Effect of dimerized thrombin fragment TP508 on acute myocardial ischemia reperfusion injury in hypercholesterolemic swine

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Abbreviations

AMI-RI, acute myocardial ischemia followed by reperfusion injury

LV, left ventricular

LAD, left anterior descending artery

ABG, arterial blood gas

MAP, mean arterial blood pressure

LVP, left ventricular pressure

HR, heart rate

AAR, area-at-risk

%SS, percentage of segmental shortening

TTC, triphenyl tetrazolium chloride

ADP, adenosine diphosphate

SNP, sodium nitroprusside

Bcl-2, B-cell lymphoma 2

PARP, poly-ADP ribose polymerase

AIF, apoptosis inducing factor

eNOS, endothelial nitric oxide synthase

TUNEL, dUTP nick-end labeling
Abstract

The thrombin related peptide, TP508, is a 23-amino acid monomer that represents a portion of the receptor binding domain in the thrombin molecule. TP508 is also known to readily convert to a dimer in an aqueous environment. In this study the dimeric form of TP508 was investigated in a porcine model of acute myocardial ischemia reperfusion injury (and compared to its monomer). Twenty four hypercholesterolemic pigs underwent 60 min of mid-left anterior descending coronary artery occlusion followed by 120 min of reperfusion and received either vehicle (n = 6) or TP508 monomer (n = 6) or two different doses of dimer (n = 6, respectively). Infarct size was significantly reduced in the monomer and two dimer groups compared to vehicle. Improvement in both endothelium-dependent and -independent coronary microvascular relaxations was also observed in treated groups. In addition, the expression of 27-kDa heat shock protein, αB-crystalline and phosphorylated Bcl-2 (Ser70) in the ischemic area-at-risk were higher in treated groups than vehicle, whereas cleaved poly-ADP ribose polymerase was lower in treated groups. Finally, there were fewer apoptotic cells in treated groups than vehicle. This study suggests that TP508 dimer provides a myocardial protective effect on acute ischemia reperfusion injury in hypercholesterolemic swine similar to TP508 monomer by up-regulating cell survival
pathways or down regulating apoptotic pathways.
Introduction

The thrombin fragment TP508, also known as rusalatide acetate or Chrysalin, is a 23-amino acid peptide that represents a portion of the highly conserved, catalytic site of the receptor binding domain in the native thrombin molecule. TP508 is known to have an effect on cells and tissues, accelerating dermal wound healing (Carney et al., 1992), fracture repair (Wang et al., 2005), bone formation (Sheller et al., 2004) and stimulating angiogenesis (Carney et al., 1992; Stiernberg et al., 2000). Previous studies demonstrated that intravenously administered TP508 decreased myocardial necrosis and apoptosis after ischemia reperfusion injury in normcholesterolemic and hypercholesterolemic pigs (Osipov et al., 2009a; Osipov et al., 2009b). TP508 is a monomer, and it is known that TP508 dimerizes spontaneously in a saline solution. Since TP508 contains a single cysteine residue, it is capable of forming a dimer chemically via formation of a disulfide bond (Capstone Therapeutics, personal communication) [Figure 1]. According to an in vitro experiment, dimerized form of TP508 is much more stable than monomer in saline (Capstone Therapeutics, personal communication) [Figure 2A]. Thus, we first performed in vitro experiments to examine the stability of TP508 monomer and dimer in pig plasma over the course of 120 min which is the length of infusion in the protocol. Second, we performed in vivo
experiments to assess the effect of two doses of TP508 dimer in the setting of acute myocardial ischemia followed by reperfusion injury (AMI-RI) in a swine model, compared with standard doses of TP508 monomer. Since a majority of patients presenting with AMI have endothelial dysfunction secondary to dyslipidemia, diabetes and hypertension, we elected to utilize hypercholesterolemic swine as a model of endothelial dysfunction in assessing the effect of TP508.
Methods and Materials

Stability of TP508 monomer and dimer (In vitro)

First, we assessed the dosing solution stability of TP508 monomer. TP508 monomer was prepared at two concentrations in sterile saline (0.0625 mg/mL and 2.5 mg/mL). These concentrations were selected because they were the lowest and highest infusion concentrations used in the in vivo experiments as described below. The solutions were incubated at room temperature in plastic syringes and analyzed by high-pressure liquid chromatography (HPLC) to determine the extent of dimer formation. Each dosing solution was diluted to 0.200 mg/mL using 0.1% trifluoroacetic acid and analyzed by HPLC.

Next, we assessed the plasma stability of TP508 monomer and dimer respectively. First, TP508 monomer was added to pig plasma (Bioreclamation, Inc., Liverpool, NY) at a concentration of 70 µg/mL (30.6 mM). This concentration was selected because it represented the highest concentration expected after the bolus dose was complete (60 mg bolus dose into a 20 kg pig with ~ 850 mL circulating plasma volume). Degradation of TP508 monomer into smaller peptide fragments was observed over the course of 120 min. Second, TP508 dimer was added to pig plasma (Bioreclamation, Inc.) at a concentration of 26.6 mM. Monomer and dimer
concentration was measured over the course of 120 min. Each plasma sample was maintained at 37 °C for 120 min, precipitated using an equal volume of 20 % trichloroacetic acid, vortexed for 20 sec and then centrifuged using a microcentrifuge for 10 min at 14,000 rpm. The supernatant was analyzed by HPLC. In vitro experiments were performed by Capstone Therapeutics Research Operations (Tempe, AZ).

