Vitamin B1 Analogue Benfotiamine Prevents Diabetes-Induced Diastolic Dysfunction and Heart Failure through Akt/Pim-1 Mediated Survival Pathway

Katare et al. Benfotiamine prevents diabetic cardiomyopathy

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Total Words: 5958

**Background:** The increasing incidence of diabetes mellitus (DM) will result in a new epidemic of heart failure (HF) unless novel treatments able to halt diabetic cardiomyopathy early in its course are introduced. The present study aimed to determine whether the activity of the Akt/Pim-1 signaling pathway is altered at critical stages of diabetic cardiomyopathy and whether supplementation with vitamin B1 analogue Benfotiamine (BFT) helps sustain the above pro-survival mechanism, thereby preserving cardiomyocyte viability and function.

**Methods and Results:** Untreated streptozotocin-induced type-1 or leptin-receptor mutant type-2 diabetic mice showed diastolic dysfunction evolving to contractile impairment and cardiac dilatation and failure. BFT (70mg/kg/day) improved diastolic and systolic function and prevented left ventricular end-diastolic pressure elevation and chamber dilatation in both diabetic models. Moreover, BFT improved cardiac perfusion and reduced cardiomyocyte apoptosis and interstitial fibrosis. In hearts of untreated diabetic mice, the expression/activity of Akt/Pim-1 signaling declined along with O-GlcNAc modification of Akt, inhibition of pentose phosphate pathway, activation of oxidative stress and accumulation of glycation end-products. Furthermore, diabetes reduced STAT3 phosphorylation independently of Akt. BFT inhibited these effects of DM, thereby conferring cardiomyocytes with improved resistance to high glucose-induced damage. The PI3K inhibitor LY294002 and dominant-negative Akt inhibited BFT’s anti-apoptotic action and Pim-1 upregulation in high glucose-challenged cardiomyocytes.

**Conclusions:** These results demonstrate that BFT protects from DM-induced cardiac dysfunction through pleiotropic mechanisms culminating in the activation of pro-survival signaling pathway. Thus, BFT merits attention for application in clinical practice.

**Keywords**

Diabetes mellitus; Cardiomyopathy; Diastolic dysfunction; Benfotiamine; Apoptosis
Diabetes mellitus (DM) is a potent and prevalent risk factor for heart failure (HF), independently of coronary artery disease or hypertension. Diabetic cardiomyopathy has an insidious onset and remains therefore undiagnosed and untreated in a large number of patients. Furthermore, recent studies showed evidence of diastolic dysfunction in up to 75% of young, asymptomatic patients with type-1 or type-2 DM. The association of diastolic dysfunction and microangiopathy synergistically increases the risk of HF, thus pointing out the urgent need of early mechanistic treatment.

A variety of molecular alterations have been associated to diabetic cardiomyopathy, including defects in calcium homeostasis and substrate metabolism, accumulation of advanced glycation end-products (AGE), activation of the hexosamine pathway and oxidative stress leading to cardiomyocyte apoptosis. However, early-stage mechanisms remain mostly unknown.

The pivotal role of the phosphoinositide-3-kinase (PI3K)/Akt/Proviral integration site for Moloney murine leukemia virus-1 (Pim-1) signaling pathway in the control of cardiac contractile function and cardiomyocyte growth and survival is well established. Of note, the heart of mice with long-standing DM shows decreased levels of activated Akt and, intriguingly, signal transducer and activator of transcription 3 (STAT3, an upstream modulator of Pim-1)-deficient mice spontaneously develop a form of dilated cardiomyopathy similar to that occurring in diabetic mice. However, to the best of our knowledge, no information exists on whether altered glucose metabolism may dampen the activity of the STAT3/Akt/Pim-1 trio from early phases of cardiomyopathy and whether pharmacological manipulation of such kinases could help halting DM-induced cardiac damage.

The conversion of glucose to pentose is hampered in DM because of the inhibition of pivotal enzymes of the pentose phosphate pathway, like transketolase and glucose-6-phosphate dehydrogenase (G6PD), resulting in depletion of reducing agents and accumulation of glycolysis end-products. Inhibition of transketolase activity was ascribed to deficit of its coenzyme thiamine. This situation is further aggravated by the fact that increased reactive oxygen species (ROS) induce compensatory activation of...
the DNA repairing enzyme poly(ADP-ribose) polymerase, which in turn inhibits the activity of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a crucial enzyme of glycolysis. Intermediate metabolites of glycolysis are consequently forced down to alternative pathways, including AGE formation and hexosamine pathway, which are responsible for protein modification/inactivation and cardiovascular damage.17, 18

The aim of the present study was twofold: (1) to verify whether high glucose might alter STAT3/Akt/Pim-1 expression and activity from early stages of cardiomyopathy and (2) to determine whether Benfotiamine (BFT) supplementation can sustain pro-survival signaling and prevent cardiac dysfunction in DM. The rationale of BFT supplementation is to correct thiamine deficit and thereby provide a shunt for glucose through the pentose pathway.

Results of the present study demonstrate the chronological alterations in the pro-survival signaling during the progression of diabetic cardiomyopathy and its correction by treatment with BFT.
Material and Methods

Details are provided online in Expanded Materials and Methods.

Ethics

Experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals (the Institute of Laboratory Animal Resources, 1996) and with approval of the British Home Office and the University of Bristol. Type-1 diabetes was induced in male CD1 mice (Charles River, UK) by injection of streptozotocin (STZ; 40 mg/kg body weight i.p. per day for 5 days). Age-matched animals that received STZ-vehicle served as non-diabetic (healthy) control. In addition, male obese leptin-receptor mutant BKS.Cg-+/Lepr<sup>db</sup>/OlaHsd mice (Harlan, UK) were used as a model of insulin-resistant type-2 DM. Elevations of blood glucose begin at four to eight weeks in these mutant mice. Age-matched lean mice (BKS.Cg-m+/Lepr<sup>db</sup>/OlaHsd) were used as control.

Treatment protocol

The experimental protocols are summarized in Supplemental Fig. 1A&B. Type-1 DM mice were randomly assigned to receive BFT (70mg/kg body weight per day) or vehicle (1mMol/L HCl) in drinking water starting at 4 weeks from DM induction (last day of STZ injection) throughout the study. Type-2 DM mice were similarly randomized to treatments starting from 9 weeks of age. The elected BFT dosage reportedly produces a 4-fold increase in plasma thiamine. Gender- and age-matched healthy (for type-1 DM) or db/+ (for type-2 DM) mice of the same genetic background of diabetic ones were given vehicle or BFT and used as reference.

Biochemical Measurements

Confirmation of DM was achieved by measurements of blood glucose levels through the study (Supplemental Fig. 2). Transketolase, G6PD and GAPDH activities in peripheral blood erythrocytes and LV tissue were measured at sacrifice as described.
AGE levels were measured by ELISA (Cosmobio, Japan) (n=5 mice per group).\textsuperscript{16, 17, 21}

**Echocardiography**

Dimensional and functional parameters were measured using a high-frequency, high resolution echocardiography system (Vevo 660, Visual Sonics, Toronto, Canada). Left ventricular (LV) chamber dimensions, LV ejection fraction (LVEF) and fractional shortening (LVFS) were determined as described.\textsuperscript{23}

**Measurement of LV pressures and cardiac dimensions**

LV pressure (LVP) was measured in anesthetized mice (n=10 to 12 per group) before sacrifice, using a high-fidelity 1.4F transducer tipped catheter (Millar Instruments, Houston, TX, USA).

**Measurement of blood flow**

Myocardial blood flow (BF) was assessed by the use of fluorescent microspheres, which were injected into the LV cavity (n= 4 to 6 mice per group).\textsuperscript{24}

**Immunohistochemistry**

Immunohistochemical techniques were used to identify myocardial endothelial cells (isolectin B-4 staining) and smooth muscles cells (α-smooth muscle actin), fibrosis (sirius red), apoptosis (TUNEL) and hydroxyl radicals (8-OH-DG). Superoxides were revealed using dihydroethidium staining of LV cryostat sections.

