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European Journal of Pharmacology 456 (2002) 59–68

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A Novel nonpeptidic caspase-3/7 inhibitor, (*S*)-(+)-5-[1-(2-methoxymethylpyrrolidinyl)sulfonyl]isatin reduces myocardial ischemic injury

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Received 11 July 2002; received in revised form 20 September 2002; accepted 26 September 2002

Abstract

The efficacy of a novel, nonpeptidic, caspase 3/7-selective inhibitor, (*S*)-(+)-5-[1-(2-methoxymethylpyrrolidinyl)sulfonyl]isatin (MMPSI) for reducing ischemic injury in isolated rabbit hearts or cardiomyocytes was evaluated. MMPSI (0.1–10 μM) evoked a concentration-dependent reduction in infarct size (up to 56% vs. control; $IC_{50} = 0.2$ μM). Furthermore, apoptosis (DNA laddering, soluble nucleosomes) was reduced in the ischemic area-at-risk. MMPSI inhibited recombinant human caspase-3 with an $IC_{50} = 1.7$ μM. Apoptosis in H9c2 cells after 16-h simulated ischemia and 2-h simulated reperfusion was significantly reduced by MMPSI in a concentration-dependent manner ($IC_{50} = 0.5$ μM); similar effects were observed in isolated adult rabbit cardiomyocytes ($IC_{50} = 1.5$ μM). These data support an important role for caspase-3/7 in mediating myocardial ischemic injury. Furthermore, these data indicate that cardioprotection via caspase-3/7 inhibition is attainable via a small molecule (nonpeptidic) inhibitor, a necessary step in making this approach therapeutically viable.

Keywords: Apoptosis; Cardiomyocyte; Caspase; Infarct; Ischemia; Reperfusion

1. Introduction

Apoptosis, or programmed cell death, is the result of cellular biochemical events carried out by several mediators, in particular, the family of cysteine proteases referred to as caspases. Compelling evidence indicates that apoptotic cell death plays an important role in a variety of cardiovascular diseases including myocardial infarction, heart failure, and atherosclerosis (Haunstetter and Izumo, 1998). Cardiomyocyte apoptosis has been observed experimentally in whole hearts with either ischemia alone (Black et al., 1998) or ischemia–reperfusion (Black et al., 1998; Gottlieb et al., 1994). In addition, peptidic caspase inhibitors both reduce infarct size (Holly et al., 1999; Huang et al., 2000; Mocanu et al., 2000; Yaoita et al., 1998) and inhibit apoptosis (Holly et al., 1999; Yaoita et al., 1998); several studies have specifically implicated caspase-3 or a caspase-3-like protease (e.g., caspase-7) in mediating myocardial ischemic damage and apoptosis (Bialik et al., 1999; Black et al., 1998; Condorelli et al., 2001; de Moissac et al., 2000; Holly et al., 1999; Piot et al., 1999). Nevertheless, the commonly used peptidic inhibitors display varying degrees of selectivity (both for different caspases and related proteases), and because they are peptides, are of limited utility in a clinical setting. For caspase inhibition to be therapeutically viable for providing cardioprotection, particularly in a chronic setting, small molecule inhibitors need to be identified and demonstrated to be efficacious.

Recently, a class of *nonpeptidic* isatin sulfonamides was reported to selectively inhibit caspase-3/7 activity (Lee et al., 2000). One of these compounds, (*S*)-(+)-5-[1-(2-methoxymethylpyrrolidinyl)sulfonyl]isatin (MMPSI), dem-

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PII: S0014-2999(02)02484-6

onstrated 30-fold selectivity for caspase-3 vs. caspase-9 and greater than 100-fold selectivity vs. caspases-1, 2, 4, 6, and 8. However, to our knowledge, these nonpeptidic caspase inhibitors have yet to be examined for cardioprotective efficacy. Therefore, we postulated that MMPSI would reduce myocardial ischemic injury, and provide additional support for a role for caspase-3/7 in mediating both this injury and cardiomyocyte apoptosis. The cardioprotective and anti-apoptotic efficacy of MMPSI were thus examined in isolated heart and cardiomyocyte models of ischemic injury.

2. Materials and methods

This investigation conforms to the *Guide for the care and use of Laboratory Animals* published by the Institute of Laboratory Animal Resources (NRC, 1996) and was approved by our institutional animal care committee.

2.1. Caspase-3 activity

Inhibition of caspase-3 activity was measured by incubating 10 ng purified human recombinant caspase-3 (Upstate Biotech, Lake Placid, NY) with 10 μM of fluorescent caspase-3/7 substrate Ac-DEVD-AMC (BIOMOL, Plymouth Meeting, PA) in 100 μl assay buffer consisting of 0.1% CHAPS (3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulphonate), 50 mM KCl, 5 mM β-Mercaptoethanol, 25 mM K^+ HEPES, pH 7.5 and various concentrations of inhibitor. After 30-min incubation at 37 °C, fluorescence increases were measured with a SPECTRAmax Gemini XS spectrofluorimeter (Molecular Devices, Sunnyvale, CA) using excitation and emission wavelengths of 400 and 505 nm, respectively. IC_{50} values were determined by nonlinear regression analysis using a four-parameter logistic equation. Reactions omitting enzyme were used as baseline values, while reactions without inhibitor were used as maximum values.

