Cardioprotective effects of P7C3 in diabetic hearts via Nampt activation.

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ABSTRACT

Diabetes is associated with increased cardiac injury and sudden death. Nicotinamide phosphoribosyltransferase (Nampt) is an essential enzyme for the NAD\(^+\) salvage pathway and dysregulated in diabetes. **Hypothesis:** Nampt activation results in rescued NADH/NAD\(^+\) ratios and provides pharmacological changes necessary for diabetic cardioprotection. Computer docking shows that P7C3 allows for enhanced Nampt dimerization and association. **Methods:** To test the pharmacological application, we utilized male leptin receptor-deficient (db/db) mice and treated with Nampt activator P7C3 (1-\((3,6\text{-Dibromo-carbazol-9-yl})\text{-3-phenylamino-propan-2-ol}\)). The effects of four-week P7C3 treatment on cardiac function were evaluated along with molecular signaling changes for p-AKT, p-eNOS, and SiRT-1. **Results:** The cardiac function evaluated by ECG and Echo were significantly improved after four-weeks of P7C3 treatment. Biochemically, higher NADH/NAD\(^+\) ratio in diabetic heart were rescued by P7C3 treatment. Moreover, activities of Nampt and Sirt1 were significantly increased in P7C3 treated diabetic hearts. P7C3 treatment significantly decreased the blood glucose in diabetic mice with 4-week treatment as noted by glucose tolerance test and fasting blood glucose measurements compared with vehicle treated mice. P7C3 activated Nampt enzymatic activity both *in vitro* and in the 4-week diabetic mouse hearts demonstrates the specificity of the small molecule. P7C3 treatment significantly enhanced the expression of cardioprotective signaling; p-AKT, p-eNOS, and Beclin 1 in diabetic hearts. Nampt activator P7C3 allows for decreased infarct size with decreased Troponin I and LDH release, which is beneficial to the heart. **Conclusions:** Overall, the present study shows that P7C3 activates Nampt and Sirt1 activity, decreases NADH/NAD\(^+\) ratio, resulting in improved biochemical signaling providing cardioprotection.

**KEY WORDS:** Heart, Diabetes, Nampt, NAD\(^+\), Sirt1, Myocardial infarction, Ischemia-reperfusion
Significance Statement
We show that P7C3 is effective in the treatment of diabetes and cardiovascular diseases. The novel small molecule is anti-arrhythmic and improves the ejection fraction in diabetic hearts. The study successfully demonstrated that P7C3 decreases the infarct size in heart during myocardial infarction and ischemia-reperfusion injury. Biochemical and cellular signaling show increased NAD⁺ levels, along with Nampt activity involved in upregulating protective signaling in the diabetic heart. Based on the cardioprotective properties P7C3 has high therapeutic potential for rescuing heart disease.
INTRODUCTION

Cardiovascular disease (CVD) and diabetes mellitus (DM) remain major causes of mortality and morbidity in the United States (Liu et al., 2016). Primarily characterized as the occurrence of high blood sugar, diabetes is a progressive disease of hepatic and peripheral insulin resistance, β cells dysfunction and decreased insulin secretion (Hofmann et al., 2002). Diabetes increases the incidences of cardiac arrhythmias, myocardial infarction and sudden death (Das et al., 2015; Tate et al., 2016). Cardiac electrophysiological abnormalities play an important role in diabetic cardiomyopathy (Li et al., 2008), and ventricular arrhythmias are a major cause of diabetes associated death in patients. Electrically, QT prolongation is an increased risk factor for ventricular arrhythmias and sudden cardiac death leading to increased mortality in diabetic patients (Lu et al., 2013).

Although various genetic and environmental factors affect cardiac health and arrhythmia, emerging evidence indicates that pyridine nucleotides; nicotinamide adenine dinucleotide (NAD⁺) and reduced nicotinamide adenine dinucleotide (NADH), are major regulators of cardiac electrical activity (Kilfoil et al., 2013). Nampt (Nicotinamide phosphoribosyltransferase) is an essential enzyme for intracellular NAD⁺ salvage (Wagner et al., 2022), which is dysregulated in diabetes (Revollo et al., 2007). Alteration of Nampt leading to a decrease in NAD⁺ and subsequent increase in NADH, can serve as a substrate for diabetic arrhythmogenesis (Garten et al., 2015), with potential to modulate electrical activity in the heart (Chapalamadugu et al., 2015).

P7C3 was initially identified as a neuroprotective aminopropyl carbazole agent and tested in animal models of brain disorders (Pieper et al., 2010; Blaya et al., 2014; Kemp et al., 2015; Latchney et al., 2015; Walker et al., 2015). P7C3 binds to, and enhances, Nampt activity resulting in a significant increase in intracellular NAD⁺ levels (Wang et al., 2014), suggesting that P7C3 stimulation of Nampt could be adapted to mitigate intracellular rises in NADH/NAD⁺ ratio in stressed cells of the diabetic heart. In a recent report, we demonstrated that Nampt activation by P7C3 protects the diabetic skeletal muscle (Manickam et al., 2022), however, the cardioprotective effects of P7C3 has not been investigated. We hypothesized that Nampt activation by P7C3 rescues diabetic cardiac function. In the present study, utilizing P7C3 as a Nampt activator in db/db mice, we show that P7C3 mitigates cardiac dysfunction by successfully enhancing key signaling molecules downstream of Nampt to promote cardioprotective features. The roles of NAD⁺ and Nampt were previously investigated as anti-ischemic (Hsu et al., 2014; Zhang and Ying, 2019) and anti-aging factors (van der Veer et al., 2007; Imai, 2010; Yoshida et al., 2019), however, the effects in cardiac ischemia-reperfusion injury along with metabolic basis and signaling of Nampt activation by P7C3 remains unknown. In the present study, we evaluated the activity of Nampt in diabetic hearts and identify pharmacological benefits that P7C3 offers in the diabetic heart via upregulating protective metabolism.
signaling targets and links were studied to probe the status of a classic cardioprotective signaling mediators such as pAkt, and NAD⁺, which is Nampt dependent.

**MATERIALS AND METHODS**

**Ethical Approval**

*Animals, research protocol approval and treatment.* Male, B6.BKS(D)-Leprdb/J (db/db) and C57Bl/6J (wildtype), mice were purchased from Jackson laboratory, stock number: 000697 and 000664, respectively (Bar Harbor, ME, USA) at the age of 10 weeks and housed with *ad libitum* food and water for 4-6 weeks and were used at ages 12-16 weeks. Nampt activator; P7C3 (1-(3,6-Dibromo-carbazol-9-yl)-3-phenylamino-propan-2-ol) was purchased from Cayman Chemical (Ann Arbor, MI, USA). P7C3 was prepared by a previously reported method (Latchney et al., 2015). P7C3 was dissolved in 2.5% dimethyl sulfoxide (DMSO; Sigma-Aldrich, MO, USA) in PBS containing 10% Kolliphor EL (Sigma-Aldrich, MO, USA). The db/db mice received daily intraperitoneal injections (i.p.) of P7C3 (10mg/kg body weight) or an equivalent volume of the vehicle, for 4 weeks. A total of 14 vehicle control and 14 P7C3 treated db/db mice, aged 12-16 weeks, and 3 vehicle treated and 3 P7C3 treated C57Bl/6J wildtype mice aged 16 weeks were utilized for this study. Additionally, a total of 8 C57Bl/6J wildtype vehicle controls were utilized as non-diabetic mice. Mouse hearts used for Western blotting, received insulin 5 minutes prior to tissue collection as discussed further in Western blot section. All experimental animal protocols were approved in advance by the Institutional Animal Care and Use Committee (IACUC) at the University of South Florida (Tampa, FL, USA), and conform to National Institutes of Health (NIH) standards.

