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Mitigation of Injury from Myocardial Infarction by Pentamidine, an Inhibitor of the Acetyltransferase Tip60

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Abstract

Purpose: There is an urgent unmet need for new pharmacologic approaches that promote re-muscularization and repair following myocardial infarction (MI). We previously reported that genetic depletion of the acetyltransferase Tip60 after MI in a mouse model activates the CM cell-cycle, reduces scarring, and restores cardiac function, and that these beneficial effects are mimicked by the Tip60-selective inhibitor TH1834. Here, we investigated whether the FDA-approved anti-microbial agent pentamidine, a Tip60 inhibitor from which TH1834 is derived, also protects from the damaging effects of MI.

Methods: Adult (10–14 weeks old) C57Bl/6 mice were subjected to permanent left coronary artery ligation to induce MI. Subsequently, echocardiography, electrocardiography, cardiac

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All authors contributed to the study conception and design. Data collection and analyses were performed by Xinrui Wang, Katherine Kulik, Tina Wan, and Zhuocheng Qu. The first draft of the manuscript was written by Xinrui Wang and all authors commented on subsequent versions of the manuscript prepared by John Auchampach. All authors read and approved the final manuscript.

Competing Interests:

The authors have no relevant financial or non-financial interests to disclose.

Ethics Approval:

This study adhered to the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals (NIH Pub. Nos. 85–23, Revised 1996). All protocols were approved by the Medical College of Wisconsin's Institutional Animal Care and Use Committee (IACUC). The IACUC has Animal Welfare Assurance status from the Office of Laboratory Animal Welfare (A3102–01).

Consent to Participate:

Not applicable. This study did not involve human subjects.

Consent to Publish:

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The authors declare that the data supporting the findings in this study are available within the paper and its Supplementary Information files. Should any raw data filed be needed in another format they are available from the corresponding authors upon reasonable request.

staining, and molecular analyses were performed to monitor the effects of treatment with pentamidine on cardiac injury and function.

Results: We report that transient systemic administration of pentamidine on days 3–16 post-MI at a daily dose of 3 mg/kg efficiently improved cardiac function for up to ten months. This was accompanied by improved survival, diminished scarring, and increased activation of cell-cycle markers in CMs located in the infarct border zone in the absence of hypertrophy. Histological assessments suggested that post-MI treatment with pentamidine reduced site-specific acetylation of the minor histone variant H2A.Z at lysines K4 and K7 in CMs, indicative of the dedifferentiation process which must occur prior to CM proliferation. Treating mice with pentamidine post-MI produced no prominent electrophysiological changes.

Conclusions: These findings support the translational potential of pentamidine for treatment of MI, and provide evidence that functional improvement is mediated, in part, by CM renewal due to inhibition of the acetyltransferase activity of Tip60.

Keywords

Pentamidine; Myocardial Infarction; Cardiomyocyte Dedifferentiation; Cell-Cycle Activation; Regeneration; Tip60

INTRODUCTION

Prolonged myocardial ischemia leads to the death of cardiomyocytes (CMs), which causes a reduction in muscle cell mass, impaired contractile performance, adverse remodeling, and ultimately heart failure. Shortly after birth, CMs enter a state of proliferative senescence, losing their ability to regenerate and re-muscularize the damaged myocardium. Therefore, identifying pathways enabled after birth that restrain proliferative capacity of CMs is crucial for developing treatments for myocardial infarction (MI).

We have focused on the lysine acetyltransferase (Kat) known as Tip60 (Tat-interactive protein, 60 kD). Our interest in Tip60 is grounded in findings largely from the field of cancer biology, which demonstrate that it acetylates histone and non-histone proteins that govern various cellular processes including stress response, apoptosis, cell-cycling, and DNA repair [1–5]. For example, Tip60-mediated acetylation of Ataxia-telangiectasia mutated (Atm) initiates the DNA damage response (DDR) to induce cell-cycle arrest [1–8]. Tip60 also site-specifically acetylates p53, enabling its ability to activate pro-apoptotic genes [9, 10]. In terms of histones, Tip60 is primarily recognized for acetylating the core histones H4 and H2A to mediate chromatin-transcription factor interactions for gene regulation. In addition, evidence is emerging that Tip60 acetylates H2A variants including H2A.Z [11–15]. Intriguingly, recent studies have linked Tip60-specific acetylation of H2A.Z with hematopoietic [11, 12], neuronal [13, 14], and skeletal muscle cell [13] fate specification, which has raised the idea that Tip60 may be a universal driver of cell lineage establishment and transitioning.

To investigate the function of Tip60 in CMs, wherein it is highly expressed [16, 17], we generated a line of mice containing *LoxP* sites in the *Kat5* gene [18], enabling its specific knock-out (KO) in CMs via the tamoxifen-activated Myh6-driven *merCremer*

transgene. Using this model, we have found that depletion of Tip60 in the adult heart preserves left ventricular systolic function and reduces scarring after MI [19, 20], which was accompanied by CM cell-cycle re-activation. More recently, we observed that Tip60 KO dramatically reduces acetylation of the minor histone variant H2A.Z at lysines 4 and 7 (i.e., H2A.Z^{K4/K7}) in association with transcriptional changes indicative of CM dedifferentiation [15]. Collectively, our results support the hypothesis we continue to pursue that genetic removal of Tip60 allows CMs to dedifferentiate, re-enter the cell-cycle, and proceed through the process of cell division, thereby improving re-muscularization following MI.

To extend these findings to a therapeutic context, we are also investigating whether administration of small molecule acetyltransferase inhibitors provides beneficial effects. We began by testing TH1834, a small molecule inhibitor of the Tip60 histone acetyltransferase (HAT) domain derived via molecular modeling from the anti-microbial agent pentamidine [21, 22], which we found to mimic the beneficial effects of Tip60 depletion [23]. Because pentamidine also inhibits Tip60 and is currently FDA-approved for use in humans for the treatment of fungal and parasitic diseases in humans, in this study we examined whether pentamidine protects against the damaging effects of MI.

MATERIALS AND METHODS

Animal Care & Experimentation

These experiments adhered to the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals (NIH Pub. Nos. 85–23, Revised 1996). All protocols were approved by the Medical College of Wisconsin's Institutional Animal Care and Use Committee (IACUC). The IACUC has Animal Welfare Assurance status from the Office of Laboratory Animal Welfare (A3102–01). For all experiments, C57Bl/6 mice were purchased from The Jackson Laboratory.

Echocardiography

Echocardiography was performed prior to induction of MI or sham surgery and at subsequent intervals using a VisualSonics 3100 high-frequency ultrasound imaging system. This was performed on mice lightly anesthetized with isoflurane delivered via a nose cone (1.0–1.5%). Parasternal long-axis, short-axis, and apical 4-chamber views were obtained using a transducer (MX550D) operating at 30–40 mHz. Short-axis views in M-mode assessed left ventricular anteroposterior internal diameter (LVID), anterior wall thickness (LVAW), and posterior wall thickness (LVPW) at end-diastole (d) and end-systole (s) at the mid-ventricular level. Long-axis views in B-mode assessed left ventricular internal area (LVA) and length (L) at end-diastole and end-systole. Three short axis views (evenly spaced around a mid-level view at the papillary muscles, one towards the apex or distal portion of the heart and one towards the base or proximal portion of the heart) assessed internal area at end-diastole and end-systole. Left ventricular systolic function was assessed by the following: (i) fractional shortening (FS %), calculated as $([LVIDd - LVIDs]/LVIDd) \times 100$; (ii) fractional area change (FAC %) = $([LVA d - LVA s]/LVA d) \times 100$, and (iii) ejection fraction (EF %) = $(\text{end-diastolic volume} - \text{end-systolic volume})/\text{end-diastolic volume}$, wherein volumes were estimated by B-mode: $4\pi/3 \times L/2 \times (LVA \div \pi(L/2))$. In addition, global

left ventricular function was assessed by calculating the myocardial performance index: $MPI = (\text{isovolumic contraction time} + \text{isovolumic relaxation time}) / \text{ejection time}$ [24–27]. Time intervals were obtained from pulsed Doppler waveforms of mitral valve inflow and aortic valve outflow obtained from apical 4-chamber views.