2.2. Isolation of adult cardiomyocytes

Male New Zealand White rabbits (3–4 kg; Covance, Denver, PA) were anesthetized by i.v. administration of sodium pentobarbital (30 mg/kg), followed by intubation and ventilation with 100% O_2 using a positive pressure ventilator. A left thoracotomy was performed and the heart was removed and quickly placed in warmed (37 °C) DMEM/F12 (Dulbecco's Modified Eagle Medium/F12) media containing 100 U/ml sodium heparin. The heart was then quickly mounted on a Langendorff apparatus and washed for 5 min by retrograde perfusion via the aorta with calcium-free oxygenated J-MEM (Joklik Minimum Essential Medium) at 37 °C. After 5 min, the heart stopped beating and the solution was changed to J-MEM containing 0.8 mg/ml collagenase (type 2) (Worthington Biochemical, Lakewood, NJ) and 1 mg/ml bovine serum albumin. The heart was perfused (with recirculation) for 30 min in the collagenase solution at 37 °C. The ventricles were then minced and incubated an additional 15 min with gentle agitation in the same solution at 37 °C. After gentle trituration with a wide mouth pipette, the tissue was filtered through sterile gauze and centrifuged for 2 min at $300 \times g$. The resulting cell pellet was washed once with J-MEM (1 mg/ml bovine serum albumin) and then resuspended in 50 ml of J-MEM (1 mg/ml bovine serum albumin). The suspension was placed into a 37 °C water bath where the calcium concentration was brought slowly to 1.25 mM by addition of sterile 250 mM $CaCl_2$ solution over a 5-min period. The cells were then pre-plated in a 75-cm^2 culture flask for 2 h at 37 °C. Finally, the nonadherent cells were collected by centrifugation, resuspended in DMEM/F12 media containing 0.1 mg/ml bovine serum albumin, and plated in 100 mm (DNA laddering) or six-well (ELISA; Enzyme-Linked ImmunoSorbent Assay) culture dishes precoated with mouse laminin (20 μg/ml). This procedure resulted in >85% rod shaped, striated adult rabbit cardiomyocytes. The cells were allowed to attach for 2 h in a 5% CO_2/37 °C incubator, and then the media was carefully replaced with fresh media either with or without appropriate concentrations of inhibitor. To obtain control cells, some plates were scraped at this point, the myocytes collected by centrifugation, and snap frozen in liquid nitrogen. The remaining plates were placed in an incubator overnight (16 h), during which time the cardiomyocytes underwent apoptosis. For DNA laddering, the cardiomyocytes were scraped from the dishes, collected by centrifugation, snap frozen in liquid nitrogen, and stored at −70 °C until assayed. For detection of soluble nucleosomes, the dishes were centrifuged at $3000 \times g$ for 10 min, the media aspirated, and the cells lysed directly in the dishes, without freezing.

2.3. Induction of apoptosis in H9c2 cells

H9c2 (2-1) cells (rat embryonic cardiac-derived myoblast, ATCC, Manassas, VA) were grown in 100 mM or six-well culture plates in high glucose DMEM containing 10% fetal bovine serum. When cells reached 75% confluency, cells were preincubated for 30 min with various concentrations of inhibitor. This was followed by exposure to simulated ischemia by placing the culture dishes in a chamber purged with 95% N2/5% CO_2, and replacing the media with serum and glucose-free DMEM, containing 20 mM 2-deoxyglucose, with or without inhibitor. After 16 h, the cells were "reperfused" by replacing the culture media with serum-containing high glucose DMEM, with or without inhibitor, and returning the cells to a normoxic environment for an additional 2 h. H9c2 cells were then processed for DNA laddering or soluble nucleosome ELISA as described for the cardiomyocytes.

2.4. Langendorff preparation

Male New Zealand White rabbits (3–4 kg; Covance) were anesthetized by i.v. administration of sodium pentobarbital (30 mg/kg), followed by intubation and ventilation with 100% O_2 using a positive pressure ventilator. A left thoracotomy was performed, the heart exposed, and a snare (2–0 silk) placed loosely around a prominent branch of the left coronary artery. The heart was rapidly removed from the chest, mounted on a Langendorff apparatus, and maintained by retrograde perfusion (nonrecirculating) with a modified Krebs solution (NaCl 118.5 mM, KCl 4.7 mM, Mg SO_4 1.2 mM, KH_2PO_4 1.2 mM, $NaHCO_3$ 24.8 mM, $CaCl_2$ 2.5 mM, and glucose 10 mM) at a constant pressure of 80 mm Hg and a temperature of 38.5 °C. Perfusate pH was maintained at 7.4–7.5 by bubbling with 95% O_2/5% CO_2. The temperature of the heart was maintained by suspending it in a heated, water-jacketed organ bath. A fluid-filled latex balloon was inserted in the left ventricle and connected by stainless steel tubing to a pressure transducer; the balloon was inflated to provide a systolic pressure of 80–120 mm Hg, and a diastolic pressure ≤ 10 mm Hg. Heart rate, left ventricular systolic and diastolic pressures, and left ventricular developed pressures were recorded using a PO-NEMAH Data Acquisition and Archive System (Gould Instrument Systems, Valley View, OH). Total coronary flow rate was determined using an in-line flow probe (Transonic Systems, Ithaca, NY); coronary flow was normalized for heart weight. Each heart was allowed to equilibrate for 30 min and if stable left ventricular pressures within the parameters outlined above were not observed, the heart was discarded. Pacing was not used unless the heart rate fell below 180 beats/min prior to the 30-min period of regional ischemia; in this case, the heart was paced at 200 beats/min, which was the average spontaneous rate observed.

