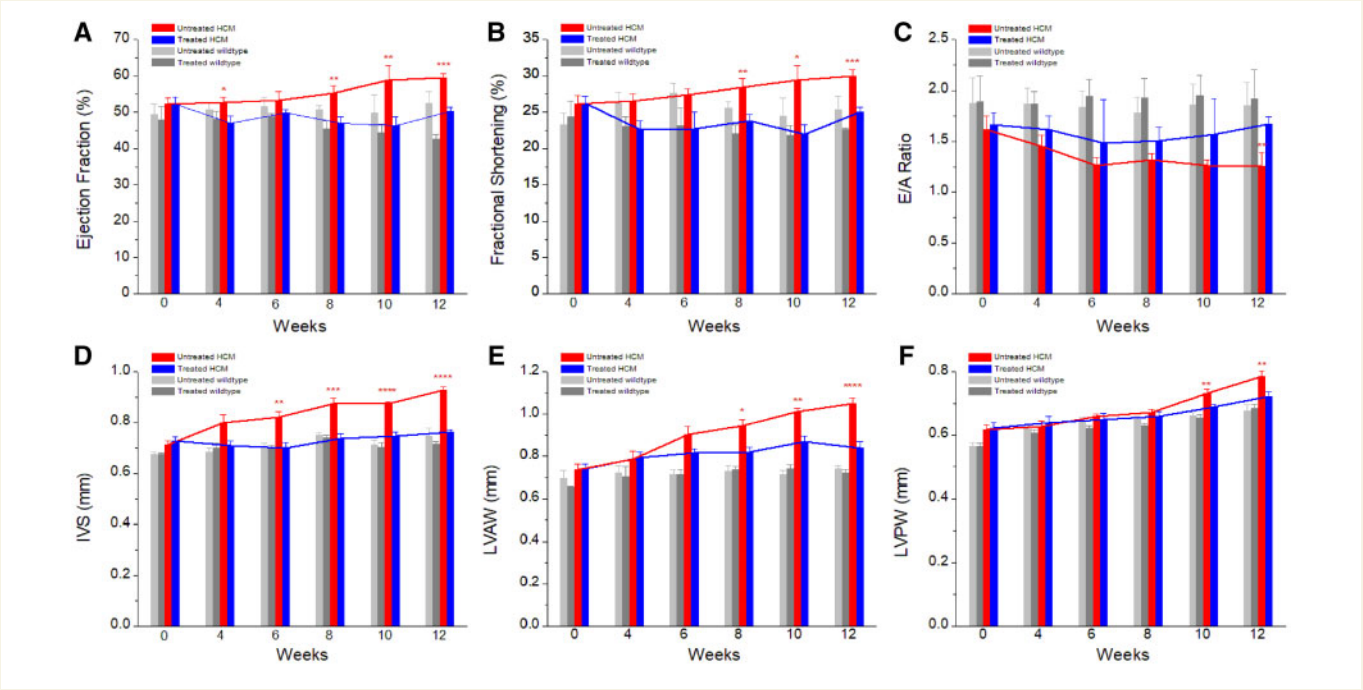


**Figure 3** Changes in cardiac function and left ventricular (LV) wall thickness during treatment with fropofol. (*A*) Changes in ejection fraction (EF). EF remained relatively constant over the course of treatment in both wild-type and HCM fropofol-treated groups but increased significantly over that period in untreated HCM mice. (*B*) Changes in fractional shortening (FS) over the course of fropofol treatment in wild-type and HCM mice. (*C*) Changes in E/A ratio in HCM and wild-type mice at the end of the study. (*D–F*) Changes in wall thickness in fropofol-treated and untreated HCM mice during the course of the study. These values were obtained during diastole. IVS, interventricular septum; LVAW, left ventricular anterior wall; LVPW, left ventricular posterior wall. *P*-values from ANOVA (fropofol-treated vs. untreated): **P* < 0.05, ***P* < 0.01, ****P* < 0.001, *****P* < 0.0001, *n* = 20 in each group.

