JPET Fast Forward. Published on June 6, 2006 as DOI: 10.1124/jpet.106.104455
This article has not been copyedited and formatted. The final version may differ from this version.
JPET Fast Forward. Published on June 6, 2006 as DOI:10.1124/jpet.106.104455

Chronic Matrix Metalloproteinase Inhibition Following Myocardial Infarction in Mice: Differential Effects on Short and Long Term Survival

Francis G. Spinale, G. Patricia Escobar, Jennifer W. Hendrick, Leslie L. Clark, Sarah S. Camens, Joseph P. Mingoia, Christina G. Squires, Robert E. Stroud, John S. Ikonomidis

Division of Cardiothoracic Surgery
Medical University of South Carolina, Charleston, South Carolina, and
The Ralph H. Johnson Veteran's Affairs Medical Center

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 19, 2024

Copyright 2006 by the American Society for Pharmacology and Experimental Therapeutics.

JPET Fast Forward. Published on June 6, 2006 as DOI: 10.1124/jpet.106.104455
This article has not been copyedited and formatted. The final version may differ from this version.

Running Title: Survival and MMP Inhibition

Corresponding author:
Francis G. Spinale
Cardiothoracic Surgery
Medical University of South Carolina
114 Doughty Street, Suite 625
Charleston, South Carolina 29425, USA
843-876-5186 (PH)
843-876-5187 (FAX)
Email: wilburnm@musc.edu

Number of text pages: 25
Number of tables: 0
Number of figures: 5
Number of references: 36
Number of words in the abstract: 225
Number of words in the introduction: 506
Number of words in the discussion: 1,728

LV: left ventricle
MI: myocardial infarction
MMP: matrix metalloproteinase
MMPi: MMP pharmacological inhibition
TIMP: tissue inhibitor of matrix metalloproteinase

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 19, 2024

JPET Fast Forward. Published on June 6, 2006 as DOI: 10.1124/jpet.106.104455
This article has not been copyedited and formatted. The final version may differ from this version.

ABSTRACT

Left ventricular (LV) remodeling occurs following myocardial infarction (MI) and the matrix metalloproteinases (MMPs) contribute to adverse LV remodeling following MI. Short term pharmacological MMP inhibition (MMPi;days to weeks) in animal models of MI have demonstrated a reduction in adverse LV remodeling. However, the long term effects (months) of MMPi on survival and LV remodeling following MI have not been examined. MI was induced in adult mice (n=131) and at 3 days post-MI assigned to MMPi (MI-MMPi:PD200126, 7.5 mg/day/PO, n=64) or untreated (MI only, n=67). Unoperated mice (n=16) served as controls. The median survival in the MI only group was 5 days whereas median survival was significantly greater in the MI-MMPi group at 38 days ($p<0.05$). However, with prolonged MMPi (>120 days), a significant divergence in the survival curves occurred in which significantly greater mortality was observed with prolonged MMPi ($p<0.05$). LV echocardiography at 6 months revealed LV dilation in the MI-only and MI-MMPi groups (154$\pm$14, 219$\pm$24 µL) compared to control (67 $\pm$ 4 µL, $p<0.05$) with a greater degree of dilation in the MI-MMPi group ($p<0.05$). MMPi conferred a beneficial effect on survival early post-MI, but prolonged MMPi (>3 months) was associated with higher mortality and adverse LV remodeling. These unique results suggest that an optimal temporal window exists with respect to pharmacological interruption of MMP activity in the post-MI period.

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 19, 2024

JPET Fast Forward. Published on June 6, 2006 as DOI: 10.1124/jpet.106.104455
This article has not been copyedited and formatted. The final version may differ from this version.

INTRODUCTION

Myocardial infarction (MI) evokes changes within the architecture of the left ventricular (LV) wall leading to chamber dilation. This process, which is termed post-MI remodeling, has been shown to be an independent predictor of morbidity and mortality in several large clinical trials. Thus, identifying and interrupting cellular and molecular pathways which contribute to LV structural remodeling post-MI hold significant clinical and scientific interest. While the LV remodeling process evokes changes within both the cellular and extracellular compartment, recent studies have demonstrated that changes in extracellular structure and composition occur within the MI region as well as surviving myocardium.(Frangogiannis et al., 2002; Schellings et al., 2004; Thompson and Squire, 2002; Wainwright, 2004) Specifically, the induction and activation of a family of matrix proteases, termed the matrix metalloproteinases (MMPs) have been demonstrated to occur in patients and animal models of myocardial injury and remodeling.(Bradham et al., 2002; Creemers et al., 2001; Kaden et al., 2003; Nian et al., 2004; Mukherjee et al., 2003; Tziakas et al., 2004; Wainwright, 2004; Yarbrough et al., 2003) The MMPs are a large family of proteolytic enzymes and can degrade a number of matrix proteins, process biologically active signaling molecules and growth factors, and degrade a number of transmembrane proteins.(Brinckerhoff and Matrisian, 2002; Coussens et al., 2002; McCawley and Matrisian, 2001; McDonnell et al., 1999; Overall et al., 2004; Steffensen et al., 2001; Tsuruda et al., 2004; Woessner, 1998) Utilizing murine transgenic constructs, genetic deletion of a specific MMP type can favorably modify the post-MI remodeling process whereas deletion of endogenous inhibitors of MMPs (TIMPs) can accelerate adverse LV remodeling.(Creemers et al., 2000; Ducharme et al., 2000; Ikonomidis et al., 2005; Matsumura et al., 2005) In other studies, it has been demonstrated that pharmacological strategies which provide for inhibition of active MMPs within the myocardium, can significantly attenuate the post-MI remodeling process.(Creemers et al., 2001; Ikonomidis et al., 2005; Mukherjee et al., 2003; Yarbrough et al., 2003) Thus, a cause-effect relationship between induction of MMP activity and LV remodeling post-MI has emerged. While the MMP gene gain/loss of function studies will provide important mechanistic insight into the regulation and activity of myocardial

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 19, 2024

JPET Fast Forward. Published on June 6, 2006 as DOI: 10.1124/jpet.106.104455
This article has not been copyedited and formatted. The final version may differ from this version.

MMPs in the remodeling process, it is likely that a pharmacological MMP inhibition will be the approach to be considered in clinical application. However, several outstanding issues regarding the utility of pharmacological MMP inhibition exist. First, initial clinical studies demonstrated adverse systemic side effects with prolonged MMP inhibition. (Bloomston et al., 2002; Coussens et al., 2002; Overall and Lopez-Otin, 2002; Vihinen and Kahari, 2002) Therefore, the duration of treatment with MMP inhibition following MI must be considered. Second, the effects of long term MMP inhibition on LV myocardial structure and function post-MI have not been examined. Finally, while past studies have demonstrated a favorable effect of MMP inhibition on LV structure and function in animal models of MI, these have been of short duration.(Creemers et al., 2000; Creemers et al., 2001; Ikonomidis et al., 2005; Mukherjee et al., 2003; Yarbrough et al., 2003) Thus, the direct effects of MMP inhibition on survival following MI induction remains to be established. The present study was designed to address these issues by surgically inducing an MI in a large sample of mice, and performing a long term survival study (6 months) following treatment with a continuous MMP inhibition post-MI. LV structure and function were examined in the surviving mice in order to assess the effects of prolonged MMP inhibition in this murine post-MI model.

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 19, 2024

JPET Fast Forward. Published on June 6, 2006 as DOI: 10.1124/jpet.106.104455
This article has not been copyedited and formatted. The final version may differ from this version.