Experimental design (In vivo)

Animals were housed individually and provided with laboratory chow and water ad libitum. All experiments were approved by the Beth Israel Deaconess Medical Center animal care and use committee and the Harvard Medical Area standing committee on animals. The experiments conformed to the US National Institutes of Health guidelines regulating the care and use of laboratory animals (NIH publication 5377-3, 1996).

Twenty four intact (non-castrated) male Yucatan mini-swine (20-22 week-old) were fed with a high-fat/high-cholesterol diet for 4-6 weeks (Sinclair Research Center, Columbia, MO) (Spurlock and Gabler, 2008). All animals were subjected to regional left ventricular (LV) ischemia by left anterior descending (LAD) arterial occlusion distal to the second diagonal branch for 60 min. Animals received intravenously either vehicle (V, n = 6) or TP508 monomer (M-1, n = 6) as a bolus of 0.5 mg/kg.
(0.21µmol/kg) 50 min into ischemia followed by a continuous infusion of 1.25 mg
(0.54µmol/kg/h) during the entire period of reperfusion, or equivalent dose of TP508
dimer to M-1 expressed in mole (DD, n = 6) or half dose of TP508 dimer as DD (D, n =
6) (Harvard Apparatus, Hollistone, MA) (Table 1). To assess the dose-dependent effect
of TP508 monomer, twelve pigs were added and divided into two groups and received
1/10 times M-1 (M-1/10, n = 6) or 4 times M-1 (M-4, n = 6), respectively.

Arterial blood gas (ABG), arterial blood pressure, hematocrit (Hct), LV
pressure, heart rate (HR), EKG, O2 saturation, and core body temperature were
measured and recorded. Myocardial segmental shortening in the long-axis (parallel to
the LAD) and short-axis (perpendicular to the LAD) were recorded at baseline prior to
the onset of the ischemia and prior to harvest. At the completion of the protocol, the
heart was excised and tissue samples from ischemic and non-ischemic left ventricle
were collected for analysis as described below. Thus, each pig served as its own control
with regard to molecular studies.

Surgical protocol

Swine were sedated with ketamine hydrochloride (20 mg/kg, intramuscularly,
Abbott Laboratories, North Chicago, IL) and anesthetized with a bolus infusion of
thiopental sodium (Baxter Healthcare Corporation, Inc., Deerfield, IL; 5.0-7.0 mg/kg
intravenously), followed by endotracheal intubation. Ventilation with a volume-cycled ventilator (model Narkomed II-A; North American Drager, Telford, PA; oxygen, 40%; tidal volume, 12.5 ml/kg; ventilation rate, 9-11 breaths/min; positive end-expiratory pressure, 3 cm H$_2$O; inspiratory to expiratory time, 1:2) was used. General endotracheal anesthesia was established with 3.0% isoflurane (Ultane; Abbott Laboratories) at the beginning of the surgical preparation and maintained with 1.0% throughout the experiment. One liter of Lactated Ringer’s intravenous (IV) fluid was administered after induction of anesthesia and continued throughout the surgical protocol at 150 ml/h.

Right groin dissection was performed and the femoral vein and common femoral artery were isolated and cannulated utilizing 6 French sheaths (Cordis Corporation, Miami, FL). The right femoral vein was cannulated for intravenous access and the right common femoral artery was cannulated for arterial blood sampling and continuous intra-arterial blood pressure monitoring (Millar Instruments, Houston, TX). A median sternotomy was performed. A catheter-tipped manometer (Millar Instruments, Houston, TX) was introduced through the apex of the heart to record LV pressure. Segmental shortening in the area-at-risk (AAR) was assessed utilizing a sonometric digital ultrasonic crystal measurement system (Sonometrics Corp., London, ON, Canada) using four 2 mm digital ultrasonic probes implanted in the subepicardial layer approximately
10 mm apart within the ischemic LV area. Cardiosoft software (Sonometrics Corp., London, ON, Canada) was used for data recording (LV \(dP/dt\), segmental shortening, arterial blood pressure and heart rate) and subsequent data analysis to determine myocardial function. Baseline hemodynamic, functional measurement, ABG analysis and hematocrit were obtained. ABG analysis was performed at the baseline, 30 min after occlusion and 30 min after reperfusion. All animals received a bolus of lidocaine (1.5mg/kg) as prophylaxis against ventricular dysrhythmia, as well as 80 units/kg of intravenous heparin bolus prior to occlusion of the LAD. The LAD coronary artery was occluded 3 mm distal to the origin of the second diagonal branch utilizing a Rommel tourniquet. Myocardial ischemia was confirmed visually by regional cyanosis of the myocardial surface. The Rommel tourniquet was released 60 min after the onset of acute ischemia and the myocardium was reperfused for 120 min. At the end of the reperfusion period, hemodynamic and functional measurements were recorded as described above, followed by religation of the LAD and injection of monastryl blue pigment (England Corp., Louisville, KY) at a 1:150 dilution in PBS into the aortic root after placement of an aortic cross-clamp distal to the coronary arterial ostia to demarcate the AAR. The heart was rapidly excised just after 50mL injection of blue pigment and the entire LV, including the septum, was dissected free. The LV was cut into
approximately 1 cm thick slices perpendicular to the axis of the LAD from the LV apex to the point of ligation, which resulted in four slices. The AAR was clearly identified by lack of blue pigment staining. The AAR of the second slice proximal to the LV apex was isolated and divided for use in western blotting and for paraffin section. The other three slices were used for infarct size measurement as described below. Ventricular dysrhythmia (ventricular fibrillation or pulseless ventricular tachycardia) events were recorded and treated with immediate electrical cardioversion (100-150J, internal paddles).