**In vitro Studies**

*Pro-survival effect of BFT on high glucose-challenged cardiomyocytes*

HL-1 cardiomyocytes (a gift from Prof. William Claycomb, Louisiana State University Medical Center, New Orleans, USA) were cultured in the presence of high D-glucose (HG, 30mM) or normal glucose (5mM) added with 25mM D-mannitol as
osmotic control. After 72h, cells were supplemented with either BFT (150μM) or vehicle (1mM HCl) for further 24h. Optimal BFT concentration was decided on the basis of pilot titration studies (Supplemental Fig 3A). In addition, a fixed dosage of BFT was tested to contrast the apoptotic effect of increasing doses of D-glucose (Supplemental Fig 3B). At the end of the experiments, cardiomyocytes were collected for measurement of caspase-3/7 activity (Caspase-Glo® 3/7, Promega), performed in 6 wells per each condition and repeated 3 times.

Inhibition of PI3K, Akt or STAT3

To verify the involvement of PI3K/Akt in BFT-induced pro-survival effects, we used either the PI3K inhibitor LY-294002 (50μM, Sigma Aldrich) or HA-tagged dominant negative mutated form of Akt (Ad.DN-Akt, K179M) or control Ad.Null (both at 100MOI). In a separate study, HG-treated HL-1 cardiomyocytes were exposed for 30min to STAT3 inhibitor peptide (Ac-PpYLKTK-OH, 1mM, Calbiochem), a cell permeable compound which reduces the levels of active STAT3 and inhibits DNA-binding activity of STAT3 by forming an inactive STAT3-peptide complex. This is followed by treatment with BFT or vehicle. After additional 24h, cells were used for caspase-3/7 activity and WB analyses as described above. WB were performed in triplicates and repeated three times and caspase-3/7 activity was performed in 6 wells per each condition and repeated 3 times.

Expression Studies

Western blot analyses were performed on LV samples and HL-1 cardiomyocytes to verify the effects of DM, HG and treatment on total and pSTAT3 (Tyr705), Protein phosphatase 2A (PP2A), total and peNOS (Ser1177), total and pAkt (Ser473), Pim-1, total and pBad (Ser112), Bcl-2, total and pFOXO-1 and cleaved caspase-3 expression.

To assess the O-GlcNAc modification of Akt, LV extracts were immunoprecipitated with anti-Akt antibody (Cell signaling). The precipitate was then immunoblotted with antibodies for O-GlcNAc (1:10000, a gift from Prof. G.W. Hart, Johns Hopkins University School of Medicine, Baltimore, USA) and for total and pAkt. Akt activity
in protein extracts was measured with a commercial kit (Assaydesigns/Stressgen, USA).

Quantitative RT-PCR (qPCR) for mouse Pim-1 and 18s rRNA (for normalization) was performed in a LightCycler.

All the expression analyses were performed on 4 biologic replicates.

**Statistical Analysis**

Results are represented as mean±standard deviation. The hemodynamic and echocardiographic measurements were compared by use of repeated measures Two-Way ANOVA (factorial design: two independent variables, (1) treatment and (2) presence or absence of diabetes), followed by pair-wise comparison using the Holm-Sidak method.

For the histological, biochemical and morphometric analysis, difference between multiple groups were analyzed using one-way ANOVA and difference between two groups using t-test (paired or unpaired as appropriate). For blood flow and molecular expression studies, when normality test fails, differences between groups were analyzed using Siegel-Tukey test. Survival curves were analyzed by the Kaplan-Meier method and comparisons were made with the Gehan-Breslow log-rank test using SigmaStat statistical software. A P value of <0.05 is considered statistically significant for all the parameters.
Results

BFT attenuates DM-induced cardiac remodeling and dysfunction

The murine models studied here showed distinct phases in the evolution of cardiomyopathy. The initial phase consisted of diastolic dysfunction, as indicated by the significant decrease in E/A ratio at 4 weeks from type-1 DM induction (Fig. 1A). A similar figure was observed in 9 week-old type-2 diabetic mice (Fig. 2A). BFT partially rescued the reduced E/A ratio and prevented the further deterioration of diastolic function with duration of DM in both diabetic models (Fig. 1A and 2A, P<0.01 versus healthy for both comparisons).

The second phase consisted of impaired contractile performance and cardiac dilatation. In type-1 diabetic mice, systolic function started declining from 12 weeks of DM, as indicated by the progressive reduction in LVEF and LVFS (Fig. 1B). The late stage was characterized by LV chamber enlargement, LV wall thinning and critical fall in cardiac output, which are typical features of HF (Fig. 1B and Supplemental Fig 4A).

Analysis of LV pressure indices and PV loops further verified the marked dysfunction of diabetic hearts (Fig. 1C). In type-2 diabetic mice, systolic dysfunction was precocious, being already apparent at 9 weeks of age and rapidly advancing toward HF (Fig. 2 and Supplemental Fig. 4B). Importantly, BFT treatment resulted in the global improvement of LV performance, pressure indices and volumes in both diabetic models (Fig. 1, Fig. 2 and supplemental Fig. 4, P<0.01 versus vehicle-treated diabetic for all parameters), but did not affect cardiac function in healthy mice (data not shown). Inotropic and lusitropic responses to adrenergic stimulation were blunted in type-1 diabetic mice (P<0.01 versus healthy), but improved by BFT (P<0.01 versus vehicle-treated diabetic, Supplemental Fig. 5).

BFT improves survival rate

Type-1 diabetic animals survived less than healthy controls, but survival rate was remarkably improved by BFT (Supplemental Fig. 6, P<0.001 versus vehicle-treated).
BFT improves myocardial BF

As shown in Figure 3A, myocardial perfusion was reduced by DM, with this defect being prevented by BFT in both diabetic models (P<0.01 versus vehicle-treated diabetic).

Concordantly, capillary density was reduced in the LV of diabetic mice (P<0.001 versus healthy), and partially conserved by BFT (P<0.01 versus vehicle-treated diabetic, Fig. 3B). Fractional analysis of arteriole density revealed a marked decrease in small (<30 μm of diameter) arterioles in the LV of both diabetic models (P<0.001 versus healthy for both comparisons), which was inhibited by BFT (P<0.001 versus vehicle-treated diabetic for both comparisons, Fig. 3C).

Histological validation of BFT effects

Cardiomyocyte cross sectional area was reduced in type-1 diabetic mice (66±8 versus 83±6 μm² in healthy, P<0.01) and preserved by BFT (79±7 μm², P<0.01 versus vehicle-treated diabetic).

BFT remarkably prevented cardiomyocyte apoptosis and interstitial fibrosis in both diabetic models (Fig. 4, P<0.01 for both comparisons).

BFT activates the pentose phosphate shunt pathway and reduces oxidative stress

We found that the activity of transketolase, G6PD and GAPDH is reduced in diabetic hearts (Fig. 5A-C) and associated to marked increase in AGE (Fig. 5D) and O-GlcNAc protein modification (vide infra). BFT prevented these effects and additionally avoided the increase in ROS levels in the heart of both diabetic models (Fig. 5E and Supplemental Fig. 7, P<0.01 versus vehicle-treated diabetic for both comparisons).