METHODS

Animal Model and Surgical Induction of MI

The mice used in these studies were adult male inbred 129 Sv mice of 10-12 weeks of age. Only male mice were utilized in this study in order to avoid the potential confounding effect of gender on MI survival. Under isoflurane anesthesia (3% in oxygen), mice were placed in supine position and the trachea intubated with a 1.1mm steel intubation tube. The mice were then placed on a rodent ventilator and ventilated at a tidal volume of 1ml and 200 cycles/minute. Using sterile technique, a left thoracotomy was performed in the fourth intercostal space. After opening the pericardium, the left coronary artery was ligated near its origin using 6-0 prolene and an atraumatic needle (Ethicon, K801). The incisions were then closed. After extubation, the mice were given buprenorphine (2-2.5 mg/kg IP), placed on oxygen by mask and a warming blanket. At 3 days post-operatively, a transthoracic echocardiogram was obtained in order to confirm the presence of an MI by clear defects in LV posterior wall motion. For these studies, the mice were anesthetized with isoflurane (1.5-2% in oxygen) and maintained at ambient body temperature with a heating blanket. Heart rate was determined from a surface electrocardiogram and our results have confirmed that this regimen maintains an ambient heart rate of 400-500 bpm and the mice remain normothermic throughout the procedure. Two-dimensional targeted M-mode echocardiographic recordings were obtained using a high band linear 15.7 mHz transducer (Sonos 5500, Hewlett Packard/Agilent Tech, MA). From these post operative screening echocardiographic studies, any mice that did not display a clear LV wall motion abnormality were excluded from the study. The exclusion rate was 5%. All mice were then treated to the experimental protocol described below. All animals were treated and cared for in accordance with the National Institutes of Health "Guide for the Care and Use of Laboratory Animals" (National Research Council, Washington, 1996).

Experimental Design

At 3 days post-MI, 131 mice were assigned to undergo MMP inhibition using a broad spectrum MMP inhibitor (MI-MMPi:PD200126: 7.5 mg/day/PO - (formerly PD166793,

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 19, 2024

Parke-Davis, MI) Pfizer, CT) or to remain untreated. Oral administration of this MMP inhibitor in animal models of LV remodeling have been described and characterized previously.(Ikonomidis et al., 2005; Mukherjee et al., 2003; Peterson et al., 2001; Spinale et al.,1999) The mice were coded by ear tag, and assigned in an alternating fashion to the MMP inhibitor or untreated groups. The treatment assignments and codes were not broken until the completion of the study. The dose of MMP inhibitor used in this study achieved a steady state plasma level of 8.5$\pm$ 2.2 mg/mL which has been demonstrated previously in *ex-vivo* studies to achieve a significant broad spectrum MMP inhibitory effect and has been used in rodents to achieve MMP inhibition.(Ikonomidis et al., 2005; Peterson et al., 2001) While this compound effectively inhibits MMP activity, it does not affect other metalloproteases such as angiotensin converting enzyme, neutral endopeptidases, or tumor necrosis factor alpha converting enzyme.(Mukherjee et al., 2003; Peterson et al., 2001; Spinale et al., 1999) With respect to MMP inhibitory specificity, the inhibitory potency of PD166793 for the MMP catalytic domain (effective inhibitory concentration; EC_{50}) ranges from 7.9 μmol/L for MMP-9 to 0.008 μmol/L for MMP-13.(Peterson, 2001; Spinale, 1999) The plasma levels achieved in the present study significantly exceeded the EC_{50} for all of the major MMP types by approximately 10-fold. In a past study, it has been demonstrated that this MMP inhibitor has a high myocardial penetrance and significantly inhibited myocardial MMP activity.(Mukherjee, 2003; Spinale, 1999) The rationale for initiating MMP inhibition at 3 days post-MI was 2-fold. First, this allowed for screening and confirmation of an MI in this murine model prior to treatment assignment. Second, the intent of this study was to examine post-MI remodeling and not to interfere with the acute phase of the myocardial wound healing process. The mice were examined in the morning and afternoon of each day. The duration of this study was 6 months.

LV Function, Histomorphometric and Biochemical Measurements at 6 Months

Terminal studies were performed in all of the surviving mice at 6 months. A cohort of age matched male mice (n=16) was included in these studies in order to serve as reference controls. LV function was first assessed by anesthetizing the mice as

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 19, 2024

JPET Fast Forward. Published on June 6, 2006 as DOI: 10.1124/jpet.106.104455
This article has not been copyedited and formatted. The final version may differ from this version.

described in the previous section and 2-dimensional echocardiography performed in order to obtain LV end-diastolic volumes (μL) and ejection fraction (%) using conventional methods. (Collins et al., 2003) Following the echocardiographic study, and under 5% isoflurane, a sternotomy was performed and 0.5 mL of 0.1 mM cadmium chloride was injected into the LV in order to achieve mechanical arrest in diastole. The heart was quickly removed, placed in iced saline and the LV trimmed away and weighed. The LV was then divided into 2 equivalent sections along the long axis with transected the MI region. One LV section was then fixed in a 4% formalin solution overnight, embedded in paraffin and used for histomorphometrics. The second LV section was rapidly frozen in a dry ice slurry and maintained at –70degC until used for biochemical analysis.

For the histomorphometry studies, sections (5 μm) were stained with hematoxylin and eosin for measurement of MI size and myocyte cross-sectional area using computer assisted methods described previously.(Creemers et al., 2000; Ikonomidis et al., 2005; Spinale et al., 1999) Briefly, MI size was based upon performing computer based planimetry (Sigma Scan, Media Cybernetics) on the entire endocardial and epicardial borders of the LV section and demarcating the MI region. The MI size was expressed as a percent of the total LV area.(Ikonomidis, 2005) For myocyte cross-sectional area, the myocyte profiles were digitized using a final magnification of 60X and a minimum of 100 profiles were measured from each LV section.(Spinale, 1999) Additional LV sections were stained with picro-sirius red for fibrillar collagen and the percent area of collagen within the remote and MI regions of the LV was computed.(Ikonomidis et al., 2005; Mukherjee et al., 2003; Spinale et al., 1999; Yarbrough et al., 2003)

In order to examine whether relative changes in MMP or TIMP profiles occurred in the treated and untreated MI groups at 6 months, substrate zymography was performed in order to assess the relative content of the gelatinases, MMP-2 and MMP-9.(Chapman 2003 et al.; Heymans et al., 1999; Peterson et al., 2001) Immunoblotting was performed for MMP-13 as well as for TIMP-1and -4 using methods described previously.(Ikonomidis et al., 2005; Mukherjee et al., 2003; Peterson et al., 2001;

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 19, 2024

JPET Fast Forward. Published on June 6, 2006 as DOI: 10.1124/jpet.106.104455
This article has not been copyedited and formatted. The final version may differ from this version.

Yarbrough et al., 2003) The previously frozen full thickness LV myocardial sections were homogenized using ice-cold MMP extraction buffer (10mM cacodylic acid, 150mM NaCl, 0.01mM $ZnCl_2$, 20mM $CaCl_2$, 2mM NaN_3, and 0.1% Triton-X-100, pH 5.0). For zymography, the myocardial homogenates (10 ug total protein) were subjected to electrophoretic separation containing a denatured collagen substrate (1 mg/mL type III gelatin, Sigma, St. Louis). For immunoblotting, myocardial extracts (10 µg) were loaded onto 4-12% BisTris gels and subjected to electrophoretic separation. The separated proteins were then transferred to a nitrocellulose membrane. Following a blocking and washing step, the membranes were incubated for 1 hour in anti-sera (1:5000 dilution) corresponding to MMP-13 (AB8114, Chemicon, Temecula, CA); TIMP-1(RP2T1, Triple Point Biologics, OR) or TIMP-4 (AB816, Chemicon, Temecula, CA). The membranes were then washed and incubated with a secondary antibody (1:5000, Vector Laboratories, Burlingame, CA) conjugated with horseradish peroxidase. Signals were detected by chemiluminescence (Western Lightning, Perkin Elmer, Boston, MA). The zymograms and immunoblots were digitized, and analyzed (Gel Pro Analyzer, Media Cybernetics, Silver Spring MD). Recombinant standards (Chemicon) were included in all zymograms and immunoblots as positive controls and to standardize the digital analysis.