**Electrocardiography (ECG).** Mouse ECG recordings were obtained utilizing 2-3% isoflurane/oxygen anesthesia in lead II configuration. ECGs were acquired for a total duration of 15 min, with 1 min recordings obtained at 5-minute intervals. Heart rate was monitored, while ECG traces were acquired using PowerLab system operated with LabChart 7.2 software (AD Instruments, UK). Data were analyzed offline using the ECG module of LabChart 7.2 software, as reported elsewhere (Chapalamadugu et al., 2015; Tur et al., 2016). The intervals (ms) of QTc, JT, and ST elevation (mV) were measured. QT interval was measured from the start of the Q peak to the point where the T wave returns to the isoelectric baseline (TP baseline), and heart rate corrected QT (QTc) interval was obtained using the formula (QTc = QT / (RR/100)¹/²) (Chapalamadugu et al., 2015; Tur et al., 2016; Tur et al., 2017).

**Echocardiography.** Serial transaortic echocardiography was conducted under 2-3% isoflurane/oxygen anesthesia using a Visualsonic Vevo 770 system equipped with 30MHz linear signal transducer (Toronto,
Ontario, Canada). The mice were depilated as required for imaging and before placing on a 37°C heated platform. Echo measurements were taken from at least three different cardiac cycles for each mouse. M-mode imaging from short-axis of the left ventricle (LV), using the papillary muscles for reference, was used to obtain measurements. Fractional shortening (FS%) and ejection fraction (EF%) were calculated as previously described (Tur et al., 2016).

**Fasting blood glucose.** Mice were fasted overnight, and the tail vein blood was used for measuring the blood glucose levels of the diabetic mice treated with either Vehicle (db/db Veh) or P7C3 (db/db P7C3) for 4 weeks. The blood glucose levels were measured using the ACCU-CHECK blood glucose meter (Roche Diagnostics, Mannheim, Germany).

**Glucose tolerance test.** The intraperitoneal glucose tolerance test (GTT) was performed in the overnight fasted db/db Veh and the db/db P7C3 treated (10 mg/kg body weight/day, i.p.) mice, and injected with 2g/kg body weight of D-(+)-glucose (Sigma-Aldrich, MO, USA). Blood samples were obtained by the submandibular puncture technique. Serum glucose levels were assessed at 0, 15, 30, 60, and 120 minutes of glucose administration using the glucose oxidase peroxidase kit (Pointe Scientific Inc., MI, USA) according to the manufacturer’s instructions. The index of total glucose shift between the treatment groups were calculated as the area under the curve (AUC) by using the trapezium method.

**Ex vivo ischemia reperfusion protocol.** Mice were injected with heparin (360 USP units, Sigma-Aldrich, MO, USA) and euthanized with Somnasol™ (pentobarbital, 50mg/kg i.p. body weight). Hearts were then excised and mounted on to the Langendorff apparatus immediately and perfused with Krebs-Hanseleit buffer containing (in mM): 119 NaCl, 25 NaHCO3, 4 KCl, 1 MgCl2, 1.8 CaCl2, 1 MgCl2, 10 glucose and 2 Sodium pyruvate, pH 7.4, that was constantly bubbled with carbogen gas and maintained at 37°C as described previously (Kilfoil et al., 2019). Perfusion was maintained at a constant flow of ~2.2 ml/min. Hearts were stabilized for 30 min before proceeding to the ischemic phase. Hearts that underwent P7C3 (3µM) treatment during the Langendorff procedure were allowed to stabilize for 10 mins then a 20 min perfusion with P7C3 (3µM) (within Krebs-Hanseleit buffer) as pre-ischemic dosing. Cardiac ischemia was induced by the pause in Krebs-Hanseleit buffer perfusion for 45 min. Reperfusion was induced with the induction of Krebs-Hanseleit buffer at a constant flow of ~2.2 ml/min for 2 hours. Hearts that underwent P7C3 (3 µM) treatment received 2-hour reperfusion with P7C3 within the Krebs-Hanseleit buffer. Hearts were then removed from the Langendorff apparatus and immediately placed in -20°C freezer for 30 minutes. Hearts were then placed on a sectioning block and sliced into (10 micron) slices. Heart sections were subjected to 2,3,5-Triphenyltetrazolium chloride (TTC) staining for 30 minutes. Hearts were then imaged.
and scanned for ischemic damage assessment. Image J software was utilized to quantify the ischemic areas for several sections per heart, which were then averaged and divided by the total area of the heart section and multiplied by a factor of x100 (%) to determine the overall damage percentage per heart.

Hearts undergoing LY294002 (Sigma L9908, 2-(4-Morpholinyl)-8-phenyl-1(4H)-benzopyran-4-one hydrochloride) (15µM) exposure were performed with a modified ex vivo ischemia reperfusion protocol (Mocanu et al., 2002), used as PI3K inhibitor. The C57Bl/6J wildtype mice were injected with heparin (360 USP units, Sigma-Aldrich, MO, USA) and euthanized with Somnasol™ (pentobarbital, 50mg/kg i.p. body weight). The hearts were then excised and mounted on to the Langendorff apparatus immediately, and perfused with Krebs-Hanseleit buffer containing (in mM): 119 NaCl, 25 NaHCO3, 4 KCl, 1 MgCl2, 1.8 CaCl2, 1 MgCl2, 10 glucose and 2 Sodium pyruvate, pH 7.4, that was constantly bubbled with carbogen gas and maintained at 37°C. Perfusion was maintained at a constant flow of ~2.2 ml/min (Kilfoil et al., 2019). Hearts were stabilized for 10 min before proceeding to pre-treatment phase. Control hearts were perfused with vehicle for 15 minutes followed by P7C3 (3µM) for an additional 15 minutes. Hearts that underwent LY294002 treatment during the Langendorff procedure were allowed to stabilize for 10 mins then a 15 min perfusion with LY294002 (15µM) followed by P7C3 + LY294002 for an additional 15 minutes. Cardiac ischemia was induced by the pause in Krebs-Hanseleit buffer perfusion for 45 min. Reperfusion was induced with the induction of Krebs-Hanseleit buffer at a constant flow of ~2.2 ml/min for 2 hours with P7C3 (3µM). Hearts were then removed from the Langendorff apparatus and processed for Western blot assessment (see below).

Hearts undergoing ex vivo ischemia reperfusion protocol post-exposure with P7C3 (3 µM) were performed as defined above. Hearts were stabilized for 30 min before proceeding to the ischemic phase. Cardiac ischemia was induced by the pause in Krebs-Hanseleit buffer perfusion for 45 min. Reperfusion was induced with the induction of Krebs-Hanseleit buffer at a constant flow of ~2.2 ml/min for 1 hour. Hearts that underwent P7C3 (3µM) treatment received 1-hour reperfusion with P7C3 (3µM) within the Krebs-Hanseleit buffer.

