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# Neuroprotective antioxidant STAZN protects against myocardial ischemia/reperfusion injury

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ABSTRACT

*Background:* Protecting the myocardium from ischemia-reperfusion injury has significant potential to reduce the complications of myocardial infarction and interventional revascularization procedures. Reperfusion damage is thought to result, in part, from oxidative stress. Here we use a novel method of percutaneous coronary occlusion to show that the potent antioxidant and neuroprotective free-radical scavenger, stilbazulenyl nitrone (STAZN), confers marked cardioprotection when given immediately prior to reperfusion.

*Methods and results:* Physiologically controlled male Sprague-Dawley rats were anesthetized with isoflurane, paralyzed with pancuronium and mechanically ventilated. A guide wire was introduced via the femoral artery and advanced retrogradely via the aorta into the left coronary artery under fluoroscopic guidance. Rats with established coronary ischemia (85 min after occlusion) were given STAZN 3.5 mg/kg or its vehicle 5 min before and 2 h after reperfusion, and were subjected to functional and histopathologic studies at 3 days. Ischemia-associated Q wave amplitude was reduced by 73% in STAZN-treated rats ($P = 0.01$), while infarct-related ejection fraction, fractional shortening and severe regional wall-motion impairments were improved by 48%, 54% and 37%, respectively, relative to vehicle-treated controls ($P = 0.05$). Total myocardial infarct volume in STAZN-treated rats was correspondingly reduced by 43% ($P < 0.05$), representing a sparing of 14% of the total left ventricular myocardium.

*Conclusions:* STAZN, a second-generation azulenyl nitrone with potent neuroprotective efficacy in brain ischemia, is also a rapidly acting and highly effective *cardio*protective agent in acute coronary ischemia. Our results suggest the potential for clinical benefit in the setting of acute coronary syndromes.



## 1. Introduction

Thrombolytic therapy and percutaneous coronary interventions reduce mortality from acute myocardial infarction (AMI), and it has become standard practice to administer these treatments as early as possible [1]. However, adjunctive therapeutic strategies are needed because significant mortality and disability ensue despite the timely administration of these therapies, which are intended to restore blood flow following ischemia. Elevated levels of oxidizing free radicals

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following reperfusion provide a rationale for the testing of antioxidants in models of myocardial ischemia/reperfusion injury [2]. Previous animal studies have shown therapeutic cardioprotective effects for various antioxidants. The lack of successful translation of these strategies to the clinic to-date [3] may relate, not only to possible inter-species differences in drug efficacy, but also to the fact that many previous experimental studies have involved treatments administered only at the onset of ischemia and have failed to substantiate a beneficial effect at longer survival periods [4].

Controversy as to the role of reactive oxygen species (ROS) in cardioprotection also stems from observations of others that the cardioprotective effects of acute preconditioning [5] and of preconditioning-mimetics such as opioids [6] or volatile anesthetic agents [7] are blocked by ROS scavengers, suggesting that ROS are actually necessary for cardioprotective signaling. It is possible that complete neutralization of oxidative stress might reduce both its salutary and damaging influences.

Stilbazulenyl nitrone (STAZN) is a novel, second-generation azulenyl-nitrone free-radical scavenger that confers enduring neuroprotection in models of transient focal cerebral ischemia [8]. In pharmacokinetic and biodistribution studies, STAZN exhibited a long circulating half-life and attained significant myocardial tissue levels. Given a common role of free radicals in cerebral and myocardial reperfusion injury, we hypothesized that STAZN might be cardioprotective in a model of AMI in rats.

## 2. Materials and methods

### 2.1. Chemical synthesis of STAZN

STAZN was synthesized from guaiazulene in an efficient five-step procedure that has been published in detail, and its purity was confirmed via reference to archived spectroscopic data for this compound [9].

### 2.2. Animal preparation

All studies were approved by the University of Miami's Animal Care and Use Committee. Twelve male Sprague-Dawley rats ($492 \pm 34$ g (S.D.), age ~5 months) were allowed rat chow and water ad libitum before surgery. Anesthesia was induced with 4% isoflurane, 70% nitrous oxide and a balance of oxygen delivered into a closed jar. To permit controlled ventilation, rats were orotracheally intubated (2.1 mm o.d. × 45 mm B&D Insyte catheter tubing; Becton Dickinson Infusion Therapy Systems Inc., Sandy UT), and ventilated with a rodent respirator (Stoelting Co., Wood Dale, IL) on a mixture of 70% nitrous oxide, 1.0–2.0% isoflurane and a balance of oxygen passed through a humidifier containing 1 ml of Mucomyst-10 (acetylcysteine) in water. Animals were immobilized with pancuronium bromide (0.75 mg/kg i.v.). The left femoral arteries and veins were cannulated with PE-50 polyethlene tubing. Arterial blood pressure was continuously monitored (model RS3400 polygraph; Gould Instrument Systems Inc., Cleveland, OH). Arterial blood gases ($pO_2$, $pCO_2$, pH) were measured (model ABL 330; Radiometer America Inc., Westlake, OH) and maintained in the normal range by ventilatory adjustments. With rats in a supine position, ECG needle electrodes were placed subcutaneously in all four limbs. The precordial (chest) lead was placed (under fluoroscopic guidance) to the left of the sternum between the fourth and fifth ribs, approximately at the mid-clavicular line. Three limb leads, the corresponding unipolar leads and the precordial lead were continuously monitored (ECG switch box, Bio Amp, and Chart 4 software, ADInstruments Inc., Colorado Springs, CO). Rectal temperature was monitored by a thermistor and maintained at $36.5 \pm 0.5$ °C by a heating pad or heating lamp (CMA/150 Temperature Controller; CMA/Microdialysis AB, Stockholm, Sweden).

