Inhibition of JNK by novel curcumin analog C66 prevents diabetic cardiomyopathy with a preservation of cardiac metallothionein expression

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Wang Y, Zhou S, Sun W, McClung K, Pan Y, Liang G, Tan Y, Zhao Y, Liu Q, Sun J, Cai L. Inhibition of JNK by novel curcumin analog C66 prevents diabetic cardiomyopathy with a preservation of cardiac metallothionein expression. Am J Physiol Endocrinol Metab 306: E1239–E1247, 2014. First published April 8, 2014; doi:10.1152/ajpendo.00629.2013.—The development of diabetic cardiomyopathy is attributed to diabetic oxidative stress, which may be related to the mitogen-activated protein kinase (MAPK) c-Jun NH2-terminal kinase (JNK) activation. The present study tested a hypothesis whether the curcumin analog C66 [(2E,6E)-2,6-bis(2-trifluoromethyl)benzylidene) cyclohexanone] as a potent antioxidant can protect diabetes-induced cardiac functional and pathological changes via inhibition of JNK function. Diabetes was induced with a single intraperitoneal injection of streptozotocin in male C57BL/6 mice. Diabetic and age-matched control mice were randomly divided into three groups, each group treated with C66, JNK inhibitor (JNKi), or vehicle (1% CMC-Na solution) by gavage at 5 mg/kg every other day for 3 mo. Neither C66 nor JNKi impacted diabetic hyperglycemia and inhibition of body-weight gain, but both significantly prevented diabetes-induced JNK phosphorylation in the heart. Compared with basal line, cardiac function was significantly decreased in diabetic mice at 3 mo of diabetes but not in C66- or JNKi-treated diabetic mice. Cardiac fibrosis, oxidative damage, endoplasmic reticulum stress, and cell apoptosis, examined by Sirius red staining, Western blot, and thioarbituric acid assay, were also significantly increased in diabetic mice, all which were prevented by C66 or JNKi treatment under diabetic conditions. Cardiac metallothionein expression was significantly decreased in diabetic mice but was almost normal in C66- or JNKi-treated diabetic mice. These results suggest that, like JNKi, C66 is able to prevent diabetic upregulation of JNK function, resulting in a prevention of diabetes-induced cardiac fibrosis, oxidative stress, endoplasmic reticulum stress, and cell death, along with a preservation of cardiac metallothionein expression. Diabetes; cardiac; oxidative stress; JNK; metallothionein

Although many different pathogenic mechanisms are involved in the development of diabetic cardiomyopathy (DCM) (6, 31), a well-known complication of long-standing diabetes, the major contributing factor may be oxidative stress (5, 38). Hyperglycemia-induced production of reactive oxygen and/or nitrogen species (ROS and/or RNS) is a major risk factor for the development of cardiac and endothelial cell dysfunction (28, 35). Increased cellular levels of ROS and RNS within the myocardium have been associated with oxidative damage (22, 50), including the apoptotic cell death that plays an important role in the development of structural and mechanical changes associated with DCM (4, 51). Therefore, preventive interventions to reduce oxidative stress on the diabetic heart could prevent and/or delay the development and progression of DCM.

The endoplasmic reticulum (ER) is an essential organelle involved in the intrinsic pathway of apoptosis (14). It provides a specialized environment for the folding of secretory and membrane proteins (3). Various conditions, such as hyperglycemia, and instances where there is increased oxidative stress, can result in ER stress by disrupting the organelle’s ability to function properly inside the cell. In the past, studies from our laboratory and others’ as well have consistently implicated ER stress in the pathogenesis of DCM (8, 10, 20). In pathological conditions, several signaling pathways are activated to cope with ER stress, which are termed the unfolded protein response (UPR) (3). One major pathway of the UPR is to upregulate the expression of resident calcium-dependent chaperone proteins, such as glucose-regulated protein-78 (GRP78, also known as BIP), which can contribute to the repair of unfolded proteins (20). The UPR may play a critical role in the pathophysiology of the heart because some UPR pathways can lead to cell death (16). When ER stress becomes increased or when the UPR is compromised, intracellular cell death signals are immediately activated. This activation is mediated by three ER-resident proteins: protein kinase R-like ER kinase [(PERK), which acts upstream to activate transcription factor 4 (ATF4)], inositol-requiring kinase-1 (IRE-1), and activating transcription factor 6 (ATF6) (25). ER stress-mediated cell death signaling is involved in the activation of caspase-12 (26) and, consequently, activation of caspase-3 (25). CHOP (C/EBP homologous protein) is the proapoptotic basic leucine zipper transcription factor that is regulated by the ATF4 pathways (26).