2.5. Langendorff experimental protocols

After a 30-min equilibration period, MMPSI, ZVAD-fmk, DEVD-fmk, or YVAD-cmk was perfused through the hearts for 5 min followed by a 10-min wash-out. Regional ischemia was then elicited for 30 min by tightening the snare around the branch of the coronary artery. At the end of this period, the snare was released and the heart reperfused for an additional 120 min. Control hearts did not receive any drug. At the completion of the experiment, hearts to be analyzed for apoptosis were removed from the Langendorff apparatus, and the left ventricle area at risk and right ventricle free wall (nonrisk area) were dissected, snap frozen in liquid nitrogen, and stored at -70 °C until assayed. Hearts used for infarct size determination were treated as described below.

2.6. Assessment of apoptosis by DNA electrophoresis

The DNA was extracted from frozen samples by standard techniques. Briefly, 100 μg of tissue from the Langendorff preparation was powdered with a mortar and pestle in liquid nitrogen, then resuspended in 1 ml digestion buffer consisting of 100 mM NaCl, 25 mM EDTA, 0.5% SDS, 10 mM Tris, pH 8.0 containing 50 μg/ml RNase (DNase free). One milliliter digestion buffer was directly added to the adult rabbit cardiomyocytes and H9c2 samples from the 100-mm dishes. The samples were then incubated for 1 h at 37 °C with gentle agitation. Proteinase K (0.1 mg) was added and the samples

Table 1
Cardiac function and coronary flow data from isolated rabbit hearts

Group	*n*	Preocclusion			Post-30-min occlusion		
		Heart rate beats/min	Coronary flow, ml min^{-1} g^{-1}	Left ventricular developed pressure, mm Hg	Heart rate beats/min	Coronary flow, ml min^{-1} g^{-1}	Left ventricular developed pressure, mm Hg
Control	5	199 ± 9	6.3 ± 0.2	98 ± 5	183 ± 8	4.0 ± 0.3*	54 ± 7*
ZFA-fmk							
1 μM	5	211 ± 7	6.5 ± 0.3	95 ± 5	209 ± 3	4.9 ± 0.5*	62 ± 5*
10 μM	7	210 ± 7	7.4 ± 0.5	102 ± 4	208 ± 6	6.3 ± 0.5*	79 ± 5*
ZVAD-fmk							
1 μM	5	202 ± 7	6.5 ± 0.3	105 ± 3	201 ± 8	4.8 ± 0.4*	76 ± 4*
10 μM	9	203 ± 2	6.9 ± 0.2	101 ± 3	204 ± 3	5.2 ± 0.3*	69 ± 4*
DEVD-fmk							
2 μM	6	212 ± 8	6.8 ± 0.2	104 ± 3	207 ± 10	5.3 ± 0.3*	77 ± 4*
20 μM	5	214 ± 5	7.6 ± 0.2	103 ± 2	211 ± 8	6.2 ± 0.3*	78 ± 3*
YVAD-cmk							
20 μM	5	216 ± 16	7.1 ± 0.2	105 ± 6	204 ± 17	5.1 ± 0.3*	65 ± 9*
MMPSI							
0.1 μM	5	207 ± 7	5.9 ± 0.7	87 ± 7	192 ± 7	3.2 ± 0.4*	45 ± 3*
1 μM	5	218 ± 12	5.4 ± 0.3	101 ± 5	213 ± 11	3.2 ± 0.1*	52 ± 8*
10 μM	5	215 ± 6	6.0 ± 0.3	95 ± 5	203 ± 6	3.9 ± 0.2*	52 ± 10*

Values are means ± S.E.M. *n*, number of rabbit hearts.
* $P < 0.05$ vs. preocclusion values.

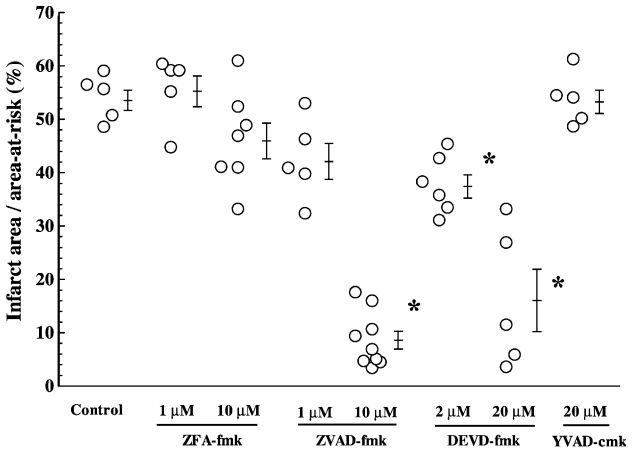

Fig. 1. Effect of peptidic caspase inhibitors on infarct size in isolated rabbit hearts subjected to ischemia–reperfusion injury. Each drug was perfused through the heart for 5 min followed by a 10-min wash-out prior to 30 min of regional ischemia and 120 min of reperfusion, as described in Materials and methods. Infarct area and area-at-risk were determined by image analysis and infarct area was normalized for area-at-risk (%IA/AAR). Data from each heart are presented, along with the mean ± S.E.M. for each group; $n = 5–9$; *significantly different ($P < 0.05$) from control.