Data Analysis

For the survival portion of the study, survival curves were constructed utilizing Kaplan-Meier probability estimates. The median survival time, that is the time at which 50% of the sample group died, was compared between the 2 groups utilizing a Chi-Square analysis. In addition, comparisons of survival were compared utilizing a stratified log rank test. LV function and geometry were compared between the 2 groups utilizing a t-test. For the morphometric data, the measurements of cross-sectional area and collagen area were first confirmed to conform to a Gaussian distribution, subjected to analysis of variance and finally to Tukey's test for mean separation. For the zymography and immunoblotting studies, all measurements were performed in duplicate, and the zymographic/immunoreactive signals were analyzed using densitometric methods (Gel Pro Analyzer, Media Cybernetics) to obtain 2-dimensional

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 19, 2024

JPET Fast Forward. Published on June 6, 2006 as DOI: 10.1124/jpet.106.104455
This article has not been copyedited and formatted. The final version may differ from this version.

integrated optical density (IOD) values. The IOD values were then computed as a percent of non-MI control values where the control values were set to 100% and comparisons performed by a separate t-test. Results are presented as mean ± SEM. Values of $p<0.05$ were considered statistically significant. All statistical procedures were performed using the STATA statistical software package (Statacorp, College Station, TX).

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 19, 2024

JPET Fast Forward. Published on June 6, 2006 as DOI: 10.1124/jpet.106.104455
This article has not been copyedited and formatted. The final version may differ from this version.

RESULTS

The constructed Kaplan-Meier post-MI survival curves for the 6 month observation period is shown in Figure 1. The initiating time point (time 0) for was at the point of treatment assignment. Significant mortality occurred in the untreated, MI only group early in the follow-up period with a median survival of 5 days. However, median survival was 7-fold longer in the MI-MMPi group (Chi Square statistic:4.27, $p<0.05$). In the mice that survived for a longer duration, a much different effect was observed. Specifically, the MI-MMPi survival curves crossed at approximately 45 days, and by 120 days the MMPi group demonstrated a significantly greater mortality compared to MI only (Chi Square statistic:9.71, $p<0.05$). Post-mortem analysis revealed that approximately 15% of the deaths were due to myocardial rupture at the LV apical region, 60% were due to occult cardiac decompensation as evidenced by significant serous fluid accumulation within the thoracic space, and 25% revealed no significant transudate or serosanginuous fluid in the thoracic space and therefore the deaths were presumed to be of a arrhythmic origin. There were no differences in these post-mortem findings between treatment groups. Thus, an improvement in early post-MI survival was observed in the MMPi group, but with longer treatment durations a worsening survival occurred.

LV function and geometry by echocardiography were assessed in the mice surviving to 6 months in the MI only group (n=27) and the MI-MMPi group (n=17) and were compared to an age matched reference control group (n=16). Representative LV echocardiograms are shown in Figure 2. LV end-diastolic volume increased in the MI only and MI-MMPi groups (154 ± 14, 219 ± 24 µL) compared to control (67 ± 4 µL, $p<0.05$), with a greater degree of dilation in the MI-MMPi group ($p<0.05$). LV ejection fraction was reduced to the same degree in the MI-MMPi and MI only groups (30 ± 3 vs 30 ± 3 %) compared to control (61 ± 1 %, $p<0.05$).

Representative longitudinal LV sections from reference control, MI only and MI-MMPi at the 6 month treatment interval are shown in Figure 2. The remote, viable myocardium and the region encompassing the MI could be clearly differentiated.

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 19, 2024

JPET Fast Forward. Published on June 6, 2006 as DOI: 10.1124/jpet.106.104455
This article has not been copyedited and formatted. The final version may differ from this version.

Computed MI size was equivalent between the MI only and MMPi groups (Figure 3). Relative collagen content was over 90% in the MI region of both the MI only and MI-MMPi groups. Representative photomicrographs of complete LV sections stained for fibrillar collagen are shown in Figure 4. Within the remote, viable myocardium, relative collagen content was reduced from control values within the MI only group, but this did not reach statistical significance ($p=0.25$). However, relative collagen content within the viable, remote region was increased from both control and MI only values in the MI-MMPi group (Figure 3). Myocyte cross-sectional area within the viable myocardium was increased from control values in both MI groups (Figure 3), but was reduced from MI only values in the MI-MMPi group. LV mass increased in both the MI only and MI-MMPi groups (172 ± 6 and 191 ± 14 mg, respectively) compared to control (139 ± 6 mg, $p<0.05$).

Representative MMP/TIMP zymograms and immunoblots are shown in Figure 5. Relative MMP-2(72 kDa band) and MMP-9 (92 kDa band) levels were determined from zymography and MMP-13, TIMP-1 and TIMP-4 determined from immunoblotting. Myocardial MMP-2 levels appeared increased in the MI only group from reference controls, but did not reach statistical significance ($131 \pm 16\%$, $p=0.30$). MMP-2 levels increased from control and MI only values in the MI-MMPi group ($198 \pm 17\%$,$p<0.05$). Relative MMP-9 levels increased in a similar direction in the MI only and MI-MMPi group compared to reference controls ($684 \pm 139\%$ and $639 \pm 115\%$ respectively, $p<0.05$). In contrast, MMP-13 levels concordantly decreased in both the MI and MI-MMPi groups from reference control values ($65 \pm 17\%$ and $61 \pm 11\%$, $p<0.05$). TIMP-1 levels were similar to reference controls in the MI and MI-MMPi groups ($123 \pm 32\%$ and $148 \pm 50\%$, $p>0.40$). TIMP-4 levels were unchanged from reference control values in the MI only and MI-MMPi groups ($94 \pm 18\%$ and $96 \pm 22\%$, $p>0.70$).

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 19, 2024

JPET Fast Forward. Published on June 6, 2006 as DOI: 10.1124/jpet.106.104455
This article has not been copyedited and formatted. The final version may differ from this version.

DISCUSSION

The unique findings of the present study were 2-fold. First, MMP inhibition imparted an early survival benefit in mice post-MI, but with prolonged treatment (~3 months) a worsening survival was observed. Second, chronic MMP inhibition resulted in a greater degree of LV dilation, hypertrophy of surviving myocytes, and increased collagen content within the remote myocardium. Taken together, these results as well as results from past reports (Creemers et al. , 2001; Ducharme et al. , 2000; Heymans et al. , 1999; Ikonomidis et al., 2005; Matsumura et al., 2005; Mukherjee et al., 2003; Yarbrough et al., 2003) would suggest that MMP inhibition early post-MI can provide beneficial effects on LV remodeling and survival, but prolonged broad-spectrum MMP inhibition is associated with adverse effects on LV remodeling and survival. Thus, a specific temporal window for therapeutic intervention likely exists with respect to regulating MMP activity in the post-MI period.

A number of past studies have examined the effects of MMP inhibition in animal models of post-MI remodeling.(Creemers et al., 2001; Ikonomidis et al., 2005; Matsumura et al., 2005; Mukherjee et al., 2003; Yarbrough et al., 2003) These studies have been focused upon the early post-MI period (up to several weeks post-MI) and have uniformly documented a reduction in the degree of LV dilation. Moreover, these past studies have demonstrated that the relative reduction in the adverse remodeling process which was achieved by MMP inhibition was not due to differences in the degree of initial injury (ie infarct size), but rather due to modifying the proteolytic processes which occurred following MI. The present study was designed to address several potential confounding factors that would independently have influenced remodeling and survival in the post MI period. First, mice underwent MMP inhibition following confirmation of an equivalent wall motion abnormality post MI. Our past studies have documented a relatively uniform early MI size is achieved through the surgical procedure employed in the present study (Creemers et al., 2003; Ikonomidis, et al., 2005). MI sizes were identical in the surviving mice assigned to the 2 groups, thus it is unlikely that differences in MI size significantly influenced the findings with respect to survival and LV remodeling. Second, only male mice were utilized in the

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 19, 2024

JPET Fast Forward. Published on June 6, 2006 as DOI: 10.1124/jpet.106.104455
This article has not been copyedited and formatted. The final version may differ from this version.

present study, since gender can influence LV function and remodeling in the post-MI period in mice.(Cavasin et al., 2004) Third, MMP inhibition was instituted at 3 days post-MI. This time point was chosen in order to avoid interference with the initial wound healing response that may have occurred with early MMP inhibition.(Creemers et al., 2000; Frangogiannis et al., 2002; Nian et al., 2004; Steffensen et al., 2001; Thompson and Squire, 2002)