Metallothionein (MT) is a small, cysteine-rich, metal-binding protein with biological functions in essential metal homeostasis, heavy metal detoxification, and cellular antioxidative defense (10, 11). MT is also important for cell survival, angiogenesis, apoptosis, and proliferation (46). MT antioxidant activity may attenuate local oxidative stress. Results from chemically induced MT synthesis in various organs have revealed that MT has a protective effect on organs exposed to radiation and chemical-induced oxidative stress. This result parallels the well-demonstrated finding that MT protects against diabetes-induced cardiovascular injury (9, 10, 39).
Curcumin, a natural polyphenol, has been widely investigated in its anti-inflammatory, antioxidant, and antimicrobial effects (36, 41). However, previous authors have summarized the limitations of its use, including malabsorption, and other adverse physiological effects (17). Thus, more studies are needed to evaluate the efficacy and safety of curcumin, especially the derivative of natural active curcumin, before it can be approved for human use. We have synthesized a few novel curcumin analogs from native curcumin (29, 30). Among these analogs, C66 (21, 29) was found to effectively protect diabetic rats from diabetes-induced kidney injury via inhibition of renal inflammatory responses and macrophage infiltration induced by high serum glucose (29). Furthermore, the renal protection from diabetes-induced injury of C66 was accompanied by significant inhibition of JNK (29). Considering the important role of JNK in ER stress and apoptotic cell death (18, 44), we hypothesized that inhibition of C66 might prevent diabetes-induced cardiac apoptosis via inhibition of ER stress and activation of endogenous antioxidant mechanisms such as MT expression.

Therefore, the present study was undertaken to investigate whether C66 can prevent the development of and/or delay the progression of diabetes-induced cardiac pathogenesis along with discovery of the underlying mechanism behind these results. To this end, we used a type 1 diabetic mouse model induced with a single dose of streptozotocin (STZ). Diabetic and age-matched control mice were treated with C66 for 3 mo. In addition, a portion of these C66-treated diabetic and age-matched control mice were also treated with JNK inhibitor (JNKi) for 3 mo. This was done in order to determine whether C66 protection against diabetes-induced cardiac pathogenesis is mediated by inhibition of JNK.

MATERIALS AND METHODS

Animals. C57BL/6J male mice, 8–10 wk of age, purchased from the Jackson Laboratory (Bar Harbor, Maine), were housed in the University of Louisville Research Resources Center at 22°C, on a 12:12-h light-dark cycle, free access to standard rodent feed, and free access to tap water. The Institutional Animal Care and Use Committee at the University of Louisville approved all experimental procedures for these animals, which complied with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

Diabetes model. To establish a type 1 diabetes model, C57BL/6J male mice were injected with a single intraperitoneal dose of STZ [Sigma-Aldrich, St. Louis, MO; dissolved in 0.1 M sodium citrate (pH 4.5)] at 150 mg/kg. Aged-matched control mice (Ctrl) received multiple injections of sodium citrate buffer at 150 mg/kg. Three days after injection, hyperglycemia (blood glucose levels >250 mg/dl) was noted in STZ-injected mice. Both diabetic and age-matched controls were randomly divided into three groups, each group treated by gavage with C66, JNKi, or vehicle (C66 and JNKi dissolved in 1% CMC-Na solution) for 3 mo. All solutions were distributed at 5 mg/kg every other day for 3 mo.

Noninvasive blood pressure monitoring. Blood pressure (BP) was measured by tail cuff manometer using a CODATM noninvasive BP monitoring system (Kent Scientific, Torrington, CT), based on our previous study (2). Briefly, the BP was measured for 10 acclimation cycles followed by 20 measurement cycles. After sufficient training, formal BP measurements were performed and BP data were collected.

Echocardiography. Transthoracic echocardiography (Echo) for anesthetized mice at rest was performed using a high-resolution imaging system for small animals (Vevo 770, Visual Sonics, Canada) equipped with a high-frequency ultrasound probe (RMV-707B). More details on the protocol were as described before (2). Two-dimensional and M-mode echocardiography was used to assess wall motion, chamber dimensions, and cardiac function. The final data represent averaged values taken from 10 mouse cardiac cycles.