were further incubated for 2 h at 50 °C with gentle agitation. The samples were centrifuged for 30 min at 14,000 × *g* and 4 °C. Supernatants were extracted three times with an equal volume of phenol/chloroform/isoamy alcohol (24:24:1). DNA was precipitated by adding cold isopropanol (60% of final volume) and sodium acetate, pH 5.2 (0.1 M final concentration), and standing at −20 °C overnight. DNA was pelleted by centrifugation, washed with 75% ethanol and then resuspended in TE (Tris–EDTA) buffer. 10 μg DNA was electrophoresed through a 1.8% agarose TAE (Tris–acetate EDTA) gel. The gel was stained with SYBR Green (Molecular Probes, Eugene, OR) overnight in TAE and visualized by UV transillumination.

2.7. Determination of apoptosis by nucleosome ELISA

Soluble nucleosomes were detected with a Cell Death Detection ELISA^{plus} (Roche Molecular Biochemicals, Indianapolis, IN). Frozen heart samples were prepared for the assay by powdering 100 mg tissue in liquid nitrogen with mortar and pestle, and then solubilizing in 1 ml lysis buffer for 30 min at room temperature. The samples were then centrifuged for 20 min at 14,000 × *g*, the supernatant diluted 1:50 with phosphate-buffered saline (PBS), and then 20 μl assayed as per kit instructions. Adult rabbit cardiomyocytes and H9c2 cells samples were prepared by aspirating the culture media and adding 1 ml of the lysis buffer directly to each well of the six-well dishes. Cells were allowed to solubilize for 30 min at room temperature, followed by centrifugation for 20 min at 14,000 × *g*, and 20 μl assayed as per kit instructions. For all samples, protein concentrations were determined by the BCA method (Pierce, Rockford, IL) with bovine serum albumin as the standard.

2.8. Determination of infarct size

After completion of each experiment, and with the heart suspended and perfused on the Langendorff apparatus, the coronary artery snare was re-tightened and a 0.5% suspension of fluorescent zinc cadmium sulfide particles (1–10 μm) was perfused through the heart to delineate the area-at-risk (nonlabeled) in the left ventricle for infarct development. The heart was removed from the Langendorff apparatus, blotted dry, weighed, wrapped in aluminum foil and stored overnight at −20 °C. Frozen hearts were sliced into 2-mm transverse sections and incubated with 1% triphenyl tetrazolium chloride in phosphate-buffered saline for 20 min at 37 °C to delineate non-infarcted (stained) from infarcted (nonstained) left ventricular tissue. The infarct area and the area-at-risk were calculated for each slice of left ventricle using video-captured images and ETC3000 image analysis software (Engineering Technology Center, Mystic, CT), followed by adding the values for each tissue slice to obtain the total infarct area and total area-at-risk for each heart. To normalize the infarct area for differences in the area-at-risk between hearts, the infarct size was expressed as the ratio of infarct area vs. area-at-risk (%IA/AAR).

2.9. Assessment of apoptosis by TUNEL staining

Hearts to be processed for TUNEL (Terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end-labeling) were perfusion fixed with 10% buffered formalin for 10 min, removed from the Langendorff apparatus and routinely processed for light microscopy. Five-micron-thick sections from regions of the heart encompassing the area-at-risk were

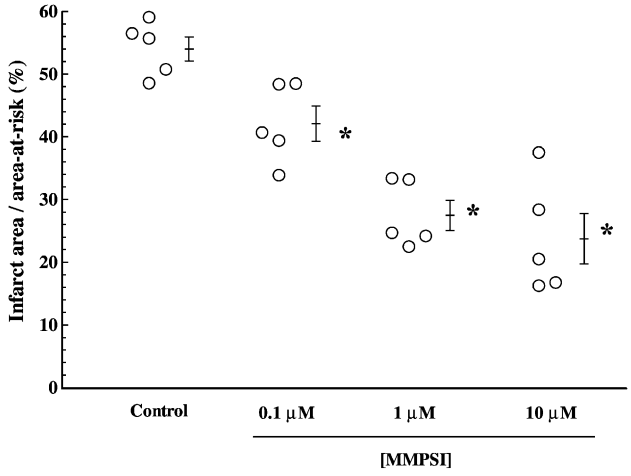

Fig. 2. Effect of MMPSI on infarct size in isolated rabbit hearts subjected to ischemia–reperfusion injury. MMPSI was perfused through the heart for 5 min followed by a 10-min wash-out prior to 30 min of regional ischemia and 120 min of reperfusion, as described in Materials and methods. Infarct area and area-at-risk were determined by image analysis and infarct area was normalized for area-at-risk (%IA/AAR). Data from each heart are presented, along with the mean ± S.E.M. for each group; $n = 5$; *significantly different ($P < 0.05$) from control.