Myocardial Infarction

MI or sham surgery were induced in 10–12-week-old male mice under general anesthesia with isoflurane (1.5–2.0%) and mechanical respiration (model 845, Harvard Apparatus) via an endotracheal tube with room air supplemented with 100% O₂. Rectal temperature was maintained at 37°C using a servo-controlled heating pad. Following anesthesia onset, mice were subcutaneously injected with sustained release meloxicam (4 mg/kg) to manage post-operative pain. A left-sternal thoracotomy was performed to expose the heart, and the pericardium was opened. To target the MI to the lower half of the ventricle, an 8.0 nylon suture was threaded beneath the left main coronary artery at a level below the tip of the left atrium, with the aid of a microscope. Ischemia was induced by tying a permanent suture with a double knot. Coronary occlusion was verified by visually observing blanching of the myocardium distal to the ligature, and by elevation of the ST segment on the ECG. For sham experiments, the sutures were placed as described above but they were not tightened to occlude the artery. The chest wall was closed using polypropylene suture. Recovery was monitored until mice became fully ambulatory.

Electrocardiograms

ECGs were continuously recorded during surgery and at subsequent intervals post-MI via needle electrodes under general anesthesia with isoflurane (1.5–2.0%) using the lead II configuration. For the analyses, 10 or more beats were averaged to give a precise representation of the ECG waveform; each line marker is manually adjusted to meet the standard waveform as previously described [28–30]. At least 10 averaged recordings (more than 100 total beats) were corrected and analyzed per heart for final average values. The Formula described in Mitchell et al [31] is used for QT correction.

Administration of Pentamidine and Heart Collection

Administration of pentamidine began on the third day post-MI/sham surgery via daily intraperitoneal injections of vehicle (1x PBS; controls) or PBS containing pentamidine (3 mg/kg; C₁₉H₂₄N₄O₂; MW 340.4). Pentamidine isethionate salt was purchased from Sigma (cat. #P0547). Daily injections were performed for 14 consecutive days, i.e., from the third through the sixteenth day post-surgery. On the day before harvest, mice were intraperitoneally injected with BrdU (1 mg). On the day of harvest (10 or 28 days post-MI), mice were euthanized with CO₂ after which hearts were removed and processed for immunostaining as described below.

Terminal Deoxynucleotidyl Transferase dUTP nick end-labeling (TUNEL)

TUNEL staining was assessed using the DeadEnd Fluorometric TUNEL System (Promega #G3250) per the manufacturer's instructions. CM identity was verified by co-immunostaining cytoplasm with cTnT, and by imaging CM boundaries with WGA. Total

numbers of TUNEL-positive CMs in the border and remote zones were manually counted at 400x magnification. TUNEL signal was counted only if confined within a DAPI-positive nucleus, and, nuclei were scored as TUNEL-positive only if at least 50% of the nucleus contained fluorescent signal. Apoptosis was also determined by immunostaining cleaved caspase-3 as described below.

Myocardial Scarring

Scarring was assessed in Masson trichrome-stained transverse sections (4 μ m thick) of hearts removed at 0.3 mm intervals along the axis between the apex and ~1 mm above the ligation site. Trichrome-stained sections were photographed at 10x magnification on a Nikon SMZ800 microscope and MIQuant software was used to quantitate infarct size, as previously described [23]. Results are expressed as the average percentage of area, and midline length, around the left ventricle.

Wheat Germ Agglutinin (WGA) Staining

WGA staining was performed using Thermo-Fisher #W21405 Texas Red-X conjugate. Sections were stained with 50 μ g/ml WGA in PBS for 10 minutes at room temperature, followed by thorough washing. Images of CMs in transverse orientation were photographed at 400x magnification and processed using ImageJ software to determine numbers of CMs, and numbers of pixels per CM, as respectively indicative of CM density and CM size. Briefly, the Texas Red channel displaying CMs outlined in cross-section was isolated, followed by thresholding to fill-in spaces occupied by CM cytoplasm, then adjusting settings to acquire particle sizes in the 600-infinity range having a circularity of 0.25–1. After results were obtained, particles representing CMs that were non-transversely sectioned, or blood vessels, were removed. Data were analyzed in blind and averaged from >300 CMs per zone in each heart.

Immunostaining

On the day before harvest, mice were injected with 1 mg 5'-bromo-2'-deoxyuridine (BrdU; Sigma #B9285). Following removal, hearts were perfused with ice-cold cardioplegic solution and atria were removed. Ventricles were fixed overnight in fresh ice-cold 4% paraformaldehyde/PBS, processed through ethanol series, and embedded in paraffin. Sections (4 μ m thick) mounted on microscope slides were de-waxed, subjected to antigen retrieval (100° C in 10 mM trisodium citrate pH6.0/0.05% Tween-20 for 20 minutes) followed by 30 minutes' cooling at RT, and blocked with 2% goat serum/0.1% Triton-X-100 in PBS. Primary antibodies were diluted in blocking buffer and applied overnight at 4°C; secondary antibodies were applied for one hour in the dark. Combinations and dilutions of primary and secondary antibodies used to immunostain each target antigen are listed in Supplemental Table 1.

Microscopy was performed on a Nikon Eclipse 50i microscope equipped with a Nikon DSU3 digital camera. For counting cell-cycle markers, at least 1,000 CMs were evaluated in 5–6 random 200x photomicrographic fields; to count other markers, at least 300 CMs were evaluated in 5–6 random 400x photomicrographic fields. CM identity was assessed by co-immunostaining cytoplasm with cTnT, and cellular outline with WGA. CMs in the border

and remote (the area ~2 mm distal to the infarct boundary) zones relative to the infarction site were separately counted.

Quantitative RT-PCR (qPCR)

Heart tissue, previously disrupted by homogenization with a motorized (Kimble 749,540–0000) Teflon pestle and stored at -80°C in TRIzol reagent, was thawed. RNA was purified using PureLink RNA Mini-Kits (ThermoFisher #12183018A), including a genomic DNA removal step (PureLink DNase kit for on-column protocol, Thermo-Fisher #12185–010), according to the manufacturer's instructions. RNA yield and quality were determined via 260/280 ratio using an Eppendorf Biophotometer Plus instrument.