**Table 1 Parameters of cardiac function as assessed by transthoracic echocardiography**

| Parameters | Untreated | | Fropofol-treated | |
|---|---|---|---|---|
| | 0 Weeks | 12 Weeks | 0 Weeks | 12 Weeks |
| Systolic | | | | |
| EF (%) | 52.3 ± 1.6 | 60.0 ± 1.4* | 55.3 ± 1.7 | 49.1 ± 1.1# |
| FS | 25.8 ± 1.1 | 30.0 ± 1.0* | 26.1 ± 1.0 | 25.1 ± 0.7 |
| LV mass (mg) | 83.4 ± 2.7 | 107.3 ± 3.2* | 93.2 ± 3.4 | 94.8 ± 4.1# |
| LVID, end-systolic (mm) | 2.54 ± 0.07 | 2.49 ± 0.06 | 2.61 ± 0.08 | 2.63 ± 0.08 |
| LVID, end-diastolic (mm) | 3.66 ± 0.09 | 3.65 ± 0.09 | 3.72 ± 0.09 | 3.73 ± 0.08 |
| LVV, end-systolic (μL) | 23.7 ± 1.5 | 22.7 ± 1.6 | 25.2 ± 2.1 | 26.1 ± 2.3 |
| LVV, end-diastolic (μL) | 59.3 ± 3.1 | 59.2 ± 3.4 | 65.5 ± 4.1 | 64.4 ± 3.27 |
| Diastolic | | | | |
| E/A | 1.81 ± 0.11 | 1.31 ± 0.07* | 1.63 ± 0.07 | 1.65 ± 0.06# |
| E′/A′ | 0.99 ± 0.04 | 0.66 ± 0.03* | 1.03 ± 0.06 | 1.28 ± 0.19# |
| E/E′ | 17.62 ± 0.78 | 21.72 ± 0.80* | 18.56 ± 1.14 | 18.03 ± 0.72# |
| IVRT (ms) | 16.8 ± 3.0 | 20.3 ± 1.0 | 17.1 ± 0.86 | 17.5 ± 0.95 |

Values are presented as mean ± standard error.
E/A, ratio of peak transmitral Doppler flow velocity in early diastole (E) to that of late diastole due to atrial contraction (A); E′/A′, ratio of early tissue Doppler diastolic mitral annular velocity (E′) to late tissue Doppler diastolic mitral annular velocity (A′); E/E′, ratio of peak transmitral Doppler flow velocity in early diastole to early tissue Doppler diastolic mitral annular velocity; EF, ejection fraction; FS, fractional shortening; IVRT, isovolumic relaxation time; LV, left ventricle; LVID, left ventricular inner diameter; LVV, left ventricular volume.
**P* < 0.05 vs. 0 weeks.
#*P* < 0.05 vs. untreated 12 weeks, *n* = 20.