Western blot analysis. Regular Western blot analysis was performed as described in our previous studies (10). The primary antibodies used included anti-phospho-SAPK/JNK (p-JNK; 1:1,000 dilution, Cell Signaling, Boston, MA), anti-SAPK/JNK (JNK; 1:1,000 dilution, Cell Signaling), connective tissue growth factor (CTGF; Santa Cruz Biotechnology, Santa Cruz, CA, 1:1,000), transforming growth factor-β1 (TGFβ1; 1:500 dilution, Santa Cruz Biotechnology), plasminogen activator inhibitor 1 (PAI-1; 1:1,000 dilution, BD Biosciences), anti-3-nitrotyrosine (3-NT; 1:1,000 dilution, Millipore), anti-glucose-regulated protein-78 (GRP78; 1:1,000 dilution, Cell Signaling), anti-inositol-requiring enzyme 1 (IRE-1; 1:1,000 dilution, Abcam), anti-phospho-IRE-1 (p-IRE-1; 1:1,000 dilution, Thermo scientific), anti-CHOP (1:1,000 dilution, Santa Cruz Biotechnology), anti-activating transcription factor 4 (ATF4; 1:1,000 dilution, Abcam), anti-cleaved caspase-12 (c-Cas12; 1:1,000 dilution, Exalpha Biologicals), anti-Bax (1:1,000 dilution, Cell Signaling), anti-Bcl-2 (1:1,000 dilution, Cell Signaling), and anti-Bcl-2 (1:1,000 dilution, Cell Signaling), and anti-β-actin (1:2,000, Santa Cruz Biotechnology).

MT expression was detected by a modified Western blotting protocol referenced in our previous study (40). Briefly, heart proteins were treated with DTT at a final concentration of 20 mM at 56°C for 30 min, followed by addition of iodoacetamide (Sigma Chemical) at 50 mM at room temperature for 1 h in the dark, and centrifuged to collect protein suspensions. In addition, after electrophoresis on 16% SDS-PAGE gel, proteins were transferred to nitrocellulose membrane with the transfer buffer, including 2 mM CaCl2, blocked in 3% BSA for 2 h at room temperature. The monoclonal antibody against human MT (Dako North America, Carpinteria, CA) was used at 1:1,000 dilution in 3% BSA at 4°C overnight. Since the transfer buffer contained CaCl2, blots for MT could not be stripped and reported for β-actin analysis. Therefore, a parallel gel was used for β-actin analysis using the same process referenced for other proteins.

Real-time qPCR. Real-time qPCR was as described in our previous studies (2). Primers of MT and GAPDH for PCR were purchased from Applied Biosystems (Carlsbad, CA). Real-time qPCR was carried out in a 20-μl reaction buffer that included 10 μl of TaqMan Universal PCR Master Mix, 1 μl of primer, and 9 μl of cDNA with the ABI 7300 Real-Time qPCR system. Fluorescent intensity of each sample was measured at each temperature to monitor the amplification of the target gene. Comparative cycle time (Ct) was used to determine fold differences between samples.

Quantitative analysis of lipid peroxides. The lipid peroxide concentration was detected by measuring TBA reactivity, reflected by the amount of malondialdehyde (MDA) formed during acid hydrolysis of the lipid peroxide compound. The reaction mixtures contained 50 μl of protein sample, 20 μl of 8.1% sodium dodecyl sulfate, 150 μl of 20% acetic acid solution (pH 3.5), and 210 μl of 0.571% TBA. Each sample was duplicated. The mixtures were incubated at 90°C for 1 h, cooled on ice, infused with 100 μl of distilled water, and centrifuged at 4,000 rpm for 15 min. After centrifugation, a 150-μl supernatant of each sample was taken out to measure the absorbance at 540 nm. The lipid peroxide (MDA) level was expressed in nanomoles of MDA per milligram tissue.

Sirius red staining. Cardiac fibrosis was assessed, using 0.1% Sirius red staining by the detection of collagen accumulation in cardiac myocytes. Briefly, 5-μm tissue sections were used for Sirius red staining with 0.1% Sirius red F3BA and 0.25% Fast Green FCF. Sections stained for Sirius red were then examined for the presence of collagen by using a Nikon Eclipse E600 microscopy system.

Statistical analysis. Data were collected from several animals (n = 3–5 per group) and presented as means ± SD as indicated. We used
A diabetes was induced (3M), as shown in Fig. 1, glucose, body weight and BP were measured before diabetes mice by a single intraperitoneal injection of STZ. The blood Type 1 diabetes was induced in treatment with C66 and JNKi.