The present study is the first to demonstrate that a time dependent effect on modifying myocardial MMP activity exists within the post-MI period with respect to survival and LV remodeling. However, there have been a number of past studies which have examined the short term effects of modifying MMP induction/activity in the early post-MI period.(Creemers et al., 2000; Creemers et al., 2001; Creemers et al., 2003; Ducharme et al., 2000; Ikonomidis et al., 2005; Matsumura et al., 2005; Mukherjee et al., 2003; Yarbrough et al., 2003) For example, deletion of either the MMP-9 or MMP-2 gene in mice reduced the relative incidence of myocardial rupture and LV dilation in the first 2 weeks following MI.(Ducharme et al., 2000; Heymans et al., 1999; Matsumura et al., 2005) We have reported previously that TIMP-1 gene deletion, which would reduce the relative degree of endogenous MMP inhibition, was associated with an acceleration of adverse LV remodeling within the first 14 days post-MI(Creemers et al., 2003; Ikonomidis et al., 2005) Using the same MMP inhibitor employed in the present study, this laboratory has demonstrated previously that broad spectrum MMP inhibition significantly attenuated the degree of infarct expansion in a porcine model of MI in which the greatest effect was observed within the first 30 day period.(Mukherjee et al., 2003) These past results along with the findings of the present study, would suggest that MMP activation holds the greatest biological significance within the first 30 days post-MI with respect to favorably affecting both LV structure, function and survival.

A clear-cause effect relationship has been established between the early LV dilation which occurs following MI and MMP activation.(Creemers et al., 2001; Heymans et al., 1999; Ikonomidis et al., 2005; Matsumura et al., 2005; Mukherjee et al., 2003; Rohde

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 19, 2024

JPET Fast Forward. Published on June 6, 2006 as DOI: 10.1124/jpet.106.104455
This article has not been copyedited and formatted. The final version may differ from this version.

et al., 1999; Yarbrough et al., 2003) While these initial results provided the rationale for aggressively pursuing the MMP system as a therapeutic target in the post-MI period, the results from the present study provide the first results to suggest that this approach may not necessarily be associated with favorable effects on the LV remodeling process when MMP activity is modified for an extensive period of time post-MI. In the surviving mice that had undergone 6 months of MMP treatment, the relative degree of LV dilation was greater than time matched untreated post-MI mice. This finding was unexpected since short term MMP inhibition studies have uniformly demonstrated a significant attenuation in LV volumes post-MI.(Bloomston et al., 2002; Ikonomidis et al., 2005; Mukherjee et al., 2003; Spinale et al., 1999; Yarbrough et al., 2003) While the mechanisms for this effect remain unclear, it must be recognized that the myocardial remodeling is a multifactorial process and that targeting a single proteolytic pathway will likely be insufficient to completely abrogate post-MI remodeling.(Frangogiannis et al., 2002; Nian et al., 2004; Schellings et al., 2004; Thompson and Squire, 2002; Wainwright, 2004) Additional evidence that prolonged MMP inhibition differentially and adversely affected the LV remodeling process post-MI was that myocyte cross-sectional area was reduced and relative collagen content within the viable myocardial regions was increased. The LV chamber dilation coupled with the reduction in myocyte cross-sectional area would suggest that increased myocyte length occurred with prolonged MMP inhibition. In short term post-MI studies, MMP inhibition was not associated with increased myocardial collagen accumulation.(Ikonomidis et al., 2005; Mukherjee et al., 2003; Peterson et al., 2001) Taken together, these observations imply that prolonged MMP inhibition post-MI failed to maintain the favorable effects on LV remodeling that can be achieved with short term MMP inhibition.

The present study utilized a broad spectrum MMP inhibitor, defined as one that inhibits all major classes of MMPs.(Brinckerhoff and Matrisian, 2002; Coussens et al., 2002; McDonnell et al., 1999; Woessner, 1998) This MMP inhibitor has been characterized previously in rodent and large animal models of LV remodeling and dysfunction.(Mukherjee et al., 2003; Peterson et al., 2001) However, it is unlikely that

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 19, 2024

JPET Fast Forward. Published on June 6, 2006 as DOI: 10.1124/jpet.106.104455
This article has not been copyedited and formatted. The final version may differ from this version.

broad spectrum MMP inhibition will be utilized clinically due to systemic effects associated with prolonged use.(Peterson et al., 2001; Spinale et al., 1999) It is now recognized that a differential profile of MMPs in the post-MI period.(Bloomston et al., 2002; Overall and Lopez-Otin, 2002; Vihinen and Kahari, 2002) Thus, pharmacological strategies that selectively target those MMP types which are induced post-MI and are likely contributory towards adverse LV remodeling would hold biological and clinical significance. Indeed, more selective MMP inhibitors have been employed in post-MI animal models and have attenuated the degree of adverse LV remodeling.(Yarbrough et al, 2003) Thus, whether and to what degree more selective MMP inhibitors would exert the same effects as those observed in the present study warrants investigation. Additional experimental design considerations/limitations of the present study also deserve comment. Sham control (non-MI) mice with and without prolonged MMP inhibition were not included as a study cohort. Thus, whether and to what degree surgical manipulation or drug treatment alone, in the absence of an MI may have influenced long term survival or LV remodeling could not be addressed. In our initial dose determination studies, preliminary results revealed no adverse effects (systemic or cardiac) of MMP inhibition in non-MI mice with up to 6 months of treatment. However, it cannot be ruled out that prolonged MMP inhibition caused systemic toxicity that may have contributed to the differences in long term survival.

In the present study, relative levels of the predominant interstitial collagenase, MMP-13 were reduced in both the untreated and MMP inhibition groups at 6 months post-MI where as MMP-9 levels were increased. The relative reduction in MMP-13 at late post-MI time points would favor collagen accumulation- particularly in the MI region. In the MMP inhibition group, the reduction in MMP-13 coupled by local inhibition of MMP activity in general, would be one mechanism for the increased collagen accumulation in the remote region which was observed in the present study. While expressed by a large number of cell types, MMP-9 is abundantly expressed in inflammatory cells.(McDonnell et al., 1999; Thompson and Squire, 2002; Wainwright, 2004; Woessner, 1998) Thus, the persistent increase in MMP-9 is likely reflective of localized

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 19, 2024

JPET Fast Forward. Published on June 6, 2006 as DOI: 10.1124/jpet.106.104455
This article has not been copyedited and formatted. The final version may differ from this version.

inflammation and wound healing. What was noted in the present study was that chronic MMP inhibition was associated with a relative increase in MMP-2 levels when compared to untreated MI values. In human myocardial fibroblast systems, we have demonstrated previously that prolonged exposure to the MMP inhibitor utilized in the present study could induce a relative increase in MMP-2 abundance.(Chapman et al., 2003) However, the present study did not analyze relative mRNA MMP levels, and therefore whether and to what degree prolonged term MMP inhibition affected MMP levels at the transcriptional level remains to be established. While further studies are warranted, the present findings suggest that prolonged broad spectrum MMP inhibition may interfere with a feedback mechanism which regulates MMP-2 expression and/or synthesis. Nevertheless, the relative increase in MMP-2 myocardial levels which occurred in the MMP inhibitor group would not result in “pharmacological escape” since previously performed pharmacokinetic studies demonstrated that the dose utilized in the present study would still provide significant inhibition of MMP-2.(Peterson et al., 2001; Spinale et al., 1999) Results also demonstrated that relative TIMP levels were unaffected by prolonged MMP inhibition. Thus, the effects of pharmacological MMP inhibition appear to be MMP type specific.

