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Cell Permeable Protein Kinase C Epsilon Peptide Inhibitor Mitigates Myocardial Ischemic-Reperfusion Injury

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Abstract

Percutaneous coronary intervention is the primary treatment for acute myocardial infarction (MI). Paradoxically, restoration of blood flow causes myocardial ischemia reperfusion (MIR) injury, principally due to the generation of reactive oxygen species (ROS). Protein Kinase C epsilon (PKCε) is known to play a key role in ROS production. Our PKCε inhibitor conjugated to myristic acid (Myr), YT-001, has shown efficacy in reducing MIR injury in both *ex vivo* and *in vivo* animal models.

This study aims to evaluate the cardioprotective effects of a dual-conjugated version of our PKCε inhibitor conjugated to Myr and a Transactivator of transcription (Tat), N-Myr-Tat-PKCε (YT-002), in *ex vivo* rat and *in vivo* porcine models of MIR injury.

Ex vivo rat hearts were subjected to 30 minutes of global ischemia followed by 50 minutes of reperfusion. *In vivo* porcine hearts underwent 1 hour of regional ischemia followed by 3 hours of reperfusion. YT-002, or a scrambled peptide control of YT-002, or a saline control was administered at reperfusion onset. Cardiac parameters were measured throughout reperfusion and infarct size was assessed post-mortem.

In the *ex vivo* model, YT-002 (100nM) significantly decreased infarct size to 9.3±1.8%, (n=5, p<0.01) compared to saline control (23.4±3.3%, n=5) and significantly improved left ventricular function compared to saline and scrambled YT-002.

In the *in vivo* model, YT-002 (0.2mg/kg) significantly restored ejection fraction at the end of reperfusion (59.4±1.2%) to the baseline (59.4±0.8%, n=3, p=0.50) and reduced infarct size (10.0±2%, n=4) compared to scrambled YT-002 (29±9%, n=3; p<0.05).

These findings indicate that YT-002 can reduce cardiac infarct size and preserve cardiac function after MI. Since heart failure can correlate with infarct size, decrease MI-induced damage to the heart has the potential to decrease the severity of heart failure thus improving patient outcomes post-MI.

Abbreviations

Area at risk (AR); Area of necrosis (AN); Cardiac output (CO); Calcium (Ca^{2+}); Diacylglycerol (DAG); Dihydrobiopterin (BH_2); Ejection Fraction (EF); Endothelial-derived nitric oxide (eNOS); Intravenously (IV); Ischemia reperfusion (I/R); Left anterior descending (LAD); Left ventricular developed pressure (LVDP); Left ventricular end-diastolic pressure (LVEDP); Left ventricular end-diastolic volume (LVEDV); Left ventricular end-systolic pressure (LVESP); Left ventricular end-systolic volume (LVESV); Maximal rate of decrease in left ventricular pressure (dP/dt_{min}); Maximal rate of increase in left ventricular pressure (dP/dt_{max})., Myocardial infarction (MI); Myocardial Ischemia Reperfusion (MIR); Myristic acid (Myr) Myr-PKCε inhibitor (YT-001); Myr-Tat-PKCε inhibitor (YT-002); Nitric Oxide (NO); Protein Kinase C (PKC); Protein Kinase C Epsilon (PKCε); Pulmonary capillary wedge pressure (PCWP); Reactive Oxygen Species (ROS); Receptor for activated C kinase 1 (RACK1) Superoxide (SO); Tetrahydrobiopterin (BH_4); Transactivator of transcription (Tat); Tumor Necrosis Factor Alpha (TNFa).

Introduction

Myocardial infarction (MI) is a major global health concern and burden, with approximately 800,000 heart attacks occurring annually in the United States [1–3]. MI is caused by partial or complete blockage of a coronary artery, and it can be fatal or lead to long-term health consequences. Arterial blockage prevents oxygenated blood from reaching the distal regions of the myocardium (i.e., apex), causing ischemia. Prolonged ischemia leads to dysfunction of ATP production from mitochondria, leading to dysregulation of Na^+/K^+ATPase that ultimately causes an increase in intracellular calcium (Ca^{2+}), which results in permanent muscle hypercontracture called rigor[4-7]. This rigor condition compresses local blood vessels, further limiting blood flow to the affected ischemic region, and it is principally responsible for initial cardiac muscle infarction [8,9]. Reperfusion, is the reintroduction of oxygenated blood to the myocardium and the first priority of clinicians treating MI. While restoring blood flow reduces the total amount of heart infarct, it paradoxically still causes additional infarct damage [8,10]. This damage is known as myocardial ischemia reperfusion (MIR) injury, and it is currently thought to be responsible for approximately 50% of the infarct damage to the heart [11,12]. Therefore, identifying therapeutic agents that can block/inhibit reperfusion injury and mitigate damage from MIR is critical to improving the outcomes after MI.