For synthesis of cDNA, 1 μg of RNA was suspended in 14 μl of nuclease-free water (NFDW), to which 4.0 μl 5x VILO reaction mixture (ThermoFisher #11754050) were added. To start the reverse transcription reaction, 2.0 μl 10x SuperScript Enzyme Mix were added, followed by transfer to an Applied Biosystems Veriti 96-well Thermocycler programmed as follows: 10 min at 25°C $\rightarrow$ 60 min at 42°C $\rightarrow$ 5 min at 85°C . cDNA templates were diluted with NFDW to a concentration of 3.125 ng/ μl and stored at -20°C .

qPCR was carried out by subjecting each biological replicate (i.e., sample from each individual heart) to triplicate determinations. Each reaction was performed in a total volume of 10 μl in 96-well arrays, each well containing 1x Taqman Fast-Advanced Master Mix (ThermoFisher #4444557), 1x Tagman Probe Kit (Supp. Table 2), and 12.5 ng cDNA template. The arrayed samples were amplified in a Bio-Rad CFX96 Real Time System (C1000 Touch) programmed as follows: 2 min at 50°C $\rightarrow$ 20 sec at 95°C $\rightarrow$ 3 sec at 95°C $\rightarrow$ 30 sec at 60°C ; the last two steps were repeated 39 times. Results were processed using Bio-Rad CFX Manager 3.1 software.

Statistics

All determinations were performed in blinded fashion and are reported as means $\pm$ SEM. Echocardiography and ECG data were analyzed by a two-way repeated measures ANOVA to determine whether there was a main effect of time, treatment, or a time-treatment interaction. If global tests showed an effect, post hoc contrasts between baseline and subsequent timepoints within experimental groups were compared by a Dunnett's multiple comparison *t* test; differences between treatment groups at each timepoint were compared by Student's *t* testing with the Bonferroni correction for multiple comparisons. All other data were compared by an unpaired, two-tailed Student's *t* test with Welch's correction. $P < 0.05$ was considered statistically significant.

RESULTS

Experimental Scheme

Following its identification as a Tip60 inhibitor in 2010 [32], pentamidine (Fig. 1A) has been applied in molecular modeling studies as a model compound for antagonizing the acetyltransferase domain of Tip60 [22]. Our aim was to assess whether systemic administration of pentamidine would mimic the cardioprotective effects of genetically

depleting Tip60 in CMs [20]. As shown in the experimental timeline depicted in Figure 1B, which is similar to the strategy previously employed to pharmacologically inhibit Tip60 using TH1834 [20], we assessed the effect of daily intraperitoneal injection of 3 mg/kg pentamidine to wild-type adult mice, beginning on day 3 after MI/sham surgery, for 14 consecutive days, ending on day 16 post-MI/sham. MI was induced by permanent ligation of the left main coronary artery to generate uniform infarctions in the distal half of the ventricle. In order to specifically assess pentamidine impact during post-MI remodeling, administration was not initiated until 3 days after induction of MI. Mice receiving PBS served as vehicle-treated controls. Electrical conduction of the heart was monitored by electrocardiography and echocardiography was performed to assess cardiac function at the intervals indicated in Figure 1B. On days 10 and 28 post-MI, hearts were removed for histological assessment. Quantitative RT-PCR was performed to assess the expression of genes we previously demonstrated to be dysregulated in the infarcted heart by genetic depletion of Tip60 [15, 19, 20]. Among these targets, *Mapt*, *Osmr*, *Runx1*, *Tnni1*, and *Trp53* (p53) were significantly altered in pentamidine-treated hearts at day 10 post-MI (Fig. 1C), indicating that administration of pentamidine at a dose of 3 mg/kg/day effectively antagonized Tip60 activity. Data were obtained from multiple experiments consisting of small cohorts of 4–5 mice randomly assigned to treatment with pentamidine or vehicle to achieve a minimum sample size of four for histological analyses at the two different timepoints.

Pentamidine Improved Recovery of Cardiac Function Post-MI and Prolonged Survival.

As expected, at the onset of treatment on day 3 post-MI, control and pentamidine-treated groups exhibited left ventricular dysfunction across all echocardiographic parameters monitored (Fig. 2A–E). By day 10 post-MI, fractional shortening (FS; Fig. 2B) and stroke volume (SV; Fig. 2E) were preserved in pentamidine-treated mice, persisting until hearts were collected for histology at day 28. By day 19 post-MI, ejection fraction (EF), myocardial performance index (MPI), and fractional area change (FAC) were also significantly improved by pentamidine administration (Fig. 2A, C, D), which persisted at day 28. Preservation of function was consistent with improved post-MI survival of pentamidine-treated mice during the 28-day timeline (Fig. 2F). In a separate subset of mice followed for ~1.5 years (n= 4/group), functional improvement persisted for at least 40 weeks- post-MI resulting in improved long-term survival (Fig. 3). Importantly, pentamidine had no effect on any echocardiographic indices of heart function in sham-operated mice (Supp. Fig. S1). Compared to treatment with vehicle, pentamidine had no effects on electromyographic parameters of cardiac conduction in infarcted mice, including standard time intervals (PR, QRS, QTc, RR) and ST segment height, with the exception that the cQT interval was reduced in infarcted animals at the 28-day timepoint (Table 1). Pentamidine had no effect on heart and body weight measurements taken at days 10 and 28 post-MI (Supp. Fig. S2).

Post-MI Treatment with Pentamidine reduced Scarring.

To assess myocardial damage directly, the extent of scarring was quantified by trichrome staining of cross-sections taken at intervals of 0.3 mm from the area between the ventricular apex and the ligation site (Fig. 4A, left). Consistent with enhanced function observed by

echocardiography, pentamidine-treated hearts exhibited diminished scarring at 28 days post-MI, as indicated by significant reductions in both left ventricular area and midline length (Fig. 4A, right). To examine whether reduced scarring might be attributed to prevention of cell death, apoptosis was assessed by TUNEL (Fig. 4B, left) and cleaved caspase-3 (Fig. 4C). Although enumeration of TUNEL-positive nuclei in pentamidine-treated mice at day 28 post-MI revealed a trend toward reduced numbers of apoptotic cells in the infarct border zone (Fig. 4B, right), no discernable effects on percentages of cleaved caspase-3-positive CMs (Fig. 4C) were observed.

Post-MI Treatment with Pentamidine Activated the Cell-Cycle in CMs.

Immunostaining of tissue sections for phosphorylated histone H3 (pH3, Fig. 5A), 5'-bromo-2'-deoxyuridine (BrdU, Fig. 5B), and Ki67 (Fig. 5C) was performed to assess cell-cycle activation in CMs. BrdU incorporates into nuclear DNA as a thymidine analog during S-phase and pH3 identifies cells in early M-phase. At days 10 and 28 post-MI, percentages of CMs exhibiting both markers were significantly increased in the infarct border zone of hearts in pentamidine-treated mice (Fig. 5A & 5B). Also, trends toward increased numbers of CMs expressing Ki67, a marker that monitors all cell-cycle phases, were observed in the infarct border of hearts in pentamidine-treated mice (Fig. 5C).

To assess the possibility that cell-cycle activation in pentamidine-treated mice promoted CM hypertrophy, CM size was evaluated in transversely sectioned CMs outlined by WGA staining. As shown in Figure 6, CM size was reduced in both the infarct border and remote zones of hearts in pentamidine-treated mice, most prominently at the 10-day timepoint. These findings suggest that pentamidine treatment may induce CM renewal, a notion supported by the increased density of CMs observed in pentamidine-treated hearts (Fig. 6).

Post-MI Treatment with Pentamidine Reduced Acetylation of H2A.Z in CMs.