Several clinical studies have demonstrated a distinct temporal profile of plasma levels of MMPs and TIMPs in patients post-MI.(Bradham et al., 2002; Kaden et al., 2003; Tziakas et al., 2004) Basic studies have demonstrate that the myocardial induction of MMPs is both time and type specific in the post-MI period.(Bradham et al., 2002; Mukherjee et al., 2003; Wilson et al., 2003; Yarbrough et al., 2003) Further, recent animal studies have demonstrated the possibility of visualizing MMP activity within the intact cardiovascular system.(Chen et al., 2005; Schafers et al., 2004) The present study provides the first results to suggest that a specific temporal window exists with respect to achieving a beneficial effect of broad spectrum MMP inhibition on survival. Thus, it may be possible to integrate surrogate markers of MMP induction as well as direct imaging methods of MMP activity in order to identify maximal myocardial MMP activity in the post-MI period and thereby deploy pharmacological inhibition in a manner to achieve the greatest potential benefit with respect to the prevention of

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 19, 2024

JPET Fast Forward. Published on June 6, 2006 as DOI: 10.1124/jpet.106.104455
This article has not been copyedited and formatted. The final version may differ from this version.

adverse LV remodeling and increased survival. While this issue remains speculative and requires further investigation, what is clear from the present study is that long term treatment with broad spectrum MMP inhibition in the post-MI period may have deleterious effects on LV remodeling and survival.

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 19, 2024

JPET Fast Forward. Published on June 6, 2006 as DOI: 10.1124/jpet.106.104455
This article has not been copyedited and formatted. The final version may differ from this version.

References

Bloomston M, Zervos EE, Rosemurgy AS 2nd (2002) Matrix metalloproteinases and their role in pancreatic cancer: a review of preclinical studies and clinical trials. *Ann Surg Oncol* 9:668-674.

Bradham WS, Gunasinghe H, Holder JR, Multani M, Killip D, Anderson M, Meyer D, Spencer WH 3rd, Torre-Amione G, Spinale FG (2002) Release of matrix metalloproteinases following alcohol septal ablation in hypertrophic obstructive cardiomyopathy. *J Am Coll Cardiol* 40:2165-2173.

Brinckerhoff CE, Matrisian LM (2002) Matrix metalloproteinases: a tail of a frog that became a prince. *Nat Rev Mol Cell Biol* :207-214.

Cavasin MA, Tao Z, Menon S, Yang XP. (2004)Gender differences in cardiac function during early remodeling after acute myocardial infarction in mice. *Life Sci* 75:2181-2192.

Chapman RE, Scott AA, Deschamps AM, Lowry AS, Stroud RE, Ikonomidis JS, Spinale FG (2003) Matrix metalloproteinase abundance in human myocardial fibroblasts: effects of sustained pharmacologic matrix metalloproteinase inhibition. *J Mol Cell Cardiol* 35:539-548.

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 19, 2024

JPET Fast Forward. Published on June 6, 2006 as DOI: 10.1124/jpet.106.104455
This article has not been copyedited and formatted. The final version may differ from this version.

Chen J, Tung CH, Allport JR, Chen S, Weissleder R, Huang PL. (2005) Near-infrared fluorescent imaging of matrix metalloproteinase activity after myocardial infarction. *Circulation* 111:1800-1805.

Collins KA, Korcarz CE, Lang RM (2003) Use of echocardiography for the phenotypic assessment of genetically altered mice. *Physiol Genomics* 13:227-239.

Coussens LM, Fingleton B, Matrisian LM (2002) Matrix metalloproteinase inhibitors and cancer: trials and tribulations. *Science* 295:2387-2392.

Creemers E, Cleutjens J, Smits J, Heymans S, Moons L, Collen D, Daemen M, Carmeliet P (2000) Disruption of the plasminogen gene in mice abolishes wound healing after myocardial infarction. *Am J Pathol* 156:1865-1873.

Creemers EE, Cleutjens JP, Smits JF, Daemen MJ (2001) Matrix metalloproteinase inhibition after myocardial infarction: a new approach to prevent heart failure? *Circ Res* 89:201-210.

Creemers EE, Davis JN, Parkhurst AM, Leenders P, Dowdy KB, Hapke E, Hauet AM, Escobar PG, Cleutjens JP, Smits JF, Daemen MJ, Zile MR, Spinale FG (2003) Deficiency of TIMP-1 exacerbates LV remodeling after myocardial infarction in mice. *Am J Physiol Heart Circ Physiol* 284:H364-371.

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 19, 2024

JPET Fast Forward. Published on June 6, 2006 as DOI: 10.1124/jpet.106.104455
This article has not been copyedited and formatted. The final version may differ from this version.

Ducharme A, Frantz S, Aikawa M, Rabkin E, Lindsey M, Rohde LE, Schoen FJ, Kelly RA, Werb Z, Libby P, Lee RT (2000) Targeted deletion of matrix metalloproteinase-9 attenuates left ventricular enlargement and collagen accumulation after experimental myocardial infarction. *J Clin Invest* 106:55-62.

Frangogiannis NG, Smith CW, Entman ML (2002) The inflammatory response in myocardial infarction. *Cardiovasc Res* 53:31-47.

Heymans S, Luttun A, Nuyens D, Theilmeier G, Creemers E, Moons L, Dyspersin GD, Cleutjens JP, Shipley M, Angellilo A, Levi M, Nube O, Baker A, Keshet E, Lupu F, Herbert JM, Smits JF, Shapiro SD, Baes M, Borgers M, Collen D, Daemen MJ, Carmeliet P (1999) Inhibition of plasminogen activators or matrix metalloproteinases prevents cardiac rupture but impairs therapeutic angiogenesis and causes cardiac failure. *Nat Med* 5:1135-1142.

Ikonomidis JS, Hendrick JW, Parkhurst AM, Herron AR, Escobar PG, Dowdy KB, Stroud RE, Hapke E, Zile MR, Spinale FG (2005) Accelerated LV remodeling after myocardial infarction in TIMP-1-deficient mice: effects of exogenous MMP inhibition. *Am J Physiol Heart Circ Physiol* 288:H149-158.

Kaden JJ, Dempfle CE, Sueselbeck T, Brueckmann M, Poerner TC, Haghi D, Haase KK, Borggrefe M (2003) Time-dependent changes in the plasma concentration of matrix metalloproteinase 9 after acute myocardial infarction. *Cardiology* 99:140-144.

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 19, 2024

JPET Fast Forward. Published on June 6, 2006 as DOI: 10.1124/jpet.106.104455
This article has not been copyedited and formatted. The final version may differ from this version.

Matsumura S, Iwanaga S, Mochizuki S, Okamoto H, Ogawa S, Okada Y (2005) Targeted deletion or pharmacological inhibition of MMP-2 prevents cardiac rupture after myocardial infarction in mice. *J Clin Invest* 115:599-609.

McCawley LJ, Matrisian LM (2001) Matrix metalloproteinases: they're not just for matrix anymore! *Curr Opin Cell Biol* 13:534-540.

McDonnell S, Morgan M, Lynch C (1999) Role of matrix metalloproteinases in normal and disease processes. *Biochem Soc Trans* 27:734-740.

Mukherjee R, Brinsa TA, Dowdy KB, Scott AA, Baskin JM, Deschamps AM, Lowry AS, Escobar GP, Lucas DG, Yarbrough WM, Zile MR, Spinale FG (2003) Myocardial infarct expansion and matrix metalloproteinase inhibition. *Circulation* 107:618-625.

Nian M, Lee P, Khaper N, Liu P (2004) Inflammatory cytokines and postmyocardial infarction remodeling. *Circ Res* 94:1543-1553.

Overall CM, Lopez-Otin C (2002) Strategies for MMP inhibition in cancer: innovations for the post-trial era. *Nat Rev Cancer*: 657-672.

Overall CM, Tam EM, Kappelhoff R, Connor A, Ewart T, Morrison CJ, Puente X, Lopez-Otin C, Seth A (2004) Protease degradomics: mass spectrometry discovery of

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 19, 2024

JPET Fast Forward. Published on June 6, 2006 as DOI: 10.1124/jpet.106.104455
This article has not been copyedited and formatted. The final version may differ from this version.

protease substrates and the CLIP-CHIP, a dedicated DNA microarray of all human proteases and inhibitors. *Biol Chem* 385:493-504.

Peterson JT, Hallak H, Johnson L, Li H, O'Brien PM, Sliskovic DR, Bocan TM, Coker ML, Etoh T, Spinale FG (2001) Matrix metalloproteinase inhibition attenuates left ventricular remodeling and dysfunction in a rat model of progressive heart failure. *Circulation* 103:2303-2309.