**RESULTS**

Some metabolic changes in diabetic mice before and after treatment with C66 and JNKi. Type 1 diabetes was induced in mice by a single intraperitoneal injection of STZ. The blood glucose, body weight and BP were measured before diabetes was induced by giving STZ as a basal line (0M) and 3 mo after diabetes was induced (3M), as shown in Fig. 1, A–C. At 3 mo after diabetes onset, the heart weight-to-tibia length ratio was also measured to determine whether the heart became hypertrophic. Diabetes significantly increased blood glucose levels (Fig. 1A) and decreased weight gain (Fig. 1B) compared with control mice. There was no significant difference for the BP either between 0M and 3M or between diabetes and control (Fig. 1C), suggesting that a 3-mo course of diabetes had no effect on the BP. The heart weight/tibia length ratio (Fig. 1D) was significantly decreased in diabetic mice compared with control mice. The above parameters were also measured after treatment with C66 in diabetic and control mice. Our results showed that treatment with C66, at 5 mg/kg every other day for 3 mo, had no significant effect on blood glucose levels, body weight gains, BP, and the heart weight/tibia length ratio in diabetic or control mice. Since C66’s protection against diabetes-induced kidney injury was reportedly associated with JNK inhibition (29), we also explored whether inhibition of JNK using its inhibitor (JNKi) could protect against diabetes-induced cardiac injury and subsequent development of DCM. Like the treatment with C66, inhibition of JNK had no effect on blood glucose levels, body weight gains, BP, and heart weight/tibia length ratios in diabetic or control mice (Figs. 1, A–D).

*C66 inhibited diabetic activation of JNK phosphorylation, as did JNKi, in the heart.** JNK function in the hearts of diabetic and control mice was measured based on JNK phosphorylation level, using Western blotting. A significant increase in JNK phosphorylation level was seen in the hearts of diabetic mice but not in the hearts of diabetic mice treated with C66 or JNKi, respectively (Fig. 2). This demonstrated that, like JNKi, C66 has the capacity to function as a strong inhibitor of JNK.

*C66 prevented diabetes-induced cardiac dysfunction and remodeling.* DCM is characterized by ventricular dilatation, thinning of the ventricular wall, and decreased or preserved systolic function in the presence of diastolic dysfunction. In this study, cardiac performance parameters derived from echocardiography are shown in Fig. 3. The following indexes, indicative of left ventricular systolic function and cardiac structural change, were decreased in diabetic mice [ejection fraction (EF%), Fig. 3A; fractional shortening (FS%), Fig. 3B; left ventricular posterior wall (LVPW) thickness, Fig. 3C; and intraventricular septal wall thickness (IVS), Fig. 3D]. Treatment with either C66 or JNKi every other day for 3 mo completely prevented cardiac malfunctioning and remodeling from occurring.

*C66 prevented diabetes-induced cardiac fibrosis, oxidative stress, and cardiac apoptosis.* We examined the diabetes-induced fibrotic changes that occur in the heart after a long-standing course of the disease. Using Western blotting, we identified fibrotic markers such as CTGF (Fig. 4A), TGFβ1 (Fig. 4A), and PAI-1 (Fig. 4B). These markers were all significantly increased in diabetic mice but remained unchanged in diabetic mice treated with C66 or JNKi. The presence of diabetes-induced cardiac fibrosis was further confirmed by...
Sirius red staining for collagen accumulation in the diabetic mouse heart (Fig. 4C). A significant amount of collagen was identified, predominantly in interstitial areas, but also in perivascular areas as well. Treatment with either C66 or JNKi every other day for 3 mo greatly decreased the amount of diabetes-induced collagen accumulation that can accrue over many years of the disease.

Since oxidative stress has been suggested to play an essential role in the pathogenesis of diabetes-induced cardiac disease (7), our next study investigated whether C66 could protect the heart from oxidative stress by measuring cardiac 3-NT (Fig. 5A) expression as an index of nitrosative damage with Western blotting. In addition, as an index of lipid peroxidation, the amount of MDA (Fig. 5B) was measured by TBA reactivity. Diabetes-induced accumulation of 3-NT and increase in MDA contents were significantly prevented by treatment with either C66 or JNKi.