BFT prevents DM-induced downregulation of STAT3/Akt/Pim-1

Surprisingly, at initial stages, type-1 diabetic hearts showed increased pAkt and peNOS levels, but low Pim-1 protein (Fig. 6) and mRNA expression (Supplemental
Importantly, Pim-1 continuously declined with progression of cardiomyopathy, in parallel with accruing changes in its upstream modulators. In temporal sequence, the first change consisted of the decrease in the activated form of STAT3 (STAT3-p-Tyr705), which reportedly induces Pim-1 expression.29 This was followed by increase in protein phosphatase 2A (PP2A), which is known to induce Pim-1 mRNA downregulation and protein degradation (data not shown).30 At a later stage, when HF was overtly manifested, the diabetic myocardium showed reduced pAkt (Fig. 6B) and Akt activity (Fig. 6J) as well as increased O-GlcNAc modification of Akt, which was previously associated with Akt inhibition (Fig. 6K).31 Decreased were also peNOS and pFOXO-1 levels in the failing heart of type-1 diabetic mice (Fig. 6C&D).

Previous studies showed that both Akt and Pim-1 phosphorylates the pro-apoptotic Bad, resulting in the formation of Bad-(14-3-3) protein homodimer, which leaves Bcl-XL and Bcl-2 free to inhibit apoptosis.11, 32, 33 We found that downregulation of Akt/Pim-1 signaling by type-1 DM is associated to reduced pBad (Ser 112) and Bcl-2 levels and increased cleaved caspase-3 (Fig. 6G-I).

The heart of type-2 diabetic mice exhibited a marked decrease in STAT3/Pim-1 and modification of Pim-1 downstream effectors but, at variance with type-1 DM, also showed an early reduction in Akt phosphorylation and activity (Fig. 7). The global depression of STAT3/Akt/Pim-1 was mirrored by a more rapid evolution of cardiomyopathy.

Importantly, BFT prevented the downregulation of STAT3 and Pim-1 as well as the O-GlcNAc modification of Akt, thereby preserving Akt activity and downstream targets in both diabetic models (Fig. 6 and Fig. 7). In contrast, myocardial PP2A levels remained elevated in BFT-treated diabetic mice (data not shown).

Finally, to verify the direct action of BFT on cardiomyocytes, we performed in vitro assays, in which adult cardiomyocytes were cultured in HG or NG in the presence of BFT or vehicle. Consistent with in vivo experiments, HG increased cardiomyocyte apoptosis, this effect being prevented by BFT (Supplemental Fig. 9A). The anti-apoptotic action of BFT was paralleled by conservation of Pim-1 at mRNA
(Supplemental Fig. 8C) and protein level (Supplemental Fig. 9B) and inhibition of HG-induced effects on pBad and Bcl-2 (Supplemental Fig. 9C&D). Furthermore, BFT contrasted HG-induced decreases in pSTAT3 (Supplemental Fig. 9E), pAkt and Akt activity (Supplemental Fig. 9F&G) and peNOS and pFoxo-1 (Supplemental Fig. 9H&I). Of note, HG reduced the nuclear localization of Akt, which is necessary for Akt to induce Pim-1 expression and phosphorylate/inhibit FOXOs, but BFT restored proper nuclear Akt levels (Supplemental Fig. 9J).

Infection of cardiomyocytes with Ad.DN-Akt, verified by assessing the expression of HA-tag (data not shown), abrogated the stimulating action of BFT on pAkt, without affecting STAT3. Furthermore, Ad.DN-Akt significantly inhibited BFT’s anti-apoptotic action under HG and contrasted BFT’s effects on Pim-1, pBad and Bcl-2 (Supplemental Fig. 10A-F). Prevention of apoptosis by BFT was similarly reduced by the PI3K inhibitor LY294002 (Supplemental Fig. 10G). Treatment of cardiomyocytes with STAT3 inhibitor reduced basal and BFT-stimulated pSTAT3 levels, without affecting pAkt (Supplemental Fig. 10H&I). STAT3 inhibition potentiated Pim-1 downregulation and cardiomyocyte apoptosis under HG (Supplemental Fig. 10J&M). It also contrasted BFT in preserving Pim-1 expression and to a lesser extent pBad and Bcl-2 (Supplemental Fig. 10J-L) but was ineffective in inhibiting BFT’s anti-apoptotic action, suggesting that pAkt induction by BFT could compensate for STAT3/Pim-1 deficit to support cardiomyocyte viability under HG conditions.
Discussion

In a previous report in STZ mice, a 2-week administration of BFT preserved in vitro contractile properties and intracellular Ca\(^{2+}\) kinetics of cardiomyocytes.\(^{35}\) To the best of our knowledge, however, this is the first study to demonstrate the therapeutic potential of long-term treatment with BFT for prevention of cardiomyopathy in models of type-1 and type-2 DM. This is particularly relevant in the light of the prevalent incidence of cardiomyopathy in patients with type-2 DM. The effect of BFT was documented by echocardiography follow-up of LV function, Millar transducer measurement of LVP and cardiac morphometry. Furthermore, we newly report that BFT prevents myocardial microvascular rarefaction, thereby improving myocardial perfusion of diabetic hearts. The role of microcirculation in the pathogenesis of diabetic cardiomyopathy has been highlighted by a study in which gene therapy with VEGF-A prevented capillary rarefaction and cardiac dysfunction in mice.\(^{36}\) Our in vitro studies, however, indicate a direct action of BFT on cardiomyocyte survival, thus the improved myocardial BF might additively contribute to preservation of cell viability and cardiac performance.

Another novel contribution of the present study is the first documentation of chronologic alterations in components of pro-survival signaling during progression of cardiomyopathy and of BFT’s impact on these molecular changes. Our results of reduced myocardial pAkt levels in failing diabetic hearts are consistent with earlier reports in mice with long-standing DM,\(^{37,38}\) yet the mechanism responsible for Akt inhibition was not clear. Emerging evidence indicates that HG-induced activation of hexosamine pathway plays a role in the etiology of diabetic cardiomyopathy through O-GlcNAc modification of transcription factors and proteins involved in cardiomyocyte function.\(^{39}\) Here, we demonstrate for the first time the combination of reduced Akt activity and increased O-GlcNAc modification of Akt in myocardium of diabetic mice. Inactivation of Akt may account for increased cardiomyocyte apoptosis in failing diabetic hearts, since Akt directly controls the phosphorylation and sequestration of the Bad and FOXO transcription factors.\(^{40}\) Of note, BFT, which reportedly reduces the glucose flux to the hexosamine pathway,\(^{17}\) abrogated DM-induced O-GlcNAc
modification of Akt, thereby restoring Akt activity as well as Bad and FOXO-1 phosphorylation levels.

One intriguing aspect of our results consists of increased Akt activity at early stages of cardiomyopathy in type-1 diabetic mice, which is possibly a compensatory attempt to combat DM-induced damage. On the other hand, chronic Akt activation could have detrimental effects on the heart through inhibition of IRS/PI3K signaling and disruption of the coordinated association between adaptive cardiac hypertrophy and microvascular growth. Of note, while confirming the reduction of microvascular density in failing diabetic hearts, our data show no evidence of hypertrophic remodeling during initial elevation of Akt in type-1 diabetic hearts. The time duration of Akt activation might have been too short to sustain LV hypertrophy in our experimental setting. Furthermore, the dual action of Akt on cardiomyocyte survival and growth could depend on the specific intracellular location of activated Akt.

Many of the cardiac actions of Akt are ascribed to be Pim-1 dependent. For instance, Akt activation induces Pim-1 expression, but forced expression of Akt failed to protect the infarcted myocardium of Pim-1–deficient mice. Pim-1-induced cardioprotection is reportedly mediated by upregulation of Bcl-2 and Bcl-XL as well as phosphorylation/inactivation of Bad. Our in vitro findings showing that PI3K/Akt inhibition abrogates the action of BFT on cardiomyocyte survival under HG and contrasts the stimulatory effect of BFT on Pim-1 and Bcl-2 indicate that Akt is a crucial facet of cardioprotection exerted by this compound. This concept is reinforced by the finding that BFT-induced Akt upregulation in HG-challenged cardiomyocytes is sufficient for prevention of apoptosis under STAT3 inhibition.