### 2.3. Left coronary artery occlusion (LCAo)

The left coronary artery was reversibly occluded for 90 min by a novel percutaneous method employing the intraluminal insertion of a guidewire. A custom-designed, coated microcatheter (O.D. 940 μm, I.D. 760 μm, VasCon LLC, Miami FL) with a smooth bullet-shaped head and loaded with a 360 μm guidewire (Choice PT-Plus, Boston Scientific, Maple Grove, MN) was introduced via the right femoral artery by inguinal cut-down. Under X-ray fluoroscopic guidance, the microcatheter was advanced retrogradely into the descending aorta; the wire was then used to navigate the bend of the aortic arch while the catheter was advanced over it and thence into the ascending aorta until it abutted against the aortic valve. The microcatheter was then retracted slightly and served as a guide for the wire. The wire was advanced outside the microcatheter until it reached the left aortic sinus.

To reduce the incidence of fatal ventricular tachycardia and fibrillation subsequent to arterial occlusion, two preventive strategies were employed: moderate hypotension and prophylactic lidocaine. By briefly increasing the inspired isoflurane from 2.0% to 4.0%, it was possible to induce moderate hypotension ($77 \pm 5$ mmHg, range: 71–89 mmHg) at the time of the onset of ischemia. The rats received a prophylactic dose of lidocaine (10 mg/kg, i.v., 1 mg/kg/min) starting 5 min before insertion of the wire and occlusion of the artery, and continuing 5 min thereafter.

Under fluoroscopic guidance, the wire was introduced into the left coronary artery and was advanced until changes were observed on the ECG, indicating successful coronary occlusion. The duration of occlusion was 90 min. Blood pressure and ECG were continuously monitored. Blood gases were measured 15 and 85 min after insertion of the wire and were maintained in the normal range by ventilatory adjustments. Rats were randomized to receive two doses of either STAZN (3.5 mg/kg i.v. in 30% Solutol HS 15 and 70% isotonic saline at 2 ml/h, $n = 6$) or vehicle (0.37 ml/kg 30% Solutol HS 15 and 70% isotonic saline at 2 ml/h, $n = 6$). These agents were infused i.v. over 5 min, beginning at 85 min of arterial occlusion and again at 2 h of reperfusion, respectively. After 90 min of occlusion, the wire was withdrawn, the catheters removed, the incisions closed, and rats returned to their cages. Weight, temperature and ECG were recorded 1, 2 and 3 days afterwards by an investigator blinded to the treatment.

### 2.4. Electrocardiography

ECG's were recorded using PowerLab with an ECG BioAmp (ADInstruments, Colorado Springs, CO). Signal-averaged ECG's were generated by the superposition of six waveforms and the calculation of the mean amplitude at each data point.

### 2.5. Echocardiography

Echocardiography was performed in all rats (a) at baseline within a week prior to myocardial ischemia; and (b) again on the third day following ischemia. For each of these studies, anesthesia was induced with 2% isoflurane and maintained with 0.5% isoflurane and oxygen delivered by face mask. The heart was studied with a 7.5–10 MHz transthoracic echocardiographic tranducer (Toshiba Powervision 7000). Rats were imaged by B-mode in short- and long-axis views of the ventricle to evaluate ventricular wall-motion defects. Next, in the long-axis view in M-mode, the interventricular septal thickness, posterior wall thickness and the left ventricular dimensions were evaluated in systole and diastole. The echocardiographic data included heart rate, left ventricular chamber in end-diastole, left ventricular chamber in end-systole, ejection fraction, and fractional shortening. To quantify and localize ventricular wall-motion abnormalities, we developed a Wall Motion Score system in which 2D cross-sectional images of the short-axis view, at a level slightly apical to the papillary muscle, were divided into four sectors (left and right anterior and left and right posterior) and each quadrant was assigned a score between 0 and 2, where normokinesia = 2 and akinesia = 0.

### 2.6. Histopathology

Three days after LCAo, animals were deeply anesthetized with isoflurane and perfused (via a polyethylene catheter ligated in the inferior vena cava, just superior to the renal veins) with isotonic saline for 3–5 min followed by FAM (40% formaldehyde, glacial acetic acid and absolute methanol, 1:1:8 by volume) for 20 min, at a perfusion pressure of 100–120 mmHg. Hearts were immersed in FAM and stored for 48 h at 4 °C. Hearts were cut transversely into 5-mm blocks within a rat-heart matrix and were paraffin-embedded (Leica TP1050 tissue processor; Leica Inc., Deerfield IL). From apex to base, 16 transverse sections, each 10 μm thick, were stained by hematoxylin and eosin (H&E) and examined by brightfield microscopy. Comparisons of anatomical landmarks assured that similar sections were taken from all animals.