In vivo myocardial infarction (MI) protocol: The 16-weeks-old C57Bl/6J wildtype male mice were injected with a single bolus of 10 mg/kg body weight P7C3, i.p. or with an equivalent dose of vehicle control 30 minutes prior to the permanent ligation of the left anterior descending (LAD) coronary artery for generating the MI. In brief, mice were anaesthetized with CO2, and intubated orotracheally and ventilated on a positive-pressure ventilator. All surgical procedures were carried out in aseptic conditions. A left thoracotomy incision was performed at the fourth intercostal space to expose the heart and ligating the LAD coronary artery with an 8-0 polypropylene suture to induce MI as described previously (Xuan et al., 2017).
Myocardial ischemia was confirmed with both the development of pallor myocardium distal to the ligation in the left ventricle and with the ST-segment elevation on the ECG. The skin and muscle incisions were closed with a 6-0 polypropylene suture, and the mice was administered a single dose long-acting analgesic Buprenorphine SR s.c. at 0.5-1.5mg/kg body weight. The mice were monitored for any signs of pain or discomfort and its feeding and drinking behavior. After 24 hours of occlusion of the LAD coronary artery, the mice were euthanized as per the approved IACUC protocol, and the heart was excised for further processing with 2,3,5-Triphenylterrazolium chloride (TTC) (Sigma-Aldrich, MO, USA). The whole heart was cut into six parallel short-axis sections and stained with 1% TTC for 90 minutes and fixed overnight in 10% neutral-buffered formalin (Sigma-Aldrich, MO, USA). The TTC stained heart sections were then scanned to determine the total infarct size as the ratio of the infarcted area, i.e., not stained with TTC to the ventricular area of the 4 sections from the apex to the site of occlusion (n=3 mice/group).

**Pyridine nucleotide measurements.** NADH/NAD⁺ ratios were determined in frozen db/db heart tissues (from the vehicle and P7C3 treated mice) using commercially available EnzyChrome kit (Bioassays, Hayward, CA, USA) as reported before (Chapalamadugu et al., 2015). Following manufacturer’s instructions, cardiac tissue was homogenized in 100 µl of NAD⁺ or NADH extraction buffer for NAD⁺ and NADH determination, respectively. Samples were heated at 60°C for 5 minutes on a heat block and neutralized by adding 100µl of the opposite extraction buffer. Samples were then mixed by brief vortexing and centrifuged at 14,000 rpm for 5 minutes. 40 µl of supernatant from each sample was dispensed in duplicates in a 96-well plate, and 80 µl of working reagent constituted with supplier provided assay buffer, lactate dehydrogenase, lactate and MTT reagent was added to each well. Samples were gently mixed and optical density at 565 nm was acquired immediately (0 min) and after 15 minutes, at room temperature using a microplate reader. A standard curve was utilized for calculating NADH/NAD⁺ levels in each sample.

**Nampt enzymatic activity.** Nampt enzymatic activity was measured both in vitro and in vivo using the commercially available CycLex Nampt Colorimetric Assay Kit (MBL international, MA, USA) (Laiguillon et al., 2014). The db/db mice treated with vehicle and P7C3 for 4 weeks was utilized for harvesting the whole hearts. Briefly, the cardiac tissue was homogenized for protein extraction using T-per protein extraction buffer (Thermo Fisher Scientific, MA, USA). Following manufacturer’s instructions, a 50µg protein was used for Nampt activity measurement in each sample. OD values were obtained at 450 nm at 0 and 30 min after sample incubation at 30°C, and Nampt activity was measured.
Sirt1 deacetylase activity. Sirt1 deacetylase activity was measured in the whole heart lysates from the vehicle and P7C3 treated db/db mice according to the manufacturer's protocol using a deacetylase fluorometric assay kit (Sigma-Aldrich, MO, USA). The fluorescence intensity was measured at 440 nm (excitation, 340 nm).

Lactate Dehydrogenase activity. Briefly, coronary effluents were collected at varying time points (baseline, 30 minutes post-reperfusion, 60 minutes, and 120 minutes) and analyzed for LDH activity from vehicle and P7C3 treated wildtype hearts according to the manufacturer's protocol using LDH assay kit (Sigma-Aldrich, MO, USA). The absorbance intensity was measured at 450 nm.

Troponin I activity. Briefly, coronary effluents were collected at varying time points (baseline, 30 minutes post-reperfusion, 60 minutes, and 120 minutes) and analyzed for Troponin I activity from vehicle and P7C3 treated db/db and wildtype hearts according to the manufacturer's protocol using the Troponin I kit (Sigma-Aldrich, MO, USA).

Western blotting. For studies aimed to assess the insulin stimulated AKT phosphorylation, following four-week P7C3 treatment, mice were injected with Novolin R human insulin (1 Unit/kg body weight, i.p.), and hearts were collected after 5 minutes for Western blot analysis (Shao et al., 2000). Protein lysates for Western blotting were prepared from mouse hearts as described previously (Panguluri et al., 2013; Chapalamadugu et al., 2015). Equivalent amounts of protein were loaded and resolved using 12% precast polyacrylamide gels (BioRad, CA, USA). Successful transfer of proteins was detected using Ponceau S (Sigma-Aldrich, MO, USA), and blots were probed with mouse monoclonal primary antibody for phospho AKT\textsuperscript{Ser473} (p-AKT), total AKT (t-AKT), phospho eNOS\textsuperscript{Ser1177} (p-eNOS), total eNOS (t-eNOS), Beclin1, and GAPDH (Cell signaling, MA, USA). HRP conjugated Rabbit anti-mouse antibody (Millipore) was used as a secondary antibody. Target protein band densities were quantified using Image J software (Tur et al., 2016).

Computational Modeling. Using the methodology described below, docking was performed using X-Ray crystal structures of Nampt obtained from the Protein Databank (www.rcsb.org). Although these structures consist primarily of co-crystalized complexes with inhibitors bound (and no activator complexes yet deposited) it has been demonstrated that activators and inhibitors share overlapping binding sites (Gardell et al., 2019). Protein structure coordinates were obtained from the Protein Data Bank (PDB) (Bernstein et al., 1977; Berman et al., 2000). Models were generated from PDB entry 4WQ6 (Zak et al., 2015), the X-Ray structure used in a previously reported in silico study on NAMPT activators via computational docking.
(Elekofehinti et al., 2018). The Schrödinger Inc. software suite (Schrödinger, 2021c) was used as the computational workflow for these studies. Protein model systems of Nampt were prepared using Schrödinger’s Protein Preparation Wizard [ACS Citation of Software (Schrödinger, 2021b) (Sastry et al., 2013)].

**Protein Structure Refinement with Molecular Dynamics (MD).** MD simulations were executed with the GPU accelerated Desmond MD program (Bowers et al., 2006; Guo et al., 2010; Shivakumar et al., 2010; Schrödinger, 2021a), on two Nvidia GeForce GTX 1080 Ti video cards. A cubic simulation box was generated and extended at least 10Å from the protein with imposed periodic boundary conditions using TIP3P waters (Zak et al., 2015) as solvent. The OPLS-3 all-atom force field (Harder et al., 2016) was then applied to all atoms. Simulations were run at a temperature of 310 K and a constant pressure of 1 atm. All systems were energy minimized using multiple restrained minimizations to randomized systems before equilibration and final simulation. Production MD was subsequently performed on all systems for 250 ns.