reacted for TUNEL (S-7165, Intergen, Purchase, NY) according to the manufacturers instructions. As a final step, the sections were counterstained with Hoechst 33342 (Molecular Probes) and examined on a Nikon FXA microscope. Nonoverlapping fields totally surrounding the left ventricle were collected and analyzed using Optimus image analysis software (Media Cybernetics) for total nuclei as well as apoptotic nuclei.

2.10. Electron microscopy

Langendorff hearts were perfusion fixed for 10 min with 10% buffered formalin. Identical portions of the left ventricle from control and ischemia-reperfused hearts (area-at-risk) were routinely processed for transmission electron microscopy. Thin sections were examined on a JEOL 1200 microscope.

2.11. Data expression and analysis

Data are expressed as the mean ± S.E.M. Between group comparisons of areas-at-risk expressed as a percent of left ventricular areas (%AAR/LV) were compared using ANOVA. Cardiac function/coronary flow parameter comparisons were performed by *t*-test, while %IA/AAR values were compared using a Mann–Whitney test; a Bonferroni correction was applied to multiple comparisons. A *P* value of less than 0.05 was considered statistically significant.

2.12. Drugs and drug preparation

(*S*)-(+)-5-[1-(2-Methoxymethylpyrrolidinyl) sulfonyl]isatin (MMPSI) was synthesized at Pfizer Global Research and Development (Groton, CT). The peptide-based inhibitors (ZVAD-fmk, DEVD-fmk, YVAD-cmk) were from Enzyme