### 2.7. Morphometry and image-analysis

Microscopic sections were also imaged by a high-resolution CCD camera interfaced to an MCID image-analysis system (Imaging Research, Inc., St. Catherines, Ontario, Canada). Image-analysis was conducted by an operator blinded to the treatment-group assignment. Brightfield microscopy revealed infarcts composed of a central core of coagulation necrosis surrounded by a region of infiltrating neutrophils. The infarcted area was quantified along with the total area of the left ventricle and septum. Myocardial volumes were then computed across transverse levels by numerical integration.

### 2.8. Determining the area at risk

In a separate series of four male Sprague-Dawley rats ($512 \pm 45$ g), LCAo was performed as described above. Fifteen minutes after insertion of the occluding wire, an Evans Blue solution (5 ml/kg 4% Evans Blue in isotonic saline) was administered i.v. Five minutes later, the heart was arrested by intravenous injection of 1 ml/kg of saturated potassium chloride in saline. The heart was removed, washed with PBS and stored at −80 °C for 2 days. Eight transverse sections were taken every 2 mm (each 10 μm thick). Microscopic sections were imaged by the MCID image-analysis system to reveal the area of Evans Blue staining as well as total myocardial area. The area at risk was defined as the region unstained by Evans Blue.

### 2.9. Statistical analysis

Data are reported as mean ± S.D. Data were analyzed by analysis of variance (ANOVA) with repeated-measures when appropriate. Inter-group differences were considered significant at $P = 0.05$.

## 3. Results

### 3.1. Model development

Proximal occlusion of the left coronary artery in rats often quickly results in fatal ventricular arrhythmias [10]. In preliminary studies to validate the efficacy of our percutaneous coronary occlusion model, 80% of initially studied animals (not reported here) developed ventricular fibrillation (VF) and sudden cardiac death within the first 10 min of ischemia. To reduce the mortality rate, we next investigated the use of prophylactic lidocaine (10 mg/kg infused for 10 min at 1 mg/kg/min beginning 5 min prior to arterial occlusion). Lidocaine is used to reduce the incidence of VF during acute myocardial ischemia both clinically as well as in experimental models [11]. Although this reduced the incidence of VF, the mortality rate remained approximately 50%. Upon review of animals which survived, we found that *moderate hypotension* (60–80 mmHg) protected against VF, in accordance with previously published results [12]. The combination of prophylactic lidocaine (10 mg/kg) and moderate hypotension (60–80 mmHg) completely prevented VF in the animals used for this study. Initial studies (not reported here) suggested that 90 min of ischemia afforded larger, more consistent infarcts than 60 min.

### 3.2. Study groups

In this study, we attempted to simulate the time-course of clinical ischemia and reperfusion events by administering treatment at 85 min of ischemia. Twelve rats were included in the main study. Six rats received STAZN (3.5 mg/kg dissolved in 30% Solutol and 70% isotonic saline), and six rats received an

**Table 1 – Physiological variables**

| | STAZN ($n = 6$) | Vehicle ($n = 6$) |
|---|---|---|
| Before LCAo (15 min) | | |
| Rectal temperature (°C) | 37.1 ± 0.2 | 37.1 ± 0.2 |
| Arterial pH | 7.32 ± 0.05 | 7.35 ± 0.02 |
| $PaO_2$ (mmHg) | 131 ± 30 | 145 ± 15 |
| $PaCO_2$ (mmHg) | 38.0 ± 1.2 | 37.4 ± 1.5 |
| MABP (mmHg) | 87 ± 6 | 86 ± 4 |
| Plasma glucose (mg/dl) | 293 ± 47 | 267 ± 24 |
| Body weight | 488 ± 41 | 495 ± 30 |
| After LCAo (90 min) | | |
| Rectal temperature (°C) | 37.0 ± 0.1 | 37.0 ± 0.1 |
| Arterial pH | 7.31 ± 0.06 | 7.30 ± 0.01 |
| $PaO_2$ (mmHg) | 107 ± 9 | 114 ± 21 |
| $PaCO_2$ (mmHg) | 40.9 ± 4.5 | 41.1 ± 3.0 |
| MABP (mmHg) | 91 ± 8 | 83 ± 6 |
| During survival period | | |
| Rectal temperature (°C) – 1 day | 38.1 ± 0.4 | 37.9 ± 0.4 |
| Body weight – 1 day | 476 ± 37 | 478 ± 27 |
| Rectal temperature (°C) – 2 days | 38.2 ± 0.3 | 38.0 ± 0.7 |
| Body weight – 2 days | 472 ± 39 | 463 ± 24 |
| Rectal temperature (°C) – 3 days | 37.7 ± 0.7 | 37.9 ± 0.4 |
| Body weight – 3 days | 470 ± 40 | 454 ± 26 |

Value are mean ± S.D.; LCAo, left coronary artery occlusion; MABP, mean arterial blood pressure.

equivalent volume of vehicle (0.37 ml/kg) (Three additional rats that received vehicle died prematurely (two within 24 h and one within 48 h) and were discarded.) ST-segment elevation was observed in all animals following insertion of the wire into the coronary artery. Physiological variables were not significantly different between STAZN-treated and vehicle-treated groups, as shown in Table 1.