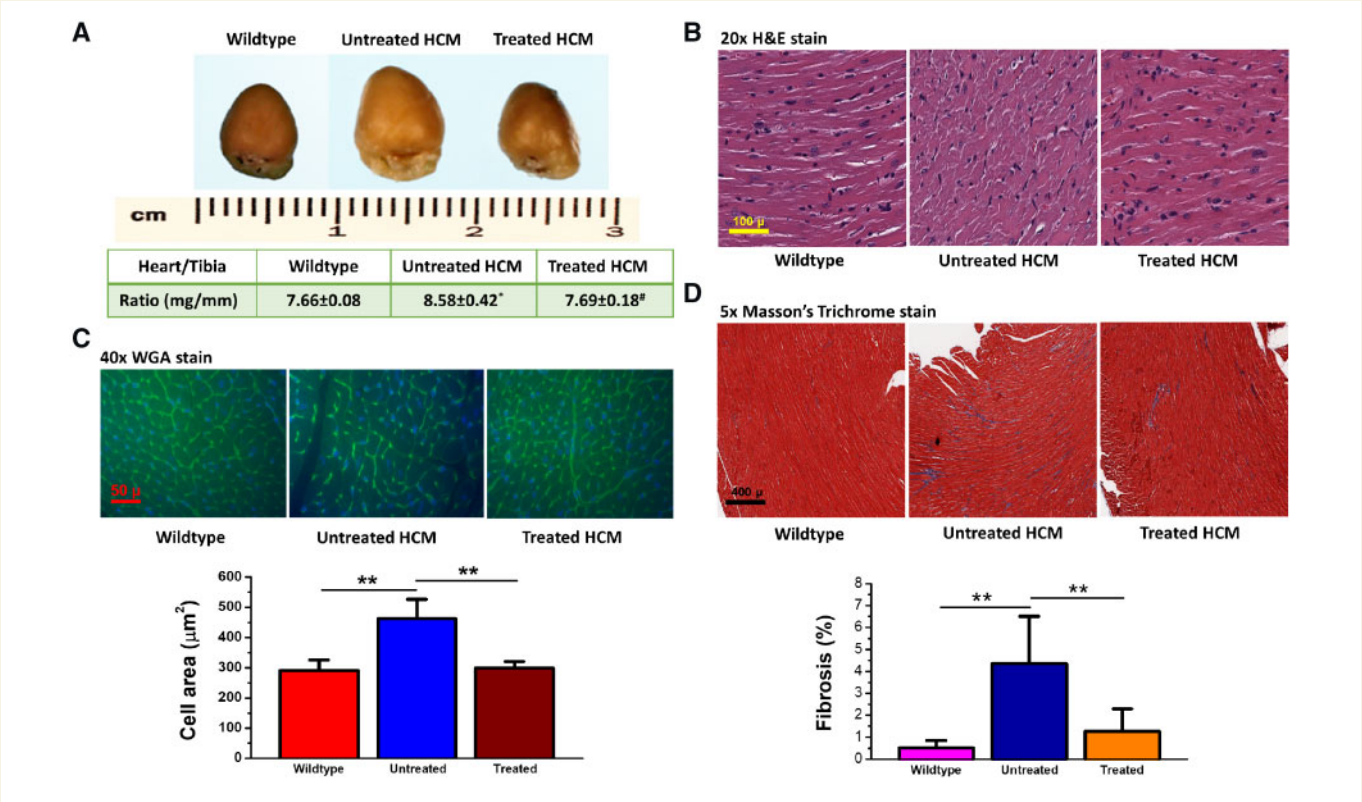


**Figure 4** Chronic fropofol treatment prevents hypertrophy development, cell size increases, myofilament disarray, and fibrosis. (*A*) Representative photographs of hearts from a wild-type mouse, an untreated HCM mouse, and a fropofol-treated HCM mouse. *P*-values from ANOVA: $^{*}P < 0.05$ vs. wild-type; $^{\#}P < 0.05$ vs. untreated, $n = 3$ hearts in each group. (*B*) Haematoxylin and eosin (H&E) staining of tissue samples from wild-type, untreated, and fropofol-treated HCM hearts. Note the disarray of myocytes in the untreated HCM heart. Scale bar = 100 µm. (*C*) Wheat germ agglutinin (WGA) staining of heart tissue samples from wild-type, untreated HCM, and fropofol-treated HCM groups (*upper panel*). Two sections were obtained from each wall of the left ventricle (anterior, posterior, and septal), and samples from three hearts of each group were used for the analysis. The pooled data from 18 sections from 3 hearts in each group are shown as means ± S.E.M. (*lower panel*). *P*-value from ANOVA: $^{**}P < 0.001$ vs. the indicated group. Scale bar = 50 µm. (*D*) Masson's trichrome staining of heart tissue samples from wild-type, untreated HCM, and fropofol-treated HCM groups (*upper panel*). Two sections were obtained from each wall of the left ventricle (anterior, posterior, and septal), and samples from three hearts of each group were used for the analysis (scale bar = 400 µm). Pooled data for quantification of the fibrosis from five histological fields at random at a magnification of ×40 on each slide at the same level on each heart are shown (*lower panel*) as means ± S.E.M. *P*-value from ANOVA: $^{**}P < 0.001$ vs. the indicated group, $n = 3$ hearts in each group.

measuring force and intracellular $Ca^{2+}$ in intact trabecular or papillary muscles from hearts of the αMHC 403/+ HCM mice. Fropofol-treated muscles exhibited less force development than did untreated muscles, without significant differences in intracellular $Ca^{2+}$ (*Figure 6A–C*). In addition, the relaxation of force was significantly shortened in fropofol-treated muscles at external $Ca^{2+}$ concentrations above 2 mM (*Figure 6D*).