We further examined apoptotic death of cardiac cells in the heart by measuring the expression of antiapoptotic protein Bcl-2, proapoptotic protein Bax, and cleaved caspase-3 via Western blot analysis. As shown in Fig. 6, marked increases in the Bax-Bcl-2 expression ratio (Fig. 6A) and caspase-3 cleavage (Fig. 6B) were seen in diabetic mice. However, treatment with either C66 or JNKi significantly reversed the diabetes-induced apoptotic death of the cardiac cells in diabetic mice.

C66 prevented diabetes-induced ER stress as well as the associated ER cell death pathway. There is increasing evidence suggesting that ER stress and associated apoptotic cell death are involved in the development of DCM (20, 48). In the present study, Western blotting revealed significant increases in GRP78 (Fig. 7A), IRE-1 (Fig. 7B), ATF4 (Fig. 7C), CHOP (Fig. 7D), and cleaved caspase-12 (Fig. 7E) in diabetic mice; however, these changes were not seen in C66- or JNKi-treated diabetic mice.

C66 upregulated the expression of MT. Since oxidative stress is considered an important mediator of various cardio-
vascular complications of diabetes (42), we assumed that the above cardiac fibrosis and cardiac apoptosis in diabetic mice might be attributed to the increased oxidative stress. Concomitantly, the protective effect by C66 or JNKi on diabetes-induced cardiac pathology may be mediated by the upregulation of endogenous antioxidant MT. Thus, in our next study, MT expression was examined at mRNA level with RT-qPCR (Fig. 8A) and at the protein level level with Western blotting (Fig. 8B). There was a significant decrease in the expression of MT in diabetic mice compared with control mice, but not in either C66- or JNKi-treated diabetic mice.

DISCUSSION

In the present study, we have provided experimental evidence to show the protective effect of curcumin analog C66 on
diabetes-induced cardiac pathogenic changes. Significantly increased cardiac fibrosis, oxidative damage, ER stress, and apoptosis were found in STZ-induced type 1 diabetic mice compared with control mice. These pathological changes were significantly prevented by both C66 and JNKi treatments. These results suggest that C66 is able to prevent diabetes-induced cardiac injury, most likely mediated by inhibition of JNK, which reduces ER stress and associated cell death and increases cardiac MT expression.

This study defined the role of JNK inhibition in the prevention of diabetes-induced cardiac injuries. We demonstrated that expression of p-JNK (Fig. 2) was significantly increased in the heart of diabetic mice. Significant increases in cardiac oxidative damage (Fig. 5), apoptosis (Fig. 7), ER stress (Fig. 6), and remodeling (Figs. 3 and 4) were also seen in diabetic mice. These pathological alterations were significantly reversed by treatment with C66 and JNKi in diabetic mice. Therefore, we hypothesize that C66 may protect from DCM by the inhibition of JNK.

Evidence has shown that under hyperglycemic conditions the JNK function is activated (12, 29), accompanied by increased ROS generation and ER stress (12). It is well documented that the protective effect of curcumin from cardiovascular disease occurs through the inhibition of JNK (13, 15, 32, 49). Consistent with these findings, we also demonstrated that C66, accompanied with inhibition of JNK, is able to reduce the high glucose-induced inflammatory changes seen in the diabetic kidney (29).

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**Fig. 6.** Protective effects of C66 on diabetes-induced cardiac apoptosis. Expression of Bax/Bcl2 ratio (A) and cleaved caspase-3 (B) in cardiac tissue was detected by Western blotting assay. This was followed by semiquantitative analysis. Data are presented as means ± SD (n = 3–5). *P < 0.05 vs. corresponding Ctrl; #P < 0.05 vs. corresponding DM.

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**Fig. 7.** Diabetes-induced cardiac endoplasmic reticulum (ER) stress. ER stress-associated markers were examined by Western blotting assay for the expression of GRP78 (glucose-regulated protein-78; A), IRE1 (inositol-requiring kinase-1; B), ATF4 (activating transcription factor 6; C), CHOP (C/EBP homologous protein; D), and cleaved caspase-12 (C-Cas12; E). This was followed by semiquantitative analysis. Data are presented as means ± SD (n = 3–5). *P < 0.05 vs. corresponding Ctrl; #P < 0.05 vs. corresponding DM.
Reports from the literature have shown that curcumin as an antioxidant is able to scavenge ROS products (17), which may explain why it provided a beneficial effect on the hearts of diabetic rats via the Akt/GSK3β signaling pathway to attenuate fibrosis, oxidative stress, inflammation, and cell death (50) and also prevented ER stress-induced apoptosis in experimental autoimmune myocarditis rats (26). Recently, a novel water-soluble curcumin derivative (NCD) was shown to protect the heart from diabetes (1). However, to the best of our knowledge, there is no study that has mentioned the relationship among C66 and cardiac oxidative and ER stress-mediated progression in type 1 diabetes models.