### 3.3. *Relative risk of infarction greater for myocardium at the apex than at the base*

In the four rats studied for area at risk, the myocardium at risk of infarction was defined as that tissue-section area from which Evans Blue dye was excluded. By integrating the unstained area and the total area of eight sections, taken equidistantly along the length of the heart from apex to base,

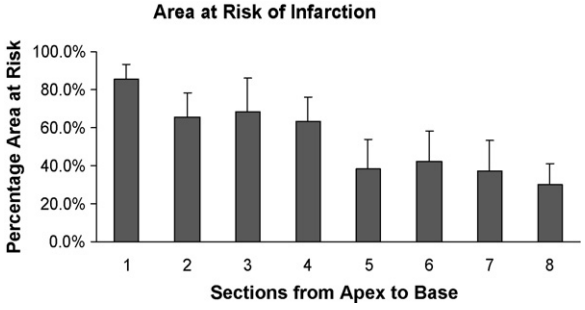


**Fig. 1 – Myocardial areas at risk of infarction, as demonstrated by Evans Blue exclusion, expressed as a percentage of total myocardial areas (mean ± S.D.). Sequential levels from apex (section 1) to base (section 8); the inter-section interval was 2 mm.**

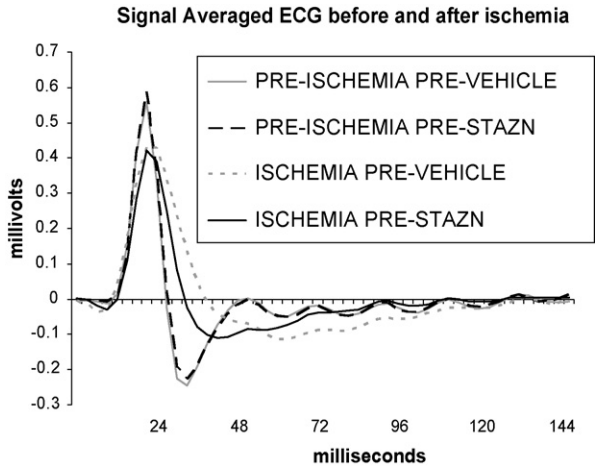


**Fig. 2 – Signal-averaged electrocardiograms in rats subjected to myocardial ischemia, acquired at 85 min of ischemia, just prior to treatment with either STAZN ($n = 6$) or vehicle ($n = 6$).**

we calculated that, after 15 min of intraluminal arterial occlusion, 50 ± 15% of the myocardium was hypoperfused and at risk of infarction. Consistent with observations in other models of acute myocardial ischemia, the percentage area at risk was greater at apical than at basal levels (Fig. 1) [13,14]. Thus, the average area at risk constituted 86 ± 8% at the most apical level, 63 ± 13% in the fourth, middle section (apex versus middle, $P < 0.05$), and 30 ± 11% in the eighth section near the base (middle versus base, $P < 0.05$).

### 3.4. *STAZN reduces electrocardiographic changes after reperfusion*

ECG's were recorded before, during, and up to 3 days after myocardial ischemia. The data are presented in Fig. 2 as two sets of signal-averaged electrocardiograms, in six STAZN-treated and six vehicle-treated rats. The baseline ECG prior to the onset of ischemia did not differ significantly between the two groups. In all rats of both groups, ischemia led to significant and equivalent ST-segment elevation prior to treatment. In rats destined to receive STAZN, the ST-segment (identified as per the method of Fukazawa et al. [15]) was elevated from −0.244 to −0.168 mV ($P < 0.05$); and in animals that subsequently received vehicle, the ST-segment was elevated from −0.254 to −0.134 mV ($P < 0.01$; NS for differences between treatment groups).

As early as 2 h after the removal of the wire and the onset of recirculation, pathological Q-waves were observed in lead I with significantly greater amplitude compared to baseline ($P < 0.001$, repeated-measures ANOVA) (Fig. 3). Throughout the 72-h recirculation period, Q-wave amplitude was significantly reduced in STAZN-treated rats compared to the vehicle-treated group (repeated-measures ANOVA, $P = 0.01$). At 72-h post-ischemia, mean Q-wave amplitude in the STAZN group was 73% less than in the vehicle group (Fig. 3).

The heart rate calculated from the ECG did not differ significantly between treatment groups, either during

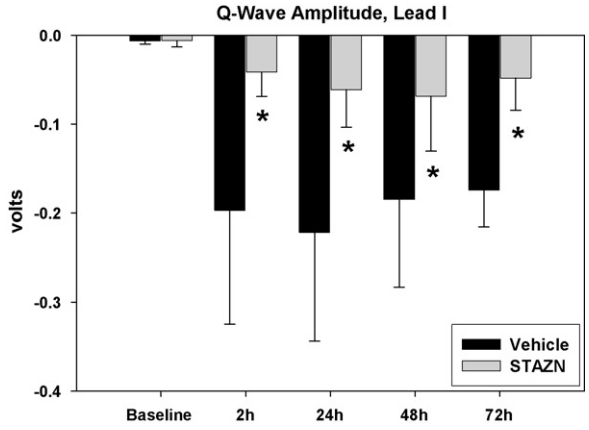


**Fig. 3 – Q-wave amplitude in ECG lead I in STAZN- and vehicle-treated rats at baseline and at 2–72 h following termination of myocardial ischemia (mean ± S.D.). (*) different from vehicle-treated group, $P < 0.05$, two-way repeated-measures ANOVA followed by Holm-Sidak test.**

ischemia or after recirculation. All values fell within one standard deviation of the overall average, 378 ± 43 bpm.

