Sarcomere neutralization in inherited cardiomyopathy: small-molecule proof-of-concept to correct hyper-Ca\textsuperscript{2+}-sensitive myofilaments

Brian R. Thompson, Joshua Martindale, and Joseph M. Metzger
Department of Integrative Biology and Physiology, University of Minnesota Medical School, Minneapolis, Minnesota
Submitted 29 December 2015; accepted in final form 5 May 2016

Thompson BR, Martindale J, Metzger JM. Sarcomere neutralization in inherited cardiomyopathy: small-molecule proof-of-concept to correct hyper-Ca\textsuperscript{2+}-sensitive myofilaments. Am J Physiol Heart Circ Physiol 311: H36–H43, 2016. First published May 13, 2016; doi:10.1152/ajpheart.00981.2015.—The sarcomere is the functional unit of the heart. Alterations in sarcomere activation lead to disease states such as hypertrophic and restrictive cardiomyopathy (HCM/RCM). Mutations in many of the sarcomeric genes are causal for HCM/RCM. In most cases, these mutations result in increased Ca\textsuperscript{2+} sensitivity of the sarcomere, giving rise to altered systolic and diastolic function. There is emerging evidence that small-molecule sarcomere neutralization is a potential therapeutic strategy for HCM/RCM. To pursue proof-of-concept, W7 was used here because of its well-known Ca\textsuperscript{2+} desensitizer biochemical effects at the level of cardiac troponin C. Acute treatment of adult cardiac myocytes with W7 caused a dose-dependent (1–10 μM) decrease in contractility in a Ca\textsuperscript{2+}-independent manner. Alkalosis was used as an in vitro experimental model of acquired heightened Ca\textsuperscript{2+} sensitivity, resulting in increased live cell contractility and decreased baseline sarcomere length, which were rapidly corrected with W7. As an inherited cardiomyopathy model, R193H cardiac troponin I (cTnI) transgenic myocytes showed significant decreased baseline sarcomere length and slowed relaxation that were rapidly and dose-dependently corrected by W7. Langendorff whole heart pacing stress showed that R193H cTnI transgenic hearts had elevated end-diastolic pressures at all pacing frequencies compared with hearts from nontransgenic mice. Acute treatment with W7 rapidly restored end-diastolic pressures to normal values in R193H cTnI hearts, supporting a sarcomere intrinsic mechanism of dysfunction. The known off-target effects of W7 notwithstanding, these results provide further proof-of-concept that small-molecule-based sarcomere neutralization is a potential approach to remediate hyper-Ca\textsuperscript{2+}-sensitive sarcomere function.

NEW & NOTEWORTHY

We show here, as proof-of-concept, small-molecule-based Ca\textsuperscript{2+} desensitization for sarcomere neutralization therapy in models of acquired and inherited hyper-Ca\textsuperscript{2+}-sensitive disease states. At both the cellular and organ level, contractile dysfunction in both models was acutely corrected with W7, a sarcomere Ca\textsuperscript{2+} desensitizer.

HYPERTROPHIC CARDIOMYOPATHY (HCM) is the most prevalent inherited heart disorder, affecting 1 in 500 individuals. HCM is characterized by left ventricular hypertrophy, diastolic dysfunction, outflow tract obstructions, and arrhythmias (21). HCM is a leading cause of death in competitive athletes and young adults. Therapeutic strategies for HCM include surgical myectomy, internal cardiac defibrillators, and pharmaceuticals aimed at reducing arrhythmias, such as Ca\textsuperscript{2+} channel blockers and sodium channel blockers. Although therapeutic strategies have increased the life expectancy of some patients, the underlying cause of HCM has not been addressed by current therapies (12, 13).