**Measurement of global and regional function**

Global myocardial function was assessed by calculating the maximum positive first derivative of LV pressure over time (+dP/dt). Regional myocardial function was determined by using subepicardial 2mm ultrasonic probes to calculate the percentage segment shortening (%SS), which was normalized to the baseline. Measurements were taken during a period of at least three cardiac cycles in normal sinus rhythm and then averaged. Digital data were inspected for the correct identification of end-diastole and end-systole. End-diastolic segment length (EDL) was measured at the onset of the positive dP/dt, and the end-systolic segment length (ESL) at the peak negative dP/dt. Measurements were taken at baseline (Pre) and then every 30 min (30 min after
occlusion: O1, 60 min after occlusion: O2, 30 min after reperfusion: R1, 60 min after reperfusion: R2, 90 min after reperfusion: R3, 120 min after reperfusion: R4

throughout the protocol using a Sonometrics system as previously described (Osipov et al., 2009a; Osipov et al., 2009b).

Quantification of myocardial infarct size

Three LV slices were immediately immersed in 1% triphenyl tetrazolium chloride (TTC, Sigma Chemical Co., St.Louis, MO) in phosphate buffer (pH 7.4) at 38 °C for 30 min. The infarct area (characterized by absence of staining), the non-infarcted area-at-risk (characterized by red tissue staining), and the non-ischemic portion of the LV (characterized by purple tissue staining) were sharply dissected from one another.

The percentage of the AAR was defined as: (infarct mass + non-infarct AAR mass)/total LV mass x 100. Infarct size was calculated as a percentage of AAR to normalize for any variation in AAR size using the following equation: (infarct mass/ total mass AAR) x 100.

Coronary microvascular reactivity studies

Coronary microvascular reactivity was examined in the ischemic territory, as previously described (Osipov et al., 2009a; Osipov et al., 2009b). Briefly, coronary arterioles were dissected with a 40x microscope. Microvessels were mounted on
dual-glass micropipettes and examined in a pressurized, isolated microvessel chamber. Adenosine diphosphate (ADP: 1 nM–100 µM), substance P (0.1 pM–10 nM) and sodium nitroprusside (SNP: 1 nM–100 µM) were applied extraluminally after precontraction by 25–50% of the baseline diameter with the thromboxane A2 analog U-46619 (0.1–1 µM).

**Western blotting**

Whole-cell lysate were made from homogenized AAR myocardial samples with RIPA buffer (Boston Bioproduct, Worcester, MA) and centrifuged at 12,000 x g for 10 minutes at 4 °C to separate soluble from insoluble fractions. In the myocardial tissue lysate, the protein concentration was measured spectrophotometrically at 595 nm with a DC protein assay kit (BioRAD, Hercules, CA). Twenty to sixty micrograms of total protein were fractionated by 4-20%, 8-16%, or 12% gradient, SDS polyacrylamide gel electrophoresis (Invitrogen, San Diego, CA) and transferred to PVDF membranes (Millipore, Bedford, MA). Each membrane was incubated overnight at 4 °C with the following antibodies: total and phosphorylated B-cell lymphoma 2 (Bcl-2) (1:200 dilution, Cell Signaling Technology, Beverly, MA), total and cleaved caspase-3 (1:300 dilution, Cell Signaling Technology), total and cleaved poly-ADP ribose polymerase (PARP) (1:300 dilution, Cell Signaling Technology), 27-kDa heat shock protein
(HSP27) (1:1000 dilution, Stressgen) and αB-crystallin (1:1000 dilution, Stressgen) were assessed. The membranes were subsequently incubated for 45 minutes in diluted appropriate secondary antibody (1:2000 dilution, Cell signaling Technology). Immune complexes were visualized with the enhanced chemiluminescence detection system (Amersham, Piscataway, NJ). Bands were quantified by densitometry of radioautograph films. Ponceau staining was performed to confirm equivalent protein loading.

TUNEL staining

The apoptotic cells were identified by dUTP nick-end labeling (TUNEL) using an apoptosis detection kit according to the manufacturer’s protocol (Millipore Corporation, Billerica, MA). At least 1 cm$^2$ of tissue from the AAR was analyzed from each animal (4 per group). The nuclei were viewed and manually counted by an observer blinded to the experimental conditions. The number of TUNEL-positive cardiomyocytes, indicating apoptosis, was divided by the surface area and expressed in the number per 100 µm$^2$.

Statistical Methods

Functional and microvascular reactivity data were analyzed using two-way repeated-measures ANOVA. Frequency of VT/VF was analyzed using chi-square test.
Infarct size and densitometry in western blot data were analyzed using one-way ANOVA. All results were expressed as mean ± SEM and a $p$ value of less than 0.05 was considered statistically significant (Systat, San Jose, CA). Immunoblots were expressed as a ratio of protein to loading band density and were analyzed after digitization and quantification of X-ray films with ImageJ 1.33 (National Institutes of Health, USA).
Results

Dosing solution stability of TP508 monomer (In vitro)

Over the course of 120 min, TP508 dimer was observed to increase from a starting value of ~0.7% to 1.9% and 1% in the low and high concentration samples respectively [Figure 2B]. This demonstrates that TP508 monomer dimerizes in a saline solution over time, however dimer formation in the dosing solution of the present study is likely to be minimal throughout 120 min infusion period.