### 3.5. *STAZN preserves myocardial function post I/R injury*

Prior to myocardial ischemia, baseline ejection fraction (EF) averaged 92 ± 3%, and fractional shortening (FS) was 59 ± 5%; values in the STAZN and vehicle groups were identical. Three days after reperfusion, decreases in both ejection fraction and fractional shortening were observed in all animals with respect to baseline values, but the extent of decrease was significantly less for STAZN-treated animals. STAZN reduced the I/R-mediated drop in EF by 48% (post I/R EF = 81% versus 69% for vehicle, $P = 0.046$) and attenuated the decrease in FS by 54% (post-I/R FS 46% versus 35% for vehicle, $P = 0.031$) (Fig. 4).

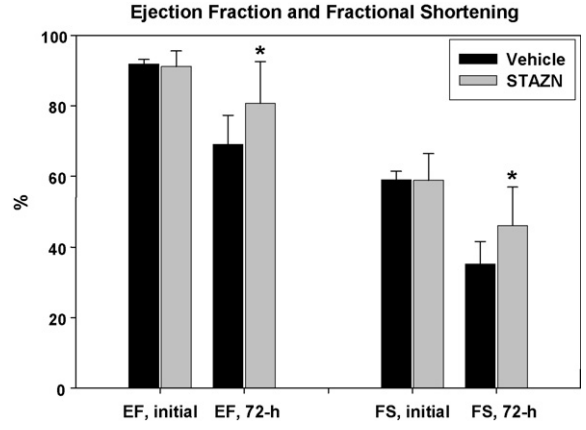


**Fig. 4 – Echocardiographic findings in rats with myocardial ischemia treated with either STAZN ($n = 6$) or vehicle ($n = 6$). Ejection fraction (EF) and fractional shortening (FS) measured before and 3 days following myocardial ischemia. (*) different from vehicle-treated group, $P < 0.05$, Student t-test.**

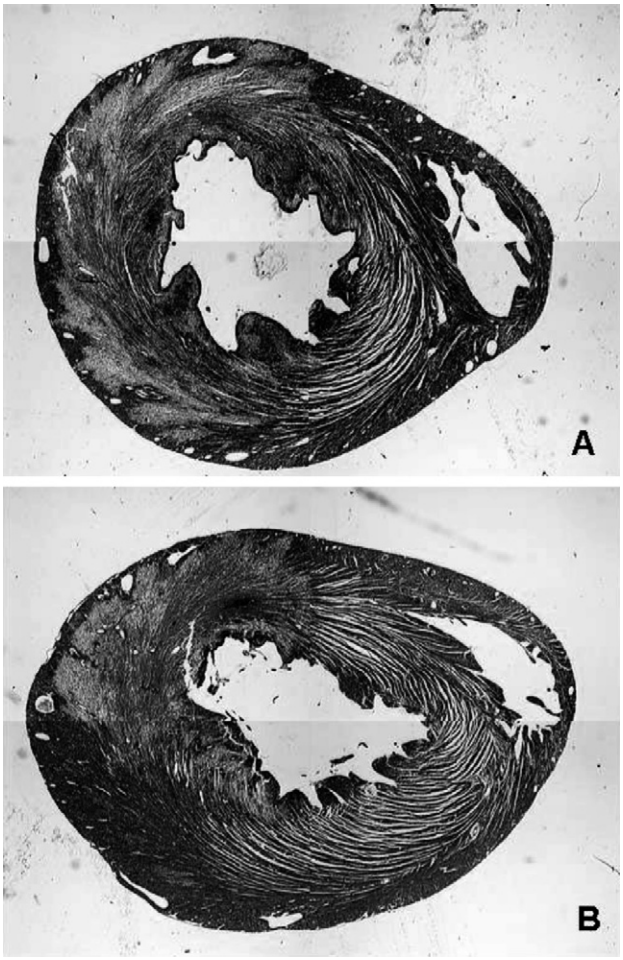


**Fig. 5 – H&E stained myocardial sections at level 8 (between apex and base) 3 days following myocardial ischemia. Specimens were selected from the animal with the greatest total percentage infarct in each group. (A) Vehicle-treated rat. Infarct size, 52.3%. (B) STAZN-treated rat. Infarct size, 42.9%.**

Wall-motion scores in the left anterior quadrants showed that STAZN-treated rats had significantly less severe motion defects than vehicle-treated animals (25% versus 67%, $P = 0.05$, Fisher exact test), demonstrating that regional as well as global ventricular function post MI was reduced in the vehicle group.