HCM is a disease of the sarcomere, with >50% of HCM patients having known sarcomeric gene mutations. To date, there are 11 causative genes for HCM, with >1,400 mutations within those genes (21). Mutations in myosin heavy chain, myosin-binding protein C, tropomyosin, cardiac troponin T, cardiac troponin I (cTnI), actin, and cardiac troponin C (cTnC) have all been implicated as disease causing. Like HCM, restrictive cardiomyopathy (RCM) is a genetic disorder of the sarcomere with many of the same genes being causative and even the same mutations resulting in HCM in one patient and RCM in another (24, 34). RCM mutations lead to a more severe disease characterized by diastolic dysfunction and heart failure. With such a diverse population of mutations in many genes, leading to multiple disease states, it is critical to determine the final common pathways of disease.

At the molecular level, HCM and RCM mutations have been shown to increase the Ca\textsuperscript{2+} sensitivity of the myofilament (4, 25). In vitro thin filament reconstitution experiments, with the only variable being the mutated protein, have shown that HCM and RCM mutations increase Ca\textsuperscript{2+} sensitivity, with RCM mutations causing a larger increase than HCM mutations (2, 4, 34). From a molecular standpoint, these two diseases are a continuum of the same disease mechanism. In general, RCM mutations result in a larger increase of myofilament Ca\textsuperscript{2+} sensitivity causing a more severe phenotype (4, 12, 20). At the cellular level, myofilament mutations show altered sarcomere relaxation and/or heightened contractility and increased diastolic tone manifest by a pronounced precontractile state (5). Further examination shows common sarcomere dysfunction, Ca\textsuperscript{2+} transient alterations secondary to increased Ca\textsuperscript{2+} buffering by troponin, energetic inefficiency, and arrhythmia potential. Both the Ca\textsuperscript{2+} buffering and focal energy deprivation can lead to arrhythmias and are phenocopied with Ca\textsuperscript{2+}-sensitizing agents, as well as reduced with Ca\textsuperscript{2+}-desensitizing agents (3, 16). These studies highlight that most HCM/RCM mutations cause altered sarcomere Ca\textsuperscript{2+} sensitivity, making it a critical avenue to pursue for new therapies.

Sarcomere Ca\textsuperscript{2+} sensitivity is a critical determinant of cardiac function and normally resides within a tightly regulated physiological zone (6). Factors that increase or decrease Ca\textsuperscript{2+} sensitivity of the sarcomere outside this physiological zone lead to disease. The concept of sarcomere neutralization therapy has the primary aim of normalizing Ca\textsuperscript{2+} sensitivity back to within this physiological zone. HCM and RCM are hyper-Ca\textsuperscript{2+}-sensitive disease states; therefore, shifting the sensitivity back to the physiological zone is the final common pathway to disease.
back to the physiological zone with a small-molecule Ca\textsuperscript{2+} desensitizer is a therapeutically relevant strategy.

Sarcomeric Ca\textsuperscript{2+} sensitivity is determined by many protein-protein interactions. This includes dynamics in the sarcomere that converge on cTnC, the protein that binds Ca\textsuperscript{2+} to initiate sarcomere activation (20). Given the central role of cTnC in terms of sarcomeric Ca\textsuperscript{2+} activation, it is an ideal target for sarcomere neutralization therapy. Whereas gene and cell-based approaches are attractive methods to redesign sarcomeric structure and performance as implemented by us and others (6), we focused here on a small-molecule approach to target cTnC. Small molecules have the potential to titrate sarcomere performance acutely, which could benefit a range of inherited and acquired cardiomyopathies.

Presently, there are several small-molecule cTnC Ca\textsuperscript{2+} sensitizers in clinical application (17, 27). In contrast, very few small-molecule Ca\textsuperscript{2+} desensitizers have been identified and studied. In a recent review on potential therapeutics for sarcomeric cardiomyopathies, Tardiff et al. (12) highlight the experimental studies of epigallocatechin gallate (EGCG) and W7 decreases intact cardiac myocyte contractility in a Ca\textsuperscript{2+} sensitive manner. This includes dynamics in the sarcomere activation, which could benefit a range of inherited and acquired cardiomyopathies.