During steady-state activations, fropofol-treated muscles exhibited a rightward shift of the force-$[Ca^{2+}]_i$ relationship (*Figure 6E*). These results indicate that fropofol treatment normalizes the excitation-contraction coupling process in HCM mice.

## 4. Discussion

In this study, we showed that fropofol, a derivative of propofol that lacks anaesthetic efficacy, directly depressed myofilament activity in cardiac muscles from both WT and αMHC 403/+ HCM mice *in vitro*. *In vivo*, fropofol treatment of αMHC 403/+ HCM mice slowed the development of hypertrophy, diastolic dysfunction, and fibrosis and altered contraction. Our data strongly support the notion that increased cross-bridge power output is the primary trigger for development of the HCM phenotype and the associated secondary changes in myofilament properties and force development. The findings also show that direct inhibition of cross-bridge force development by the small molecule fropofol is effective and safe.

HCM was first recognized as hypertrophic obstructive cardiomyopathy in the late 1950s with four key features: (i) LV hypertrophy, (ii) familial autosomal dominant transmission, (iii) sudden cardiac death, and (iv) haemodynamic obstruction of the LV outflow track.[21] Because of these important features, increased contractility (or hypercontractile state) in HCM was perceived early on, and pharmacologic treatment with negative inotropic agents such as β-blockers was begun almost immediately after the clinical recognition of this disease.[22] The *molecular* mechanism for the increased contractility was appreciated when the αMHC 403/+ mouse model of HCM was created.[13] To date, many studies have shown increases in myofilament sensitivity to $Ca^{2+}$, velocity of HCM myosin molecules, and cross-bridge force generation.[23] Thus, our data support the idea that increased contractile function/myofilament sensitization is

**Table 2 Effect of fropofol on body and heart weight**

| Mouse genotype | Body weight (g) | Heart weight (g) | Ratio (heart/body) |
|---|---|---|---|
| Untreated WT ($n = 5$) | 24.32 ± 1.71 | 0.146 ± 0.002 | 0.0061 ± 0.00012 |
| Fropofol-treated WT ($n = 5$) | 24.17 ± 1.92 | 0.144 ± 0.003 | 0.0059 ± 0.00016 |
| Untreated HCM ($n = 20$) | 24.91 ± 2.34 | 0.163 ± 0.008* | 0.0066 ± 0.00024* |
| Fropofol-treated HCM ($n = 20$) | 24.44 ± 3.12 | 0.146 ± 0.003 | 0.0060 ± 0.0001 |

HCM, transgenic hypertrophic cardiomyopathy mice; WT, wild-type mice.
*$P < 0.05$ vs. fropofol-treated HCM.

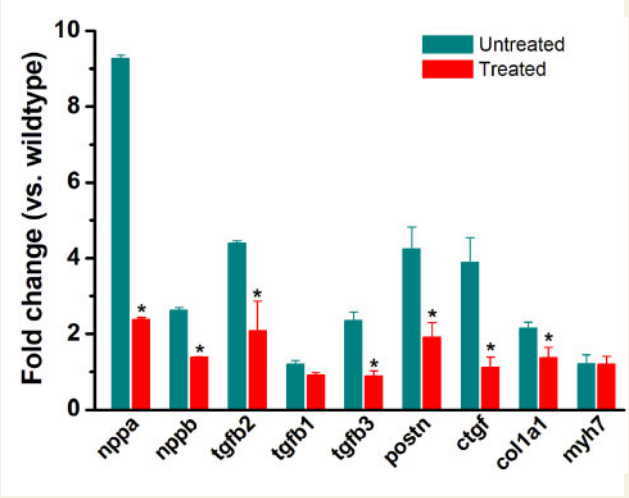


**Figure 5** Fropofol treatment suppresses hypertrophic and profibrotic genes in HCM mice. Nine genes known to be significantly enhanced in the αMHC 403/+ mouse model were selected. These genes are expressed in myocytes and fibroblasts of the HCM heart. *P*-value from ANOVA: *$P < 0.05$ between treated and untreated hearts, $n = 6$.