**Computational Docking.** After MD equilibration was confirmed using a hierarchical average linkage clustering method, based on RMSD, an average representative structure for the equilibrated Nampt system was obtained. This representative Nampt structure was then used for docking the putative Nampt activator P7C3 using the Schrödinger software suite’s GLIDE rigid receptor docking protocol with standard precision (SP) settings (Friesner et al., 2004; Halgren et al., 2004; Schrödinger, 2021c).

**Statistical Analysis.** Data are presented as mean ± SEM. The unpaired two-sided Student’s t test was used to compare the vehicle to the corresponding P7C3 treated groups. A one-way-ANOVA followed by Dunnett’s post hoc test was conducted in NAD⁺ (%) when three groups were utilized to identify significant mean differences across the groups. A $p \leq 0.05$ value was considered statistically significant.

**RESULTS**

**P7C3 enhanced Nampt activity in vitro and in vivo and improves NADH/NAD⁺ ratio.** Nampt based NAD⁺ generation results in cardioprotection (Hsu et al., 2009), however, the use of P7C3, a small molecule as Nampt activator in heart remains unknown. Early examination of the P7C3 small molecule has demonstrated the ability to bind and significantly enhance the enzymatic activity of Nampt. Diabetes remains a prominent risk factor for cardiovascular disease and likely plays a role in the deregulation of intracellular NAD⁺/NADH levels. Nampt remains a major regulator of the intracellular NAD⁺-SiRT1 axis and is a key regulator of pyridine nucleotide ratios. Therefore, we set out to examine the effect of P7C3
A four-week treatment with P7C3 (10 mg/kg body weight/day, i.p.) significantly increased cardiac Nampt activity in db/db mice compared with vehicle treated mice (Fig. 1A). The observed increase of Nampt activity in db/db P7C3 treated mice was higher than wildtype control mice as well. We therefore sought to confirm the addition of P7C3 with an in vitro Nampt enzymatic activity assay, which resulted in enhanced activity (Fig. 1B and C). Indeed, Nampt activity was increased with P7C3 at both 0.5 µM and 1 µM concentrations compared with the vehicle DMSO (0 µM P7C3), and 1 µM P7C3 without the recombinant Nampt enzyme (Fig. 1B). The Nampt activity was also measured by the absorption change per minute and was demonstrated as an increased overall activity with recombinant Nampt activity at 0.5 µM and 1 µM P7C3 concentrations (Fig. 1C).

**Nampt active site with P7C3 docking and visualization of interactions.** Computational docking and visualization of the Nampt active site shows key amino acid residues in proximity to P7C3 allowing for tight interaction. Figure 2A shows the carbon structure of P7C3 molecule with a carbazole and aromatic ring connected by a linker with chirality. As shown in Figure 2B-H, five key amino acid residues, including tyrosine, histidine, valine, serine, and asparagine interact with distinct regions of the P7C3 molecule for Nampt dimer stabilization. Figure 2B shows selected distances within a 3-5 Å range between the linker and aromatic ring regions of P7C3 with amino acids contained within the activation site (aka “tunnel”). The Glide SP based docking score calculates P7C3’s relative binding free energy as -6.7 kcal/mol. The top and side views of the activation site docked with P7C3 reveal two Nampt monomers that form two distinct Nampt dimeric interfaces (Fig. 2C-H), wherein each interface comprises both an activation site (aka “tunnel”) and an active site (aka “catalytic site”) that are solvent accessible when neither ligands or substrates are bound.

**Decreased circulating blood glucose levels in P7C3 treated db/db mice.** We recently reported that P7C3 treatment of the diabetic (db/db) mice for 4 weeks improves the circulating blood glucose levels (Manickam et al., 2022). In the present study, we show a similar significant decrease in the fasting blood glucose levels of the overnight fasted db/db mice treated with P7C3 (10 mg/kg body weight/day, i.p.) for 4 weeks compared with the vehicle treated mice (Fig. 3A). The intraperitoneal glucose tolerance test of the diabetic mice treated with P7C3 for 4 weeks displayed a decrease in circulating blood glucose from 30 minutes onwards but was found to be non-significant between the treatment groups (Fig. 3B). However, the index of the total glucose shift that was calculated as the area under curve using the trapezium method displayed a significant difference between the treatment groups variances (Fig 3C). Taken together, this confirms that P7C3 treatment enhances glucose use of the diabetic mice.
Cardiac electrical parameters are significantly reduced in P7C3 treated db/db mice. Overall in vivo cardiac function was investigated by examining key electrical parameters via surface lead ECG recordings. The QTc interval remains a critical measurement for overall cardiac health and function with diabetic patients and preclinical models demonstrating elevated QTc and JT (Vaykshnorayte et al., 2012). Here, we demonstrate that the db/db vehicle treated mice present with significantly elevated QTc and JT intervals as well as, increased ST elevation compared with their non-diabetic wildtype controls (Fig. 4A-C). These intervals were significantly attenuated in db/db P7C3 (10 mg/kg body weight/day, i.p.) treated mice, with values closely aligning with their non-diabetic wildtype controls (Fig. 4A-C). A correlative analysis clearly identifies a significant association with increased Nampt activity and lower QTc durations (Fig. 4D). Collectively, these data demonstrate that P7C3 treated db/db mice have significantly attenuated cardiac electrical parameters compared with vehicle treated mice and the values are on par with non-diabetic control mice.

Cardiac functional parameters are significantly increased in P7C3 treated db/db mice. Transaortic echocardiography was utilized to further evaluate cardiac function and to investigate overall systolic function. The db/db P7C3 (10 mg/kg body weight/day i.p.) treated mice demonstrated improved echocardiography (Fig. 5A), and significantly increased the ejection fraction (EF%) and fractional shortening (FS%) parameters post four-weeks (Fig. 5B and C). The evaluation of body weight and left ventricular heart weight demonstrated no alterations between the P7C3 and vehicle treated db/db mice (Fig. 5D and E). These data suggest that P7C3 improved the systolic function as a result of enhanced overall cardiac function, and the echo-based evaluation did not find heart weight or size changes.

Enhanced Akt and eNOS phosphorylation, and Beclin-1 signaling with increased Sirt1 enzymatic activity of P7C3 treated db/db mice. Western blot analysis of cardiac tissue from P7C3 (10 mg/kg body weight/day i.p.) treated db/db mice demonstrated significantly increased p-AKT signaling compared with vehicle treated mice (Fig. 6A). Since eNOS is a major regulator of nitric oxide production in the heart and is key for cardioprotection, and a downstream target of p-AKT signaling, we measured eNOSser1177 phosphorylation. As shown in Fig. 6B, P7C3 treatment significantly increased p-eNOS levels in cardiac tissue, suggesting that the cardioprotective benefits via pAKT by P7C3 is at least in part via eNOS stimulation. Additionally, the P7C3 treated db/db mice showed a significant increase in protein expression of pro-autophagy marker, Beclin 1, when compared with that of vehicle treated mice (Fig. 6C). Examination into the NAD⁺-SiRT1 axis demonstrated a significant increase in SiRT1 activity in cardiac tissue from db/db P7C3 treated mice compared with db/db vehicle controls (Fig. 6D). A correlation analysis further highlighted a significant positive relation with Nampt and SiRT1 activity within the P7C3 treated db/db
mice (Fig. 6E). Hearts from diabetic mice showed a 2.5-fold increase in cardiac the NAD$^+/NADH$ ratio found in P7C3 treated db/db mice compared with vehicle control (Fig. 6F).