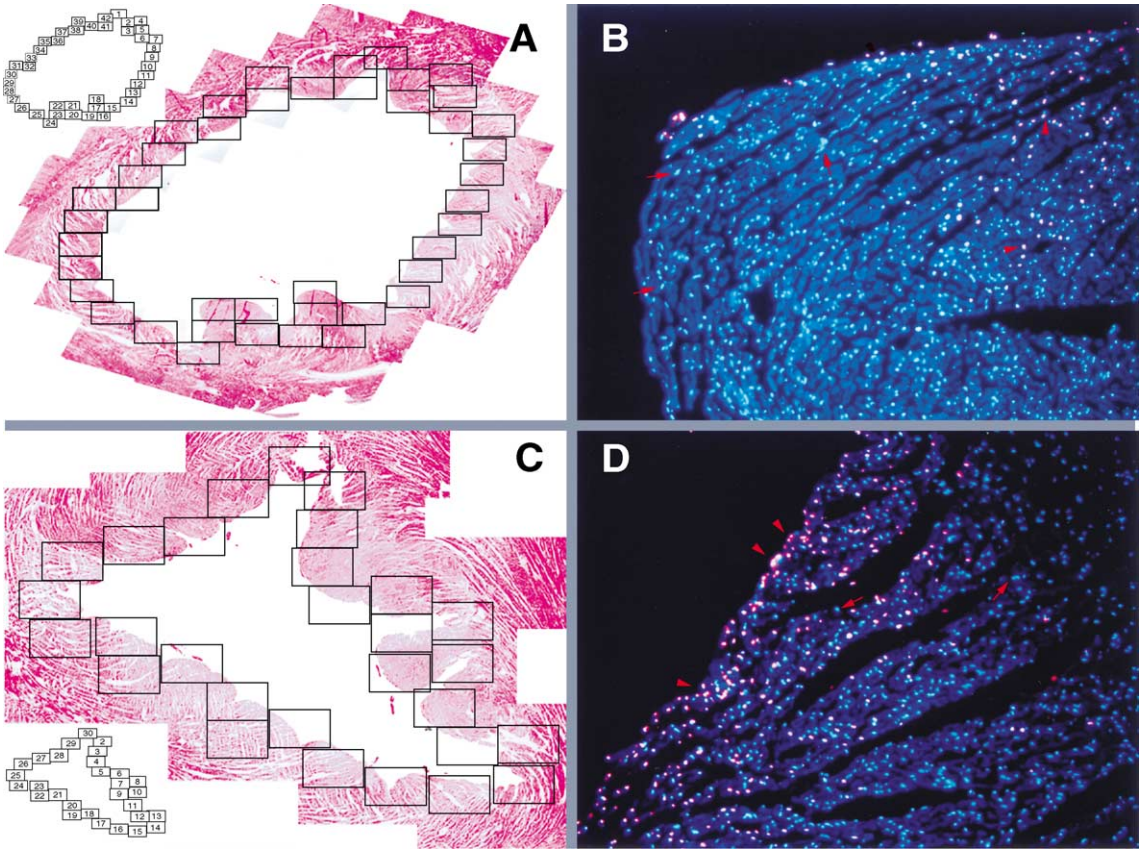

Fig. 3. (A) A representative low power montage of the left ventricle of a control rabbit heart. The boxes demarcate the fields that were quantified for apoptosis (TUNEL) as described in Materials and methods. (B) A single field (#22 in (A); 100 ×) representative of those quantified for TUNEL labeling, as described in Materials and methods. The apoptotic cells were labeled with Texas Red and appear pink (arrowheads), while the normal nuclei were labeled with DAPI and appear light blue (arrows). The unmerged figures were analyzed using Optimus software to determine total nuclei and apoptotic nuclei per mm^2. (C) A representative low power montage of the left ventricle of a rabbit heart following 30 min of ischemia and 2 h of reperfusion. The boxes demarcate the fields that were quantified for apoptosis (TUNEL) as described in Materials and methods. (D) A single field (#2 in (C); 100 ×) representative of those quantified for TUNEL labeling, as described in Materials and methods. The apoptotic cells were labeled with Texas Red and appear pink (arrowheads), while the normal nuclei were labeled with DAPI and appear light blue (arrows). The unmerged figures were analyzed using Optimus software to determine total nuclei and apoptotic nuclei per mm^2.

Systems Products (Livermore, CA). All other chemicals were of the highest grade available and obtained from Sigma (St. Louis, MO). For the Langendorff studies, MMPSI was dissolved in dimethyl sulfoxide (DMSO) and diluted in buffer; the final DMSO concentration for all experiments was less than 0.1%. This concentration of DMSO has previously been shown to have no effect on infarct size (Tracey et al., 1997).

3. Results

In isolated rabbit hearts, baseline heart rates, coronary flows and left ventricular developed pressures for each of the treatment groups were similar prior to the regional ischemia and are shown in Table 1. Left ventricular developed pressures and coronary flows were significantly ($P<0.05$) reduced in all groups by occlusion of the coronary artery, confirming that ischemia was achieved. Area-at-risk expressed as a percent of left ventricular area was $32 \pm 4\%$ ($n=5$) for the control group; other groups did not differ significantly ($P>0.05$) from the control group.

Fig. 1 illustrates the effects of peptidic caspase inhibitors on infarct development. The nonselective caspase inhibitor, ZVAD-fmk, and the caspase-3/7 selective inhibitor, DEVD-fmk, both elicited significant ($P<0.05$) reductions in infarct size compared to the control (untreated) hearts (control: $54 \pm 2\%$ IA/AAR; 10 μM ZVAD-fmk: $9 \pm 2\%$ IA/AAR; 20 μM DEVD-fmk: $16 \pm 6\%$ IA/AAR, $n=5-9$). ZFA-fmk (negative control) and the caspase-1 selective inhibitor, YVAD-cmk, did not significantly reduce infarct size in this study (10 μM ZFA-fmk: $46 \pm 3\%$ IA/AAR; 20 μM YVAD-cmk: $54 \pm 2\%$ IA/AAR, $n=5-7$).

The effects of MMPSI on infarct size in the isolated heart are shown in Fig. 2. MMPSI elicited a significant ($P<0.05$), concentration-dependent reduction in infarct size (IC_{50}: 0.2 μM); at 10 μM, MMPSI reduced infarct size by