the trigger for development of certain HCM phenotypes. However, several studies have shown that mutations in human β-myosin heavy chain (MyHC) are associated with reduced force generation in skinned single myocytes, single myofibrils, and single cross-bridges.[24–27] These contrasting observations may be explained by the differences in MyHC isoforms (i.e. β-MyHC in human vs. α-MyHC in mice)[27,28] and by the presence of reduced myofibrillar density and significant myofibrillar disarray (which might also mask the true behaviour of force generation).[29] Recent experiments with human β-MyHC showed increased $Ca^{2+}$ sensitivity of acto-ATPase activity in troponin T (TnT) mutants with HCM phenotype and increased single cross-bridge force production in human β-MyHC mutation-induced HCM (i.e. R453C[7,8] and myosin binding protein C).[30]

β-adrenergic receptor blockers and $Ca^{2+}$ channel blockers have been the mainstay therapy of HCM patients for decades,[9] but they do not eliminate disease progression.[31,32] As a result, patients with heart failure symptoms are often treated with invasive procedures such as catheter-based alcohol septal ablation or open heart myotomy.[2] These procedures are highly invasive and should be performed in experienced centres to minimize operative mortality.[2] These restrictions currently limit their availability to many HCM patients. Given that increased myofilament $Ca^{2+}$ sensitivity is central to the disease progress, $Ca^{2+}$-desensitizing agents, especially those that target the myofilament proteins themselves, would be more effective. However, only catechins, specifically (-)-epicatechin-3-gallate and (-)-epigallocatechin-3-gallate (EGCg), have been tested.[33] In a TnT-deletion transgenic mouse model of HCM, ΔE160cTnT-Tg, EGCg was shown to decrease myofilament $Ca^{2+}$ sensitivity in skinned papillary muscles by binding to troponin C (TnC). EGCg also enhanced intracellular $Ca^{2+}$.[33] Selective $\beta_1$ blocker nebivolol was shown to exert a direct myofilament desensitizing effect in isolated rabbit cardiac muscle.[34] But neither its mechanism nor its effect on intracellular $Ca^{2+}$ homeostasis is known. W7, a specific inhibitor of calmodulin (CaM), has been shown to decrease force development and myosin-ATPase activity in skinned cardiac muscles[35] and is believed to bind to TnC.[36] However, we do not yet know the effect of W7 on force development in intact muscles or heart. Moreover, inhibition of CaM leads to many CaM-mediated cellular effects, rendering W7 an impractical agent to use. Inhibitors of myosin–actin interaction such as blebbistatin and 2,3-butanedione monoxime[37,38] have been used extensively *in vitro* to inhibit contraction. However, because the degree of inhibition is too great and they produce unknown cellular effects, they have not been used as myofilament desensitizers *in vivo*. Nevertheless, the potential beneficial effects of these myofilament desensitizers still need to be investigated in detail, and their optimal dosing determined. More recently, Green *et al.*[10] showed that a small myosin ATPase-inhibiting molecule, MYK-461, prevented development of hypertrophy and other disease processes in a mouse model of HCM. MYK-461, however, dramatically decreased force at higher concentrations of the drug, thus raising safety concerns for patients. On the other hand, fropofol has several unique features: it directly targets cross-bridge cycling,[12] it produces modest (~30% maximum) force depression, and its action is completely reversible. All of these virtues make fropofol an ideal, safe, and effective agent that permits a precision medicine approach to interrupt the pathogenic cascade (hypertrophy, fibrosis, diastolic dysfunction, myofibrillar disarray, etc.) and even reverse these pathologic remodelling processes.

At the cellular level, altered excitation-contraction coupling is another feature of HCM disease. Previously, we found higher amplitudes and faster relaxation of $Ca^{2+}$ transients in R403Q-α-myosin mutant muscles than in normal muscles.[19] Others have shown changes in resting $Ca^{2+}$ and slowing of $Ca^{2+}$ transient rise and decline in TnT mutant myocytes.[39,40] We believe that these alterations in $Ca^{2+}$ fluxes are secondary to increased cross-bridge power (force) production. A recent study supported this notion by showing that increased tension (expressed as increased tension-time integral) *alone* is sufficient to induce hypertrophy.[41] Consistent with these findings, we also showed normalization of contraction and myofilament properties after fropofol treatment in isolated cardiac muscles (*Figure 6*). Metabolic changes such as altered mitochondrial respiration, inefficient intracellular energy transfer, and increased