The mechanism of MIR injury has been well studied. The restoration of oxygenated blood flow to the previously ischemic myocardium triggers the release of tumor necrosis factor-alpha (TNFα) within 15 minutes [13–15]. TNFα binds to its Gq-coupled

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receptors and drive the production of the second messengers, $Ca^{2+}$and diacylglycerol (DAG), upon activation [13]. These second messengers activate multiple different protein kinase C (PKC) isoforms that phosphorylate a variety of protein targets. PKC epsilon (PKCε) is especially sensitive to DAG and, when activated, promotes mitochondrial ATP-sensitive K^+ channel opening and uncoupled endothelial nitric oxide synthase (eNOS) activity. It is well-recognized that mitochondrial ATP-sensitive K^+ channels and uncoupled eNOS are two of the four primary sources of reactive oxygen species (ROS) in the myocardium, making them key players in reperfusion injury (Figure 1) [16–20]. ROS contributes to cellular damage through mechanisms such as lipid peroxidation, protein modification, and DNA damage, exacerbating inflammatory responses and promoting apoptotic pathways [21-23]. Additionally, ROS impair endothelial function, leading to increased vascular permeability and enhanced leukocyte recruitment that amplifies inflammatory cascades associated with reperfusion injury [8,24]. The opening of mitochondria ATP-sensitive K^+ channels results in greater ROS production. These ROS contribute to sustained mitochondrial permeability transition pore opening, which can cause mitochondrial swelling, more ROS production, and even cell death [8,24,25]. Normally, eNOS produces nitrous oxide (NO) from oxygen and L-arginine using the necessary cofactor tetrahydrobiopterin (BH_4). During ischemic-reperfusion (I/R) injury, ROS produced from the mitochondria and other sources oxidize BH_4 to dihydrobiopterin (BH_2). The increased BH_2/BH_4 ratio causes eNOS to enter an uncoupled state that interacts with PKCε to instead produce superoxide (SO) [16,26]. Overall, PKCε's role in ROS generation makes it an ideal therapeutic target for attenuating I/R injuries [27–29].

The restoration of blood flow after an ischemic period causes PKCε activation through DAG production. RACK1, the receptor for active C kinase specific to PKCε, translocates PKCε to the mitochondria ATP-sensitive K^+ channels and uncoupled eNOS, producing ROS.

In vitro studies of isolated mitochondria showed that PKCε only stimulates ROS production in the presence of ATP-sensitive K^+ channels [19,30]. This ROS production was then inhibited by a PKC epsilon-specific inhibitor peptide epsilonV1-1(EAVSLKPT) [27–29,31]. Endothelial-derived NO plays an important role in creating anti-thrombotic surfaces, which reduces leukocyte-endothelial interactions during inflammatory responses. Studies have shown that ischemia causes a decrease in endothelial NO production, while cell adhesion molecule expression and neutrophil infiltration are increased during reperfusion [32]. eNOS uncoupling inhibits NO production and promotes ROS generation. PKCε modulators affect NO and SO levels through their interactions with eNOS. In rat mesenteric postcapillary venules, BH_2 significantly augmented leukocyte rolling, adherence, and transmigration with or without a myristic acid (Myr) conjugated PKCε activator (*N*-Myr-HDAPIGYD, Myr-PKCε activator). A Myr-PKCε activator with BH_4 and a Myr conjugated PKCε inhibitor (*N*-Myr-EAVSLKPT, Myr-PKCε inhibitor, YT-001), with or without BH_2, both significantly reduced BH_2-induced inflammation [33,34]. YT-001 has been shown to significantly reduce polymorphonuclear leukocyte (PMN)-induced post-reperfused cardiac contractile dysfunction as well as PMN adherence, infiltration, and ICAM-1 expression in isolated rat hearts subjected to I/R conditions [28]. The reduced NO production after ischemia may be due to eNOS uncoupling. (*N*-Myr-HDAPIGYD, Myr-PKCε activator). These results corroborated an earlier I/R study using *in vivo* femoral veins [27]. They found that a Myr-PKCε activator with BH_2 significantly increased hydrogen peroxide production compared to BH_2 alone, and a Myr-PKCε activator with BH_4 reversed this effect [28]. They also found that YT-001 significantly increased NO release in rat renal veins after extracorporeal shock wave lithotripsy [29]. Previous studies demonstrated that YT-001 significantly reduced infarct size in *ex vivo* isolated perfused rat hearts and in an *in vivo* porcine MIR model [35,36].

Cardiac transplantation is the most effective treatment for end-

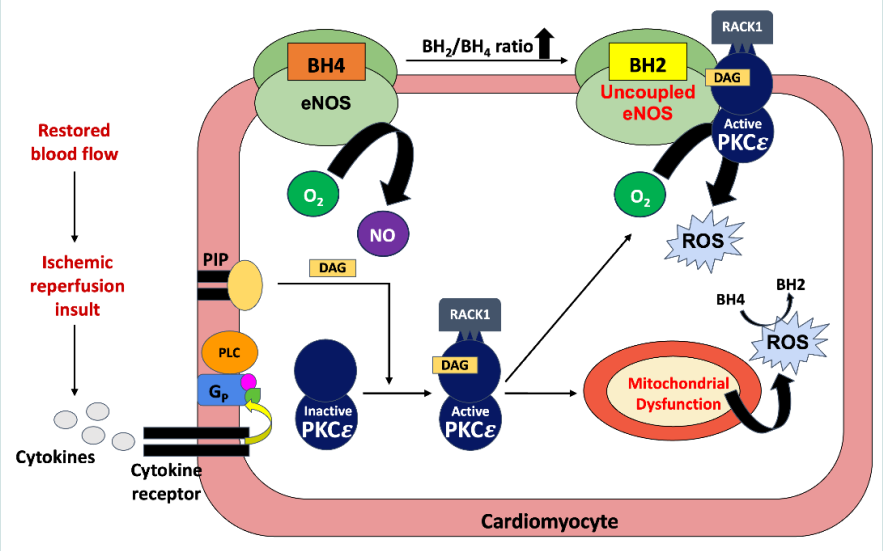

Figure 1: Mechanism of Protein Kinase C epsilon in MIR injury.