Plasma stability of TP508 monomer and dimer (In vitro)

As shown in Figure 2C, TP508 monomer rapidly degraded into smaller peptide fragments, with only ~2% of the initial monomer concentration remaining after 120 min. TP508 dimer was observed to form from the TP508 monomer, however peak concentration of the dimer only reached ~2.15 mM at 60 min. If 100% of the monomer was converted to dimer, the concentration of dimer would be expected to reach 15.3 mM. Therefore, the conversion of TP508 monomer to dimer in plasma was approximately 14%, suggesting that although dimer is formed, it reaches a maximum concentration that is a small fraction of the initial monomer concentration. Examination of total peptide recovery, which included TP508 monomer, TP508 dimer and all peptide fragments resulting from enzymatic degradation demonstrated that more than half the
monomer was degraded in the first 15 min. Total recovery dropped over time indicating that some peptide fragments were degraded to the point that they were no longer captured by the HPLC assay, or that they were bound to plasma proteins. As shown in Figure 2D, dimer concentration was observed to decrease over time, however less rapidly than was seen for TP508 monomer. Monomer was not detected. The dimer concentration was observed to decrease more slowly over time as compared to TP508 monomer. This may indicate that the dimer is also degraded in plasma, however, it is not reduced to the monomer. Although dimer concentration dropped less than was seen for monomer, the total recovery of dimer and degradation products was observed to drop more than for monomer. This may suggest that some dimer loss was due to precipitation or binding to plasma proteins, rather than enzymatic degradation.

Arterial blood gas, hematocrit, core temperature

No significant differences were observed among groups in arterial pH, $pCO_2$, $pO_2$, Hct, core temperature at any time point in the protocol.

Serum lipid profile and glucose

Levels (mg/dl) of serum total cholesterol (V: 373±67, M-1: 512±15, D: 411±63, DD: 511±63, $p = 0.33$), triglyceride (V: 12±1, M-1: 15±3, D: 12±2, DD: 19±5, $p = 0.76$), HDL (V: 89±13, M-1: 67±9, D: 73±9, DD: 92±13, $p = 0.34$), LDL (V: 202±23, M-1: 222±11, D: 227±13, DD: 232±15, $p = 0.76$).
281 ± 56, M-1: 443 ± 19, D: 335 ± 57, DD: 415 ± 55, p = 0.09) and blood glucose (V: 54 ± 4, M-1: 55 ± 2, D 59 ± 4, DD: 62 ± 5, p = 0.47) at the baseline were not significantly different among groups (Chemistry laboratory, Rhode Island Hospital).

Global and regional left ventricular function

Figure 3 (A-D) shows mean arterial blood pressure (MAP), developed LV pressure (LVP), HR and global systolic LV function as determined from LV dP/dt and from the baseline (Pre) to the end of reperfusion (R4). No significant differences were seen among groups between the baseline (pre) and the end of reperfusion (R4) in MAP (p = 0.93), HR (p = 0.61), LV dP/dt (p = 0.51) and developed LVP (p = 0.94). Regional myocardial function in the area-at-risk is shown in Figure 4 (A,B). The percentage of the segmental shortening (%SS) for horizontal axis was improved in D group (p = 0.04), whereas %SS for longitudinal axis did not show the differences among groups (p = 0.51). Regarding M-1/10 and M-4, the data (pre and R4) are shown in Table 2. There are no significant differences compared to vehicle.

Incidence of VF/VT

There was no difference in incidence of VF/VT during ischemia (V: 5/6 animals with VF/VT / total number, M-1: 5/6, D: 4/6, DD: 5/6; \( \chi^2 \) p = 0.82), or during reperfusion (V: 0/6, M-1: 1/6, D: 1/6, DD: 0/6; \( \chi^2 \) p = 0.90). As previously
demonstrated (Osipov et al., 2009a), VF/VT appeared 15-20 min after beginning of ischemia. All dysrhythmias were successfully terminated with intravenous lidocaine and electrical cardioversion. There was no mortality in the different groups.

Myocardial infarct size

The size of the ischemic AAR, expressed as a percentage of total LV mass was not significantly different among groups (V: 33.7 ± 2.0, M-1: 38.4 ± 3.8, D: 37.1 ± 3.6, DD: 33.7 ± 2.1, p = 0.59) [Figure 5A], whereas the size of the infarct area expressed as a percentage of AAR was decreased both in M-1, D and DD groups versus V (V: 60.7 ± 9.8, M-1: 41.2 ± 9.6, D: 28.3 ± 2.2, DD: 41.1 ± 8.4, p < 0.01) [Figure 5B].

M-4 group showed a significant decrease in the size of infarct area as a percentage of AAR, whereas M-1/10 group showed almost even with V (M-1/10: 57.3 ± 2.9, M-4: 44.5 ± 3.9). Dose-dependent effect of TP508 monomer and dimer on myocardial protection after AMI-RI was shown in Figure 6, respectively (M-2 was cited from previous work; M-2 receives twice M-1: n = 6) (Osipov et al., 2009a).

Coronary microvascular reactivity in the ischemic territory

The baseline diameter was 174 ± 12, 143 ± 15, 151 ± 19, 191 ± 23 µm in the group V, M, D and DD, respectively (p = 0.16). The percentage of precontraction was –33.7 ± 1.9, –34.5 ± 1.7, –32.4 ± 1.7 and –35.3 ± 2.4 in the group V, M, D and DD,
respectively ($p = 0.20$). Receptor-mediated, endothelium-dependent relaxations to ADP were improved in D and DD ($p < 0.01$) and to substance P were improved in M ($p = 0.03$), whereas endothelium-independent relaxations to SNP were improved in D among groups ($p = 0.04$) [Figure 7A-C]).

**Western blotting**

Expression of phosphorylated (Ser70) Bcl-2 showed significant differences among groups ($p < 0.01$), whereas total Bcl-2 were similar among groups ($p = 0.21$) [Figure 8A,B]. Expression of total PARP ($p = 0.52$) were not significantly different among groups, whereas cleaved PARP ($p = 0.02$) was significantly lower in M, D and DD compared to V [Figure 8C,D]. Expression of cleaved caspase-3 ($p = 0.47$) and total caspase-3 ($p = 0.33$) were not significantly different among groups [Figure 9A,B]. Expression of HSP 27 ($p < 0.01$) and αB-crystallin ($p < 0.01$) showed significantly higher in both M and D groups compared with V [Figure 9C,D].