### 3.6. *STAZN reduces myocardial histological damage after ischemia-reperfusion*

The extent of myocardial damage was assessed morphologically after 3 days of reperfusion using standard histological methods [16]. The injured tissue was readily identified as a sharply defined area of confluent coagulation necrosis surrounded by a margin of infiltrating neutrophils (Fig. 5).

Total integrated myocardial infarct volume, which averaged 412 ± 71 $mm^3$ in vehicle-treated rats, was reduced by 43%, to 234 ± 114 $mm^3$, in STAZN-treated animals – a highly significant protective effect ($P < 0.01$). Infarct size as a

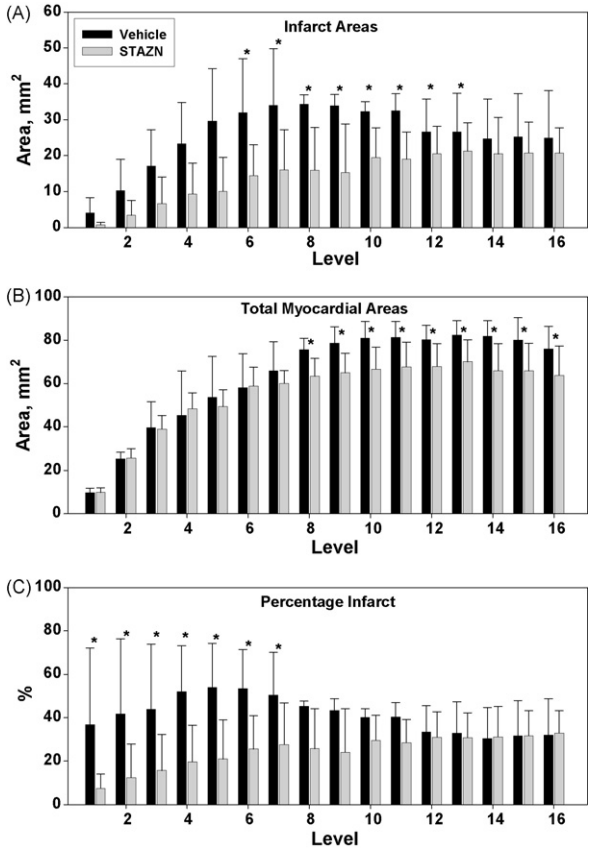


**Fig. 6 – Histological measurements on H&E-stained myocardial section 3 days following myocardial ischemia, in STAZN- and vehicle-treated rats. (A) Infarct areas (mean ± S.D.) at sequential levels from apex (section 1) to base (section 16); inter-section interval, 1 mm. (B) Total myocardial areas (mean ± S.D.). (C) Infarct areas expressed as percentages of corresponding myocardial areas (mean ± S.D.). (*) difference between vehicle-treated and STAZN-treated groups, $P < 0.05$, two-way repeated-measures ANOVA followed by Holm-Sidak tests.**

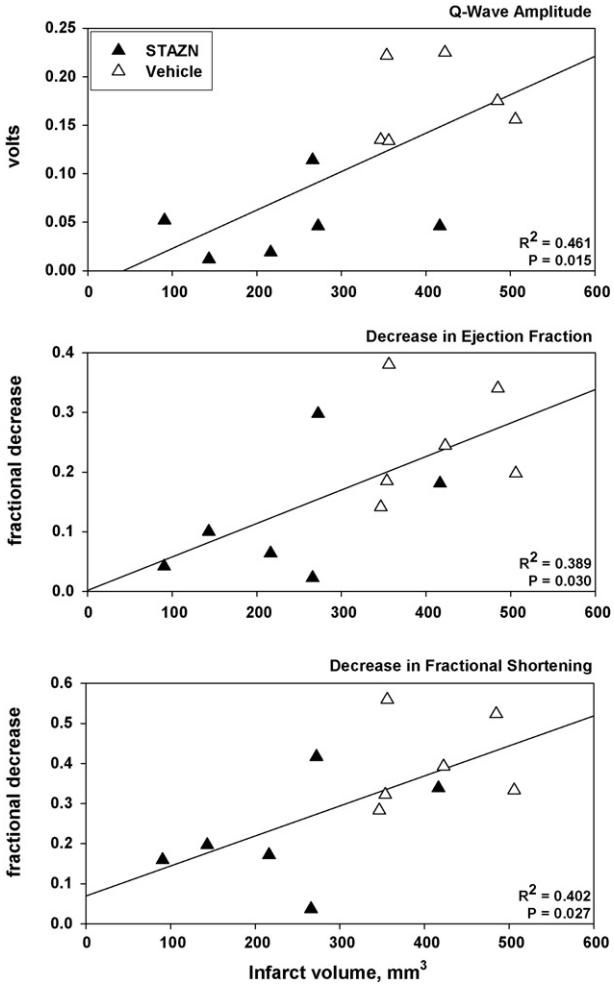


**Fig. 7 – Linear regression analysis of histological cardiac infarct volume vs. various physiological injury markers in individual STAZN- and vehicle-treated animals. Upper panel, Q-wave amplitude at 72 h; middle panel, decrease of ejection fraction at 72 h; lower panel, decrease in fractional shortening at 72 h.**

percentage of total left ventricular mass was also significantly reduced (41 ± 7% [vehicle] versus 27 ± 13% [STAZN], $P < 0.05$).