Our in vivo findings in type-1 diabetic mice showed however a situation more intricate than predicted in vitro. In the compensated phase, changes in myocardial pAkt and Pim-1 levels were not synchronous, with Pim-1 starting to decline earlier than pAkt. This pattern might suggest a crucial role of Pim-1 in early diastolic dysfunction and also indicate the contribution of mechanisms independent of Akt in Pim-1 downregulation. One possible candidate is STAT3, which upregulates Pim-1 expression by binding with
its promoter. In line, we found that pSTAT3 is reduced at compensated stages of diabetic cardiomyopathy. In addition, DM induced the expression of PP2A, which is known to dephosphorylate and inactivate Pim-1. An additional explanation for the apparent discrepancy between high pAkt and low Pim-1 levels at early stage of cardiomyopathy is that pAkt might remain sequestered in the cytosol and thus unable to upregulate Pim-1 expression. This is in line with the reduced nuclear localization of Akt in HG-challenged cardiomyocytes. Of note, BFT prevented the reduction in pSTAT3 and preserved nuclear Akt, but failed to decrease PP2A. Thus, BFT’s action seemingly impact on positive regulators of Pim-1 rather than on mechanisms of Pim-1 destabilization.

The protective action of BFT extends to other mechanisms implicated in the pathogenesis of diabetic cardiomyopathy. Hyperglycemia causes oxidative stress by inducing ROS and dampening antioxidants generation. In this study, we newly demonstrated that BFT reduces superoxide and hydroxyl radical levels in diabetic hearts by inducing the activation of pentose phosphate pathway, which regenerates the antioxidant NADPH. Furthermore, superoxide overproduction by hyperglycemia inhibits the glycolytic enzyme GAPDH, thereby diverting metabolites from glycolysis into major pathways of hyperglycemic damage. Importantly, we demonstrated that these effects of DM were inhibited by BFT as evidenced by increased GAPDH activity and reduced AGE levels. Prevention of AGE accumulation could account for inhibition of interstitial fibrosis by BFT.

In conclusion, our results illustrate fundamental mechanisms which are involved in the pathogenesis of diabetic cardiomyopathy and could be prevented by BFT (summarized in Supplemental Fig.11). Implications of the present preclinical study to the clinic field should be cautiously evaluated in the light of similarities and differences of cardiomyopathy developing in diabetic patients and animal models. Furthermore, the dosage and time schedule of BFT supplementation represent a crucial issue as for other preventive treatments, with the additional caveat that sustained activation of proto-oncogenes, like Akt and Pim-1, might increase the risk of cancer. This concern
however is not supported by our results showing no evidence of adverse effects but rather improved survival of diabetic mice treated with a high dose of BFT.
Acknowledgement

We thank Dr. Nicolle Kraenkel for her kind assistance in qPCR and Mr. Paul Salvage for his kind assistance in immunohistochemistry.

Sources of Funding

This study was supported by a project grant from Diabetes UK (RD06/0003413) and Resolve (202047). CE holds a British Heart Foundation (BHF) Basic Science fellowship (BS/05/01) and at the time of the studies, AC was a BHF PhD student (FS/05/113/19965).

Disclosures

None.
References


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Figure Legends

**Figure 1: Benfotiamine prevents ventricular dysfunction in type-1 DM.**

(A) Representative pulsed Doppler images and table showing the effect of benfotiamine (BFT) on $E/A$ ratio. $E$ and $A$ waves represent mitral valve velocity during early diastolic filling and atrial contraction, respectively (n=16 mice per group). (B) Indexes of left ventricular (LV) function assessed by echocardiography (n=16 per group). (C,i–iii) Bar graphs showing the effect of BFT on LV end diastolic pressure (LVEDP), LV end systolic pressure (LVESP) and maximum and minimum rates of developed pressure (dP/dt) at 16 weeks of treatment (n=12 per group). (C,iv) Representative pressure-volume loops obtained by integrated measurement of LV pressure (Millar catheter) and volume (echocardiography). Values are mean±SD. BFT did not affect cardiac function in healthy mice (data not shown). Results of pair-wise comparison are illustrated: *P<0.01 and **P<0.01 versus healthy; #P<0.01 and ##P<0.01 versus vehicle-treated diabetic.

**Figure 2: Benfotiamine prevents ventricular dysfunction in type-2 DM.**

(A) Representative images and table showing the effect of BFT on E/A ratio (n=10 per group). (B) Echocardiography indices of LV function (n=10 per group). (C,i–iii) Bar graphs showing the effect of BFT on LV pressure and volume indices at 8 weeks of treatment (n=10 per group). (C,iv) Representative pressure-volume loops. Values are mean±SD. BFT did not affect cardiac function in db/+ mice (data not shown). Results of pair-wise comparison are illustrated: *P<0.01 and **P<0.001 and ***P<0.0001 versus db/+; #P<0.01, ##P<0.001 and ###P<0.0001 versus vehicle-treated db/db.

**Figure 3: Benfotiamine improves myocardial blood flow and vascularization.**

(A) Bar graphs showing the effect of BFT on myocardial blood flow in type-1 (n=6 per group, 16-weeks treatment) and type-2 diabetic mice (n=4 per group, 8-weeks treatment). Age-matched untreated healthy controls for the two diabetic groups are shown for comparison (n= 4 to 6 mice) (B-C) Representative immunohistochemistry
images of microvasculature and bar graphs showing LV capillary (B) and arteriole densities (C) of diabetic mice (n=6 per group, treatment duration as above). Age-matched untreated healthy control for type-1 DM and db/+ for type-2 DM groups are shown for comparison (n=6) Scale bars are 50\(\mu\)m. Results of pair-wise comparison are illustrated: *P<0.01 and **P<0.001 versus healthy in type-1 DM or db/+ in type-2 DM; *P<0.01 and ##P<0.001 versus vehicle-treated diabetic in type-1 DM or db/db in type-2 DM.

**Figure 4: Benfotiamine prevents cardiomyocyte apoptosis and cardiac fibrosis.**

(A-B) Representative microphotographs and bar graphs showing the effect of BFT on cardiomyocyte apoptosis and interstitial fibrosis (n=5 per group). Scale bars are 50\(\mu\)m. Treatment duration and statistical analysis as in Fig. 3. *P<0.01 and **P<0.001 versus healthy in type-1 DM or db/+ in type-2 DM; *P<0.01 versus vehicle-treated diabetic in type-1 DM or db/db in type-2 DM.

**Figure 5: Benfotiamine activates the pentose phosphate pathway and inhibits ROS.**

Bar graphs showing the effect of BFT on transketolase (A), G6PD (B), GADPH (C) and AGE (D) (n=5 per group, each assay in triplicate). Representative microphotographs and bar graphs showing myocardial hydroxyl radical levels (E, n=5 per group). Treatment duration and statistical analysis as in Fig. 3. *P<0.01, **P<0.001 and ***P<0.0001 versus healthy in type-1 DM or db/+ in type-2 DM; #P<0.001, ##P<0.001 and ###P<0.01 versus vehicle-treated diabetic in type-1 DM or db/db in type-2 DM.

**Figure 6: Benfotiamine stimulates prosurvival signaling in type-1 DM.**

Representative blots (A) and bar graphs (B-I) showing the levels of pAkt, peNOS, pFOXO-1, pSTAT3, Pim-1, pBad, Bcl-2, and cleaved caspase-3 (n=4 per group). (J) Bar graphs showing myocardial Akt activity. (K) Representative immunoblots and bar graphs showing O-GlcNAC modification of Akt in diabetic hearts (n=4 per group).
Values are expressed as n-fold changes toward age-matched healthy. Siegel-Tukey test detected statistical differences as illustrated: *P<0.01 and **P<0.001 versus healthy. #P<0.01 and ##P<0.001 versus vehicle-treated diabetic.

Figure 7: Benfotiamine stimulates prosurvival signaling in type-2 DM.