**TUNEL staining**

The apoptotic cell count per 100 μm$^2$ in the AAR was higher in V compared with M, D and DD (V; 59 ± 34, M; 13 ± 3, D; 22 ± 3, DD; 23 ± 3, $p < 0.01$) [Figure 10]. As previously demonstrated (Osipov et al., 2009a; Osipov et al., 2009b), most apoptotic cells were cardiomyocytes and mainly located near the necrotic area. The non-ischemic
area was devoid of TUNEL-positive cells.
Discussion

The most significant finding of this study is intravenous therapeutic administration of TP508 dimer also has significant myocardial protective effect in response to AMI-RI as described in the previous work with TP508 monomer.

TP508 limited myocardial infarct size, likely via changing protein expression in cell survival/death and apoptosis pathways in a clinical relevant large animal model. In addition, this study is novel in that we compared the effect of TP508 monomer and dimer in vivo. Administration of two different doses of TP508 dimer in the present study significantly reduced myocardial infarct size compared to vehicle as well as TP508 monomer. The expression of cleaved PARP was significantly decreased, while the expression of phospho Bcl-2 (Ser70), HSP27 and αB-crystalline were significantly increased in groups treated with monomer and two doses of dimer compared to vehicle.

As previously demonstrated, TP508 monomer treatment is associated with higher levels of specific cell survival proteins in the ischemic myocardium, such as heat shock proteins (HSPs) (Osipov et al., 2009a). This study shows that the expression of HSP27 and αB-crystalline were significantly higher in M-1, D and DD groups compared to vehicle without any dose dependent correlation regarding two doses of dimer groups. Recent studies demonstrate that the phosphorylated form of HSP27 is a
potent anti-apoptotic molecule that may directly interfere with cell death signaling pathways (Benn et al., 2002; Stetler et al., 2009). A number of studies have shown that over expression of HSP27 reduces apoptotic cell death triggered by various stimuli, including hyperthermia, oxidative stress, staurosporine-induced apoptosis and cytotoxic drugs (Garrido et al., 1996; Garrido et al., 1997; Mehlen et al., 1996; Samali and Cotter, 1996). There are some controversies as to whether HSP27 has direct interaction with caspase-3, although HSP27 has been shown to inhibit apoptosis via the direct inhibition of caspase-3 activation (Garrido et al., 1996; Concannon et al., 2001; Garrido et al., 1999; Samali et al., 2001) and through interacting with the pro-caspase-3 molecule (Garrido et al., 1996; Pandey et al., 2000). However, this theory has been challenged by other studies showing little or no direct interaction between HSP27 and caspase-3 (Garrido et al., 1996; Pandey et al., 2000; Paul et al., 2002). This study indicates no significant difference in the expression of the total and cleaved caspase-3 between the treated and non-treated groups, while expression of HSP27 was significantly higher in all treated groups. This suggests that the higher levels of HSP27 is not associated with the caspase pathway.

αB-crystalline is known as part of the small heat shock protein (sHSP) family (Bagnéris et al., 2009; Taylor and Benjamin, 2005). The strongly cytoprotective
function of αB-crystallin and HSP27 may involve binding to specific components of apoptosis (Arrigo et al., 2007; Mao et al., 2004; Stegh et al., 2008) and autophagy (Carra et al., 2008) pathways (Bagnéris et al., 2009). This study shows significantly higher expression of αB-crystallin in treated groups compared to vehicle, suggesting that TP508 dimer may have a cytoprotective effect related to activation of sHSP such as αB-crystallin and HSP27 as well as TP508 monomer during AMI-RI [Figure 11].

PARP is known to help cells to maintain their viability and cleavage of PARP facilitates cellular disassembly and serves as a marker of cells undergoing apoptosis (Osipov et al., 2009a). In this study we found a significant decrease in cleaved PARP in all treated groups compared to vehicle, suggesting that provided treatment with TP508 monomer and dimer may have anti-apoptotic effect through cleavage of PARP [Figure 11]. Phosphorylated Bcl-2 (Ser70) is thought to be required for the enhanced anti-apototic functions of total Bcl-2 (Deng et al., 2001). This study demonstrates that phospho Bcl-2 (Ser70) was up-regulated in the treated groups, suggesting that TP508 monomer and dimer may have anti-apoptotic effects through phosphorylated Bcl-2 (Ser70) [Figure 11]. The TUNEL assay demonstrated that there were fewer apoptotic-positive cells in the TP508 treated groups. Treatment with the TP508 monomer and low and high doses of dimer may affect apoptotic signaling pathway via
Bcl-2 and PARP.