Rohde LE, Ducharme A, Arroyo LH, Aikawa M, Sukhova GH, Lopez-Anaya A, McClure KF, Mitchell PG, Libby P, Lee RT (1999) Matrix metalloproteinase inhibition attenuates early left ventricular enlargement after experimental myocardial infarction in mice. *Circulation* 99:3063-3070.

Schafers M, Riemann B, Kopka K, Breyholz HJ, Wagner S, Schafers KP, Law MP, Schober O, Levkau B (2004) Scintigraphic imaging of matrix metalloproteinase activity in the arterial wall in vivo. *Circulation* 109:2554-2559.

Schellings MW, Pinto YM, Heymans S (2004) Matricellular proteins in the heart: possible role during stress and remodeling. *Cardiovasc Res* 64:24-31.

Spinale FG, Coker ML, Krombach SR, Mukherjee R, Hallak H, Houck WV, Clair MJ, Kribbs SB, Johnson LL, Peterson JT, Zile MR (1999) Matrix metalloproteinase

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 19, 2024

JPET Fast Forward. Published on June 6, 2006 as DOI: 10.1124/jpet.106.104455
This article has not been copyedited and formatted. The final version may differ from this version.

inhibition during the development of congestive heart failure: effects on left ventricular dimensions and function. *Circ Res* 20:364-376.

Steffensen B, Hakkinen L, Larjava H (2001) Proteolytic events of wound-healing--coordinated interactions among matrix metalloproteinases (MMPs), integrins, and extracellular matrix molecules. *Crit Rev Oral Biol Med* 12:373-398.

Thompson MM, Squire IB (2002) Matrix metalloproteinase-9 expression after myocardial infarction: physiological or pathological? *Cardiovasc Res* 54:495-498.

Tsuruda T, Costello-Boerrigter LC, Burnett JC Jr (2004) Matrix metalloproteinases: pathways of induction by bioactive molecules. *Heart Fail Rev* 9:53-61.

Tziakas DN, Chalikias GK, Parissis JT, Hatzinikolaou EI, Papadopoulos ED, Tripsiannis GA, Papadopoulou EG, Tentes IK, Karas SM, Chatseras DI (2004) Serum profiles of matrix metalloproteinases and their tissue inhibitor in patients with acute coronary syndromes. The effects of short-term atorvastatin administration. *Int J Cardiol* 94:269-277.

Vihinen P, Kahari VM (2002) Matrix metalloproteinases in cancer: prognostic markers and therapeutic targets. *Int J Cancer* 10:157-166.

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 19, 2024

JPET Fast Forward. Published on June 6, 2006 as DOI: 10.1124/jpet.106.104455
This article has not been copyedited and formatted. The final version may differ from this version.

Wainwright CL (2004) Matrix metalloproteinases, oxidative stress and the acute response to acute myocardial ischaemia and reperfusion. *Curr Opin Pharmacol* 4:132-138.

Woessner FJ (1998) The matrix metalloproteinase family. In: *Matrix metalloproteinases*. Parks WC, Mecham RP, eds. Academic Press, San Diego. pp 1-141.

Yarbrough WM, Mukherjee R, Escobar GP, Mingoia JT, Sample JA, Hendrick JW, Dowdy KB, McLean JE, Lowry AS, O'Neill TP, Spinale FG (2003) Selective targeting and timing of matrix metalloproteinase inhibition in post-myocardial infarction remodeling. *Circulation* 7:1753-1759.

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 19, 2024

JPET Fast Forward. Published on June 6, 2006 as DOI: 10.1124/jpet.106.104455
This article has not been copyedited and formatted. The final version may differ from this version.

Footnotes

This study was supported by NIH grants HL59165, PO1 HL48788-08, P20 RR16434 and a Merit Award from the Veterans' Affairs Health Administration.

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 19, 2024

JPET Fast Forward. Published on June 6, 2006 as DOI: 10.1124/jpet.106.104455
This article has not been copyedited and formatted. The final version may differ from this version.

Figure Legends

Figure 1. A myocardial infarction (MI) was induced in mice and at 3 days post MI assigned to undergo matrix metalloproteinase inhibition (MMPi; n=64) or serve as an untreated MI reference group (MI only; n=67). The mice were treated and followed for 6 months and Kaplan-Meier survival curves were constructed. The median survival time (time of 50% survival) was greater in the MMPi group compared to the MI only group (38 vs 5 days; Chi Square statistic:4.27, $p<0.05$). However, in the mice that survived for a longer duration, the survival curves crossed at approximately 45 days, where the MMPi group demonstrated a greater mortality compared to MI only by 120 days (Chi Square statistic:9.71, $p<0.05$).

Figure 2. LEFT PANELS: Representative long axis views of the left ventricle (LV) at end-diastole using 2-dimensional echocardiography. The endocardial borders of the LV have been highlighted and these areas were utilized to calculate LV end-diastolic volumes. LV function and geometry was determined in all surviving mice at 6 months following myocardial infarction (MI) in both the matrix metalloproteinase inhibition group (MI-MMPi) and the MI only group. Age matched non-MI mice served as referenced controls. Significant LV dilation was observed in the MI mice, which appeared greater in the MI-MMPi group. RIGHT PANELS: Representative sections taken along the long axis of the LV which were utilized for morphometric measurements. The right ventricle (RV) was maintained on these sections for orientation purposes. The site of the MI can be readily seen as a thinning region of the myocardium along the apex of the LV. While MI sizes were equivalent in both the MI only and MMPi groups, the degree of LV dilation appeared greater in the MI-MMPi sections. Summary data are presented in the Results section. The white bars in the left panels and the black bars on the right panels indicate 2 mm.

Figure 3. A- Frequency distribution of computed MI size in the MI only (n=27) and MI-MMPi (n=17) groups from surviving mice studied at 6 months. Equivalent MI sizes were obtained between groups. B- Relative collagen volume fractions in the remote, viable myocardium were slightly reduced in the MI only group compared to controls (n=16;p=0.25). However, relative collagen content was significantly increased in the MI-MMPi group compared to reference controls and MI only values. C- Myocyte

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 19, 2024

JPET Fast Forward. Published on June 6, 2006 as DOI: 10.1124/jpet.106.104455
This article has not been copyedited and formatted. The final version may differ from this version.