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stage heart failure. Although the one-year survival rate for cardiac grafts after heart transplantation has improved, chronic rejection remains a main cause of mortality[37]. Inhibiting PKC isozymes improves cardiac graft survival after transplantation in animal models, and conjugating PKC inhibitors with Tat, a thirteen amino acid sequence (YGKKKRRQRRR), has shown improved efficacy. The positively charged components of Tat interact with the negatively charged components of the membrane, enabling peptide cargo intracellular delivery via endocytosis, whereas Myr conjugation facilitates intracellular delivery via simple diffusion [38–40]. A Tat-conjugated PKCε inhibitor (Tat-PKCε inhibitor) has been shown to extend graft survival and significantly improve functional recovery in a cardiac transplantation model [37]. It also decreased the inflammatory response by reducing T-cell and macrophage infiltration and inhibiting mononuclear inflammatory cell adhesion to the arterial wall. In models of cardiac transplantation and angiotensin-induced heart failure, Tat-PKCε inhibitors preserved the cardiac tissue architecture by reducing luminal narrowing and preventing parenchymal fibrosis[37,41]. The ability of Tat-PKCε inhibitor to improve cardiac graft and transplantation success, where ROS reduce graft cell survival, makes it an appealing conjugation to use in MIR models[37]. As a result, a combination of Tat and Myr conjugation may result in an overall increase in drug delivery compared to either conjugation alone (Figure 2).

Unconjugated peptides must enter cells via the facilitated diffusion, requiring a carrier protein. Tat conjugation enables intracellular delivery through endocytosis, and Myr conjugation enables simple diffusion. Myr-Tat conjugation aims to increase intracellular delivery by combining these cargo delivery mechanisms. (adapted [40]).

We have developed a PKCε inhibitor conjugated to both Myr and Tat (*N*-Myr-Tat-CC-EAVSLKPT), named YT-002, to test whether this dual conjugation will improve PKCε inhibition. YT-002 binds to the scaffolding protein Receptor for activated C kinase1 (RACK1), which normally transports PKCε to mitochondrial ATP-sensitive K^+ channels and eNOS [31]. As previously mentioned, these two substrates produce ROS during reperfusion, so YT-002's activity prevents ROS production (Figure 3). Previous therapeutics targeting I/R injury reduction failed primarily because they aim to scavenge already formed damaging free radicals.YT-002 instead works to prevent the further generation of new ROS.

Active PKCε is translocated by RACK1 to uncoupled eNOS, leading to excessive ROS production. These ROS contribute to heart infarct and cardiac dysfunction seen in MI. B. Pharmacological mechanism of YT-002. YT-002 binds to RACK1, preventing PKCε translocation. This inhibition reduces ROS production and mitigates its negative downstream effects. (adapted from [31]).

We hypothesize that YT-002 will show increased potency

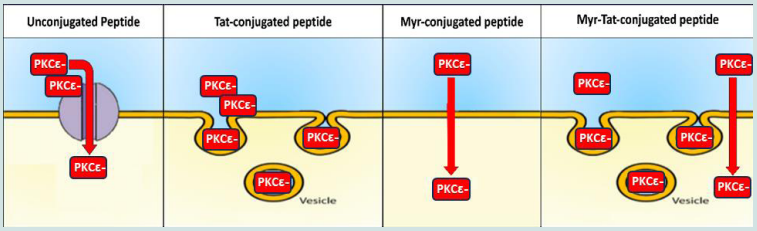

Figure 2: Proposed mechanisms of different drug conjugations in cell membrane penetration.

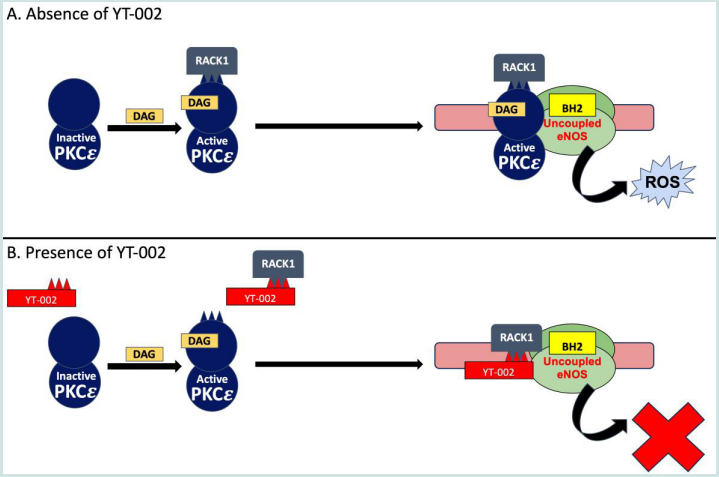

Figure 3: Mechanism of YT-002 in MIR injury. A. Normal pathophysiological response.

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compared to YT-001 or Tat-PKCε inhibitor in our *ex vivo* rat heart MIR model. We also predict that YT-002 will significantly reduce infarct size and improve cardiac parameters compared to saline control. When tested in an *in vivo* pig MIR model, we expect that YT-002 will significantly reduce infarct size and improve cardiac function compared to its scrambled version.

Methods

All experimental protocols were approved by the Institutional Animal Care and Use Committee and performed in accordance with the institutional policies of the Philadelphia College of Osteopathic Medicine and the Government Center for Medical Intervention (Veranex, formally T3 Labs)(Atlanta, GA) an Association for Assessment and Accreditation of Laboratory Animal Care-approved facility.