Tip60-mediated site-specific acetylation of lysines 4 and 7 in the minor histone variant H2A.Z – i.e., H2A.Zac^{K4/K7} – has been associated with maintenance of the differentiated state in hematopoietic stem cells (HSCs; [11, 12], neurons [13, 14], skeletal myoblasts [13], and likely CMs [15]. Because it is now accepted that adult CMs must undergo dedifferentiation prior to resuming cell-cycling and regeneration after MI [33–40], we assessed whether CMs containing H2A.Zac^{K4/K7} were reduced in pentamidine-treated mice. While modest in comparison with the reduction of H2A.Zac^{K4/K7}-positive CMs we observe after genetic depletion of Tip60 [15], pentamidine-treated mice displayed significantly decreased numbers of H2A.Zac^{K4/K7}-positive CMs within the infarct border zone at 10-days and 28-days post-MI (Fig. 7A), consistent with the dysregulation of genes associated with CM dedifferentiation and proliferation (Fig. 1C). As expected, no effects of pentamidine treatment were observed on the numbers of CMs containing bulk (i.e., acetylated plus non-acetylated) H2A.Z (Fig. 7B).

DISCUSSION

In this study, we demonstrate that pentamidine significantly reduces injury from MI induced by permanent ligation of the left coronary artery. Treatment with pentamidine improved functional recovery at all intervals tested (up to 10 months post-MI) and improved both short-term (28 days post-MI) and overall long-term survival (1.5 years). A pertinent finding of this study is that pentamidine was effective when administered transiently for only 2 weeks, beginning on the third day post-MI. We speculate that sustained functional recovery and prolongation of life by transient treatment with pentamidine was due to the observed reduction in scarring, likely mediated by formation of newly generated CMs.

Previous studies utilizing acetyltransferase inhibitors including anacardic acid [41–43], MG149 [44], C646 [45, 46], garcinol [47, 48], and curcumin [47, 49, 48, 50–53] to treat cardiac injury had positive outcomes. However, these drugs non-selectively target the conserved acetyltransferase domain in members of the MYST sub-family of KATs which includes Tip60 [47, 49, 48, 50–52, 54, 53]. By contrast, pentamidine is a relatively Tip60-specific inhibitor [22] that has not previously been tested for mitigation of cardiovascular disease.

Pentamidine is currently utilized as an anti-microbial medication to prevent or treat pneumocystis pneumonia caused by the yeast-like fungus *Pneumocystis jirovecii*, as well as to treat an array of parasitic infections [55–57]. To treat resistant pneumocystis pneumonia, pentamidine is administered by intravenous or intramuscular injection at a dose of 3–4 mg/kg per day for up to 3 weeks, which was the basis for selecting the dose of 3 mg/kg per day in our investigation. Although we initially intended to administer 10 mg/kg/day, preliminary data indicated that this dose caused distress after ~10 days. Interestingly, pentamidine was also utilized as an anti-cancer agent in a study reporting cell death in several tumors [58]. Utilizing pentamidine as a model for Tip60 binding, its interaction with acetyl-CoA was extensively assessed via computational modeling, employing *in silico* design to identify drugs that specifically target Tip60 [21]. One Tip60-specific drug, termed TH1834, was subsequently evaluated to target breast and prostate cancer cells [21]. In our previous study utilizing the *in vivo* MI mouse model employed here [23], TH1834 mimicked the beneficial post-MI effects of pentamidine reported here.

Tip60 is known to activate apoptosis [9, 59, 20], and pentamidine has been shown to repress the DDR and associated apoptosis by inhibiting Tip60-mediated acetylation of histone H2A [32]. To assess whether pentamidine reduced cell death in the infarcted myocardium, we evaluated numbers of apoptotic CMs. As shown in Figure 5, the extent of cell death was similar in vehicle- and pentamidine-treated mice at days 10 and 28 post-MI. We conjecture that the absence of an effect might have been because cell death occurred prior to post-MI day 3, since apoptosis peaks during the first week post-MI [60, 61].

Therapy with pentamidine for microbial infections has been accompanied by prolongation of the QT interval, precipitating torsade's de pointes, a pathogenic process explained by pentamidine-mediated disruption of ether-a-go-go-related gene (hERG) trafficking [62–64]. The arrhythmogenic risk of administering pentamidine for ischemic heart disease has

not been previously assessed, although pentamidine has been reported to rescue heart arrhythmicity and contractility in a myotonic dystrophy model [65]. In the present study, we obtained ECG recordings from infarcted mice during the administration of pentamidine at days 3 and 10 post-MI and found that it did not produce any changes in standard measures of conduction compared to vehicle-treated mice (Table 1), including the QT interval or the QT interval corrected for heart rate (cQT). However, it is necessary to point out that, unlike in humans, ERG is not a major repolarizing current in mice, causing differences in the shape of the ST segment when comparing human and mouse ECG waveforms [28, 30]. The ST segment in mice is not isoelectric, but instead has a characteristic J wave that represents early repolarization [66, 67]. Hence, it will be important to investigate the effects of pentamidine on MI in an animal model that more closely represents the human condition.

In conclusion, the findings reported in this study demonstrate that administering pentamidine from days 3 to 16 post-MI replicates many of the cardioprotective effects we have shown to be associated with genetic depletion of Tip60, which are highlighted by enhanced CM dedifferentiation, cell-cycle activation and density, reduced scarring, preserved function, and improved survival [15, 19, 20]. When considered in conjunction with our recently published results utilizing TH1834 [23], these findings further justify efforts to develop treatments for ischemic heart disease by targeting Tip60 with specific inhibitors, optimizing duration of treatment and dosage.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Data Availability

All data sets are presented in the published manuscript or the data supplement.

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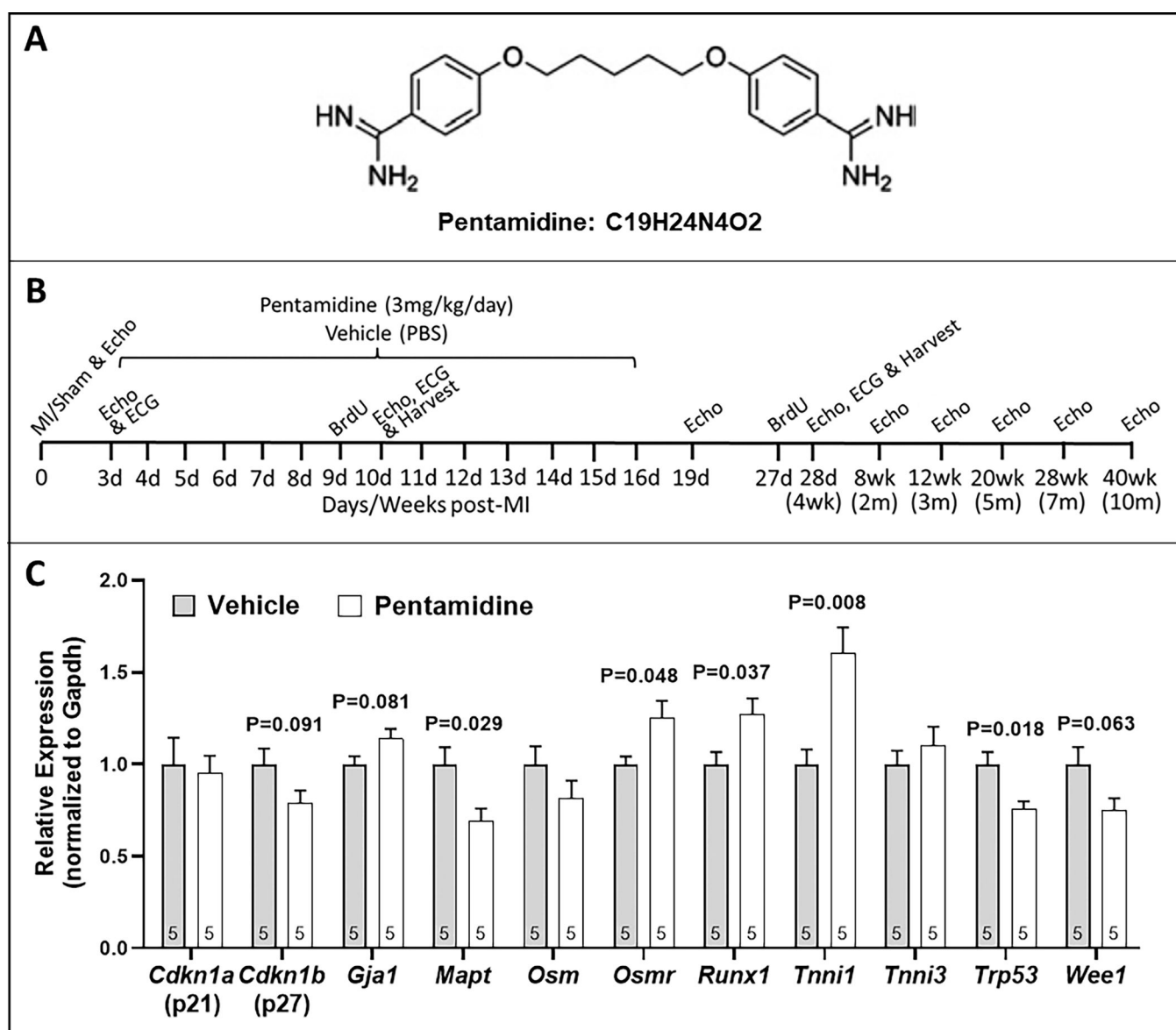