 present, several small-molecule cTnC Ca\textsuperscript{2+} sensitizers in clinical application (17, 27). In contrast, very few small-molecule Ca\textsuperscript{2+} desensitizers have been identified and studied. In a recent review on potential therapeutics for sarcomeric cardiomyopathies, Tardiff et al. (12) highlight the experimental studies of epigallocatechin gallate (EGCG) and W7 as small-molecule Ca\textsuperscript{2+} desensitizers that could lead to new therapeutics. EGCG is the most studied at the cellular and organ level in HCM and RCM models showing potential for desensitization as a therapeutic avenue. W7 has only been characterized at the biophysical and biochemical level and yet to be studied in disease models. W7 has been shown to bind to cTnC and directly decrease Ca\textsuperscript{2+} binding affinity (23). In addition, W7 has been shown to decrease Ca\textsuperscript{2+} sensitivity of force development in membrane-permeabilized myocytes (1) and to reduce contractile amplitude in intact myocytes (11). Although W7 was originally described as a calmodulin inhibitor (15), the wealth of knowledge on W7 as a sarcomere-acting Ca\textsuperscript{2+} desensitizer makes it an excellent proof-of-principle small molecule to build on the premise of sarcomere neutralization therapy.

We show here small-molecule-based Ca\textsuperscript{2+} desensitization for sarcomere neutralization therapy in models of acquired and inherited hyper-Ca\textsuperscript{2+}-sensitive disease states. Data show that W7 decreases intact cardiac myocyte contractility in a Ca\textsuperscript{2+} transient amplitude-independent manner. This, in turn, had a rapid effect to correct contractile dysfunction in both acquired (alkalosis) and inherited (RCM) forms of hyper-Ca\textsuperscript{2+}-sensitive states at the myocyte level and whole heart levels. Because of the speed of organ-level functional restitution, essentially instantaneously with W7 application, this indicates that cell intrinsic sarcomeric function, rather than organ morphological defects (e.g., altered heart growth), accounts for the physiological deficits in this RCM model. Taken together, these data provide proof-of-principle that sarcomere neutralization therapy through targeting cTnC represents a viable therapeutic strategy in acquired and inherited hyper-Ca\textsuperscript{2+}-sensitive sarcomeric disease states.

METHODS

Adult cardiac myocyte isolation and culture. All animal procedures were approved by the University of Minnesota Institutional Animal Care and Use Committee. Adult rat ventricular myocytes were isolated and cultured as previously described (33). Briefly, adult female rat hearts were enzymatically digested through retrograde perfusion with collagenase. After digestion, hearts were cut into small pieces and triturated to free single myocytes. Myocytes were plated on laminin-coated cover slips at 20,000 cells/cover slip. After 1 h, cells were bathed with M-199 media [supplemented with 10 mmol/l HEPES, 2.2 mol/l sodium bicarbonate, 0.02% bovine serum albumin, and 50 U/ml penicillin-streptomycin, with pH adjusted to 7.4, additionally insulin (5 μg/ml), transferrin (5 μg/ml), and selenite (5 ng/ml) were added (Sigma 11884); GIBCO] and cultured overnight.

Adult mouse ventricular myocytes were isolated as previously described (7). Briefly, nontransgenic and transgenic R193H cTnI mouse hearts were isolated and enzymatically digested through retrograde perfusion with collagenase. Hearts were then cut into 8 to 10 pieces and gently triturated to free single myocytes. Myocytes were plated on laminin-coated cover slips and fed with M199 media, as above. One hour after plating, cells were used for experiments.

Contractility measurements in single intact myocytes. Sarcomere length dynamics and kinetics were measured using the IonOptix system as previously described (5, 14, 33). Briefly, cover slips containing single isolated myocytes, day 1 after isolation for rat and 1 h after isolation for mouse, were placed on an inverted microscope (Eclipse TE2000; Nikon) and electrically stimulated at 0.2 Hz in a 37°C (rat) or 30°C (mouse) media bath containing modified Tyrode solution. Sarcomere length recordings were collected (1,000 Hz) using a CCD camera (MyoCam; IonOptix). Myocytes that did not follow the pacing protocol (0.2 Hz) were excluded. Sarcomere length shortening and relaxation kinetics were calculated using IonOptix software. Myocytes were initially analyzed under baseline conditions and then treated with different doses of W7 (Sigma) for 10 min and analyzed again. Ten-fifteen cells per cover slip were recorded for each treatment per myocyte prep with three to seven preps used for each dataset.