Drug Conjugation

A PKCε inhibitor peptide (EAVSLKPT) was conjugated to both Myr and Tat to create a dual-conjugated PKCε inhibitor peptide (*N*-Myr-Tat-CC-EAVSLKPT) we have named YT-002. A disulfide bond (CC) was placed between the conjugations and peptide sequence to improve RACK binding. Single-conjugated versions of this drug (YT-001 and *N*-Tat+EAVSLKPT) were also created and tested. A scrambled version of the inhibitor peptide (LSETKPAV) was also conjugated to Myr and Tat to create the scrambled version of YT-002 (*N*-Myr+Tat+LSETKPAV) to serve as a control peptide (Genemed Synthesis, Inc, San Antonio TX 78249). All drugs were diluted to the desired concentration between 100pM and 10μM using 0.03% DMSO in the perfusate.

Ex-Vivo Rat MIR Model

Male Sprague-Dawley rats (275-325 g, Charles River, Springfield, MA) were housed in a 12-hour light and 12-hour dark cycle in a temperature-controlled room. They were given continuous access to food and water. All work with these rats followed our animal-handling protocol A21-002. Rats were anesthetized via an intraperitoneal injection of sodium pentobarbital (60mg/kg) and heparin (1000 units). Hearts were excised and placed on a Langendorff perfusion machine [36] where they were perfused with Krebs' buffer at 37°C at 80 mmHg constant pressure. After a 15-minute stabilization period at which baseline measurements were made, hearts were subjected to 30 minutes of global ischemia followed by 50 minutes of reperfusion. Heart rate, left ventricular end-systolic pressure (LVESP), left ventricular end-diastolic pressure (LVEDP), the maximal rate of increase in left ventricular pressure (dP/dt_{max}), and the maximal rate of decrease in left ventricular pressure (dP/dt_{min}) were measured using an SPR-524 pressure catheter (Millar Instruments, Inc., Houston, TX) placed in the left ventricle. Data values were acquired and stored using a PowerLab/8Sp data acquisition system (AD Instruments, Colorado Springs, CO). Left ventricular developed pressure (LVDP) was found by subtracting the LVEDP from the LVESP (LVESP-LVEDP). The chosen drug or saline control was delivered during the first 5 minutes of reperfusion using a syringe pump at a rate of 1 mL/min.

After the reperfusion period, the hearts were removed from the Langendorff machine and placed in a -20°C freezer for 30 minutes. The hearts were then sectioned perpendicular to their long axis into seven 2-millimeter slices. These slices were incubated in 1% 2,3,5-triphenyltetrazolium chloride (TTC) in 0.2 M Tris buffer (pH=7.41) for five minutes to delineate infarct (pale color) and viable heart tissue (red color). They were placed in 4% paraformaldehyde solution to improve contrast and then photographed. The slices were dissected to separate the infarct and viable tissue, and the percent of infarct was found by dividing the weight of the infarct tissue by the total weight of the tissue.

All data are presented as mean±SEM. ANOVA analysis using Fisher's PSLD test was used to assess any statistical differences in infarct and cardiac parameters between groups. Probability values less than 0.05 were considered statistically significant.

In vivo porcine MIR model

Castrated Male Yorkshire pigs (35-50kg) were purchased by T3 labs. All work with these pigs followed our animal-handling protocol YF01P.Pigs were housed for at least 72 hours before surgery to allow acclimation to the environment, and each animal underwent a general visual health survey by a T3 Labs veterinarian to ensure proper health before surgery. Aspirin (anticoagulant, 300mg) and Amiodarone (antiarrhythmic, 800mg) were given once a day orally for 1 and 3 days, respectively, before surgery. Ketamine (15mg/kg) and xylazine (1mg/kg) were administered through intramuscular injection to sedate the pig. Propofol was given intravenously (IV) for laryngeal relaxation (2-4mg/kg) as needed. The pig was then intubated and maintained using Isoflurane (1.5-2.5%, inhalant) and either lactated ringer solution or 0.9% NaCl (2.5-5 mL/kg/hr, IV).

Pigs were subjected to 1 hour of regional ischemia by balloon-catheter inflation, which was then deflated for 3 hours of reperfusion A catheter with fluoroscopic guidance was used to place the balloon catheter at the level of the second diagonal branch of the left anterior descending (LAD) coronary artery, or approximately 40% of the left ventricular anterior wall distance from the apex to the base of the heart, to cause an antero-apical MI. Ten minutes before ischemia, antiarrhythmic agents' amiodarone (5mg/kg) and lidocaine (2mg/kg) were administered intravenously. End-tidal CO_2 levels, pulse oximetry, ECG, pulmonary capillary wedge pressure (PCWP), rectal temperature, heart rate, blood pressure, and depth of anesthesia were measured during ischemia. The balloon catheter was also used for drug administration and blood pressure measurement. Catheter-balloon deflation marked the onset of reperfusion, and either YT-002 or Scrambled YT-002 was administered into the LAD. A 0.2mg/kg concentration was used to approximate the 1μM concentration of YT-002 tested in our *ex vivo* rat heart model.

Additional catheters were placed in the pulmonary artery and femoral artery through external jugular vein sheaths to measure PCWP and flow, respectively. Two-dimensional electrocardiography was used to measure left ventricular end-diastolic volume (LVEDV) and left ventricular end-systolic volume (LVESV) using a modified Simpson's rule technique. Blood samples were taken from the arterial sheath to measure Troponin I and Creatine Phosphokinaselevels, and they were analyzed by Antech Diagnostics (Atlanta, GA). Electrocardiograph measurements, blood pressure measurements, and blood samples were taken at baseline (15 min before ischemia), 30 minutes into ischemia, and every hour during reperfusion.At

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the end of the reperfusion period, the chest cavity was opened, and the coronary artery, aorta, pulmonary artery, and caudal vena were occluded. We ensured to occlude the coronary artery at the same place where the balloon-catheter was placed.