Myocardial infarct areas are shown in Fig. 6A. Two-way repeated-measures ANOVA revealed a highly significant effect of group (STAZN versus vehicle, $P = 0.009$), a highly significant effect of level ($P < 0.001$), and a significant group × level interaction ($P = 0.03$). STAZN reduced infarct areas at multiple central levels (levels 6–13, $P < 0.05$, Holm-Sidak tests). Total cardiac areas are shown in Fig. 6B. Repeated-measures ANOVA revealed a highly significant overall difference with respect to group × level interaction ($P = 0.007$), denoting the trend toward greater myocardial mass in vehicle- than in STAZN-treated animals at basal levels (levels 8–16 in Fig. 6B).

Infarct areas expressed as percentages of corresponding cardiac areas are shown in Fig. 6C. Repeated-measures ANOVA again revealed an effect of treatment group ($P = 0.02$), with apical levels 1–7 showing significant STAZN-induced percentage-infarct reduction ($P < 0.05$, Holm-Sidak test).

### 3.7. *Myocardial damage-reduction correlates with functional preservation by STAZN*

In a pooled analysis of the 12 STAZN- and vehicle-treated rats, myocardial infarct volume computed from sequential area measurements in individual animals was closely correlated to postischemic measures of myocardial function, namely, to: (a) Q-wave amplitude at 72 h (Fig. 7, upper panel; $R^2 = 0.461$, $P = 0.015$); (b) the extent of postischemic decrease in ejection fraction (Fig. 7, middle panel; $R^2 = 0.389$, $P = 0.03$); and (c) the extent of postischemic decrease in fractional shortening (Fig. 7, lower panel; $R^2 = 0.402$, $P = 0.027$).

## 4. Discussion

The results of this study establish that the azulenyl-nitrone antioxidant, STAZN, given at the onset of recirculation after 90 min of focal myocardial ischemia, confers significant

cardioprotection. Within 2 h after the onset of reperfusion, the Q-wave amplitude in animals treated with STAZN was significantly less than in those treated with vehicle (see Fig. 2). The cardioprotective effects of STAZN persisted 3 days later, when a significant correlation was observed between functional (ECG and echocardiograpy) and morphological indices (Fig. 7) [17].

STAZN is a member of a class of compounds possessing a nitrone group, which, although not found in natural products, can react with free radicals and prevent free-radical-mediated damage *in vivo*. The nitrone moiety can be incorporated into molecules with different structures and still maintain its ability to combine with reactive free radicals to yield more stable nitroxide products and provide therapeutic neuroprotection [18] and cardioprotection [19]. The STAZN molecule, which contains *two* nitrone moieties attached to azulene groups, was designed to have greater antioxidant potency and lipophilicity than other nitrones, in order to inhibit lipid peroxidation more effectively [9]. STAZN confers sustained, high-grade neurological and histological protection (for up to 30 days) after transient focal cerebral ischemia in rats [8,20], reduces contusion size after traumatic brain injury in rats [21], and protects against MPTP and 3-nitropropionic acid neurotoxicities in animal models of Parkinson and Huntington's diseases [22]. The neuroprotective and cardioprotective effects of STAZN are similar to those of edaravone, another free-radical scavenger which confers therapeutic protection against both stroke [23] and also AMI [24], suggesting a common role of oxidizing free radicals in ischemia/reperfusion injury in the heart and brain.

In contrast with other antioxidants, such as superoxide dismutase and catalase, which were not effective when given at the time of reperfusion [25], STAZN was cardioprotective when given at reperfusion after 90 min of ischemia. Unlike superoxide dismutase and catalase, which have a short plasma half-life and do not penetrate cell membranes [26], STAZN has a circulating half-life of ~14 h and appears in the forebrain and myocardium at 2.5% and 56% of blood levels, respectively, within 3 h after administration [8]. There are obvious clinical advantages to long-lasting agents such as STAZN that could be delivered during therapeutic reperfusion and continue to act during the period of highest risk of abrupt coronary re-occlusion.

Although the exact therapeutic mechanism of nitrones such as STAZN is not known, reduction of levels of reactive oxygen species [27] and the production of nitric oxide [28] are two possibilities. Interestingly, these two mechanisms have also been proposed for postconditioning – the phenomenon in which rapid intermittent interruptions of blood flow applied at the onset of reperfusion result in cardioprotection, as demonstrated both in animal models and humans [29]. By understanding the mechanisms of postconditioning, it is possible that pharmacological agents may be discovered that are able to mimic or enhance postconditioning. This would be advantageous as the mechanical implementation of postconditioning is not always feasible and its efficacy is known to decline with the duration of reperfusion [30].

In seminal studies in dogs [31], despite coronary artery occlusion at the same anatomical site, wide variation was seen in the extent of the myocardium hypoperfused following occlusion and in the size of resultant infarcts. Thus, in experimental studies of myocardial infarction, measurement of the area at risk is often presented as a necessary control for each animal [32], with the infarct size being normalized and expressed as a percentage of the area at risk. Because of large inter-animal variation in infarct size in canine models, the normalization to area at risk appears reasonable in this species [33]. In humans, while the size of the occluded coronary vascular bed is a determinant of infarct size, the difficulty of measuring the size of the risk-zone in humans [34] precludes defining or normalizing clinical infarct size as a percentage of the area at risk.