Ca\textsuperscript{2+} transient measurements in single intact myocytes. Ca\textsuperscript{2+} transient analysis was conducted using the IonOptix system as described previously (5). Briefly, isolated cells were incubated with 2 μM fura 2-AM for 10 min at room temperature. They were washed and incubated in Tyrode solution for 15 min for deesterification. Cells were then monitored using the IonOptix system for their 360-to-380 nm ratio for ratiometric Ca\textsuperscript{2+} transient analysis. Experimental conditions were the same as in the contractility measurements.

Langendorff. Hearts were cannulated via the aorta and perfused with Krebs-Henseleit solution bubbled with 95% O\textsubscript{2} and 5% CO\textsubscript{2}, as described with minor modifications (8). A balloon catheter was inserted in the left ventricle (LV) to measure isovolumic LV pressure. An electrode placed at the base of the heart controlled pacing frequency. Hearts were equilibrated for 20 min while stimulated at 7 Hz. A pacing protocol was initiated by increasing heart rate by 1-Hz increments for 2 min each, ranging from 7 to 12 Hz. After hearts reached a steady state at each frequency, contractile function was measured for at least 1 min. Hearts were then returned to 7 Hz and allowed to equilibrate for 5 min. The above protocol was repeated with W7 (10 μM). Hearts were excluded if initial baseline left ventricular developed pressure was <70 mmHg or if flow was less than 1 ml/min or greater than 5 ml/min.

RESULTS

W7 effects in membrane-intact adult cardiac myocytes. W7 has been shown to bind to cTnC and decrease Ca\textsuperscript{2+}-binding affinity, resulting in Ca\textsuperscript{2+} desensitization of the myofilament. Frampton and Orchard (11) have shown that W7 reduces contractility in a Ca\textsuperscript{2+}-independent manner in adult living cardiac myocytes. To document dose-response effects, we measured sarcomere length dynamics in the presence and absence of W7. Acute W7 treatment of adult cardiac myocytes dose dependently reduced peak amplitude of contraction and increased the time-to-peak contraction without affecting the kinetics of relaxation (Fig. 1, A–E). To determine if the
reduced contractility was Ca\(^{2+}\) dependent, we measured Ca\(^{2+}\) transients with fura 2. Figure 1, F–J, shows that no parameter of the Ca\(^{2+}\) transient is changed with W7, except for increased amplitude of the transient at 10 \(\mu\text{M}\) W7. This increased Ca\(^{2+}\) transient amplitude at 10 \(\mu\text{M}\) W7 is in direct contrast to the decreased contractility, further evidence that the depressed contractility phenotype is Ca\(^{2+}\) independent. In addition, the increased Ca\(^{2+}\) transient could be the result of decreased Ca\(^{2+}\) binding to cTnC, allowing for greater sarcoplasmic reticulum load and increased transients. The Ca\(^{2+}\) independence of the contractility phenotype suggests that off-target effects of W7, i.e., calmodulin inhibition, are not significantly playing a role under these conditions. These data provide evidence for W7 as a sarcomere-acting Ca\(^{2+}\) desensitizer in living cardiac myocytes, making W7 a valid proof-of-concept small molecule for sarcomere neutralization therapy.