**Diabetic ischemia reperfusion injury is reduced by acute P7C3 treatment.** *Ex vivo* model of ischemia reperfusion (I/R) injury resulted in decreased Nampt activity and elevated NADH levels (Hsu et al., 2014; Abdellatif et al., 2021), therefore we sought to examine the level of injury the diabetic mice hearts would develop, and furthermore to test the beneficial effects of P7C3 treatment. In a subset group of db/db mice, hearts were excised and underwent the *ex vivo* I/R protocol (Fig. 7A). The db/db control hearts demonstrated profound ischemic injury with an average of 60% infarct size (Fig. 7B). The db/db hearts treated with P7C3 (3µM) just prior (15 minutes) and immediately following (2 hour reperfusion) ischemia demonstrated a significantly reduced infarct size (18%) compared with vehicle control (Fig. 7B). This elevated level of injury in db/db mice was also evident in the evaluation of cardiac effluent Troponin I levels. The db/db hearts perfused with P7C3 demonstrated a significant decrease in Troponin I levels 30 and 120 minutes post-ischemia (Fig. 7C).

**P7C3 perfusion ameliorates ischemia reperfusion injury.** Ischemia reperfusion injury remains a critical component of recovery from cardiac episodes, including myocardial infarctions. Reperfusion injury is often characterized with a return of blood supply resulting in cellular cardiac death, and are often characterized with infarct zones. Reperfusion injury has been associated with a significant increase NADH levels (Moreno et al., 2017). We demonstrated that the pre- and post- exposure to P7C3 in an *ex vivo* Langendorff system provided significant protection in db/db hearts. We therefore, sought to establish whether P7C3 perfusion has the potential to reduce all I/R injuries by utilizing a wildtype (C57Bl/6J) mouse model (Fig 8A). WT hearts perfused with P7C3 (3 µM) demonstrated a significant reduction in ischemic injury (8% vs. 55%) compared with WT controls (Fig. 8B). Biochemical examination demonstrated a significant 1.7-fold increase in cardiac Nampt activity within those hearts perfused with P7C3 (Fig. 8C). Corresponding NAD$^+/NADH$ levels demonstrated a similar significant trend with a 3-fold increase in NAD$^+/NADH$ levels observed in hearts perfused with P7C3 (Fig. 8D). Coronary effluents collected during ischemia further demonstrated significantly reduced levels of both Troponin I and LDH activity following I/R injury in P7C3 perfused hearts compared with control hearts (Figs. 8E and F). Infarct sizes demonstrated a notable increase in overall percentage when comparing db/db vehicle and C57Bl/6J wildtype mice (Provided averages 65% vs 55%, respectively), (Figs 7B and 8A). Taken together we established that the I/R injury in C57Bl/6J wildtype mice can significantly be reduced with the addition of P7C3 resulting in enhanced Nampt activity and elevated NAD$^+/NADH$ ratios coupled with significantly reduced cardiac infarct sizes and reduced cardiac injury markers, including Troponin I and LDH.
**P7C3 attenuates I/R through enhancement of p-AKT.** To further substantiate the reduced cardiac injury observed during I/R in C57Bl/6J wildtype hearts perfused with P7C3 (3µM), we sought to examine the overall cardiac p-AKT/t-AKT levels post I/R injury. Control hearts perfused with DMSO as vehicle control demonstrated an overall reduction in p-AKT/t-AKT levels compared with hearts undergoing simple perfusion. Hearts perfused with P7C3 demonstrated a significant increase in p-AKT/t-AKT levels when compared with vehicle control hearts (Fig. 9A). To further test if P7C3 indeed activates Akt and offers cardioprotection, the hearts were perfused with a PI3K inhibitor, LY294002 (15µM) just prior to inducing global ischemia (Fig. 9B). This strategy allowed to block the PI3K signaling, which is upstream of Akt. As shown in figure 9, perfusion with PI3K inhibitor LY294002 compound attenuated the P7C3 based pAkt expression (Fig 9C). These data show that P7C3 activated pAkt in the heart and provides cardioprotection.

**P7C3 post-ischemia exposure reduces ischemia reperfusion injury.** Ischemia reperfusion injury remains a critical component of recovery from cardiac episodes, including myocardial infarctions. We demonstrated that the pre- and post- exposure to P7C3 in an ex vivo Langendorff system provided significant protection in both db/db and WT hearts. We therefore, sought to establish whether P7C3 (3µM) perfusion post ischemia has the potential to reduce overall I/R injuries by utilizing a wildtype (C57Bl/6J) mouse model (Fig 10A). A similar level of ischemic injury was induced in WT hearts exposed to ischemia-reperfusion protocols with a reduced reperfusion time (60 mins vs. 120 mins) (54% vs. 55%, respectively, Figs 10B and 8A). Wildtype hearts perfused post-ischemia with P7C3 (3µM) demonstrated a significant reduction in overall ischemic injury (22% vs. 54%) compared with controls (Fig. 10B).

**P7C3 decreases in vivo myocardial infarction.** Next, we sought to determine whether P7C3 pre-treatment decreases the in vivo myocardial infarction mimicking clinical settings. The C57Bl/6J mice treated with a single bolus of P7C3 (10 mg/kg body weight/day, i.p.) 30 minutes prior to the LAD coronary artery occlusion displayed a significant reduction in the total infarct size measured 24 hours after the occlusion of the LAD coronary artery compared with control vehicle treated mice (Figs 10C and D).

**DISCUSSION**

This is the first report showing that P7C3 treatment leads to improved diabetic cardiac function. The cardioprotective benefits of Nampt activator; P7C3, are mediated by decreasing the cardiac NADH/NAD⁺ ratio and improving Nampt and Sirt1 activity. At the signaling level, activation of Nampt by P7C3 upregulated diabetic cardioprotective signaling with increased p-AKT, p-eNOS and autophagy signaling.
We previously demonstrated that P7C3 administration overcomes insulin resistance and decreases blood glucose with 4-week P7C3 treatment in diabetic mice (Manickam et al., 2022). Therefore, in the present study we evaluated the cardiovascular benefits and signaling given that diabetes is a major risk factor for myocardial ischemia and cardiovascular related deaths.

The major cardiovascular complications of diabetes include increased risk to life threatening arrhythmias and decreased cardiac function (Lu et al., 2013; Miki et al., 2013). Therefore, we evaluated the propensity of this utilizing a mouse model of diabetes and rescued by Nampt activator P7C3. The ECG based evaluation from diabetic patients show prolonged QTc, QT interval, JT interval and T wave (Robillon et al., 1999). Past studies in preclinical models such as db/db mice also confirm cardiac dysfunction in diabetes, including QTc prolongation (Lu et al., 2013), and significant systolic and diastolic dysfunction (Koka et al., 2014; Plante et al., 2014). In the present study, we show the cardioprotective benefits of Nampt activation by P7C3 in attenuating QTc durations and improved cardiac function as assessed by echocardiography. Echocardiographic data demonstrated that P7C3 treated db/db mice presented with significant increases both in the EF% and FS% parameters, suggesting a significant improvement in LV systolic function.