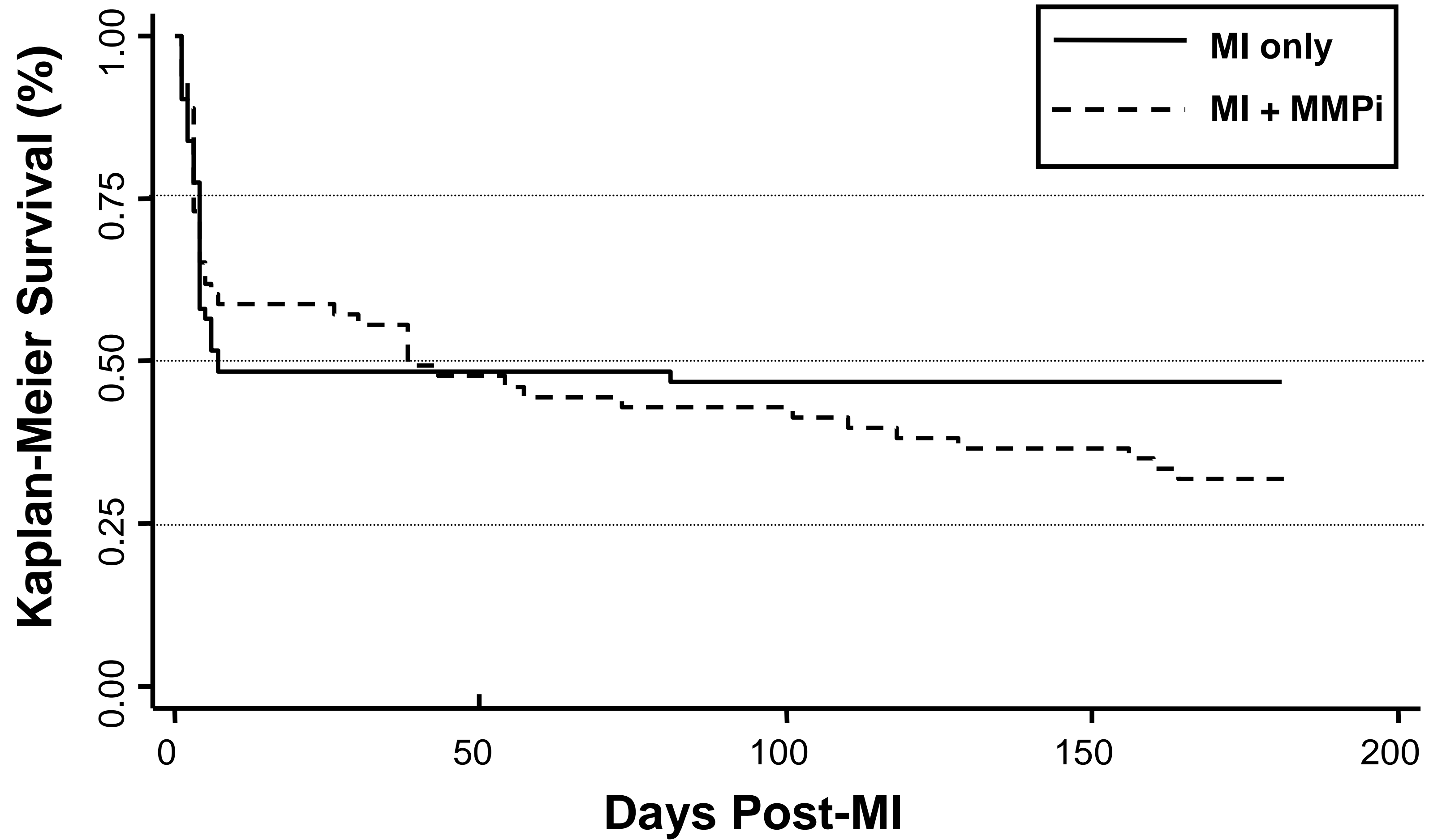
cross-sectional area was measured in the remote viable myocardium and was significantly increased in both the MI only and MI-MMPi groups compared to controls. However, myocyte cross-sectional area was reduced in the MI-MMPi group compared to MI only values. (*p<0.05 vs control; +p<0.05 vs MI only)

Figure 4. Representative photomicrographs of whole LV sections stained for fibrillar collagen under bright field and with polarizing microscopy. A: A control LV under bright field. B: The same control LV section under polarizing microscopy. An MI Only section is shown under identical bright field (C) and polarized light conditions (D).The highlighted areas marked as “R” and “I” indicate the remote and infarction regions, respectively that were used to quantify relative collagen content. These regions are shown in higher magnification in panels E and F. A representative MI-MMPi LV section is shown in the lower panels where the bright field is shown in panel G and the remote “R” and infarct “I” regions are illustrated. The respective polarized light image is shown in panel H. High power views from the remote (I) and infarct (J) for this MI-MMPi LV are shown. Relative collagen content was increased within the MI regions of both groups, but was increased significantly within the remote region in the MI-MMPi groups. Quantitative data summarized in Figure 3. Bar in photomicrographs is 50um.

Figure 5. Representative zymograms for MMP-2 and MMP-9, and immunoblots for MMP-13 and TIMP-1 and TIMP-4 in reference controls and in samples taken at 6 months following myocardial infarction (MI) with chronic matrix metalloproteinase inhibition (MI-MMPi) or MI only. The relative levels for MMP-2 were similar in the MI only group when compared to controls, but were increased in the MI-MMPi group when compared to control and MI only values. Relative MMP-9 levels were increased in both the MI and MI-MMPi groups compared to controls. An immunoreactive signal for MMP-13 could be detected for the proform (~60 kDa) and was reduced from control values in both MI groups. TIMP-1 levels appeared increased in both MI groups, but did not reach statistical significance. TIMP-4 levels were unchanged from control values in either MI group. Quantitative summary data presented in the Results section.

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 19, 2024

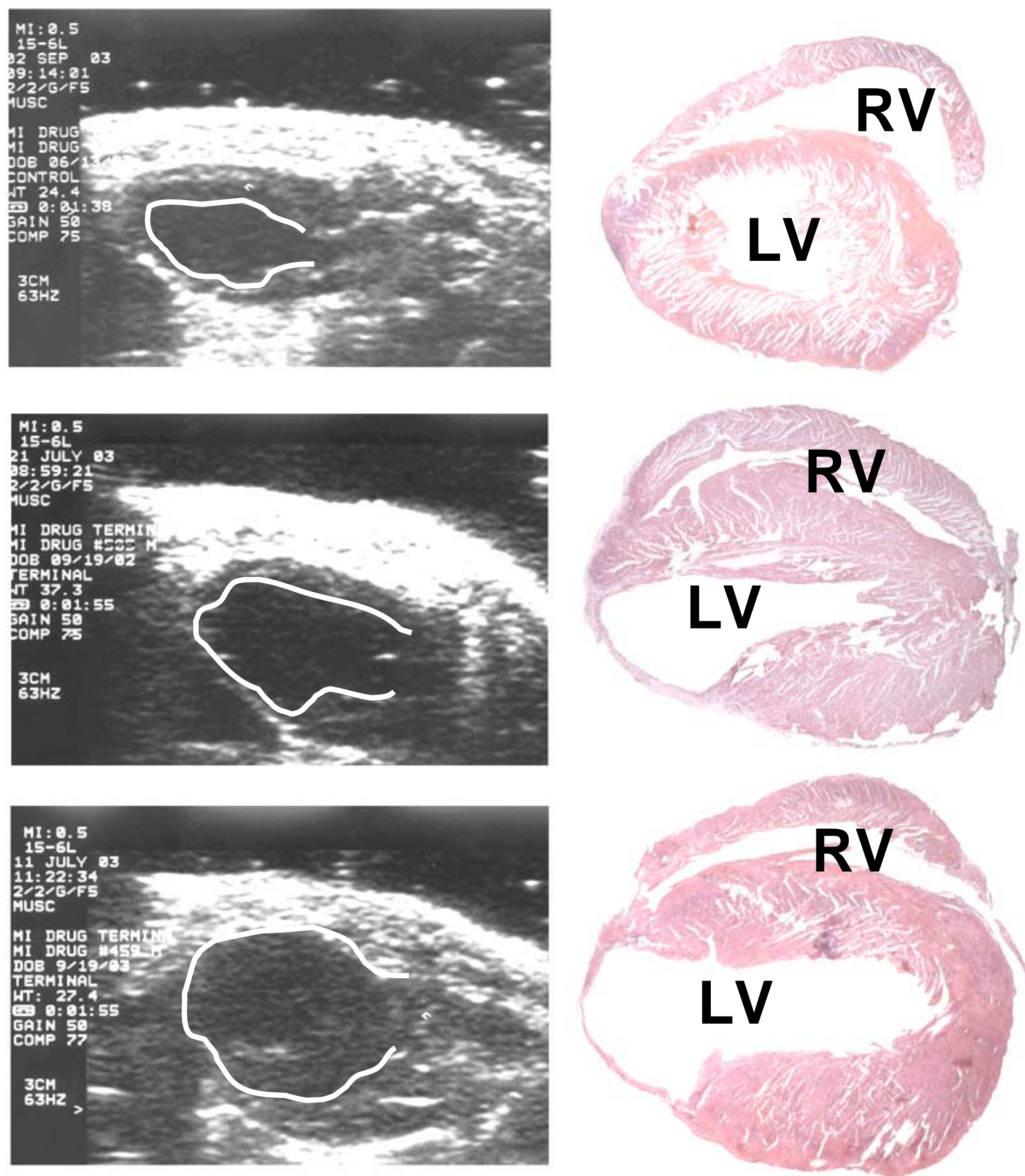
Figure 1



JPET Fast Forward. Published on June 6, 2006 as DOI: 10.1124/jpet.106.104455
This article has not been copyedited and formatted. The final version may differ from this version.