Alkalosis. One experimental model of acquired Ca\(^{2+}\) sensitivity is alkalosis. To determine if sarcomere-acting small
molecules can mitigate hyper-Ca\textsuperscript{2+}-sensitive states, we tested W7 in alkalosis. Alkalosis has been shown to increase Ca\textsuperscript{2+} sensitivity of the myofilaments and increase contractility in a Ca\textsuperscript{2+}-independent manner in cardiac myocytes (18, 31). Adult cardiac myocytes were exposed to pH 8.1 Tyrode solution for 10 min. Alkalosis increased peak amplitude of contraction and reduced baseline sarcomere length (Fig. 2). These functional outcomes are characteristic of sarcomeric-based Ca\textsuperscript{2+} sensitization in adult cardiac myocytes and evidence that alkalosis does increase Ca\textsuperscript{2+} sensitivity in membrane-intact cardiac myocytes. W7 dose dependently mitigated both phenotypes, with 5 μM W7 completely neutralizing the effects of alkalosis, restoring function back to control values. These data provide evidence that sarcomere-acting small molecules can normalize hyperactivating sarcomeres in an acquired Ca\textsuperscript{2+}-sensitive-state in vitro model (alkalosis).

**RCM causing R193H cTnI myocytes.** To determine if sarcomere-acting small molecules can neutralize sarcomeric defects in a model of inherited hyper-Ca\textsuperscript{2+}-sensitive disease we tested W7 in RCM R193H cTnI transgenic myocytes. Human R193H patients have a severe restrictive phenotype. Studies have shown this mutation increases Ca\textsuperscript{2+} sensitivity of force development in permeabilized myocytes and that membrane-intact adult myocytes exhibit severe phenotypes of reduced resting sarcomere length and slow relaxation indicative of heightened Ca\textsuperscript{2+} sensitivity (5, 7). The reduced resting sarcomere length is due to a precontractile state where cTnC is partially activated, and this can be prevented by myosin inhibition (5). As shown in Fig. 3, R193H cTnI adult mouse myocytes have short resting sarcomere lengths and severe relaxation deficits as indicated by time-to-baseline at 75%. Acute W7 treatment of these myocytes shows a rapid dose-dependent neutralization of these disease phenotypes, with resting sarcomere length almost fully recovered and time-to-baseline 75% completely restored to nontransgenic values at 10 μM W7. In addition, peak amplitude of contraction is diminished in the R193H cTnI myocytes due to the precontractile state, which is reversed by W7 treatment. This acute recovery from a severe cellular phenotype provides evidence that drug-based Ca\textsuperscript{2+} desensitization can neutralize the hyper-Ca\textsuperscript{2+}-sensitive inherited cardiomyopathic disease state.

**DISCUSSION**

Small-molecule-based sarcomere neutralization seeks to target myofilament Ca\textsuperscript{2+} sensitivity as a direct mechanism to restore normal physiological performance to the diseased myocardium. Inherited cardiomyopathies often share a final common pathway of myofilament hyper-Ca\textsuperscript{2+} sensitivity (12, 34). The main new findings here provide proof-of-concept that sarcomere-acting small molecules can rapidly and effectively restore normal cellular and organ-level function in the setting of acquired and inherited cardiac disease models caused by hyper-Ca\textsuperscript{2+}-sensitive sarcomeres. Because of the rapid organ-
Studies using membrane-permeabilized isolated TnI switch peptide domain, thus decreasing TnI’s affinity for cTnC’s hydrophobic patch (23). Mechanistically, W7’s positive charged tail (Fig. 1) is modeled to repel two arginines at the beginning of the cardiac A regulatory domain of cTnC, alters Ca\(^{2+}\) sensitivity, and decreases TnI switch peptide affinity for cTnC’s hydrophobic patch (23, 29). Studies using membrane-permeabilized isolated myocytes show that W7 decreases the Ca\(^{2+}\) sensitivity of force development (1). Taken together, W7 is the best-characterized cTnC-targeting Ca\(^{2+}\) desensitizer to date. Our new data demonstrate that W7 dose dependently decreases live cell cardiac myocyte amplitude of contraction in a Ca\(^{2+}\)-independent manner. Importantly, Ca\(^{2+}\) transient amplitude and kinetics were unchanged or increased with W7, evidence that calmodulin inhibition, another target of W7 (15), is not playing a significant role in the contractility phenotype detailed here.