Nampt is the rate-limiting enzyme in mammalian NAD⁺ salvage pathway. Nampt is key for NAD⁺ synthesis in the heart and previous research demonstrated that overexpressed Nampt increases NAD⁺ content in cardiomyocytes (Hsu et al., 2009). Nampt is downregulated at both the protein and mRNA levels under stress conditions in the heart (Hsu et al., 2009) leading to decreased NAD⁺ level in the heart (Pillai et al., 2010). Studies in rats showed that diabetes leads to a near 3-fold increase in intra-cardiac NADH/NAD⁺ ratio, measured as lactate/pyruvate ratio, when compared with control group (Ramasamy et al., 1997). Recent murine studies using pressure overload and isoproterenol models of heart failure showed that administration of NMN, which is a product of Nampt activity, leads to normalization of NADH/NAD⁺ balance in the heart and significantly attenuates cardiac hypertrophy, LV dilatation, and improved contractile function (Lee et al., 2016), suggesting significant cardioprotective benefits of NAD⁺ accretion. Previously, we and others have established pyridine nucleotides, in particular NAD⁺/NADH ratios to play a critical role in multiple regulatory pathways involved in cardiac function, including autophagy, DNA repair, antioxidant, and mitochondrial function (Tur et al., 2017; Berthiaume et al., 2019).

Molecular docking studies suggest interactions between P7C3 and Nampt. These interactions likely indicate P7C3 to be a putative small molecule Nampt activator based on its binding and affinity to the Nampt protein. Moreover, a working hypothesis for Nampt activation by P7C3 considers the ligand’s role in further stabilizing the Nampt dimeric interface, wherein upon binding of P7C3 to the Nampt “tunnel” adjacent to the catalytic site (Burgos et al., 2009) may increase the interactions between the two Nampt monomers and provides additional energetic stability to the entire Nampt dimer. Both the in vitro and in vivo evidence confirm that P7C3 activates Nampt enzyme. In addition, the present study is consistent with
the beneficial effects in diabetic heart and with evidence for increased Nampt and SiRT1 activity along with increased NAD\(^+\) levels.

The P7C3 treated db/db mice in this study demonstrated significantly improved cardiac Nampt activity and correspondingly decreased NADH/NAD\(^+\) ratio concomitant to significant cardioprotection. NAD\(^+\) is an essential cofactor for Sirtuins and regulates their deacetylase activity (Kitada and Koya, 2013; White et al., 2014), and activation of Sirtuins has been shown to protect heart from injury (Alcendor et al., 2007; Planavila et al., 2011; Koka et al., 2014; Lu et al., 2014). Further, enhancing Sirt1 activity in db/db mice has been shown to improve cardiac function (Koka et al., 2014) at least in part through NAD\(^+\) -dependent correction of proteome acetylation status and improved mitochondrial function (Koka et al., 2014; Lee et al., 2016). It is thus plausible that the cardioprotective benefits of Nampt activation by P7C3 may at least in part be mediated through improving Sirtuin activity, as noted in this study, where P7C3 treatment significantly improved Sirt1 deacetylase activity.

Administration of NMN, an intermediate of NAD\(^+\) synthesis, to female diabetic mouse has been demonstrated to elevate hepatic NAD\(^+\) and AKT phosphorylation (Yoshino et al., 2011). Although animal models of diabetes differ in the basal activity of PI3K/AKT pathway in the heart, it is however consistent that insulin resistance in diabetes is associated with significant attenuation of decreased myocardial PI3K/AKT signaling (Mazumder et al., 2004; Cook et al., 2010; Lee et al., 2010; Lu et al., 2013). Treatment with P7C3 enhanced cardiac p-AKT levels, suggesting that P7C3 alleviated cardioprotective signaling in db/db mice. Moreover, elevated p-AKT that is also suggestive of enhanced PI3K/AKT cascade in myocardium explains at least in part the QTc normalization observed in P7C3 treated db/db mice. Recent studies showed that deletion of PI3K p110α subunit prolongs action potential duration and QT interval in mice, and augmenting PI3K activity in type1 and type 2 diabetic mice significantly reversed the action potential prolongations (Lu et al., 2013). It is therefore likely that the prolongation of action potential and corresponding ECG correlates such as QTc interval in db/db mice. Further, increased eNOS\(^{ser1177}\) phosphorylation that drives cardioprotective nitric oxide production in endothelial cells is downstream of AKT, and as such increased p-AKT in human endothelial cells has been shown to increase p-eNOS levels (Haynes et al., 2000), suggesting the P7C3 induced cardioprotection involves PI3K/AKT/eNOS axis. Previous studies also reveal that Beclin 1 is dysregulated in db/db heart (Brenner et al., 2003; Han et al., 2009), therefore we investigated the status of autophagy in P7C3 treated diabetic hearts and identified an increase in Beclin 1 expression, suggesting the cardioprotective benefits.

Previous studies using anti-diabetic drugs and their utility for cardioprotection was tested for using I/R model and demonstrated protection in the heart, although the effects are comparable to P7C3, the mechanism of action for Metformin and P7C3 are different (Shi and Hou, 2021; Simanenkova et al., 2021) and remains under investigation. The present study shows that P7C3 activates Nampt allowing
cardioprotection in diabetes, or during ischemia and ischemia-reperfusion injury. Previous studies show that NADH/NAD⁺ ratio is increased in the heart with ischemia reperfusion injury (Abdellatif et al., 2021), therefore we evaluated the role of Nampt activator P7C3 in the heart by perfusing P7C3 and demonstrate that activation of Nampt is beneficial and protects the heart by decreasing the infarct size. At biochemical level the key injury maker Troponin I was decreased and the pAkt is activated with P7C3 treatment. Overall, we identified that the Nampt activator P7C3 is beneficial to the heart under diabetic conditions as well as during ischemia and ischemia-reperfusion injury. While pre-treatment of NAD⁺ may not always be feasible in a clinical setting, we further investigated the potential of P7C3 solely as a post-ischemic treatment. Strikingly, post-ischemic exposure with P7C3 in wildtype hearts was able to significantly reduce overall infarct size by 2.5-fold compared with reperfusion alone. While preliminary, this experiment highlights a clinically significant avenue of investigation for those immediately experiencing episodes of myocardial ischemia and the potential to significantly reduce overall infarct tissue damage. Although, previous studies showed increased NAD⁺ is beneficial for heart via use of Nicotinamide Riboside or Nicotinamide Mononucleotide (which supplement NAD⁺), however, in the present study we directly targeted Nampt enzyme via P7C3 small molecule. Therefore, the present study provides the cross talk between Nampt activation and its role for NAD⁺ generation and stimulation of SiRT1, along with pAkt and other beneficial markers allowing for cardioprotection (Fig. 9D). These studies confirm the specificity of Nampt activator P7C3 for the first time in a pharmacologically relevant manner.