Representative blots (A) and bar graphs (B-I) showing the levels of pAkt, peNOS, pFOXO-1, pSTAT3, Pim-1, pBad, Bel-2, and cleaved caspase-3 (n=4 per group). (J) Bar graphs showing myocardial Akt activity. (K) Representative immunoblots and bar graphs showing O-GlcNAC modification of Akt in diabetic hearts (n=4 per group). Values are expressed as n-fold changes toward age-matched healthy. Siegel-Tukey test detected statistical differences as illustrated: *P<0.01, **P<0.001 and ***P<0.001 versus db/+; #P<0.01 and ##P<0.001 versus vehicle-treated db/db.
Figure 2

A

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B

End systolic LV chamber volume

End diastolic LV chamber volume

Ejection fraction

Fractional shortening

C

(i) LVEDP

(ii) LVEDP

(iii) dP/dt

(iv) PV loop

- db/+  
- db/db  
- db/db-BFT
Figure 3

A. Cardiac Perfusion

B. Capillary density

C. Arteriole density

Healthy  Diabetic  Diabetic-BFT  db/+  db/db  db/db-BFT
Figure 4
A. Transketolase activity

Peripheral blood erythrocytes

Myocardium

B. G6PD activity

Peripheral blood erythrocytes

Myocardium

C. GAPDH activity

D. AGE levels

Legend:
- Healthy
- Diabetic
- Diabetic-BFT
- db/+  
- db/db
- db/db-BFT
E. Hydroxyl radicals

**Type-1 DM**

- Healthy
- Diabetic
- Diabetic-BFT

**Type-2 DM**

- db/+ 
- db/db 
- db/db-BFT

% of 8-OHDD positive cardiomyocyte nuclei

- Healthy
- Diabetic
- Diabetic-BFT
- db/+ 
- db/db 
- db/db-BFT

Significance:

- *: p < 0.05 compared to Healthy
- #: p < 0.05 compared to Diabetic
Figure 6i
J. Akt kinase activity

K. O-GlcNAc-Akt modification
Figure 7i

A. Age of mice

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Figure 7ii

B. pAkt/Akt

C. peNOS/eNOS

D. pFOXO-1/FOXO-1

E. pSTAT3/STAT3

F. Pim-1/Actin

G. pBad/Bad

H. Bcl-2/Actin

I. Cleaved caspase-3/Actin

(db+/ db/db db/db-BFT)

Figure 7ii

J. Akt kinase activity

![Graph showing Akt kinase activity across different ages and genotypes.](image)

K. O-GlcNAc-Akt modification

![Table and diagram showing O-GlcNAc-Akt modification at different ages and genotypes.](image)
Vitamin B1 Analogue Benfotiamine Prevents Diabetes-Induced Diastolic Dysfunction and Heart Failure through Akt/Pim-1 Mediated Survival Pathway
Rajesh G. Katare, Andrea Caporali, Atsuhiko Oikawa, Marco Meloni, Costanza Emanueli and Paolo Madeddu

_Circ Heart Fail._ published online January 27, 2010; _Circulation: Heart Failure_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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SUPPLEMENTAL MATERIAL

Expanded Methods

Echocardiography
Measurements of dimensional and functional parameters were performed at baseline, before mice entered the treatment, and every 4 weeks thereafter, using a high-frequency, high resolution echocardiography system (Vevo 660, Visual Sonics, Toronto, Canada). Briefly, mice were anesthetized using tribromo-ethanol and transferred to an imaging stage equipped with a warming pad for controlled maintenance of mouse body temperature at 37°C and a built-in electrocardiography system for continuous heart rate (HR) monitoring. Standard B mode (2D) images of the heart and pulsed Doppler images of the mitral valve inflow were acquired. The thickness of the left ventricle (LV) was measured at the level of the papillary muscles in parasternal short axis at end-systole and end-diastole. LV ejection fraction (LVEF) and fractional shortening (LVFS) were determined as described by De Simone et al.¹

Measurement of intra-ventricular pressure
Terminal measurement of left ventricular pressure (LVP) was made at 20 weeks after DM induction in type-1 diabetic mice (n=12 in each group) and at 17 weeks of age in type-2 diabetic mice (n=10 in each group). The body temperature of the mice was maintained between 36º and 37º C throughout the experiment using a homeothermic blanket warming system. A tracheotomy was made and the mouse was intubated using the 23 gauge catheter, secured in place with 6-0 silk suture.

A high-fidelity 1.4F transducer tipped catheter (Millar Instruments, Houston, TX, USA) was zeroed in 37°C saline. Calibration of the transducer was verified using a mercury manometer, as suggested by the manufacturer. The right carotid artery was isolated, and tow ties were gently pulled back, using hemostats, to block blood flow from vessel. When pulsatile flow was no longer visible, a small cut was made just below the distal tie, and the catheter was placed inside the carotid artery and secured in place. The transducer was advanced into the heart, where its
position was confirmed by the rapid deflection of the diastolic pressure wave without any change in systolic pressure. Mice were allowed to stabilize for 10 min. After stabilization, baseline data were collected, including the HR, Peak LV systolic pressure (LVESP), LV end-diastolic pressure (LVEDP), and maximal rates of LV pressure rise (dP/dt\text{max}) and fall (dP/dt\text{min}).\textsuperscript{2} For the pressure volume relationship, the recording from Millar catheter was synchronized with echocardiography measurements as per manufacturer instructions.

**Measurement of transketolase activity**

Frozen heart tissue was defrosted, chopped finely and a 10% homogenate was prepared with 0.1 M Tris-HCl buffer (pH 8.0) and centrifuged at 3,000 x g for 10 min. The lysate was kept on ice until used. For erythrocytes, peripheral blood samples were centrifuged (2,000 x g, 5 min) and the plasma and white blood cells were removed. The packed erythrocytes pellet was washed three times with PBS and lysed with ddH\textsubscript{2}O and membrane fragments sedimented (10,000 x g, 10 min, 4°C). The activity of transketolase in myocardial tissue homogenate and erythrocytes lysate was determined by the method of Chamberlain et al.\textsuperscript{3, 4} Aliquot (200 µl) of substrate cocktail (14.8 mM R-5-P, 253 µM NADH, 185 U/ml TPI, and 70 µl of 21.5 U/ml GDH in 250 mM Tris/HCl buffer, pH 7.8, all from Sigma Chemical, UK) was added to the wells of a 96-well microplate and 20 µl of a 6-fold dilution of erythrocyte lysate or tissue homogenate was added. The absorbance at 340 nm was monitored at 10 min intervals for 120 min and the rate of decrease in absorbance between 10 to 80 min was used to deduce the rate of oxidation of NADH in the GDH catalyzed reaction, which is rate limited by the transketolase catalyzed conversion of R-5-P and xylulose-5-phosphate to sedoheptulose-7-phosphate and GA3P under these conditions.

**Measurement of glucose-6-phosphate dehydrogenase (G6PD) activity**

G6PD activity was determined by measuring the rate of production of NADPH as previously described.\textsuperscript{5, 6} In brief, the samples were prepared in a similar manner explained above for transketolase activity. Aliquot (250µl) of substrate cocktail (50 mM glyglycine, pH 7.4, 2 mM D-glucose-6-phosphate, 100mM 6-phosphogluconic acid, 670 µM βNADP and 10mM MgCl\textsubscript{2})
was added to the wells of a 96-well microplate and 12.5 µl of a 6-fold dilution of myocardial tissue homogenate or erythrocyte lysate was added. The absorbance at 340 nm was monitored at 1min intervals for 5min. A second 12.5µl of a 6-fold dilution of erythrocyte lysate or tissue homogenate was added to a separate substrate cocktail (250µl) without D-glucose-6-phosphate and the absorbance was measured for 5 min. G6PD activity was calculated by subtracting the rate of change of absorbance with or without D-glucose-6-phosphate to eliminate the contribution of 6-phosphogluconate dehydrogenase (6PGD) to total NADPH production, as 6PGD also produces NADPH. Protein content in each sample was measured by BioRad assay with commercially available kit (BioRad, UK). Data are represented as units/min/ml for erythrocytes and units/min/mg of protein for myocardial homogenates.