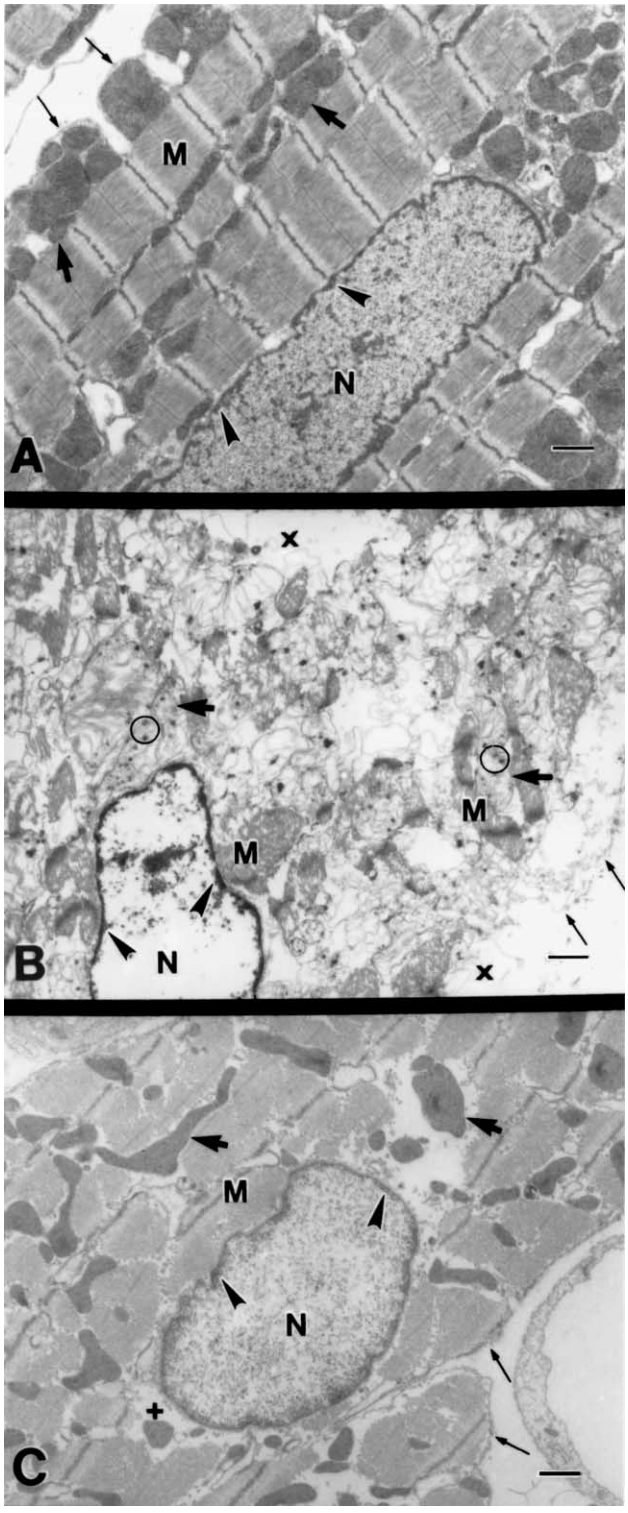

Fig. 4. (A) Electron micrograph of the left ventricle area-at-risk of a control rabbit heart (5000 ×). Typical ultrastructural morphology of cardiac myocardium can be observed. The nucleus (N) is unremarkable and contains both heterochromatin (arrowheads) and euchromatin. The muscle fibers (M) are not swollen and contain the typical banding pattern for cardiac myocardium. Interspersed throughout the cell are numerous mitochondria (thick arrows) containing well-defined cristae. The plasma membrane (thin arrows) is contiguous and does not contain any large ruptures. Bar = 1 μm. (B) Electron micrograph of the left ventricle area-at-risk of a rabbit heart following 30 min of ischemia and 2 h of reperfusion (5000 ×). Numerous ultrastructural changes that define oncosis can be seen. The nucleus (N) has lost most of its euchromatin (that remaining being inhomogeneously clumped) while maintaining a ring of heterochromatin (arrowheads). The muscle fibers (M) within the cell appear fragmented and have lost most of their typical banding pattern. The mitochondria (thick arrows) and their cristae have swollen and numerous dense bodies (circles) can be seen within the mitochondria. The plasma membrane (thin arrows) contains large ruptures (×). Bar = 1 μm. (C) Electron micrograph of the left ventricle area-at-risk of a rabbit heart treated with MMPSI (10 μM), following 30 min of ischemia and 2 h of reperfusion (5000 ×). The nucleus (N) contains a ring of heterochromatin (arrowheads) and does not appear to have lost as much euchromatin as the nontreated hearts. The muscle fibers (M) appear normal in this cross-sectioned area with only occasional spaces (+) between the bundles. The mitochondria (thick arrows) appear normal with well-defined christae. The plasma membrane does not contain any evidence of ruptures. Bar = 1 μm.

56% compared to control hearts (control: 54 ± 2% IA/AAR; 10 μM MMPSI: 24 ± 4% IA/AAR; $n = 5$) (Fig. 2).

Apoptosis was assessed in the isolated hearts by TUNEL, electron microscopy, soluble nucleosome ELISA, and DNA laddering. An increase in TUNEL positive cells was observed after 2 h of reperfusion; the percentage of TUNEL positive cells increased from 0.8% of the left ventricle area-at-risk of control hearts to 1.8% in ischemia-reperfused hearts (Fig. 3). Electron microscopy revealed primarily oncotic changes in the cardiomyocytes present in the area-at-risk, including inhomogeneously clumped nuclear chromatin and swollen mitochondria with numerous dense bodies; these ultrastructural changes were markedly reduced in MMPSI (10 μM)-treated hearts (Fig. 4). Soluble nucleosomes and DNA laddering were also detected in the area-at-risk myocardium (Fig. 5).