**Conclusion.** Our results demonstrate that Nampt activation with P7C3 offers a potential avenue for cardioprotection. Mechanistically, we identified that Nampt regulation and its activation by P7C3 in the diabetic mice is key for increased intracardiac Nampt and Sirt1 activity, decreased NADH/NAD⁺, improved p-AKT, p-eNOS and Beclin 1 expression and overall improved cardiac function (Fig 9D). The addition of P7C3 may also provide a critical prevention and treatment avenues for cardiac ischemia/reperfusion injury. Acute perfusion prior to and during reperfusion resulted in a significant decrease in total infarct size in both diabetic and non-diabetic cardiac models. The P7C3 pre-treatment also significantly decreased the myocardial infarct size in the wildtype mice. Further investigation on developing an optimal timing window may provide greater mechanistic insight and provide a more clinically relevant and meaningful conclusion.

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None

**AUTHOR CONTRIBUTIONS**

Participated in research design: Tipparaju.
Conducted experiments: Tur, Badole, Manickam, Chapalamadagu, Xuan
Performed data analysis: Tur, Manickam, Badole, Chapalamadagu, Bisht, Crews, Xuan, Guida, and Tipparaju.

Wrote or contributed to the writing of the manuscript: Tur, Manickam, Badole, Chapalamadagu, Crews, Guida, Bisht, and Tipparaju.

**Abbreviations**

AUC - area under curve  
CVD - cardiovascular disease  
DM - diabetes mellitus  
ECG - electrocardiogram  
Echo - echocardiography  
EF - ejection fraction  
FS - fractional shortening  
GTT - glucose tolerance test  
HRP - horse radish peroxidase  
i.p. - intraperitoneal  
I/R - ischemia/reperfusion  
i.v. - intravenous  
LAD - left anterior descending  
LDH - lactose dehydrogenase  
NAD⁺ - oxidized nicotinamide adenine dinucleotide  
NADH - reduced nicotinamide adenine dinucleotide  
P7C3 - (1-(3,6-Dibromo-carbazol-9-yl)-3-phenylamino-propan-2-ol)  
pAkt - phospho Akt  
p-eNOS - phospho eNOS  
s.c. - sub cutaneous  
t-Akt - total Akt  
t-eNOS - total eNOS  
TTC - Triphenyltetrazolium chloride
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Schrödinger LNY, NY, NY (2021c) ACS Citation of Software in *Maestro*, ACS Citation of Software New York, NY.


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DISCLOSURES
US Patent 11,007,178 has been issued to USF (Tipparaju SM, 2021). Another application has been on file, USF Reference No. (19A039) patent pending.

Financial Disclosures
No author has an actual or perceived conflict of interest with the contents of this article.
**Figure legends**

**Figure 1.** P7C3 treatment improves enzymatic Nampt activity. (A) Vehicle (db/db) hearts demonstrated a decrease in Nampt activity compared with wildtype control (WT), while 4-week treatment with P7C3 (10 mg/kg body weight/day, i.p.) in db/db mice resulted in significantly increased Nampt activity. (B-C) Enzymatic activity of recombinant Nampt was measured every 5 minutes for 1 hour. Negative control (DMSO), positive control (Nampt enzyme), Nampt activator (P7C3) were all utilized to assess enzymatic activity. Data was reported as mean ± SEM and * indicated a p≤0.05.

**Figure 2.** Computational modeling of P7C3 docking with Nampt active site. (A) Chemical structure of P7C3 with 2-D representation of the molecule. (B) Table provides the relative binding free energy of P7C3 when docked with the Nampt protein (from PDB code 4WQ6) and lists the projected distances (measured in angströms) between selected regions of the P7C3 compound and related regions of the Nampt binding site. (C-F) Image depicts (P7C3 ligand in black), wherein (C-D) the top view, (E-F) side view, of the Nampt binding site (from PDB code 4WQ6). Green shading illustrates the van der Waals surface of the Nampt binding site. The protein ribbon structure shown in the image is provided to emphasize the Nampt binding site (“aka tunnel”) when two Nampt monomers dimerize to form the Nampt dimeric interface. (E) Image depicts the projected interactions by GLIDE SP docked P7C3 within the Nampt binding site (from PDB code 4WQ6). As it appears the GLIDE SP docked pose of P7C3 demonstrates no specific interactions between P7C3 and the Nampt binding site. Although a single interaction does exist between P7C3 and a water molecule (depicted as a purple dot) as indicated by the hydrogen bond represented as the black dashed line. (F) Image shows the interaction between the Nampt monomers to form the active site cavity. (G) Image displays the entire Nampt dimeric protein where monomer A is displayed in green and monomer B is displayed in blue and the yellow shading illustrates the van der Waals surface of the Nampt binding site (“aka tunnel”) of each interface. (H) Image emphasizes the two active sites (i.e., catalytic sites) contained within the Nampt dimeric protein.

**Figure 3.** P7C3 treatment improves blood glucose levels of the diabetic mice. (A) Fasting blood glucose levels of the db/db mice treated with P7C3 (10 mg/kg body weight/day, i.p.) (db/db P7C3) or with an equivalent volume of vehicle (db/db Veh) (n=5 mice/group). Data are expressed as mean ± SEM, and * indicated a p≤0.05. (B) The intraperitoneal glucose tolerance test (GTT) of the overnight fasted db/db Veh and db/db P7C3 mice (n=5-6 mice/group). (C) The area under the curve of GTT obtained by using the trapezium method. There was a significant difference between the treatment groups variances * indicated a p=0.0021.

**Figure 4.** Administration of P7C3 preserves ECG in db/db mice. ECG recordings were acquired in db/db mice after a 4-week treatment period with P7C3 (10 mg/kg body weight/day, i.p.) or vehicle (10 ml/kg body weight/day i.p.) control. (A) QTc interval (B) JT interval, (C) ST elevation (mV), (D) QTc and Nampt Activity correlation. Data are expressed as means ±SEM *p<0.05; P7C3 vs. vehicle treated db/db mice. Correlation is Spearman.

**Figure 5.** P7C3 improves contractile function in db/db mice. Echocardiography acquired from db/db mice post 4-week treatment with P7C3 (10 mg/kg body weight/day, i.p.) or vehicle. (A) Representative M-mode short axis images from db/db mice treated with vehicle and P7C3. (B) Ejection fraction measurements from db/db vehicle and P7C3 (C) Fractional shortening measurements from db/db vehicle and P7C3. (D) Body weight measurements from db/db vehicle and P7C3. (E) Left ventricular heart weight measurements from db/db vehicle or P7C3. Data are expressed as means ± SEM; *p<0.05 P7C3 vs. vehicle treated db/db mice.

**Figure 6.** P7C3 treatment potentiates AKT phosphorylation and cardioprotective signaling via eNOS and autophagy upregulation with enhanced SiRT-1 activity in db/db mice. After 4-week treatment with
P7C3 (10 mg/kg body weight/day, i.p.) or vehicle, db/db mice were injected intravenously (i.v.) with 1 unit/kg Novolin R regular human insulin. After 5 minutes, hearts were collected and snap frozen in liquid N2. (A) Immunoblots of whole heart lysates were analyzed by Western blotting for cardiac levels of phosphorylated AKTser473 (p-AKT), total AKT (t-AKT) and the ratio of p-AKT/t-AKT. (B) Cardiac levels of phosphorylated eNOS (p-eNOS), total eNOS (t-eNOS) and the ratio of p-eNOS/t-eNOS. (C) Western blotting of Beclin-1, GAPDH and the ratio of Beclin-1/GAPDH. Band intensities were quantified using image J software reported as mean ± SEM, *p<0.05, ** for vehicle vs. P7C3. (D) Sirt-1 activity data are expressed as means ± SEM ***p<0.001; P7C3 vs. vehicle treated db/db mice. (E) Nampt activity and Sirt-1 activity correlation. Correlation is Spearman. (F) P7C3 treated db/db mice demonstrated elevated cardiac NAD/NADH levels compared with db/db vehicle controls.