The left atrium was incised, and 1% Evans Blue Dye (1 ml/kg) was injected into the left atrium to determine the area that was not at risk by labeling the area not at risk in blue. The pig was then euthanized using Potassium Chloride (1-2 mEq/kg) via IV injection, and the heart was excised. The left ventricle was cut axially into 8-millimeter slices and then placed in 1% TTC for 1 hour to determine the area at risk (AR). Thereafter, the area of necrosis (AN) was the area that turned pale after the TTC staining. The slices were photographed, and three blinded analysts measured the AR and AN. A percent infarct was calculated using AN/AR, and the analyst's values were averaged to obtain a final percent infarct for each heart using ImageJ analysis. Ejection fraction (EF) was calculated by dividing stroke volume (difference of end-systolic volume and end-diastolic volume) by end-diastolic volume and cardiac output (CO) was calculated by multiplying the heart rate by the stroke volume.

All data are presented as mean±SEM. Student's t-test was used to assess any statistical differences in infarct and cardiac parameters between groups. Probability values less than 0.05 were considered statistically significant

Results

Ex-vivo results

YT-002 was tested at concentrations ranging from100pM to 10μM. YT-002 significantly reduced infarct size compared tosaline control (23.5±1.8%, n=5) at concentrations of 1nM (9.7±2.3%, n=5, p=0.006), 100nM (9.3±1.8%, n=5, p=0.0002), 1μM. (5.0±2.0%, n=5, p<0.001), and 10μM (5.1±2.2%, n=5, p<0.0001). Interestingly, Scrambled YT-002 (100nM) also significantly decreased infarct (14.5±2.9%, n=5, p=0.0175) compared to control. Myr-PKCε inhibitor (10μM) (Figure 4). (14.9±2.4%, n=5, p=0.0237) significantly reduced infarct compared to control, whereas Tat-PKCε inhibitor (10μM) (17.6±2.3%, n=5) did not have the same effect (Figure 4). Comparing the PKCε inhibitor conjugations, YT-002 (1μM and 10μM) significantly reduced infarct compared to Myr-PKCε inhibitor (10μM) (p=0.0095 and p=0.0144, respectively) and Tat-PKCε inhibitor (10μM) (p=0.0018 and p=0.0030, respectively).

YT-002 1nM (9.7±2.3%, n=5), YT-002 100nM (8.3±1.8%, n=5), Scrambled YT-002 100nM (14.5±2.9%, n=5), YT-002 1μM (5.0±1.4%, n=5), and YT-002 10μM (5.1±2.2%, n=4) significantly reduced heart infarct compared to saline control (23.5±3.3%, n=5). Myr-PKCε inhibitor (Myr-PKCε-)10μM also significantly decreased heart infarct (14.9±2.4%, n=5) compared to control while Tat-PKCε inhibitor (Tat-PKCε-) 10μM (17.6±2.3%, n=5) did not. YT-002 1μM and YT-002 10μM significantly decreased infarct size compared to these singly conjugated PKCε inhibitors. * vs. control, # vs. Myr-PKCε-, † vs. Tat-PKCε-. *p=0.0237 for Myr-PKCε-, *p=0.0175 for Scrambled YT-002 100nM, ***p=0.0006 for YT-002 1nM, ***p=0.0002 for YT-002 100nM, ***p<0.001 for YT-002 1μM, ***p<0.0001 for YT-002 10μM, #p=0.0144 for YT-002 10μM, ##p=0.0095 for YT-002 1μM, ‡p =0.0030 for YT-002 10μM ‡p=0.0018 for YT-002 1μM

Two key measurements of left ventricular performance (i.e., the heart's ability to pump oxygenated blood) are dP/dt_{max} (Figure 5A) and LVDP (Figure 5B). YT-002 (100nM) significantly increased dP/dt_{max} ($p<0.05$) from 5 minutes of reperfusion until the end of reperfusion compared to saline control. YT-002 (100nM) also significantly increased dP/dt_{max} ($p<0.05$) compared to Scrambled YT-002 (100nM) and YT-002 (1μM) from the 25 minutes of reperfusion until the end of reperfusion. YT-002 (100nM) significantly increased LVDP ($p<0.05$) compared to saline control from 10 to 40 minutes of reperfusion. YT-002 (100nM) also briefly significantly increased LVDP ($p<0.05$) compared to Scrambled YT-002 (100nM) and YT-002 (1μM) in the middle of reperfusion.

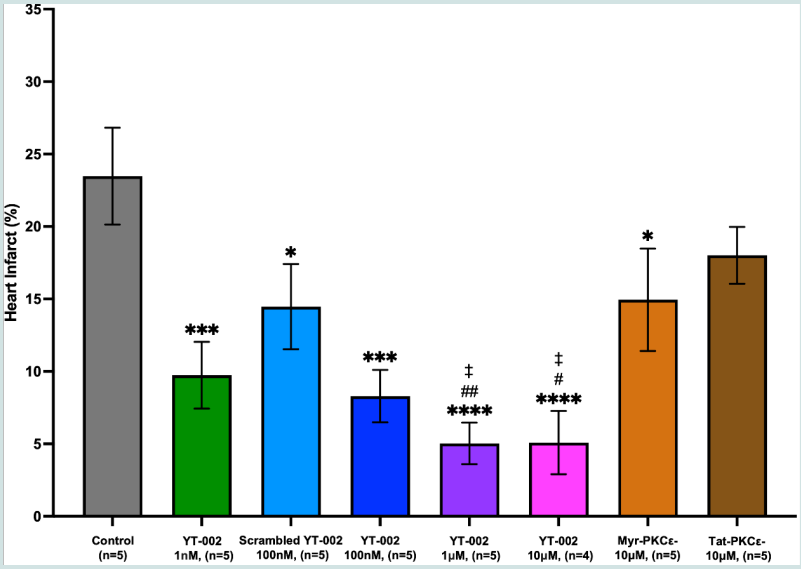

Figure 4: Heart infarct in an ex vivo rat heart MIR model.