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 19, 2024

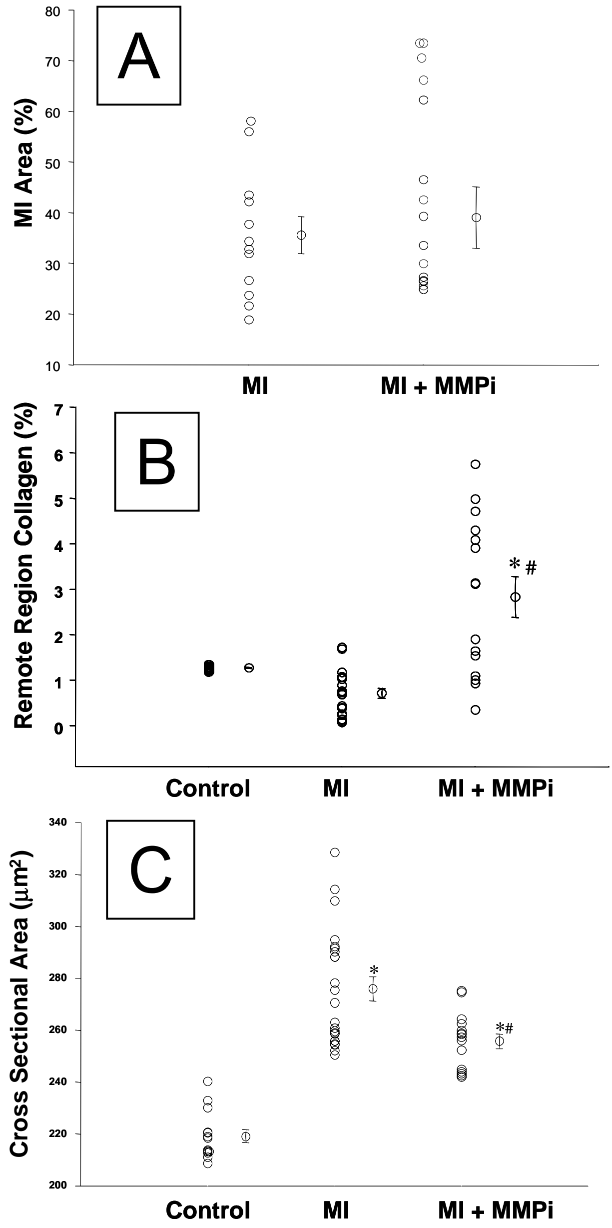
Figure 2



JPET Fast Forward. Published on June 6, 2006 as DOI: 10.1124/jpet.106.104455
This article has not been copyedited and formatted. The final version may differ from this version.

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 19, 2024

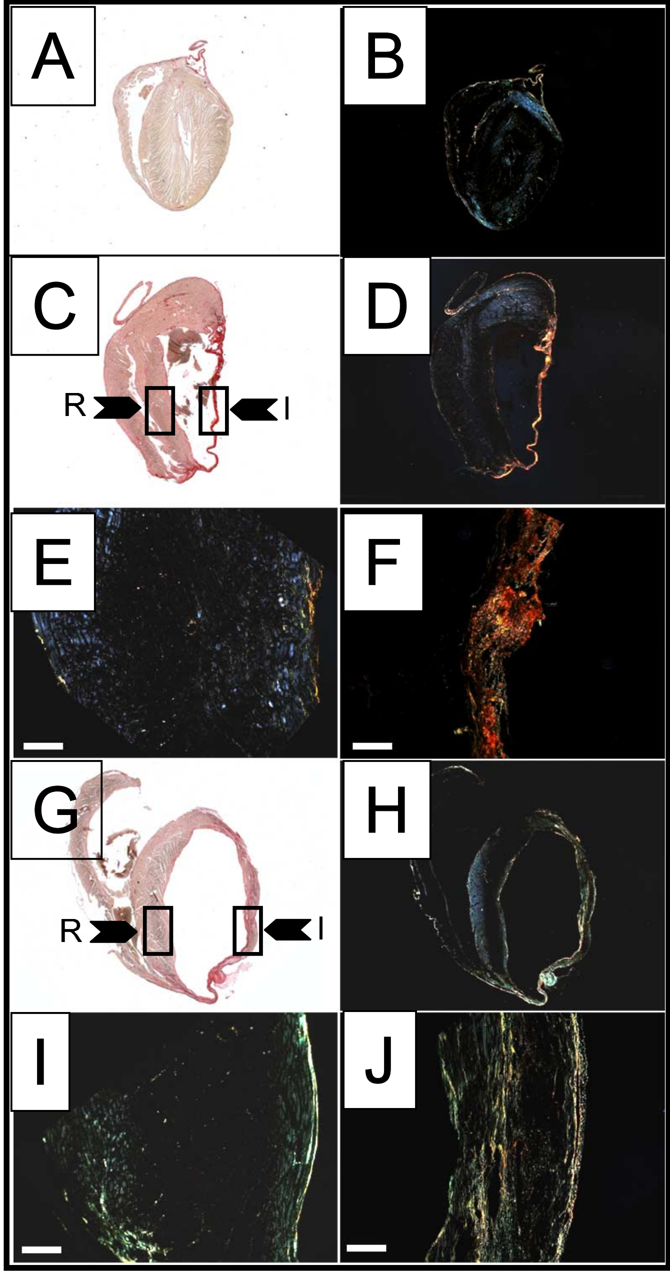
Figure 3



JPET Fast Forward. Published on June 6, 2006 as DOI: 10.1124/jpet.106.104455
This article has not been copyedited and formatted. The final version may differ from this version.

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 19, 2024

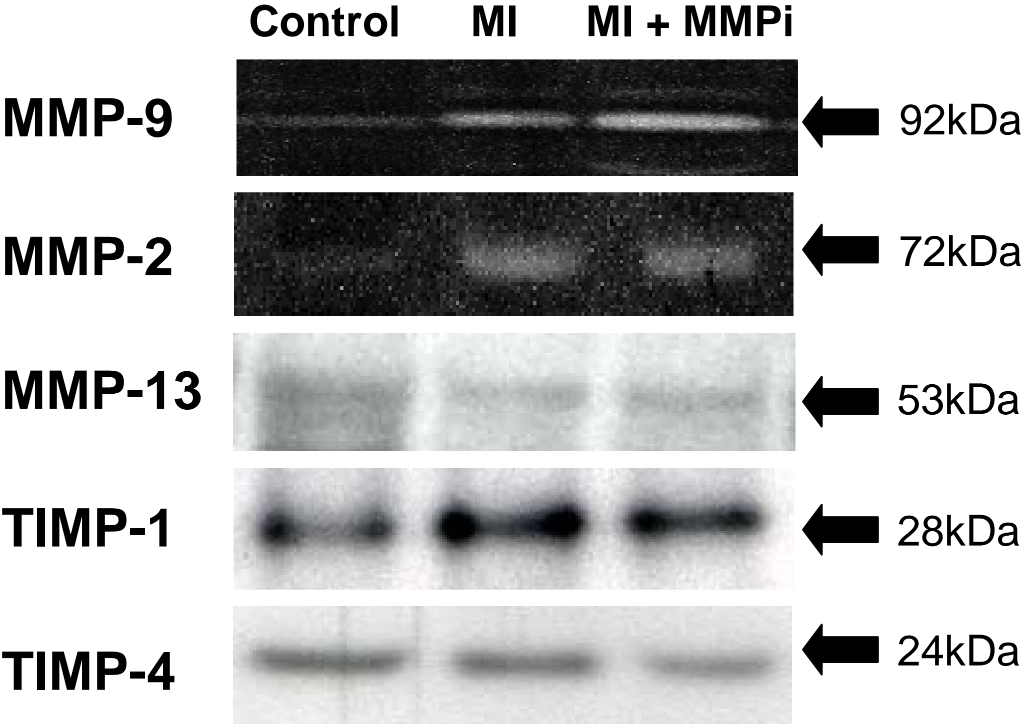
Figure 4



JPET Fast Forward. Published on June 6, 2006 as DOI: 10.1124/jpet.106.104455
This article has not been copyedited and formatted. The final version may differ from this version.

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 19, 2024

Figure 5



JPET Fast Forward. Published on June 6, 2006 as DOI: 10.1124/jpet.106.104455
This article has not been copyedited and formatted. The final version may differ from this version.

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 19, 2024