**Measurement of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) activity**

GAPDH activity was measured using the cytosolic fraction of myocardial tissue homogenate, which is prepared by centrifuging the myocardial tissue lysate at 100,000 x g at 4°C for 30 min. Enzyme activity was measured as described earlier. Briefly, 1µg of cytosolic protein was added to 200µl of assay buffer (100mM Tris/HCl pH 8.6, 1.5mM NAD, 3mM dithiothreitol, 5mM sodium arsenate, 1.5mM glyceraldehydes-3-phosphate) at room temperature. NADH formed was determined by monitoring the increase in absorbance at 340 nm at 10 sec interval for 1 min and then every minute for 60 min. Activity was expressed as units/sec/mg of protein.

**Measurement of blood flow using fluorescent microspheres**

Myocardial perfusion was measured using fluorescent microspheres. A polyethylene (PE10) catheter was inserted through the right carotid artery for the reference blood withdrawal. Microspheres, 0.02µm in diameter (Molecular Probes, CA, USA) were injected into the LV cavity over 1 min and flushed with 0.15 ml of 0.9% NaCl. Reference blood was collected via the carotid catheter starting 15 sec before to 1 min after the microsphere injection. The animals were sacrificed 2 min later and the heart was removed and separated into LV, RV and septum. The kidneys were also collected and analyzed as internal control organs to demonstrate homogenous distribution of the microspheres throughout the bloodstream. Each sample was weighed, cut into
small pieces and digested in 10 ml of 2 M ethanolic KOH containing 0.5% Tween 80 at 60°C for 48h with constant shaking. After complete digestion of tissues, the microspheres were collected by centrifugation at 2,000 x g for 20 min and sequential washing with 10 ml of deionized water with or without 0.25% Tween 80. Finally, microspheres were dissolved in 3 ml of 2-ethoxyethylacetate and the fluorescence intensity was determined using a fluorophotometer (Fluostar Optima, BMG labtech). Regional blood flow was calculated as the absolute blood flow in ml/min/g of tissue as described earlier.  

Assessment of myocardial capillary and arteriole densities
LV sections (3µm) were deparaffinized and incubated with biotinylated Isolectin B4 (Invitrogen, Molecular Probes) followed by streptavidin Alexa Fluor 488 (Invitrogen, Molecular probes) for measurement of capillary density. To measure arteriole density, the sections were incubated with anti-mouse α-smooth muscle cell actin antibody (Sigma chemicals) conjugated with Alexa Fluor 488 (Invitrogen, Molecular probes). Capillaries and arterioles were calculated in at least 20 fields at X400 magnification and the final data expressed as the number of capillaries or arterioles per square millimeter. Arterioles were also categorized according to their luminal size.  

Assessment of myocardial fibrosis
Myocardial fibrosis was analyzed by Sirius red staining followed by morphometric analysis using the Image Pro analysis software (MediaCybernetics, USA) and the data expressed as the ratio between intensity of staining and area examined.

TUNEL staining
Apoptosis was quantified on paraffin embedded LV sections (3µm) by the terminal deoxynucleotidyltransferase (TdT)-mediated dUTP nick-end labeling (TUNEL) technique (in situ cell death detection kit Fluorescein, Roche applied science, USA). Following treatment of slides with proteinase K (20 µg/ml, 30min at 37°C), TUNEL assay was performed according to the manufacturer’s instruction. The same sections were then stained with DAPI to recognize nuclei. To recognize cardiomyocytes, sections were also stained with mouse monoclonal primary
antibody for the cardiomyocyte marker α-sarcomeric actin (Dako, 1: 50, overnight at 4°C), which was revealed by counterstaining with the secondary antibody conjugated to fluorophore (Alexa 568, Invitrogen, Molecular probes). Twenty fields were randomly evaluated in each section at X400 magnification. The fraction of TUNEL positive nuclei over total cardiomyocyte nuclei was then calculated.10

In situ detection of reactive oxygen species

Dihydroethidium staining for detection of superoxide

Superoxide production in the myocardium was determined using the fluorescent dye dihydroethidium (DHE, Invitrogen, Molecular probes). LV cryosections (10µm) were incubated with 5 µmol/L DHE, at 37°C for 30 min, in a humidified chamber. Images (X100 magnification) were captured on an Olympus fluorescence microscope fitted with camera (Media cybermatics) and the mean DHE fluorescence intensity of myocyte nuclei was calculated by dividing the combined fluorescence value of the pixels by the total number of pixels in 15 randomly selected field using Image-Pro advanced software.11

8-OHGDG staining for detection of hydroxyl radicals

Myocardial production of hydroxyl radicals was determined by immunofluorescent staining of the deparaffinized LV sections (3µm) using the primary antibody for 8-hydroxy-2'-deoxyguanosine (8-OHGDG, Cosmo Bio, Japan). The nuclear localization of 8-OHGDG was detected using the goat anti-mouse secondary antibody conjugated to fluorophore (Alexa 568, Invitrogen, Molecular probes) and counterstaining the cardiomyocytes with α-sarcomeric actin and DAPI to recognize nuclei. Images (X1000 magnification) were captured using an Olympus fluorescence microscope fitted with camera (Media cybernetics, USA) and data expressed as percentage of 8-OHGDG positive nuclei.12

Immunocytochemical analysis for Akt localization

Effect of HG on localization of Akt was detected using immunocytochemical analysis. For this purpose, HL-1 cells were fixed with 4% paraformaldehyde following exposure to HG for 24h or 72h with or without treatment with BFT. After repeated washing and subsequent blocking with
serum, the cells were incubated with primary antibody against Ser473- phospho-Akt (Cell Signaling, 1:1000) at 4°C overnight. The nuclear or cytoplasmic localization of pAkt was detected using the goat anti-rabbit secondary antibody conjugated to fluorophore (Alexa 488, Invitrogen, Molecular probes). Images (X400 magnification) were captured using an Olympus fluorescence microscope fitted with camera (Media cybernetics, USA).

**Western blot analyses**

Proteins were extracted from LV using ice-cold RIPA buffer. Protein concentration was determined using the Bio-Rad protein assay reagent (Bio-Rad). Detection of proteins by western blot analysis was done following separation of whole tissue / cell extracts (50μg) on SDS-polyacrylamide gels. Proteins were transferred to polyvinylidene difluoride membranes (PVDF, Amersham-Pharmacia) and probed with the following antibodies: anti-mouse Ser1177-phospho-eNOS (Cell Signaling, 1:1000), eNOS (Cell Signaling, 1:1000), Ser473-phospho-Akt (Cell Signaling, 1:1000), Akt (Cell Signaling, 1:1000), Ser256- phospho-FOXO-1 (Upstate, UK), FOXO-1 (Cell Signaling, 1:1000), Tyr705 phospho-STAT3 (Cell signaling, 1:500), STAT3 (Cell Signaling, 1:1000), Pim-1 (Santacruz biotechnology, 1:250), Ser112-phospho-Bad (Cell Signaling, 1:1000), Bad (Cell Signaling, 1:1000), Bcl-2 (Cell Signaling, 1:1000) and cleaved-caspase-3 (Cell Signaling, 1:1000). Actin (Cell Signaling, 1:1000) was used as loading control. For detection, secondary antibody goat anti-rabbit or anti-mouse conjugated to horseradish peroxidase (both from Amersham Pharmacia, 1:5000) were used, followed by chemiluminescence reaction (ECL, Amersham Pharmacia).