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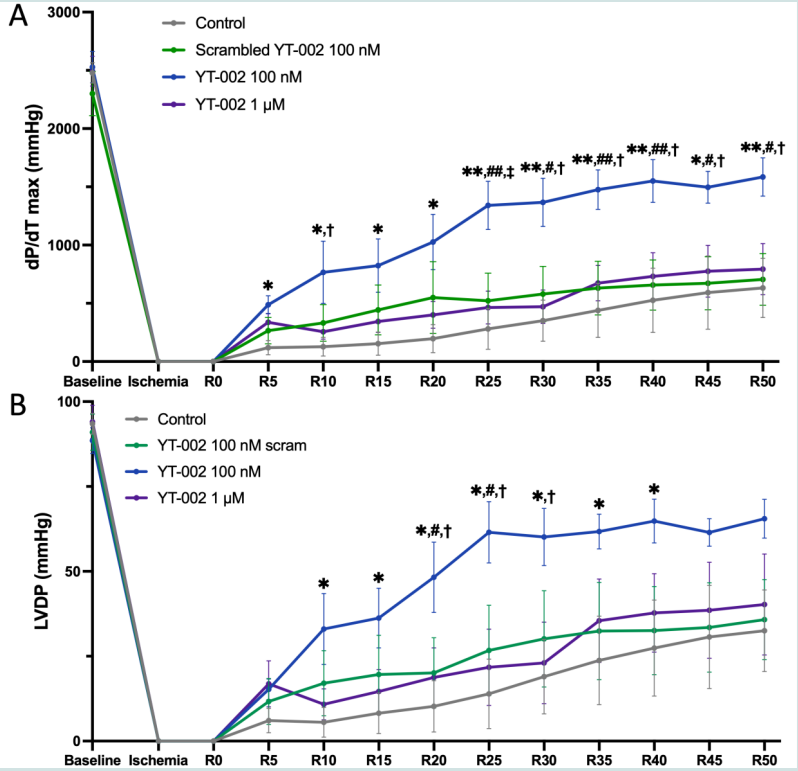

Figure 5: Cardiac function in an ex vivo rat heart MIR model. A. Time course of dP/dt_{max} values.

YT-002 (100nM) significantly increased dP/dt_{max} values compared to saline control throughout the reperfusion period. In addition, YT-002 (100nM) significantly increased dP/dt_{max} compared to scrambled (scram) YT-002 (100nM) and YT-002 (1µM) after 25 minutes of reperfusion. **B. Time course of LVDP values.** YT-002 (100nM) significantly increased LVDP compared to saline control from 10 to 40 minutes. It also significantly increased LVDP compared to scram YT-002 (100nM) and YT-002 (1µM) during the mid-point of the reperfusion period. * p<0.05, **p<0.01 for YT-002 (100nM) vs. Control; #p<0.05, ##p<0.01 for YT-002 (100nM) vs. scram YT-002 (100nM); †p<0.05, ‡p<0.01 for YT-002 (100nM) vs. YT-002 (1µM). "R#" on the x-axis indicates the reperfusion time in minutes.

***In-vivo* results**

Based on our rat heart experiments, we chose to use YT-002 1µM (i.e. 0.2mg/kg) for testing in the porcine MIR model since this was the lowest concentration that had tissue-salvaging effects without impacting cardiac function, in contrast to YT-002 10µM which depressed cardiac function relative to control (data not shown). Scrambled YT-002 (0.2mg/kg) was used as our control. In our *in vivo* pig heart experiments, YT-002 significantly reduced infarct (10.0±2.0%, p=0.039) compared to Scrambled YT-002 (29.0±9.0%.) (Figure 6). YT-002 significantly increased EF at the second hour (55.3±0.8% vs. 59.4±1.1%, p=0.02) and third hour (54.6±1.3% vs. 58.4±0.8%, p=0.04) of reperfusion compared to Scrambled YT-002 despite having similar reperfusion onset EF values (Figure 7). YT-002 also restored EF to its baseline value by the end of reperfusion (59.4±1.2% vs. 59.4±0.8%, p=0.50) while Scrambled YT-002 failed to have the same effect (62.0±0.58% vs. 55.3±0.9%, p=0.003). Heart rate, blood pressure, creatine phosphokinase, and Troponin I were not significantly different at any timepoint throughout this experiment (Table 1).

Scrambled YT-002 (A) shows greater infarct compared to YT-002(B). The area at risk (AR) is outlined in blue and the area of necrosis (AN) is outlined in white. **C. Graph of Heart Infarct.** YT-002 significantly reduced heart infarct (10.0±2.0%, n=4) compared to Scrambled YT-002 (29.0±9.0%,n=3). Heart infarct = AN/AR *p=0.039.