The coronary microcirculation, consisting of arterioles less than 175 µm in diameter, is the principal site of resistance in the coronary circulation and responds to metabolic stimuli to govern myocardial perfusion (Sohda et al., 2009). Dysfunction in this vascular bed, which is known to occur after AMI-RI (Sohda et al., 2009; Hein et al., 2003) is thought to be responsible for the impairments in myocardial perfusion observed after re-establishment of flow in the target vessels of thrombolysis, percutaneous coronary intervention, and coronary artery bypass grafting — the large epicardial coronary arteries (Ito et al., 1992). Our previous study suggested that in the setting of hypercholesterolemic swine model TP508 monomer may improve vasodilatation and enhance coronary microvascular relaxation via modulation of NO signaling (Osipov et al., 2009a). The present study shows improvements in endothelium-dependent vasorelaxation to ADP in coronary microvessels treated with both doses of TP508 dimer. The improved microvascular responses to substance P were seen only in TP508 monomer treated animals. On the other hand, only the lower dose of the TP508 dimer resulted in improvements in endothelium-independent vasorelaxation to SNP. These results indicate that dimerized TP508 may protect coronary microvasculature against ischemia reperfusion injury. As previously demonstrated, the improvements in coronary
microvascular relaxation may have significant clinical relevance. It has been suggested that up to 40% of patients may fail to regain appropriate myocardial perfusion despite achieving Thrombolysis in Myocardial Infarction (TIMI) grade 3 epicardial flow after intervention. This might be attributed partly to coronary microvascular dysfunction (Ito et al., 1992; Prasad and Gerch, 2005; Prasad et al., 2004).

Finally, we speculate that the TP508 monomer and dimer exist mostly as their own form respectively based on the in vitro findings and suggest that both TP508 monomer and dimer may have each dose-dependent effect. It has been reported that the myocardial protective effect caused by an agent may behave in a dose dependent manner (Knight et al., 2001; Niemann et al., 2002; Zuo et al., 2009). Our previous study demonstrated TP508 monomer may have a positive effect in a dose dependent manner within a certain range of administration. In the present study, we performed the additional experiments with administration of very low (1/10 times) and very high (4 times) doses of TP508 monomer to verify the dose dependent response in the more extensive ranges. We found a certain trend of dose dependent effect on myocardial protection of TP508 monomer (including the published group) (Osipov et al., 2009a) [Figure 6], suggesting that there is a certain point in which TP508 monomer will be most effective and that excessive dose may not lead to an equivalent benefit. Recent
studies demonstrate that hydrogen sulfide (Elrod et al., 2007) and gadolinium (Nicolosi et al., 2008) attenuate AMI-RI dose-dependently and the protective effect may not be increased associated with increased doses. In this study, the lower dose of TP508 dimer lead to a greater reduction in the myocardial infarct size than the higher dose. This suggests that TP508 dimer, if it is compared with the equivalent dose of TP508 monomer in mole, might provide protective effect with the lower dose than monomer. It has been suggested that TP508 may be exerting its effects through nonproteolytic interation with one of the known protease-activated receptors (PAR-1, PAR-3, or PAR-4) or through a separate non-protease-activated receptor (NPAR) (Ryaby et al., 2006; Coughlin, 2000). It is still unknown whether chemically synthesized TP508 dimer operates with the thrombin related receptor such as PAR or NPAR in vivo similarly to TP508 monomer. Further investigation on TP508 dimer will be required to detail the mechanism in vivo.

There are still several limitations in this study. Our time course for tissue harvest (3 hours after the onset of ischemia) is not able to account for long-term effects of these drugs on myocardial function and infarct extension, and conversely may miss rapid changes in the activation/phosphorylation status of certain apoptotic signaling proteins. In addition, we did not assess the activity levels of these enzymes while we
measured the protein concentration of molecules involved with signaling. Finally, the present study doesn’t provide in vivo plasma TP508, though we believe that the in vitro data using pig plasma are sufficient to indicate how the monomer and dimer exist in a blood condition. Finally, the present study measured did not measure the in vivo TP508 monomer and dimer.

In conclusion, therapeutic administration of novel chemically synthesized TP508 dimer, prior to the onset of reperfusion markedly attenuates AMI-RI. This study demonstrates that TP508 dimer also affects apoptotic signaling, limiting apoptosis similar to TP508 monomer. Both TP508 monomer and dimer have a specific dose or range which can provide the most beneficial effect on myocardial protection in the setting of AMI-RI. This study might be a trial worthy of special mention in that dimerization of an existing drug would offer an additional approach to achieve a more stable condition of the drug and to find other beneficial roles for clinical trial.
Acknowledgement

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Footnotes

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Legends for Figures

Figure 1. The sequences of TP508 monomer and dimer.

M: monomer, D: dimer

Figure 2. In vitro analysis of TP508 monomer and dimer. (A) A long time solution stability of TP508 monomer and dimer in saline. A comparison of TP508 monomer and dimer solution stability, providing purity (%) vs time (hours) with TP508 monomer, TP508 monomer + 10 μM EDTA, and TP508 dimer in a 0.625 mg/mL in sterile saline. The result at room temperature (~24 °C). Analyzed by UV-HPLC. The purity value used is determined by HPLC percent area using UV detection and the following formula: “Main Peak Area” is the peak area as integrated by the HPLC software for the analyte of interest, such as TP508 monomer or TP508 dimer. The total area is the area of the analyte and all other impurities or degradation products. The purity goes down in value as degradation peaks appear or grow in size. (B) TP508 dimer formation in dosing solution. TP508 monomer was prepared at two concentrations in sterile saline (0.0625 mg/mL and 2.5 mg/mL). These concentrations were selected because they were the lowest and highest infusion concentrations used experimentally. Over the course of 120 min, dimer formation was observed to increase from a starting value of ~ 0.7% to 1.9% and 1% in the low and high concentration samples respectively. This demonstrates
that dimer formation in the dosing solution was likely to be minimal throughout the infusion period. (C) **TP508 monomer and dimer concentration in pig plasma vs. time.** TP508 monomer was added to pig plasma in vitro at a concentration of 70 μg/mL (30.6 mM). The plasma was maintained at 37 °C for 120 min. Rapid degradation of TP508 monomer into smaller peptide fragments was observed, with only ~2% of the initial monomer concentration remaining after 120 min. Dimer was observed to form from the TP508 monomer, however peak dimer concentration reached only 14%. This indicates that although dimer is formed, it reaches a maximum concentration that is a fraction of the initial monomer concentration. Accounting for total peptide recovery, which included TP508 monomer, TP508 dimer and all peptide fragments resulting from enzymatic degradation demonstrated that more than half the monomer was degraded in the first 15 min. Total recovery dropped over time indicating that some peptide fragments were degraded to the point that they were no longer captured by the HPLC assay, or that they were bound to plasma proteins. (D) **TP508 dimer concentration in pig plasma vs. time.** TP508 dimer was added to pig plasma at a concentration of 26.6 mmol. The plasma was maintained at 37 °C for 120 min. Dimer concentration was observed to drop over time, however less rapidly than was seen for TP508 monomer. Monomer was not detected. This may indicate that the dimer is also degraded in pig
plasma, however, it is not reduced to the monomer.