Figure 1. Experimental scheme and dysregulated gene targets of Tip60 in pentamidine-treated hearts:

Panel A, Pentamidine structure. **Panel B**, Experimental Timeline: Hearts of CL57/Bl6 adult mice were infarcted or subjected to sham surgery, followed three days later by daily intraperitoneal injection of pentamidine (3 mg/kg) or vehicle (normal saline) for 14 consecutive days. MI, myocardial infarction; Echo, echocardiography; ECG, electrocardiography; BrdU, 5'-bromo-2'-deoxyuridine. **Panel C**, Heart tissue removed at 10 days post-MI was subjected to quantitative RT-PCR determinations of genes that we previously determined to be altered following genetic depletion of Tip60 [15, 19, 20]. Each biological replicate (group size indicated within each bar) is the average of three technical replicates. Data (mean $\pm$ SEM) were normalized to *Gapdh* and compared using unpaired, two-tailed Student's t-tests with Welch's correction.

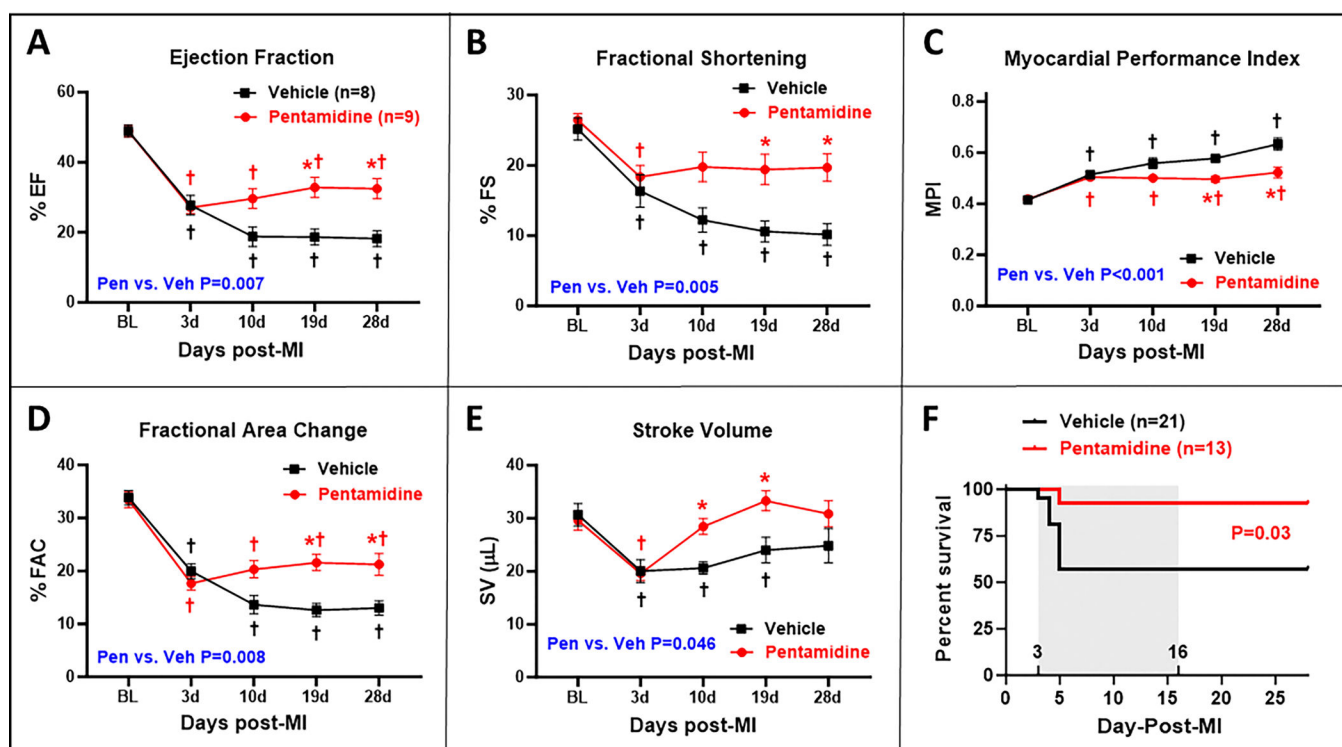


Figure 2. Administration of pentamidine improves cardiac function and survival after myocardial infarction (MI).

Echocardiography was performed at the indicated intervals up to 28 days post-MI, when hearts were removed for histological assessment. **Panels A-E** show indices of LV function, respectively Ejection Fraction (EF), Fractional Shortening (FS), Myocardial Performance Index (MPI), Fractional Area Change (FAC), and Stroke Volume (SV). Pentamidine treated mice exhibited improved function compared with vehicle-treated controls on days 10, 19, and 28 post-MI. **Panel F**, Kaplan-Meier curve indicating improved 28-day survival of pentamidine-treated mice. Shading denotes treatment window. Echocardiographic data (mean $\pm$ SEM) were analyzed by two-way repeated measures ANOVA followed by Dunnett's (effect of time) and Bonferroni's (effect of treatment) multiple comparisons. * denotes $P<0.05$ vs. Vehicle. † denotes $P<0.05$ vs baseline (BL) value on Day 0.