YT-002 (n=5) and Scrambled YT-002 (n=3) groups had similar ejection fractions at baseline and the onset of reperfusion. YT-002 significantly increased ejection fraction compared to Scrambled YT-002 at the second hour (55.3±0.8% vs. 59.4±1.1%, *p=0.02) and third hour (54.6±1.3% vs. 58.4±0.8%, *p=0.04) of reperfusion. By the end of reperfusion, YT-002 restored ejection fraction back to baseline (59.4±1.2% vs. 59.4±0.8%, p=0.50) while Scrambled YT-002 did not (62.0±0.58% vs. 55.3±0.9%, **p=0.003)

Discussion

Summary of Major Findings

Our study aimed to determine if YT-002 would mitigate MIR injury by reducing infarct size and improving cardiac function. Additionally, we explored whether the dual conjugation of YT-002

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Table 1: Cardiac markers in an in vivo porcine heart MIR model.

Timepoint	Heart Rate (bpm) (Scrambled YT-002 / YT-002)	Blood Pressure (mmHg) (Scrambled YT-002 / YT-002)	Troponin I (ng/mL) (Scrambled YT-002 / YT-002)	Creatine Phosphokinase (IU/L) (Scrambled YT-002 / YT-002)
Baseline	73±3 / 69±6	81/44±2/2 / 82/44±4/3	0.000±0.002 / 0.014±0.005	713±261 / 1004±156
Ischemia	67±1 / 61±2	85/55±9/9 / 73/40±6/4	0.046±0.016 / 0.045±0.025	793±286 / 1040±123
Reperfusion Onset	67±4 / 72±10	79/45±10/5 / 68/43±22/6	16.8±14.3 / 1.22±0.66	3205±2248 / 1418±113
Reperfusion 1 Hour	83±20 / 72±5	91/45±6/3 / 80/44±6/1	50.0±0.0 / 43.7±5.0	7082±2983 / 4576±1183
Reperfusion 2 Hour	86±24 / 64±2	90/45±13/9 / 99/53±11/7	50.0±0.0 / 50.0±0.0	10841±5953 / 6011±1814
Reperfusion 3 Hour	89±20 / 65±1	79/36±19/7 / 85/41±10/5	50.0±0.0 / 50.0±0.0	5523±6812 / 6812±2350

No significant differences were found at any time points in neither cardiac function nor cardiac markers at any timepoint between Scrambled YT-002 (n=3) and YT-002 (n=5).

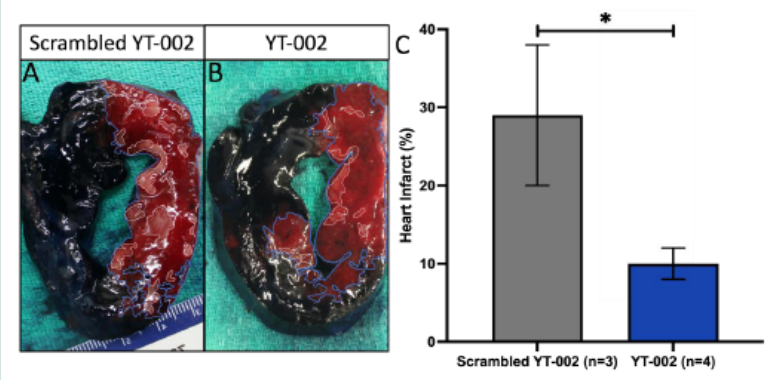

Figure 6: Heart infarct in an in vivo porcine heart MIR model. A, B Representative Heart Slices.

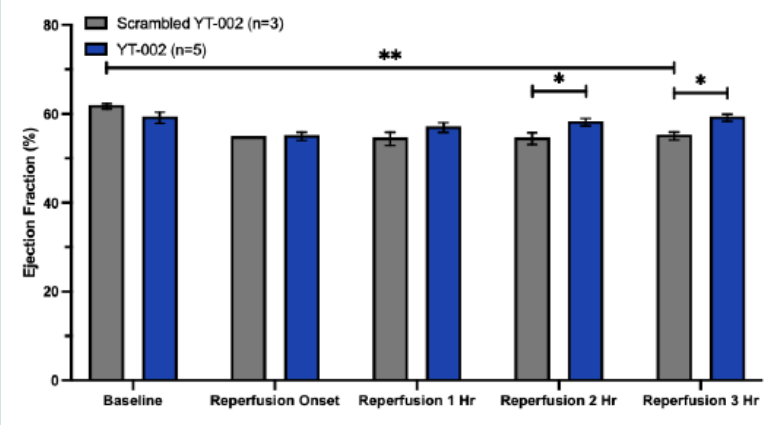

Figure 7: Ejection Fraction for in vivo porcine heart MIR model.

(Myr+Tat) would be superior in reducing MIR injury in comparison to Myr or Tat conjugation alone. We found that:

1. YT-002 demonstrated 10,000 times greater potency compared to either YT-001 or Tat-PKCε inhibitor. In the *ex vivo* rat heart model, YT-002 significantly reduced infarct size at 1nM, while YT-001only caused a similar effect at 10μM and Tat-PKCε inhibitor had no effect at 10μM.

2. YT-002 treatment significantly reduced infarct size compared to its control in both *ex vivo* and *in vivo* MIR models.

3. YT-002 treatment significantly improved cardiac function during reperfusion, as shown by improvements in dP/dt_{max} and LVDP in the *ex vivo* rat heart model and EF in the *in vivo* pig model.

4. YT-002's cardioprotective effects are amino-acid specific, as its scrambled control failed to reduce infarct and restore cardiac function as YT-002 did in the *in vivo* MIR pig model.