**Figure 3. Global and regional myocardial function.** (A) Mean arterial blood pressure (MAP). (B) Developed LV pressure (LVP). (C) Heart rate (HR). (D) Left ventricular (LV) systolic function as determined by +LV dP/dt. No significant differences were observed among groups (MAP: \( p = 0.93 \), HR: \( p = 0.61 \), LV dP/dt: \( p = 0.51 \) and developed LVP: \( p = 0.94 \); two-way repeated measures ANOVA).

Pre: pre-occlusion, O1: 30 min after occlusion, O2: 60 min after occlusion, R1: 30 min after reperfusion, R2: 60 min after reperfusion, R3: 90 min after reperfusion, R4: 120 min after reperfusion.

**Figure 4. Regional myocardial function in the area-at-risk.** The percentage of the segmental shortening (%SS) in horizontal axes (A) and in longitudinal axes (B). %SS for horizontal axis was significantly improved in D group \( (p = 0.04) \), whereas %SS for longitudinal axis did not show the differences among groups \( (p = 0.51) \) (*: \( p < 0.05 \); two-way repeated measures ANOVA).

Pre: pre-occlusion, O1: 30 min after occlusion, O2: 60 min after occlusion, R1: 30 min after reperfusion, R2: 60 min after reperfusion, R3: 90 min after reperfusion, R4: 120 min after reperfusion.

**Figure 5. Myocardial ischemic area at risk (AAR) and infarct size.** (A) AAR as a
percentage of total LV mass ($p = 0.59$). (B) Infarct size as a percentage of AAR ($p < 0.01$). (C-F) Representative images of the myocardium after triphenyl tetrazolium chloride (TTC) staining are shown in the vehicle (C), M group (D), D group (E) and DD group (F). The picture represents the forth slices cut into 1 cm thick slices perpendicular to the axis of the left anterior descending artery after TTC staining. Three zones can be differentiated: non-ischemic area (dark red), area at risk (AAR; bright red) and necrotic area (pale). The size of the infarct area expressed as a percentage of total LV mass was significantly decreased in M, D and DD groups versus V ($p < 0.01$, *: $p < 0.05$; one-way ANOVA).

**Figure 6. Dose-dependent effect of TP508 monomer and dimer on the reduction of myocardial infarct area.** Myocardial infarct sizes as a percentage of AAR for various TP508 monomer doses (upper) and the present dimer series (bottom). M-1/10, M-1, M-2 (M-2 is published data, not presented in this study) and M-4 means 0.0216 µmol/kg, 0.216 µmol/kg, 0.432 µmol/kg and 0.864 µmol/kg represented as a bolus dose, respectively. M-1, M-2, M-4, D and DD are statistically lower than vehicle ($p < 0.05$) (*:$p < 0.05$; one-way ANOVA). In the monomer series, there is the most effective dose around M-2, whereas the negative dose response effect can be observed in the dimer series.
**Figure 7.** Coronary microvascular reactivity. Responses are shown to endothelium-dependent agents, adenosine diphosphate (ADP) (A) and substance P (B). Response to endothelium-independent agents sodium nitroprusside (SNP) is shown in C. ADP was improved in D and DD ($p < 0.01$) and to substance P was improved in M ($p = 0.03$), whereas to SNP (C) was improved in D among groups ($p = 0.04$) (*: $p < 0.05$; two-way repeated measures ANOVA).

**Figure 8.** Protein levels in the AAR. Western blotting of AAR tissue for total and phospho (Ser70) Bcl-2 (A,B), total and cleaved poly-ADP ribose polymerase (PARP) (C,D). Representative western blots and ponceau staining for gels are shown. Values are mean ± SEM in arbitrary densitometry units and compare vehicle (n = 6), TP508 monomer (M-1, n = 6) and 2 doses dimer (D and DD, n = 6 respectively) groups. (*: $p < 0.05$; one-way ANOVA). Phospho Bcl-2 (Ser70) showed significantly higher in M-1, D and DD vs. V ($p < 0.01$), whereas total Bcl-2 showed no significant differences among groups ($p = 0.21$). Cleaved PARP ($p = 0.02$) was significantly lower in M-1, D and DD vs. V, whereas total PARP ($p = 0.52$) was not significantly different among groups.

V: vehicle, M-1: standard dose TP508 monomer, D: lower dose TP508 dimer, DD: higher dose TP508 dimer. P: ponceau staining

**Figure 9.** Protein levels in the AAR. Western blotting of AAR tissue for total and
cleaved caspase 3 (A,B), HSP27 (C) and αB-crystallin (D). HSP 27 ($p < 0.01$) and αB-crystallin ($p < 0.01$) showed significantly higher expression in M-1, D and DD vs. V ($*:p < 0.05$; one-way ANOVA).