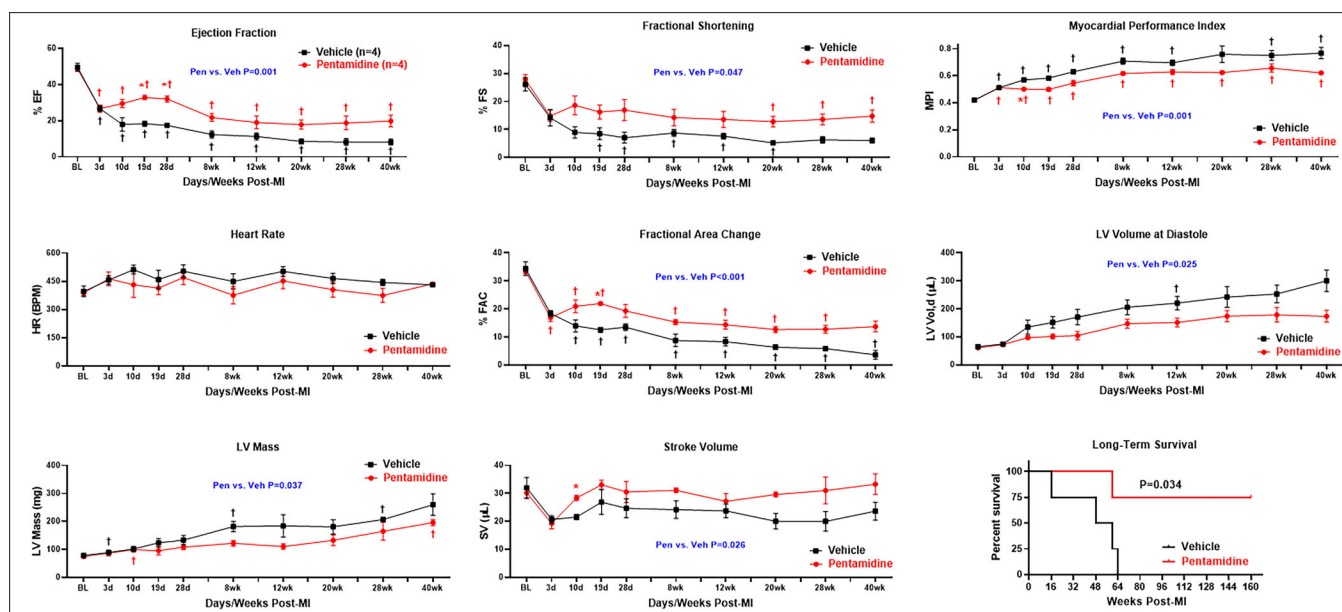


Figure 3. Administration of pentamidine sustains cardiac function for up to 40 weeks post-MI and prolongs overall survival.

A cohort of eight mice were subjected to MI and injected daily with pentamidine (3 mg/kg, i.p.; $n=4$) or vehicle (PBS; $n=4$) for 14 days beginning three days after induction of MI and followed for 1.5 years. Pentamidine-treated mice exhibited improved indices of LV function, including ejection fraction, fractional shortening, myocardial performance index, fractional area change, left ventricular diastolic volume, and stroke volume. In addition, LV mass was reduced in pentamidine-treated mice. Kaplan-Meier analysis indicated prolonged 1.5-year survival of pentamidine-treated mice. Echocardiographic data (mean $\pm$ SEM) were analyzed by two-way repeated measures ANOVA followed by Dunnett's (effect of time) and Bonferroni's (effect of treatment) multiple comparisons. * denotes $P<0.05$ vs. Vehicle. † denotes $P<0.05$ vs baseline (BL) value on Day 0.

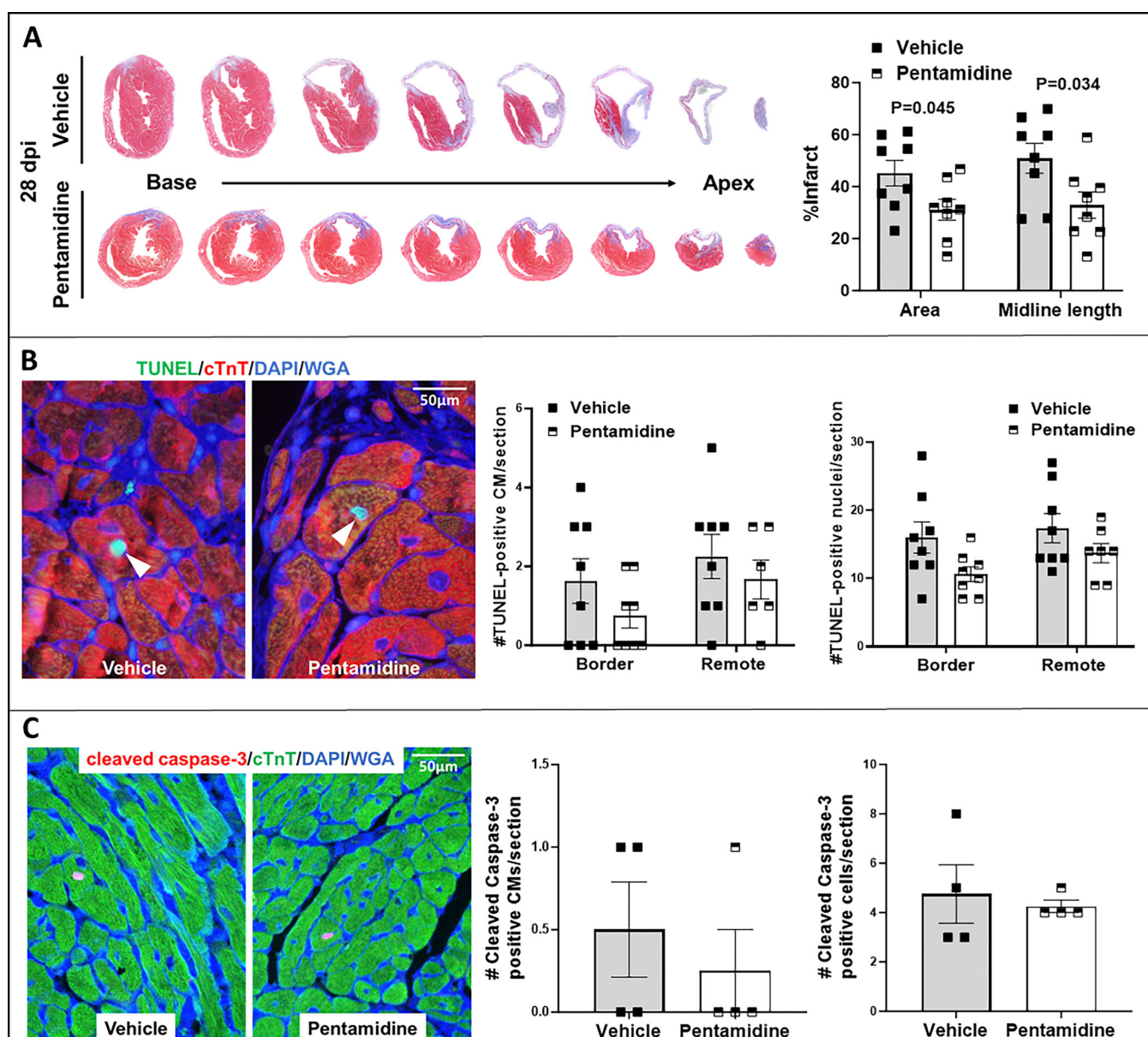


Figure 4. Effects of pentamidine treatment on scarring and apoptosis in infarcted mouse hearts at 28 days post-MI.

Panel A, Representative trichrome-stained cross-sections (left) and scar size quantified by measuring area and midline length below the ligation site. Sections were taken at 0.3 mm intervals; blue stain shows area of the scar. **Panel B**, representative TUNEL stain, and quantification of TUNEL-positive CMs and total number of TUNEL-positive cells per heart section within the border and remote zones. **Panel C**, representative immunostains of cleaved caspase-3, and quantification of cleaved caspase-3-positive CMs and total number of cleaved caspase-3-positive cells. CMs were identified by staining with cardiac troponin-T (cTnT) and wheat germ agglutinin (WGA). Arrowheads denote TUNEL-positive CMs. Data (mean $\pm$ SEM) are compared by unpaired, two-tailed Student's t tests with Welch's correction.