For this study, small-molecule W7 was investigated as a Ca\(^{2+}\) desensitizer. W7 is an excellent molecule to begin these investigations because of the wealth of biochemical data demonstrating W7 directly binds to cTnC to alter its Ca\(^{2+}\)-binding properties (23). Specifically, W7 binds to the NH\(_2\)-terminal regulatory domain of cTnC, alters Ca\(^{2+}\) affinity, and decreases cTnI switch peptide affinity for cTnC’s hydrophobic patch (23, 29). Mechanistically, W7’s positive charged tail (Fig. 1A) is modeled to repel two arginines at the beginning of the cardiac TnI switch peptide domain, thus decreasing TnI’s affinity for cTnC (29). Studies using membrane-permeabilized isolated myocytes as a model of restrictive cardiomyopathy (RCM) and chronic heightened sarcomeric Ca\(^{2+}\) sensitivity. A: representative traces of nontransgenic (NTg, black), cTnI R193H transgenic (red), cTnI R193H Tg + 5 \(\mu\)M W7 (blue), and cTnI R193H Tg + 10 \(\mu\)M W7 (green) normalized to baseline sarcomere length. B: baseline sarcomere length as a measure of cellular diastolic tone. C: peak amplitude of contraction. D: time from peak amplitude of contraction to 50% return to baseline as a measure of relaxation. E: time from stimulation to 50% of peak amplitude. F: time from peak amplitude of contraction to 75% return to baseline. NTg and cTnI R193H transgenic (R193H) adult mouse myocytes were isolated, plated on cover slips, and analyzed within 2 h of isolation. Two independent myocyte isolations with 10–15 myocytes/isolation were analyzed. W7 treatment was for 10 min at 30°C. Sarcomere length dynamics were analyzed at 0.2 Hz at 30°C. P < 0.05 based on 1-way ANOVA with Bonferroni post hoc test compared with NTg (*) and R193H (#).
line sarcomere length. This result is independent of an effect of W7 to change pH (11). In the inherited RCM model, acute W7 exposure rapidly increased baseline sarcomere lengths and corrected slow relaxation, supporting the hypothesis that it is sarcomere dysfunction, and not secondary cell/organ remodeling, that directly underlies the disease phenotype. At the organ level, R193H cTnI hearts show marked elevations in LVEDP, demonstrating diastolic dysfunction characteristic of the restrictive cardiomyopathy phenotype. Similar to the cellular findings, acute administration of W7 immediately restored LVEDP to control nontransgenic values. These organ-level results further support that sarcomere performance, independent of myocyte disarray, hypertrophy, or fibrosis, is a primary driver of disease dysfunction. In addition, the models used here induce sarcomeric hyper-Ca\textsuperscript{2+} sensitivity through distinctly different mechanisms, suggesting that a small-molecule strategy targeting cTnC may apply to a range of diseases arising from sarcomere dysfunction. This is evident with alkalosis predominately affecting contractility while the RCM model predominately affects relaxation and W7 neutralizes both phenotypes. Because HCM/RCM mutations give rise to different levels of Ca\textsuperscript{2+} sensitivity and patients present with heterologous symptoms (13), finding a therapeutic that targets a final common pathway is necessary. With hyper-Ca\textsuperscript{2+} sensitivity as the most basic mechanism underlying HCM/RCM, targeting cTnC with Ca\textsuperscript{2+} desensitizers is a viable avenue for experimental therapeutics.