**RT-PCR**

Total RNA was isolated from LV samples as well as from HL-1 cells (Trizol, Invitrogen, UK) and reverse transcribed (Sensiscript reverse transcriptase, Qiagen). Quantitative PCR (qPCR) was performed in a LightCycler (Roche, Burgess Hill, UK) using Platinum taq polymerase (Qiagen) and the primer pairs listed below. For quantification, mRNA amount of the respective gene was normalized to the amount of18S rRNA using the 2–DDCT method. Each reaction was performed in triplicate.¹³
**In vitro inhibition of PI3K and Akt**

To verify the involvement of PI3K/Akt in BFT-induced pro-survival effects, we used two protocols. In the first protocol, HG-treated HL-1 cardiomyocytes were exposed to the PI3K inhibitor LY-294002 (50µM, Sigma Aldrich)\(^\text{10}\) for 24h followed by treatment with BFT (150µM) or vehicle (1mM HCl). In the second protocol, HG-treated HL-1 cardiomyocytes were infected with an adenovirus carrying a HA-tagged dominant negative mutated form of Akt (Ad.DN-Akt, K179M) or control Ad.Null (both at 100MOI).\(^\text{10}\) After 24h, the medium was replaced with a fresh one supplemented with either BFT or vehicle. After additional 24h, cells were used for measurements of caspase-3/7 activity (6 wells per each condition and repeated 3 times) and western blot (WB) analyses (n=4 samples per group).
References


10. Caporali A, Sala-Newby GB, Meloni M, Graiani G, Pani E, Cristofaro B, Newby AC,


Supplemental Figure 1A - Experimental protocol with type-1 DM mice

Basal Echocardiography (n=140)

Injection of streptozocin (STZ, 40mg/kg/ip for 5 days)

Pre-treatment Echocardiography at 4 weeks after the last STZ injection (n=120)*

Randomization

Vehicle (n=60) at 4 weeks after STZ (1mMol/l HCl in drinking water)

Benfotiamine (n=60) at 4 weeks after STZ (in drinking water, 70mg/kg/day)

Randomization

Echocardiography at 8, 12, 16 and 20 weeks after STZ

Collection of heart for molecular biology at 8, 12 and 20 weeks after STZ

Randomization

Measurement of intraventricular pressure by pressure tip catheter (n=at least 12 in each group).

Evaluation of cardiac perfusion using fluorescent microspheres (n=6).

Sampling for histology and immunohistochemistry (n=7).
Supplemental Figure 1B - Experimental protocol with type-2 DM mice

Pre-treatment Echocardiography (n=28, 9 weeks of age)

Randomization

Vehicle (n=14)
(1mMol/l HCl in drinking water)

Benfotiamine (n=14)
(in drinking water, 70mg/kg/day)

Randomization

Echocardiography at 13 and 17 weeks of age

Collection of heart for molecular biology at 13 and 17 weeks of age

Randomization

Measurement of intraventricular pressure by pressure tip catheter (n=10, 17 weeks of age)

Evaluation of cardiac perfusion using fluorescent microspheres (n=4, 17 weeks of age)

Sampling for histology and immunohistochemistry (n=6, 17 weeks of age)
Supplemental Figure 2 - Serum glucose levels

A. Type-1 DM

B. Type-2 DM
Supplemental Figure 3 - Caspase-glo 3/7 Assay

A. Titration of Benfotiamine dosage

30mM glucose

B. Benfotiamine inhibits the apoptotic action of increasing doses of glucose

Glucose concentration (mM)
Supplemental Figure 4 - Cardiac function

A. Type-1 DM

B. Type-2 DM
Supplemental Figure 5

A

B

-0.6 -0.4 -0.2 0 0.2 0.4 0.6 0.8 1 1.2
0 4 8 12

(min)

-12 -8 -4 0 4 8 12
0 4 8 12

(min)

 Healthy  Diabetic  Diabetic-BFT

Healthy  Diabetic  Diabetic-BFT

Area under curve (X10⁴)

Area under curve (X10⁴)
Supplemental Figure 6 - Survival curve

- Healthy
- Diabetic
- Diabetic-BFT

Weeks after STZ or vehicle

Survival
Supplemental Figure 7

DHE staining for superoxides in type-1 DM heart

Healthy

Diabetic

Diabetic-BFT

Intensity/Area ratio (arbitrary units, AU)

Healthy  Diabetic  Diabetic-BFT

**  ****  **

*  ***  ##
Supplemental Figure 8

A: Pim-1 mRNA expression in type-1 DM heart

B: Pim-1 mRNA expression in type-2 DM heart

C: Pim-1 mRNA expression in HL-1 cells exposed to high glucose
Supplemental Figure 9
Molecular changes in HL-1 cells exposed to high glucose

A. Caspase-glo 3/7 Assay

B. Phospho-PLIN-1/Actin

C. Phospho-Bad/Bad

D. Phospho-Bad/Actin

E. Phospho-STAT3/STAT3

F. Phospho-Akt/Akt

G. Akt kinase assay

H. Phospho-eNOS/eNOS

I. Phospho-Foxo-1/Foxo-1

J. pAkt localization

NG, NGB, HG, HGB
Supplemental Figure 10 (i)

Inhibition of benfotiamine-induced expressional effects by Ad.DN-Akt

A

B

C

D

E

F

G. Inhibition of benfotiamine-induced pro-survival effects by LY294002

Caspase-glo 3/7 Assay

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Supplemental Figure 10 (ii)

Inhibition of benfotiamine-induced expressional effects by STAT3 Inhibitor peptide

Graphs showing the expression levels of various proteins under different conditions.
Supplemental Figure 11

Diabetes Mellitus/ Hyperglycemia

BFT → STAT3  PP2A  Akt ← BFT

Pim-1

Phosphorylation of Bad
Preservation of Bcl-2
Inhibition of apoptosis
Cell survival
Legends to Supplemental Figures

Supplemental Figure 1
Illustration of experimental protocols in type-1 (A) and type-2 (B) diabetic mice. Animals were randomized in subsequent phases for: (1) treatment with BFT or vehicle (4 weeks after the last STZ injection throughout duration of the study), (2) echocardiography/survival follow up or molecular biology assessment at indicated time points and (3) terminal measurement of intra-ventricular pressure or measurement of cardiac perfusion, or sampling of the heart for histology. *In type-1 DM protocol, 120 mice showing overt glycosuria entered the study, while the remaining 20 were discarded because of unsuccessful induction of DM.

The same protocol was carried out in parallel in (1) age-matched mice (healthy controls of type-1 DM), which were injected with the STZ-vehicle and 4 weeks later were randomly allocated to treatment with BFT or its vehicle (n=40 each) and (2) age-matched lean mice (BKS.Cg-m+/+Leprdb/OlaHsd, db/+ ) which were randomly allocated to treatment with BFT or its vehicle at 9 weeks of age (n=14 each)

Supplemental Figure 2
Scatter plots show the serum glucose levels in type-1 (A) and type-2 (B) diabetic mice during treatment with BFT or vehicle. **P<0.001 versus respective control healthy mice in type-1 DM or db/+ mice in type-2 DM; #P<0.05 or ##P<0.01 versus pre-treatment (time 0, corresponding to 9 weeks of age) in db/db mice.

Supplemental Figure 3
Bar graphs show the levels of activated caspase-3/7 in cultured adult cardiomyocytes.
A. Cardiomyocytes were cultured in normal glucose (NG) or high glucose (30mM) with different concentrations of benfotiamine (B) or vehicle (V). Values are expressed as relative units (RLU) and are mean ± standard deviation. *P<0.01 and **P<0.001 versus NG; #P<0.01 versus V.
B. Cardiomyocytes were cultured in normal (5mM D-glucose) or high glucose (15 - 35mM
D-glucose) with 150µM benfotiamine or vehicle. Values are expressed as relative units (RLU), and are mean ± standard deviation. **P<0.001 versus 5mM D-glucose and ^P<0.01 versus corresponding vehicle group.

Each experiment was performed in 6 wells per each condition and repeated 3 times.