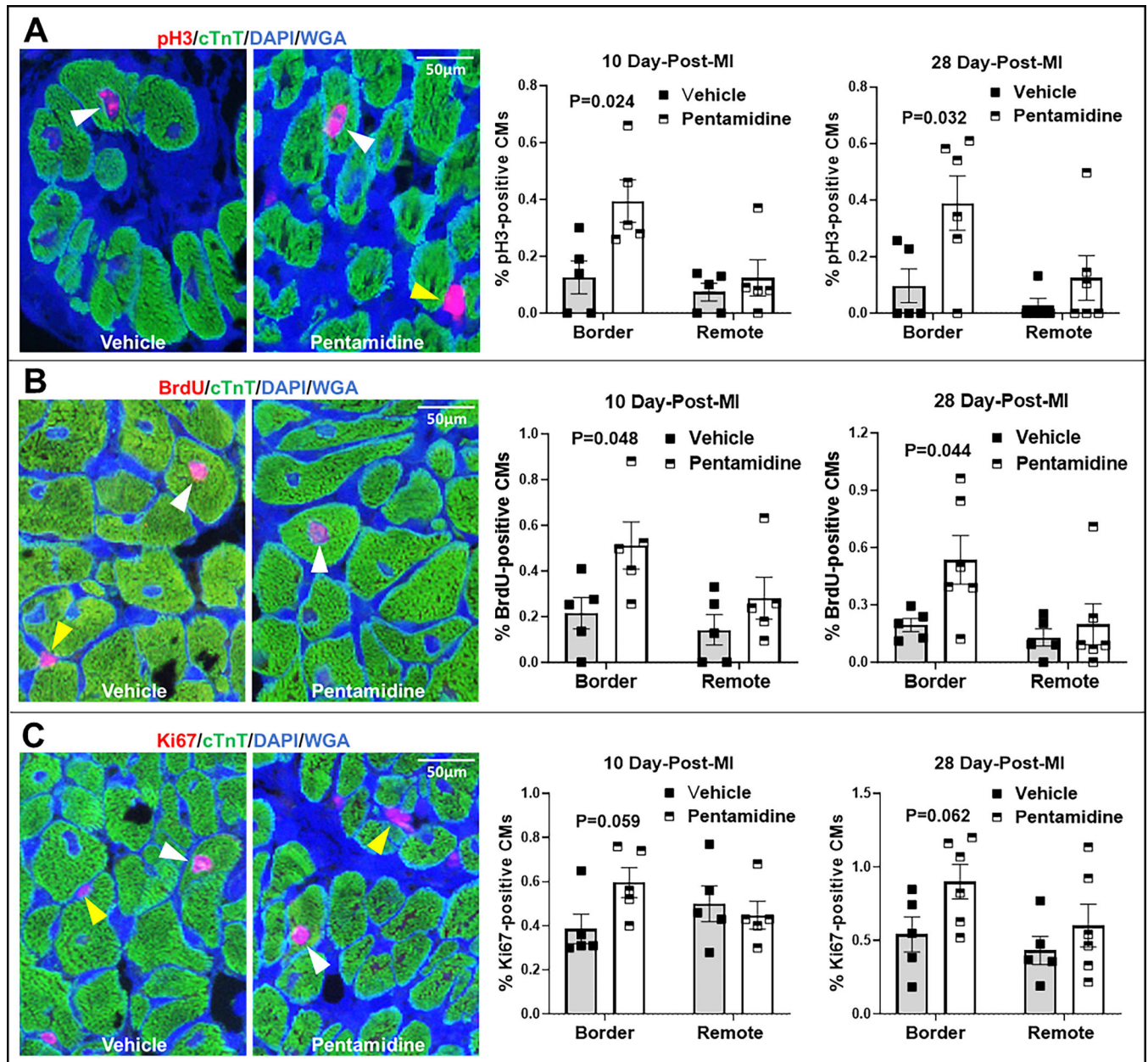
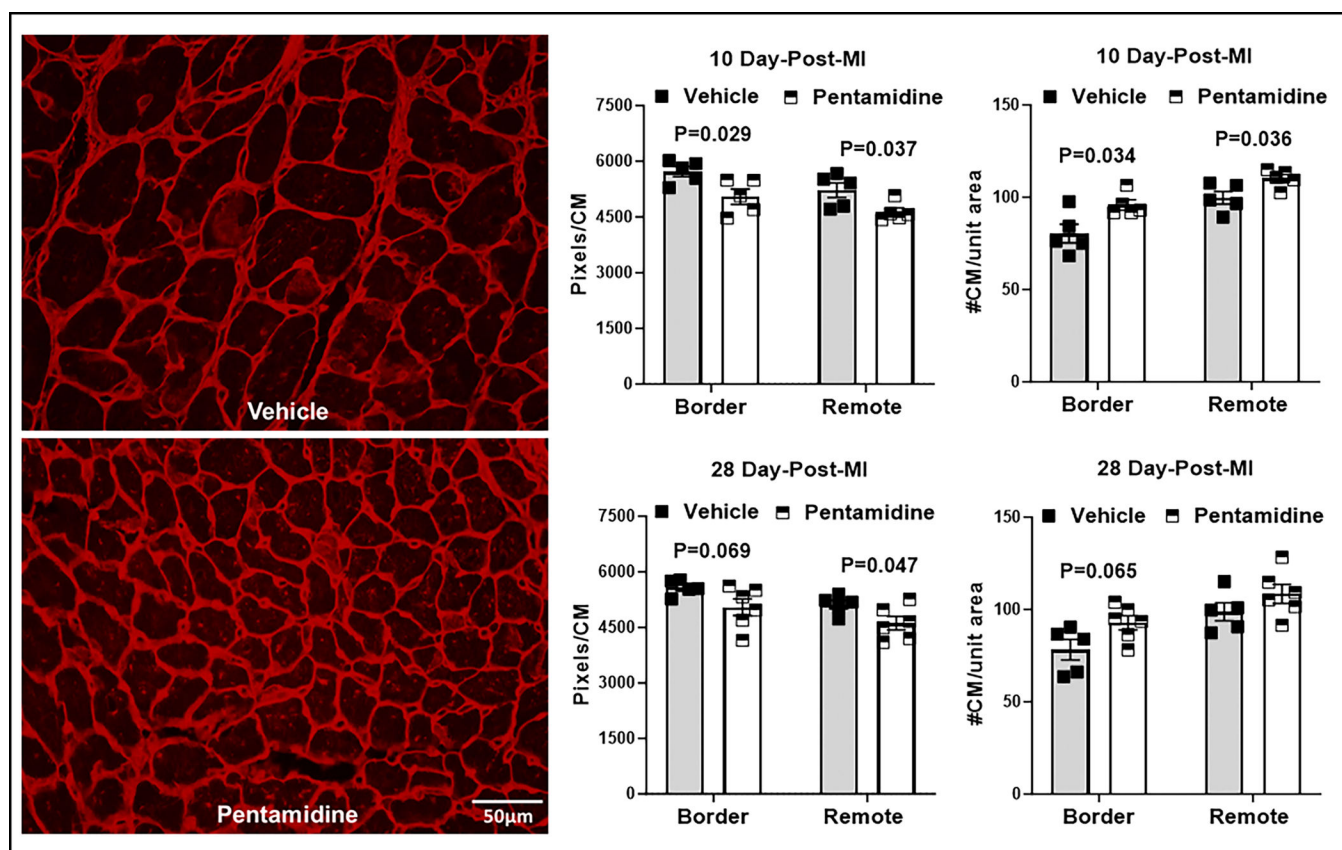


Figure 5. Increased activation of the cell-cycle in CMs within the infarct border zone of pentamidine-treated hearts.