Overgeneration of ROS could result in oxidative stress that causes the development and progression of various cardiovascular diseases, including diabetic complications (38, 43). Disulfide bond formation in the ER causes oxidative stress and leads to apoptotic cell death (23). It has been reported that increased production of ROS and nitrogen oxide (NO) is involved in the impairment of cardiac endothelial cell function in heart failure (27). Excessive production of NO by inducible nitric oxide synthase contributes to active ER stress through the depletion of ER Ca2+ stores (25). In this study, we observed that increased oxidative stress and ER stress were present in diabetic mice. Both C66 and JNKi treatments significantly decreased oxidative stress markers (Fig. 5), ER stress markers (Fig. 7), and caspase family proteins, including caspase-12 (Fig. 7E) and caspase-3 (Fig. 6B).

In addition, C66 inhibition of JNK phosphorylation and apoptotic death of cardiac cells, leading to cardiac protection from diabetic damage, may be mediated by MT upregulation. It has been widely reported that MT offers antioxidative protection from various conditions, including diabetes in our own laboratory (9, 10, 24, 37) and in others’ (39, 47). We found that diabetes decreased MT expression in the heart along with pathological cardiac damage. A previous study from our team showed that attenuation of early cardiac cell death, via suppression of mitochondrial oxidative stress, can prevent the development of DCM in cardiac-specific MT-overexpressing transgenic diabetic mice (10). In addition, we also described that MT can prevent diabetes-induced cardiac ER stress, along with its associated cell death, most likely via its antioxidant action (45).

In fact, the potential cross-talk between JNK phosphorylation and MT expression and the effect of JNK function in cadmium-induced apoptotic cell death has been implicated by a couple of previous studies (19, 34). In those studies, cadmium-resistant (Cd-R) cells were developed with cadmium-sensitive (Cd-S) rat lung epithelial cells (LEC) by stepwise exposure of LECs to cadmium chloride from 1 to 20 μM after 20 passages. All these Cd-R cells were significantly protected from cadmium-induced apoptotic death compared with Cd-S LECs (Cd-S). The basal mRNA level of MT was dramatically higher in Cd-R cells than in Cd-S cells. Treatment of both Cd-R and Cd-S cells with high levels of cadmium resulted in increasing JNK phosphorylation along with apoptotic cell death in Cd-S cells, which was significantly low and less in Cd-R cells with high MT level. A strong activator of JNK, Ro318220, could reverse the cadmium-sensitive phenotype in Cd-R cells (19, 34). Furthermore, in MT knockout fibroblasts, JNK1/2 phosphorylation was markedly increased after cadmium exposure compared with similarly treated wild-type cells (34). Another study indicated that MT suppression of JNK activation-mediated cell death is mediated by MT’s scavenging of ROS (33). These results indicate that the lesser apoptotic effect of cadmium in Cd-R cells was associated with MT overexpression, which might be linked to the specific suppression of the JNK pathway; however, whether MT directly inhibits JNK phosphorylation or not is unclear. The present study has shown that neither C66 nor JNKi impacts MT phosphorylation in nondiabetic heart but indeed prevents diabetic decrease in MT expression at both mRNA and protein levels (Fig. 8), suggesting that either C66 or JNK impact MT expression indirectly.

In summary, we have investigated whether C66 can protect the heart from diabetes-induced pathological changes. Using a type 1 diabetes model, we treated diabetic and age- and sex-matched control mice with 5 mg/kg C66 every other day for 3 mo. We concluded that treatment with C66 can prevent or greatly reduce diabetes-induced cardiac oxidative damage, fibrosis, ER stress, and cellular apoptosis in diabetic mice. The mechanism responsible for this protective effect is mediated by the inhibition of JNK. Although additional exploration is needed surrounding its underlying mechanism, we propose that JNK inhibition, mediating C66’s protective cardiac effects from diabetes-induced damages, may lead to a decrease in ER stress and an upregulation of MT expression in the cardiac myocytes. Also, the specific mechanism for the preservation of MT expression by C66 and JNKi is unclear, suggesting a need for further investigation on this topic as well. That said, the present study provides new and exciting evidence supporting...
the potential clinical applications of C66 for diabetic patients in order to prevent their long-standing diabetes-induced cardiovascular complications.

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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

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