**Figure 7. Ischemia reperfusion injury in db/db hearts treated with P7C3.** *Ex vivo* ischemia reperfusion injury was assessed via the Langendorff perfusion system. (A) Schematic of Ischemia reperfusion protocol utilized. (B) TTC staining of I/R hearts from db/db mice perfused with P7C3 (3 µM) or vehicle with representative section images. (C) Cardiac effluents were assessed for Troponin I taken at baseline, 30 minutes post Ischemia/Reperfusion, 60 minutes post, and 120 minutes. Effluent samples represent (n=4) means ± SEM *p<0.05; P7C3 vs. vehicle treated db/db mouse.

**Figure 8. Ischemia reperfusion injury in wildtype mice perfused with P7C3.** *Ex vivo* ischemia reperfusion injury was assessed via the Langendorff perfusion system. (A) Schematic of Ischemia reperfusion protocol utilized. (B) TTC staining of I/R hearts from WT mice perfused with P7C3 (3 µM) or vehicle with representative section images. Heart samples presented are (n=4) means ± SEM ****p<0.0001; P7C3 vs. vehicle treated WT mice. (C) WT hearts perfused with vehicle and P7C3 (3 µM) had Nampt activity levels determined post treatment. (D) WT hearts perfused with vehicle and P7C3 (3 µM) had cardiac NAD/NADH levels determined post treatment. (E) Troponin I measurements taken at baseline, 30 minutes post Ischemia/Reperfusion, 60 minutes post, and 120 minutes. (F) LDH measurements taken at baseline, 30 minutes post Ischemia/Reperfusion, 60 minutes post, and 120 minutes. Data was reported as mean ± SEM *p≤0.05, **p≤0.01, ***p≤0.001, ****p≤0.0001.

**Figure 9. Ischemia reperfusion injury results in dysregulated pAKT.** *Ex vivo* ischemia reperfusion injury was assessed via the Langendorff perfusion system and heart tissue was examined for pAKT. (A) Immunoblots of whole heart lysates were analyzed by Western blotting for cardiac levels of phosphorylated AKTser473 (p-AKT), total AKT (t-AKT) and the ratio of p-AKT/t-AKT. Band intensities were quantified using image J software reported as mean ± SEM, *p<0.05, for control and DMSO vs. P7C3 (3 µM). (B) Schematic of Ischemia-Ly294002 (15 µM) reperfusion and protocol utilized. (C) Immunoblots of whole heart lysates were analyzed by Western blotting for cardiac levels of phosphorylated AKTser473 (p-AKT), total AKT (t-AKT) and the ratio of p-AKT/t-AKT. Band intensities were quantified using image J software reported as mean ± SEM, ****p<0.0001, for Ly294002 +P7C3 vs. P7C3. (D) Schematic diagram showing the effects of P7C3 for cardioprotection.

**Figure 10. Ischemia reperfusion injury in wildtype mice post-ischemia perfused with P7C3.** (A) *Ex vivo* ischemia reperfusion injury was assessed via the Langendorff perfusion system. Schematic of Ischemia reperfusion protocol utilized. (B) TTC staining of I/R hearts from WT mice perfused with P7C3 (3 µM) or vehicle with representative heart tissue sections. (C) *In vivo* permanent ligation model used to assess the infarct size and benefit via using P7C3. The C57BL/6J wildtype (WT) mice were treated with a single bolus of P7C3 (10 mg/kg body weight/day, i.p.) or an equivalent dose of vehicle 30 minutes prior to ligating the left anterior descending (LAD) artery. Mice were euthanized at 24 hour time point and the heart samples were processed for the infarct size measurement using TTC staining (D). Heart samples presented are (n=3) means ± SEM **p<0.01; P7C3 vs. vehicle treated WT mice.
Figure 1

A

Nampt activity (min⁻¹)

WT db/db Veh db/db P7C3

**

B

Nampt activity (A450)

Minutes

0.5μM P7C3 (+) Nampt 1μM P7C3 (+) Nampt 1μM P7C3 (-) Nampt 0μM P7C3 (-) Nampt

C

Nampt activity (min⁻¹)

P7C3 (μM)

0.5 1.0 0.00 0.05 0.10 0.15 0.20

+ - Nampt
Figure 2

A) 

B) 

<table>
<thead>
<tr>
<th>P7C3 Region</th>
<th>Amino Acid Residue</th>
<th>Distance (Å)</th>
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<tbody>
<tr>
<td>Linker</td>
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C) 

D) 

E) 

F) 

G) 

H)
Figure 3

A. Fasting blood glucose (mg/dL)

B. Blood glucose (GTT) (mg/dL)

C. AUC
Figure 4

A. QTC Interval (ms)

B. JT Interval (ms)

C. ST Elevation (mV)

D. QTC-Nampt Activity Correlation

* indicates statistical significance.
Figure 5

A  

B  

C  

D  

E  

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Figure 6

A. Vehicle vs. P7C3 for p-AKT and t-AKT

B. Vehicle vs. P7C3 for p-eNOS and total eNOS

C. Vehicle vs. P7C3 for Beclin-1 and GAPDH

D. Sirt1 Activity (U/μM)

E. Sirt1-Nampt correlation

F. NAD/NADH Ratio

p = 0.08
Figure 7

A

Protocol A
- Stability
- Ischemia
- Reperfusion

Protocol B
- Stability
- P7C3
- Ischemia
- Reperfusion/P7C3

B

Infarct Size (%)

I/R
I/R + P7C3

C

Troponin I (ng/mL)

I/R
I/R + P7C3

Minutes

-50
0
50
100
150
Figure 9

A

<table>
<thead>
<tr>
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**p-AKT**

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<tr>
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<th>P7C3</th>
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<tbody>
<tr>
<td>t-AKT</td>
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</table>

B

Protocol A: Stability, DMSO, P7C3, Ischemia, Reperfusion/P7C3

Protocol B: Stability, Ly294002, LY294002+P7C3, Ischemia, Reperfusion/P7C3

C

P7C3+Ly294002 vs. P7C3

D

P7C3

↓ Nampt activation

↓ NAD$^+$

↓ SiRT1

↓ PI3K, pAkt, eNOS, Beclin

↓ Cardioprotection (Improved ECG, EF, FS)

Decreased infarct size

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Figure 10

A) Protocol C and Protocol D

Protocol C:
- Stability
- Ischemia
- Reperfusion

Protocol D:
- Stability
- Ischemia
- Reperfusion/P7C3

B) Infarct Size (%)

<table>
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<th>Infarct Size (%)</th>
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<tbody>
<tr>
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<tr>
<td>I/R + P7C3</td>
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C) Time course of the experiment

Baseline → LAD ligation → 24h

D) Infarct size

<table>
<thead>
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<tr>
<td>Veh</td>
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<tr>
<td>P7C3</td>
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