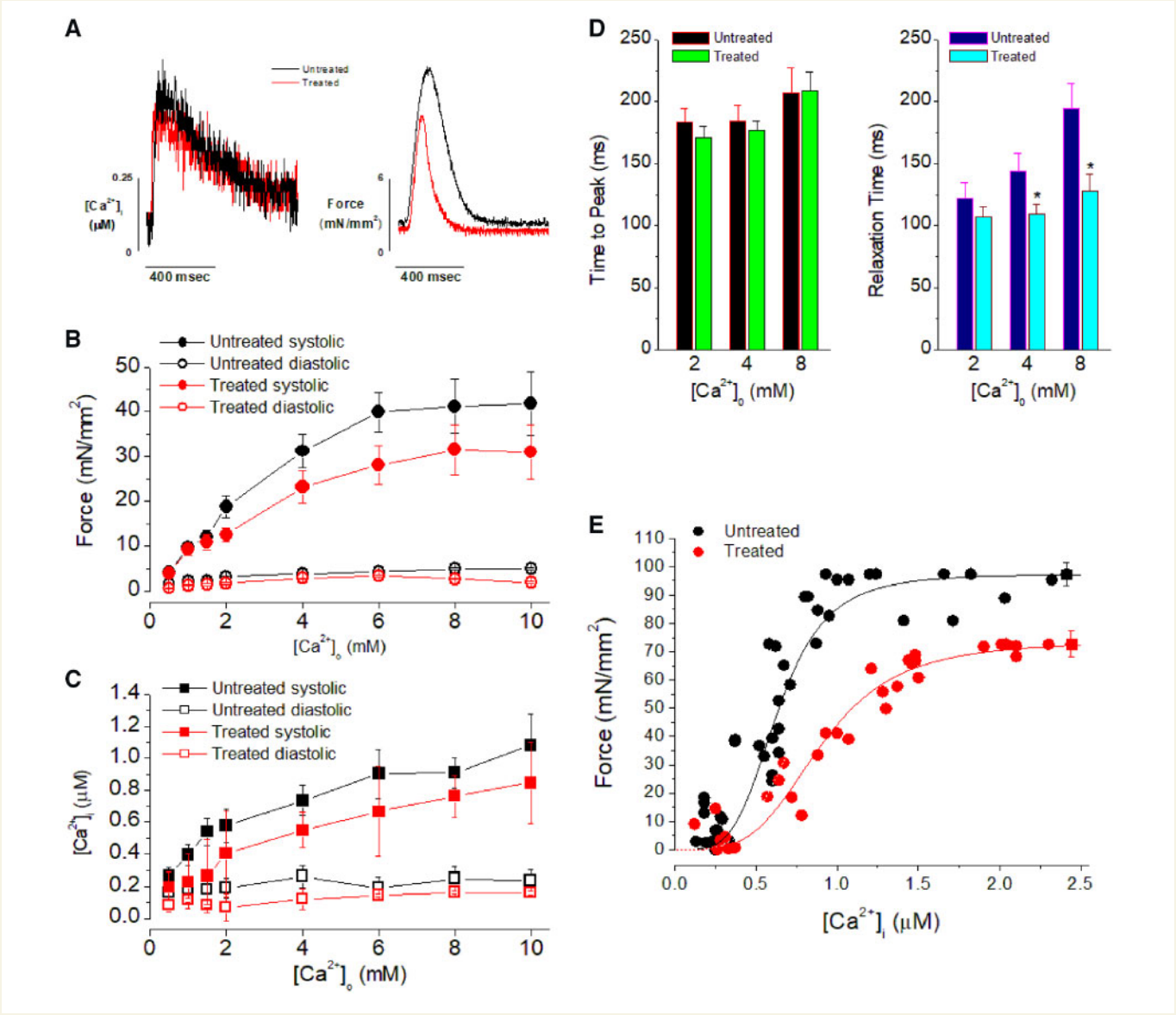


**Figure 6** Contraction and intracellular $Ca^{2+}$ in isolated HCM mouse cardiac muscles after 12 weeks of fropofol treatment. (*A*) Raw recordings of twitch force (*right panel*) and intracellular $Ca^{2+}$ transients (*left panel*) ($[Ca^{2+}]_o = 2.0$). (*B* and *C*) Pooled data of force development and intracellular $Ca^{2+}$ at varied extracellular $Ca^{2+}$ concentrations. (*D*) Dynamics of twitch force in isolated trabecular muscles after fropofol treatment. Notably, fropofol-treated muscles had accelerated relaxation of twitch force (*right*), but time-to-peak force remained unchanged (*left*). *P*-values from ANOVA: $^*P < 0.05$ between treated and untreated, $n = 7$. (*E*) Steady-state activations (i.e. tetanizations) were achieved by stimulating the muscle at 8–10 Hz in the presence of cyclopiazonic acid (50 μM) at varied $[Ca^{2+}]_o$ to obtain different levels of tetanized forces. The pooled data shown represent steady-state force-$[Ca^{2+}]$ relationships of untreated and treated trabeculae or small capillary muscles. The absolute $F_{max}$ is shown as the mean ± S.E.M. and is plotted at highest $[Ca^{2+}]_i$. All other force levels were normalized with respect to their own maximal values. The continuous line is the Hill fit based on the means of $Ca_{50}$ and Hill coefficient (untreated: $n = 8$; treated $n = 9$).

cellular oxidative stress also occur in HCM.[42] It will be interesting to investigate whether these metabolic disturbances are also normalized by fropofol treatment.

HCM mice in our *in vivo* study were subjected to 90 min of swimming twice a day. We used this protocol in order to accelerate the disease expression and progress. Inclusion of swimming can potentially be a limitation of this study because exercise can induce physiologic adaptation. Indeed, voluntary cage wheel exercise prevented disease progression in this mouse model of HCM.[43] However, these HCM mice also have impaired exercise capacity, especially during strenuous exercise such as swimming.[13] Consistent with the findings of Geisterfer-Lowrance *et al.*,[13] some mice died during and immediately after swimming (Supplementary material online, *Figure S3*). Likewise, Kazmierczak *et al.*[15] reported that the swimming protocol induced pathologic hypertrophy in HCM mice with an A57G mutation in essential myosin light chain but not in WT mice.