Supplemental Figure 4
Echocardiographic assessment of cardiac function in type-1 (A, n=16 in each group) and type-2 diabetic mice (B, n=10 in each group). Upper panels show linear measures of LV cavity captured at end-systole and end-diastole. Lower panels show LV posterior wall thickness at end-diastole and cardiac output. Values are mean ± standard deviation. BFT did not affect cardiac parameters in healthy mice (data not shown). Results of pair-wise comparison are illustrated: ^P<0.01, **P<0.001 and ***P<0.0001 versus vehicle-treated healthy mice in type-1 DM or db/+ mice in type-2 DM; #P<0.01 and ##P<0.001 versus vehicle-treated diabetic mice in type-1 DM or db/db mice in type-2 DM. End systolic LV chamber internal diameter values of healthy and diabetic-BFT mice from 4 and 12 weeks overlapped and are therefore expressed by the same line.

Supplemental Figure 5
(A) Line graphs show changes in the maximum (inotropic) and minimum (lusitropic) rates of developed pressure in response to adrenergic stimulation (epinephrine 1mg/kg/IV) in healthy and type-1 diabetic mice, given BFT or vehicle. (B) Average response to adrenergic stimulation is expressed as area under the curve. Values are mean ± standard deviation. Results of pair-wise comparison are illustrated: ^P<0.01 versus vehicle-treated healthy mice; âP<0.01 versus vehicle-treated diabetic mice. Each group consisted of 6 mice.

Supplemental Figure 6
Line graph shows the effect of BFT on survival of type-1 diabetic mice. Mice were followed until 20 weeks after STZ (n=32 vehicle-treated or BFT-treated diabetic mice) or STZ-vehicle injection (n=40 healthy mice). Diabetic mice showed an increased mortality (P<0.001 versus
healthy mice), which was prevented by BFT (P<0.001 versus vehicle-treated diabetic mice). The survival rate of type-2 diabetic mice was not determined because of the limited group size.

Supplemental Figure 7
Representative microphotographs and bar graphs show the effect of BFT on superoxide levels in myocardium at 20 weeks from STZ or STZ-vehicle injection in type-1 diabetic mice. Each group consisted of 5 mice. Values are mean ± standard deviation. Results of pair-wise comparison are illustrated: *P<0.001 and **P<0.001 versus healthy mice; ##P<0.001 versus vehicle-treated diabetic mice. Scale bars are 100µm.

Supplemental figure 8
(A-B) Bar graphs show the levels of Pim-1 gene expression in LV of type-1 (at 8, 12 and 20 weeks from STZ or STZ-vehicle injection, A) or type-2 diabetic mice (at 13 and 17 weeks of age, B). Each group consisted of 4 mice. Values are expressed as n-fold changes toward vehicle-treated healthy mice and are mean ± standard deviation. *P<0.01 and **P<0.001 versus healthy mice in type-1 DM or db/+ mice in type-2 DM; #P<0.01 and ##P<0.01 versus vehicle-treated diabetic mice in type-1 DM or db/db mice in type-2 DM. (C) Bar graphs showing the levels of Pim-1 gene expression in cultured adult cardiomyocytes. Cardiomyocytes were cultured in normal (NG) or high glucose (HG) in the presence of benfotiamine (HGB) or vehicle. Each experiment was repeated three times in triplicate. Values are expressed as n-fold changes toward NG and are mean ± standard deviation. *P<0.01 versus NG; #P<0.01 versus HG.

Supplemental Figure 9
(A-I) Bar graphs show the levels of activated caspase-3/7 (A), Pim-1 (B), pBad (C), Bcl-2 (D), pSTAT3 (E), pAkt (F), Akt kinase activity (G), peNOS (H) and pFOXO-1 (I) in cultured adult cardiomyocytes. Cardiomyocytes were cultured in normal (NG) or high glucose (HG) in the presence of benfotiamine (NGB and HGB) or vehicle. Each experiment was repeated three times in triplicate. Values are expressed as n-fold changes toward NG for all parameters, except caspase 3/7 which is expressed as relative units (RLU), and are mean ± standard deviation.
Siegel-Tukey test detected statistical differences as illustrated: *P<0.01 and **P<0.001 versus NG; #P<0.01 and ##P<0.001 versus HG. (J) Representative microphotographs showing the pAkt intracellular localization in cardiomyocytes cultured in normal (NG) or high glucose (HG) for 24 or 72 h in presence of benfotiamine (HGB) or vehicle. Scale bars are 50 µm.

**Supplemental Figure 10**

(A-F) Bar graphs show the levels of pSTAT3 (A), pAkt (B), Pim-1 (C), pBad (D) Bcl-2 (E) and activated caspase-3/7 (F). Cardiomyocytes cultured in high glucose (HG) or normal glucose (NG) were infected with Ad.DN-Akt or Ad.Null followed by another 24h culture in the presence of benfotiamine (HGB) or vehicle. Each experiment was performed in triplicate and repeated three times. For caspase-3/7 activity, assay was performed in 6 wells per each condition and repeated three times. Values are expressed as n-fold changes toward Ad.Null NG for all parameters, except for caspase 3/7, which is expressed as relative units (RLU), and are mean ± standard deviation. *P<0.01 and **P<0.001 versus NG within Ad.DN-Akt or Ad.Null groups; #P<0.01 and ##P<0.001 versus HG within Ad.DN-Akt or Ad.Null groups. $P<0.01 versus the corresponding treatment of the Ad.Null group. (G) Bar graphs show the levels of activated caspase-3/7 in cultured adult cardiomyocytes exposed to high glucose in the presence of LY 294002 (50µM) or vehicle. Each experiment was performed in 6 wells per each condition and repeated three times. Values expressed as relative units (RLU), and are mean ± standard deviation. *P<0.01 and **P<0.001 versus NG within vehicle or LY 294002 groups; #P<0.01 versus HG within vehicle group. $P<0.01 versus corresponding treatment of the vehicle group. (H-M) Bar graphs show the levels of pSTAT3 (H), pAkt (I), Pim-1 (J), pBad (K) Bcl-2 (L) and activated caspase-3/7 (M) in cultured adult cardiomyocytes. Cardiomyocytes cultured in HG or NG were preincubated with STAT3 inhibitor peptide (1mM) or vehicle for 30 min, followed by another 24 h culture in the presence of benfotiamine (HGB) or vehicle. Values are expressed as n-fold changes toward NG (vehicle) for all parameters, except for caspase 3/7, which is expressed as relative units (RLU), and are mean ± standard deviation. *P<0.01 and **P<0.001 versus NG within STAT3 inhibitor peptide or vehicle groups; #P<0.01 and ##P<0.001 versus HG within STAT3 inhibitor peptide or vehicle groups. $P<0.01 versus corresponding treatment of
Supplemental Figure 11
Schematic illustration showing mechanisms implicated in BFT induced cardio-protection in the setting of diabetes.

Diabetes or hyperglycemia inhibits the phosphorylation of STAT3, which is known to activate cell survival Pim-1 by binding to its promoter. Furthermore, diabetes or hyperglycemia induces O-GlcNAC modification of Akt, resulting in inhibition of Akt activity. BFT acts on both arms of this pathway which converges into Pim-1. We found that BFT preserves STAT3 phosphorylation in cardiomyocytes, with this effect, together with Pim-1 upregulation, being abolished by STAT3 inhibitor peptide. In addition, BFT prevents O-GlcNAC modification of Akt, thereby restoring Akt activity and Pim-1 expression. The effect of BFT on Pim-1 expression is abolished by treating cardiomyocytes with dominant negative form of Akt or PI3-kinase inhibitor LY294002. On the other hand, diabetes or hyperglycemia induced the activation of protein phosphatase 2A (PP2A), which is known to dephosphorylate and destabilize Pim-1. However, the upregulation of PP2A was not affected by BFT, suggesting that BFT mainly acts through positive regulators of Pim-1.