Many cardiac disease states have a critical component of altered Ca\textsuperscript{2+} sensitivity, including HCM/RCM, dilated cardiomyopathy, ischemia, and heart failure (6). As such, therapeutics targeting the sarcomere to neutralize altered sarcomeric function need to be investigated. Levosimendan and pimobendan target cTnC to increase Ca\textsuperscript{2+} sensitivity of the myofilaments (17). Levosimendan, in Europe, and Pimobendan, in Japan, are in use clinically to treat heart failure (17, 27, 30). Whereas several small-molecule Ca\textsuperscript{2+} desensitizers have been developed for the treatment of heart failure, little attention has been paid to myofilament Ca\textsuperscript{2+} desensitizers. EGCG and resveratrol have been suggested to bind to cTnC and decrease Ca\textsuperscript{2+} sensitivity (19, 26, 28). EGCG was recently shown to improve diastolic dysfunction in HCM and RCM mouse models, suggesting that desensitization of the myofilament is therapeutically relevant (10, 32, 35). In a cTnI model of HCM, EGCG improved relaxation in working hearts and improved Ca\textsuperscript{2+} transient kinetics in cardiac myocytes (32). In a cTnI RCM model, EGCG improved diastolic parameters in echocardiography of whole animals treated chronically as well as acute treatment in cardiac myocytes improved relaxation (35). EGCG targets many different proteins and pathways (10), making it challenging to dissect the cTnC-targeted component. Blebbistatin, a myosin inhibitor, has been used experimentally as a Ca\textsuperscript{2+} desensitizer in the context of arrhythmias in HCM (3). Although blebbistatin was able to reduce arrhythmia potential in an HCM mouse model, contractility, which would necessarily be severely impaired, was not a main focus of that work. Although not ideal therapeutic candidates, EGCG and blebbistatin do highlight the role of Ca\textsuperscript{2+} sensitivity in HCM and RCM. The acute reversal of disease phenotypes with multiple small molecules in different models is strong evidence that cell-intrinsic sarcomere dysfunction is the main disease driving insult in HCM and RCM.

Our data build upon the work with EGCG in examining the utility of Ca\textsuperscript{2+} desensitizers in HCM/RCM. The findings presented here are similar to EGCG, specifically in acutely increasing relaxation at the cellular level and decreasing diastolic dysfunction in the heart of an RCM model. The replication of phenotypic changes seen with two different small-molecule desensitizers in RCM supports the therapeutic potential of sarcomere-acting Ca\textsuperscript{2+} desensitizers.

**Perspective**

With more than 1,400 different HCM mutations of 11 sarcomeric proteins linked to inherited cardiomyopathy, targeting each one by an alternative gene-based approach, for example, would be an arduous task. With a unifying feature of altered sarcomeric Ca\textsuperscript{2+} sensitivity, a small-molecule pharmaceutical approach has some inherent advantages, including not being disease gene specific. Targeting cTnC with small molecules for sarcomere neutralization therapy is an attractive strategy but not without limitations. First, off-target effects, as
seen with first-in-class molecules, would need to be addressed. For genetic disorders, HCM/RCM patients would need lifelong treatment, so long-term safety must be addressed. In addition, treatment would most likely need to be early in the course of disease to be most efficacious. This brings up the clinical conundrum of putting genotype-positive, phenotype-negative patients on a therapy (22). Finally, a method of detecting efficacy in human patients would need to be developed. Clinical studies are aiming to find better early markers of disease progression, which would be necessary for tracking outcomes. This is critical, because desensitizing too far could result in compromised systolic function and possibly dilated cardiomyopathy. Taking into account these limitations, continued effort to develop novel Ca\(^{2+}\) desensitizers has potential merit in remediating diseases of the cardiac sarcomere.

Note that, during the review of this work, a paper was published whereby a small-molecule inhibitor of myofilament function was shown to mitigate disease presentation in transgenic cardiomyopathic mice, supporting the main conclusions of the present work (13).

ACKNOWLEDGMENTS

We thank the Metzger lab for critical review and editing of this work.

GRANTS

This work was supported by grants from the National Institutes of Health [B. R. Thompson (T32) and J. M. Metzger] and the Lillehei Heart Institute (J. M. Metzger).

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

B.R.T. and J.M.M. conception and design of research; B.R.T. and J.M. performed experiments; B.R.T. and J.M. analyzed data; B.R.T. and J.M. interpreted results of experiments; B.R.T. and J.M. prepared figures; B.R.T. drafted manuscript; B.R.T. and J.M.M. edited and revised manuscript; B.R.T., J.M., and J.M.M. approved final version of manuscript.

REFERENCES