We did not investigate the molecular mechanism of fropofol's action on HCM myofilament proteins, which is a limitation of this study. We

have recently shown that fropofol directly interacted with myosin molecule, decreasing myosin sliding velocity against actin and myosin ATPase activity in purified rat cardiac myosin.[12] We believe that fropofol interacts with myosin similarly in HCM. Our future study will focus on molecular characterization of fropofol's interaction with myosin and other myofilament proteins in HCM.

In conclusion, fropofol, a propofol derivative without anaesthetic efficacy, exerts a direct myofilament depressing effect in cardiac muscles from both WT and HCM mice *in vitro*. Chronic treatment with fropofol prevented hypertrophy, fibrosis, diastolic dysfunction, and disease progression in mice with HCM.

# Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

# Authors' contributions

YH, HL, XR, RE, FL, WB, WL, and WDG performed the experiments. YH, HL, XR, HS, KG, RA, RE, and WDG were analysed the data. YH, HL, and WDG were involved in designing the experiments; YH and HL contributed equally. WB, WPD, and RE provided fropofol for the experiments. HL, XR, RA, RE, and WDG were involved in initial experimental conception and design. RE and WDG supervised all phases of experiments and data analysis.

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## Translational perspective

Hypertrophic cardiomyopathy (HCM) has a high prevalence worldwide and treatment of HCM has had limited success. This study showed that, fropofol, a novel myofilament targeting small molecule, inhibited contraction at the myofilament level and prevented development of hypertrophy, fibrosis, and diastolic dysfunction in transgenic mice models of HCM after chronic treatment. Translationally, this study demonstrated fropofol as a unique, safe, and effective agent that will permit a precision-medicine approach to interrupt the pathogenic cascade and even reverse these pathologic remodelling processes. Our findings will ultimately help in the design of novel and effective therapies in the management of HCM.