V: vehicle, M-1: standard dose TP508 monomer, D: lower dose TP508 dimer, DD: higher dose TP508 dimer.

**Figure 10. TUNEL staining.** Terminal deoxynucleotidyl transferase dUTP-mediated nick-end labeling (TUNEL) positive cells counts per 100 µm$^2$ in control (A), M group (B), D group (C) and DD group (D) groups (4 animals per group). Panels display representative histologic images by x200 magnification. Brown nuclei are the TUNEL positive cells. M, D and DD vs. V ($p < 0.01$) ($*:p < 0.01$; one-way ANOVA).

**Figure 11. Summary diagram of TP508 monomer and dimer myocardial protective properties in the AMI-RI.** HSP = heat shock protein, PARP = poly-ADP ribose polymerase, Bcl-2 = B cell lymphoma 2.
**Table 1. List of the dosage used in the surgical protocol**

<table>
<thead>
<tr>
<th>Drug</th>
<th>Bolus</th>
<th>Infusion</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>µmol/kg (mg/kg)</td>
<td>µmol/kg/h (mg/kg/h)</td>
</tr>
<tr>
<td>M-1</td>
<td>Monomer 0.21 (0.5)</td>
<td>0.54 (1.25)</td>
</tr>
<tr>
<td>M-1/10</td>
<td>Monomer 0.021 (0.05)</td>
<td>0.054 (0.125)</td>
</tr>
<tr>
<td>M-2*</td>
<td>Monomer 0.43 (1.0)</td>
<td>3.1 (2.5)</td>
</tr>
<tr>
<td>M-4</td>
<td>Monomer 0.86 (2.0)</td>
<td>6.2 (5.0)</td>
</tr>
<tr>
<td>D</td>
<td>Dimer 0.11 (0.5)</td>
<td>0.27 (1.25)</td>
</tr>
<tr>
<td>DD</td>
<td>Dimer 0.21 (1.0)</td>
<td>0.54 (2.5)</td>
</tr>
</tbody>
</table>

Molecular weight: monomer = 2311.49, dimer = 4621.0.

*: M-2 is not presented in the method of the present study (M-2 is already published group) (Osipov et al., 2009a).
**Table 2. Global and Regional myocardial function**

<table>
<thead>
<tr>
<th></th>
<th>M-1/10 (n = 6)</th>
<th>M-4 (n = 6)</th>
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<tr>
<td>MAP (mmHg)</td>
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<tr>
<td>pre</td>
<td>78.5±4.8</td>
<td>75.5±4.9</td>
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<tr>
<td>R4</td>
<td>66.6±4.6</td>
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<tr>
<td>HR (bpm)</td>
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<tr>
<td>pre</td>
<td>117.2±8.2</td>
<td>110.3±10.3</td>
<td>NS</td>
</tr>
<tr>
<td>R4</td>
<td>102.1±3.6</td>
<td>96.9±6.1</td>
<td>NS</td>
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<tr>
<td>LV dP/dt</td>
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<tr>
<td>pre</td>
<td>1651±124</td>
<td>1579±105</td>
<td>NS</td>
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<tr>
<td>R4</td>
<td>1438±132</td>
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<tr>
<td>dev LVP</td>
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<td>pre</td>
<td>87.1±5.4</td>
<td>82.5±3.9</td>
<td>NS</td>
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<tr>
<td>R4</td>
<td>72.5±4.9</td>
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<td>%SS (H)</td>
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<td>pre</td>
<td>17.5±1.8</td>
<td>18.0±2.2</td>
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<tr>
<td>R4</td>
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<td>11.0±3.2</td>
<td>NS</td>
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<tr>
<td>%SS (L)</td>
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<tr>
<td>pre</td>
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<tr>
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<td>7.0±1.3</td>
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<td>NS</td>
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</table>

Values are mean ± SE, n = 6 per group. (NS: p > 0.05; two-way repeated measures ANOVA). BW = body weight. MAP = mean arterial pressure. HR = heart rate.

bpm = beat per minute. dev LVP = developed left ventricular pressure.
%SS = percentage of segmental shortening. H = horizontal axis. L = longitudinal axis.

pre: pre-occlusion. R4: 120 min after reperfusion. NS: not significant.
The dimer is two TP508 monomer molecules connected by a bond between the Cys amino acids.


D

Figure 2

A

Purity (%) vs. Time (hours)
- TP508
- TP508 + EDTA
- Dimer

B

Percentage of Dimer (%) vs. Time (hours)
- 0.0625 mg/mL Dosing Solution
- 2.5 mg/mL Dosing Solution

C

Concentration (mM) vs. Time (minutes)
- TP508 Monomer
- TP508 Dimer Formation

D

TP508 Dimer Concentration (mM) vs. Time (minutes)
Figure 4
Figure 7

A

% Relaxation vs. Log M [ADP]

B

% Relaxation vs. Log M [Substance P]

C

% Relaxation vs. Log M [SNP]
Figure 8

A

B

C

D

V | M-1 | D | DD

28kDa

phospho-Bcl2 (Ser70)

Densitometry Units

V | M-1 | D | DD

3.0

2.0

1.0

0.0

total bcl2

V | M-1 | D | DD

89kDa

V | M-1 | D | DD

116kDa

cleaved-PARP

V | M-1 | D | DD

total PARP
Figure 11

- Improved microvascular reactivity
- Cytoprotective effect (α B-crystalline, HSP27)
- Anti-apoptotic effect (PARP, phospho Bcl-2 (Ser70))

DIAGRAM:
- TP508 monomer
- TP508 dimer
- 0 min
- 60 min
- 120 min
- 180 min
- Ischemia
- Reperfusion

Less apoptosis
Less necrosis