In each panel, the **left column** shows representative immunostains for each cell-cycle activation marker, denoted by nuclear red fluorescence; each cell was identified as a CM per cytoplasmic expression of cardiac troponin-T (cTnT), and cellular outline per wheat germ agglutinin (WGA). White and yellow arrowheads respectively denote CMs and non-CMs. The **middle column** of each panel shows percentages of CMs expressing pH3 (**panel A**), BrdU (**panel B**), and Ki67 (**panel C**) in the infarct border and remote zones at 10 days post-MI; the **right column** shows similar results at day 28 post-MI. Data (mean $\pm$ SEM) are compared using unpaired, two-tailed Student's *t* tests with Welch's correction.



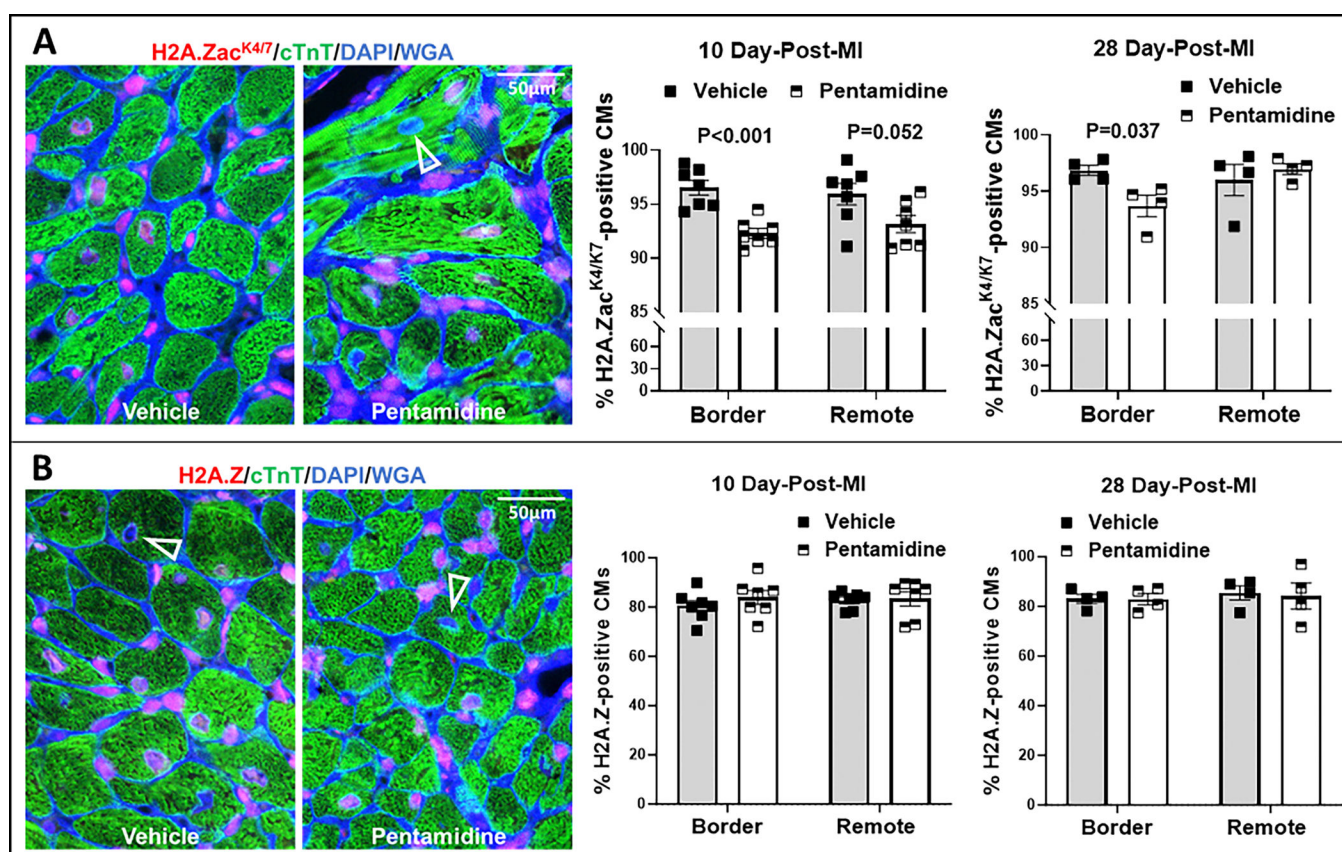


Figure 7. Administration of pentamidine reduced site-specific acetylation of histone H2A.Z in cardiomyocytes (CMs) after myocardial infarction (MI).

Panel A, representative immunostains of acetylated histone H2A.Z at lysines K4 and K7 (H2A.Zac^{K4/K7}) and quantification of H2A.Zac^{K4/K7}-positive CMs, identified by cardiac troponin-T (cTnT) and wheat germ agglutinin (WGA), in the infarct border and remote zones at days 10 (middle) and 28 (right) post-MI. **Panel B**, representative immunostaining (left) and quantification (middle & right) of CMs containing bulk histone H2A.Z. The open arrowhead in panel A denotes an H2A.Zac^{K4/K7}-negative CM; open arrowheads in panel B denote bulk H2A.Z-negative CMs. Data (mean ± SEM) are compared by unpaired, two-tailed Student's t tests with Welch's correction.

Table 1.

Effect of Pentamidine Treatment on the Electrocardiogram (ECG) after Myocardial Infarction (MI).

	Baseline		3 days post-MI		10 days post-MI		28 days post-MI	
	Vehicle N=9	Pentamidine N=9	Vehicle N=9	Pentamidine N=9	Vehicle N=8	Pentamidine N=8	Vehicle N=7	Pentamidine N=6
PR Interval (ms)	38.76±1.35	38.89±1.41	34.90±1.77	33.05±2.10 [†]	33.01±1.43 [†]	34.07±1.66	48.92±1.42 [†]	48.04±1.77 [†]
QRS Interval (ms)	9.23±0.38	9.68±0.32	17.49±1.82 [†]	16.69±2.07 [†]	14.73±1.61 [†]	12.88±0.99	17.08±0.70 [†]	13.47±0.89
QTc (ms)	37.42±2.13 [†]	36.15±1.70	59.21±3.41 [†]	60.88±2.08 [†]	66.76±1.05 [†]	61.72±1.31 [†]	70.33±1.52 [†]	59.20±5.67 ^{†*}
ST Height (μV)	69±22	79±12	-97±46 [†]	-91±46 [†]	-128±20 [†]	-105±17 [†]	-77±2.14 [†]	-62±29 [†]
RR Interval (ms)	138.6±7.9	138.4±4.5	123.0±2.6	127.8±9.1	125.5±3.6	139.2±5.0	157.7±13.4	162.2±8.5

Hearts from C57/Bl6 mice were infarcted and then injected with pentamidine (3 mg/kg) or vehicle (PBS) on 14 consecutive days beginning 3 days after induction of MI. ECGs were obtained via needle electrodes under general anesthesia with isoflurane using the lead II configuration. Data are mean ± SEM.

* P<0.05 vs. vehicle and

[†] P<0.05 vs. baseline value analyzed by two-way repeated measures ANOVA followed by Bonferroni's (effect of treatment) and Dunnett's (effect of time) multiple comparisons.