In rat models of myocardial infarction produced via coronary artery ligation, various studies have reported similar values for the percentage of the left ventricle at risk of infarction [35,36]. While some studies excluded animals if the area at risk was below a threshold (usually approximately 20%), this exclusion procedure resulted in no significant differences in area at risk between treatment groups [37,38]. Thus, in rat models of coronary ischemia, expressing infarct size as a percentage of the area at risk appears superfluous in that the area of infarction is being normalized by a value that is roughly the same for all animals irrespective of the treatment group. In contrast to our method, in which the position of the guidewire in the left coronary artery is visualized fluoroscopically, variation in the placement of the suture for external ligation may cause variation in the area at risk and even failure to occlude the left coronary artery (consistent with the need to exclude some animals with small risk regions) [39].

In our study, it was not feasible to measure the area at risk in each animal by reinserting the wire after 3 days of recovery; to do so would have entailed attempting to re-occlude the left coronary artery exactly as it had been occluded 3 days earlier. Nonetheless, in separate animals an effort was made to measure the variability in the pattern of distribution of the occluded coronary artery. Because the size of the area at risk is a determinant of infarct size, consistency in the size of the area at risk would predict consistency in the size of infarcted tissue [40]. We showed that the risk region produced by our novel method of myocardial infarction is consistent across animals – Evans Blue dye was excluded from approximately $50 \pm 15\%$ (S.D.) of the myocardium.

An intriguing aspect of the cardioprotective effects of STAZN in this study is that the relative extent of infarct size reduction was more substantial (and statistically significant) for the apical myocardium than for the base. The observation that the percentage area at risk decreased from apex to base (see Fig. 1) can be explained by coronary artery anatomy and is consistent with other studies [13,14]. Hale and Kloner found that the extent of necrosis was greater toward the apex than toward the base in an arterial ligation model in rabbits [13]. This was true in controls and in those animals receiving phenylephrine or preconditioning *before* the onset of ischemia. We observed that, when STAZN was given at the onset of reperfusion *after* 90 min of ischemia, the percentage of myocardium infarcted was significantly reduced only in apical sections 1–7 (Fig. 6C).

Although the animals in different treatment groups did not have statistically different body weight or total myocardial volume, the myocardial volume of basal sections (levels 8–15) was significantly greater in vehicle-treated rats ($641 \pm 58$ mm$^3$)

than STAZN-treated animals (532 ± 71 $mm^3$) ($P < 0.02$) (Fig. 6B). This may reflect compensatory ventricular hypertrophy. Myocyte hypertrophy has been described as early as 3 days after infarction in coronary artery ligation models [41] and can be seen within 2–3 weeks after onset of hemodynamic loading in mouse and rat models of myocardial ischemia-reperfusion injury [42,43]. Hypertrophy is a major element of ventricular remodeling after MI, and a frequent precursor of congestive heart failure. Decreasing the extent of infarction can limit ventricular hypertrophy and remodeling, and prevent the progression to heart failure. Thus, this novel animal model may be useful for further investigations of myocardial infarction, ventricular remodeling and heart failure.

An advantage of occluding the proximal left coronary artery by means of an intraluminal guidewire might be to reduce the variation in infarct size seen after external ligation of the distal artery, which results from anatomical differences in arterial branching [39]. Wire-induced ischemia, of very short duration, was earlier reported by Urasawa et al. [44]. Building on those observations, we have developed a novel, non-invasive method for producing experimental myocardial infarction, in which a percutaneous guidewire is used to occlude the lumen of the rat left coronary artery. Unlike the standard model involving external ligation of the coronary artery [45], our approach does not require a thoracotomy. After the wire is removed, the femoral artery is ligated and the small incision is closed. This greatly reduces perioperative complications and facilitates long-term survival studies. Wire-induced intra-luminal arterial occlusion may be feasible in other organs; this would allow comparisons and contrasts of ischemia/reperfusion injury in different tissues and the investigation of remote preconditioning and postconditioning.

*Study limitations*: In compliance with institutional protocols, animals were anesthetized to reduce pain and suffering. However, both clinical and experimental studies have shown that anesthetics can confer significant cardioprotection from ischemia/reperfusion injury [46]. Although anesthesia was controlled in both treatment groups, therapeutic effects may have resulted from the combination of STAZN and anesthesia.

In conclusion, the neuroprotective drug STAZN, a free-radical scavenger and potent inhibitor of lipid peroxidation, lessens functional and histological deterioration in acute myocardial infarction when it is administered at the time of recirculation. Antioxidants such as STAZN are promising adjunctive therapeutics for further research to limit reperfusion injury in stroke, AMI and other conditions in which therapy may be given effectively at the onset of blood-flow restoration in the treatment of ischemia/reperfusion injury.

## Disclosure

Drs. Becker and Ginsberg are principals in Cognitrone, Inc.

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