Peptide Conjugation

The dual conjugation of YT-002 appears to be more effective at

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penetrating cardiomyocytes and assisting the PKCε inhibitor than its single conjugation versions. A Tat conjugated PKCε inhibitor tested in Phase II clinical trials for postherpetic neuropathy and postoperative orthopedic pain showed no safety or immunogenicity concerns [38,39]. No Myr acid conjugated PKCε inhibitor has been tested in clinical trials yet. However, Myr is an endogenous cargo delivery molecule, suggesting it would present no safety concerns in a clinical trial[40]. As a result, we anticipate that YT-002 is unlikely to present any safety or immunogenic problems in clinical trials.

Mechanism of YT-002

YT-002 operates through a unique mechanism of PKCε inhibition by directly targeting the cellular mechanisms that cause oxidative stress and subsequent myocardial damage during reperfusion. It prevents the formation of ROS by stopping PKCε from interacting with eNOS and mitochondria ATP-sensitive K^+ channels [17,19,27,28]. Unlike conventional treatments that neutralize ROS after they are generated, YT-002 quenches the production of ROS [8,27–29]. By specifically targeting PKCε, whose main activity during reperfusion is in oxidative stress pathways, YT-002 provides a focused approach that preserves myocardial tissue integrity and function and minimizes off-target interactions,reducing the potential for adverse effects compared to abroader-acting pharmacological agent [27–29,33,35–37,41]. At the same time, YT-002 provides a dual mechanism of action by inhibiting two ROS-generating pathways

Comparison of YT-002 to other PKCε Inhibitors

Other PKCε inhibitors have already been tested in MIR animal models. A novel Tat conjugated PKCε inhibitor was found to significantly reduce infarct size and the number of ventricular fibrillation cases in a murine cardiac transplantation model [37,41]. Our lab previously showed that the Myr conjugated PKCε inhibitor (YT-001) significantly reduced infarct size and recovered dP/dt_{max} values in an *ex vivo* rat heart model, and the same drug was found to reduce infarct size and restore EF to baseline values in an *in vivo* pig heart model [35,36]. While our experiments with YT-002 in these same MIR models produced similar results, YT-001 had to be used at concentrations between 10μM and 20μM, more than 10,000 times the concentration of YT-002 found to be effective in our study. (Figure 4) [1nM]). These studies provide additional support that targeting PKCεis an effective way to reduce MIR injury and that YT-002's dual conjugation enables it to be effective at lower concentrations.

Potential Role YT-002 in other I/R settings

Our results demonstrate that YT-002 can mitigate I/R injury by inhibiting ROS generation in the setting of MI. PKCε has been shown to be present and even acutely increased during the I/R conditions that occur during cardiac and kidney transplantation, cerebral stroke, and extracorporeal shockwave lithotripsy, and all of these conditions involve ROS damage [29]. Our YT-001 was shown to be effective in reducing kidney I/R injury *in vivo* [42]. Given that the YT-002's mechanism of action is independent of the tissue setting, this drug has the potential to be effective in reducing I/R injury in a variety of ischemic organ settings.

Study Limitations

This study presents limitations in its attempt to provide pre-clinical knowledge. First, our Troponin I measurement tool did not have an adequately large range for our experiment, resulting in all measurements at or above 50 ng/mL being recorded as 50 ng/mL. Troponin I is widely used in the clinic for assessing MI, so this limitation prevented us from further understanding YT-002's potential in mitigating MIR injury. Pigs and rats are known to have different cardiac properties than humans, though pigs anatomically and physiologically are more similar to humans [43]. These differences provide limitations in our ability to use these animal MIR models to predict a drug's success in the clinic. Both models caused MI through immediate methods whereas MI has a gradual pathogenesisin humans [43,44]. In addition, these animal models only used PKCε inhibition to treat the induced MI, whereas our drug would likely be an adjunctive therapy to treatments currently used in the clinic, such as percutaneous coronary intervention. A MIR model that more closely resembles human MI in theseways will likely produce more translatable results.Both experiments also only used male animals, making our findings less applicable to the general population. In addition, MI has been shown to have long-term effects on patients, while all our analyses were done during or immediately after reperfusion.

Future Studies

We aim to conduct a six-month *in vivo* porcine MIR survival study to evaluate the capabilities of YT-002 to mitigate the long-term effects of MI. This study will also help determine the therapeutic window and dosing regimen that optimizes efficacy and minimizes potential adverse effects in a heart model that closely mimics human cardiac physiology. We will use the same MIR pig model presented in this manuscript, andleft ventricle EF and high-sensitivity troponin and creatine phosphokinase-MB will be measured throughout the six-month survival periodto determine if YT-002 can reduce post-MI incident heart failure.

Conclusion

In conclusion, YT-002 was cardioprotective and reduced MIR injury in *ex vivo* and *in vivo* animal MIR models. It significantly reduced infarct size and restored LV function. This effect resulted from YT-002 inhibiting PKCε-driven ROS generation at the beginning of reperfusion, suggesting it could be effective in other I/R scenarios. Our results principally highlight YT-002's potential for improving MI outcomes in the clinic. Future studies will evaluate the long-term effects of YT-002 treatment in a porcine MIR model, and we aim to enter this drug into Phase 1 clinical trials.

Highlights

- Myocardial ischemia reperfusion injury is a major contributor to myocardial infarction pathophysiology.
- YT-002 mitigates this injury by inhibiting the production of reactive oxygen species.
- YT-002 administration at reperfusion onset reduces infarct size and restores LV function in MIR.
- Phase 1 clinical trials are next in studying YT-002's potential as an MI therapeutic.

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