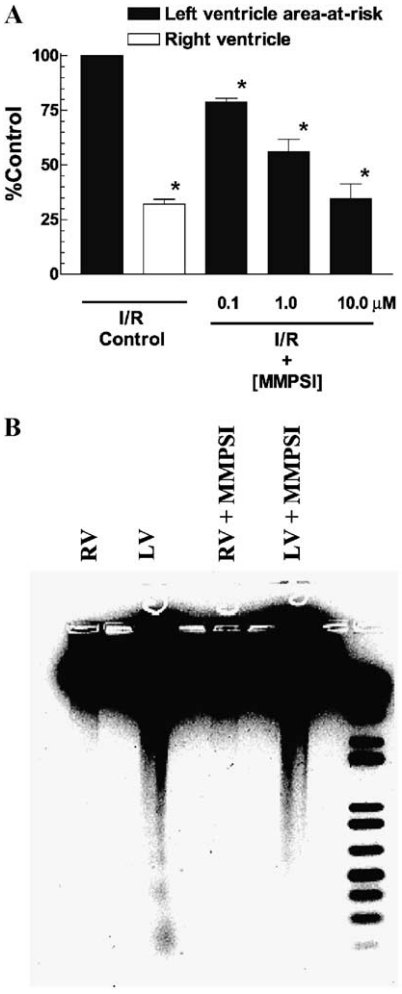

Fig. 5. (A) Effect of MMPSI on apoptosis in isolated rabbit hearts subjected to ischemia–reperfusion (I/R) injury. MMPSI was perfused through the heart for 5 min followed by a 10-min wash-out prior to 30 min of regional ischemia and 120 min of reperfusion, as described in Materials and methods. Soluble nucleosomes were quantified in cardiac tissue by ELISA. Values were normalized as percent of the signal obtained from nondrug-treated, ischemic left ventricle area-at-risk (I/R control). Data are the mean ± S.E.M., $n = 3–4$; *significantly different ($P < 0.05$) from left ventricle I/R control. I/R: ischemia–reperfusion. (B) Effect of MMPSI on apoptosis as measured by DNA laddering in isolated rabbit hearts subjected to ischemia–reperfusion injury. MMPSI (10 μM) was perfused through the heart for 5 min followed by a 10-min wash-out prior to 30 min of regional ischemia and 120 min of reperfusion, as described in Materials and methods. DNA was extracted from the ischemic left ventricle area-at-risk or the non-ischemic right ventricle, separated on a 1.8% agarose gel, and visualized with SYBR Green.

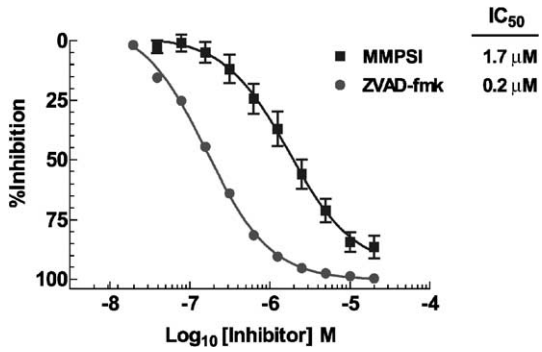

Fig. 6. Effect of MMPSI on in vitro caspase-3 activity. Cleavage of the fluorescent substrate ac-DEVD-AMC by human recombinant caspase-3 was measured in the presence or absence of MMPSI, or ZVAD-fmk, as described in Materials and methods. Data are means ± S.E.M.; $n = 6$.

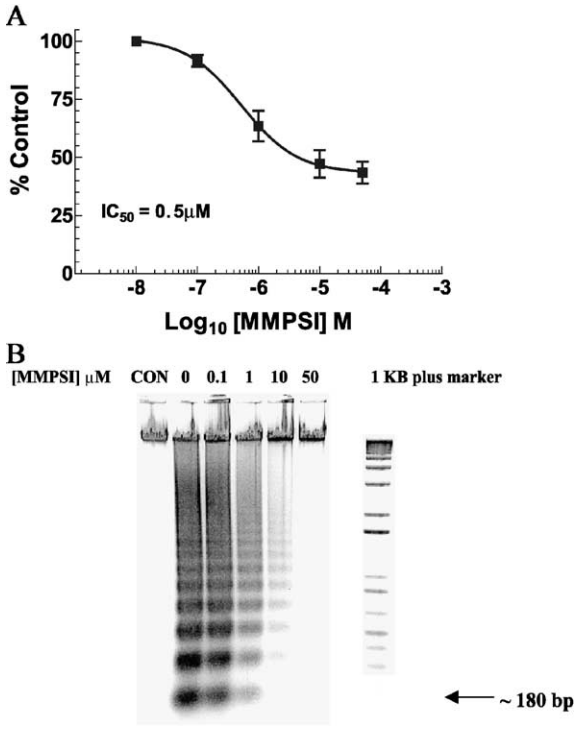

Fig. 7. (A) Effect of MMPSI on apoptosis in H9c2 cells subjected to simulated ischemia–reperfusion. H9c2 cells were treated as described in Materials and methods in the presence of various concentrations of MMPSI. Soluble nucleosomes were quantified by ELISA. Data are the mean ± S.E.M., $n = 8$. (B) Effect of MMPSI on apoptosis in H9c2 cells subjected to simulated ischemia–reperfusion and assessed by DNA laddering. H9c2 cells were treated as described in Materials and methods in the presence of various concentrations of MMPSI. DNA was extracted and separated on a 1.8% agarose gel and visualized with SYBR Green. Control cells were maintained under normoxic conditions.

MMPSI concentration-dependently inhibited apoptosis in the ischemia-reperfused hearts (Fig. 5); the extent of apoptosis (soluble nucleosomes) in the ischemic left ventricle area-at-risk of hearts treated with 10 μM MMPSI was equivalent to the basal levels observed in non-ischemic right ventricles of drug-free hearts (Fig. 5A). Fig. 5B shows a similar reduction in DNA laddering in the left ventricle area-at-risk. Inhibition of caspase-3 by MMPSI was confirmed by examining the effect of this compound on human recombinant caspase-3 activity (Fig. 6). The nonselective, peptidic caspase inhibitor ZVAD-fmk was used for comparison. MMPSI inhibited caspase-3-dependant substrate cleavage in a concentration-dependent manner with an $IC_{50} = 1.7$ μM.

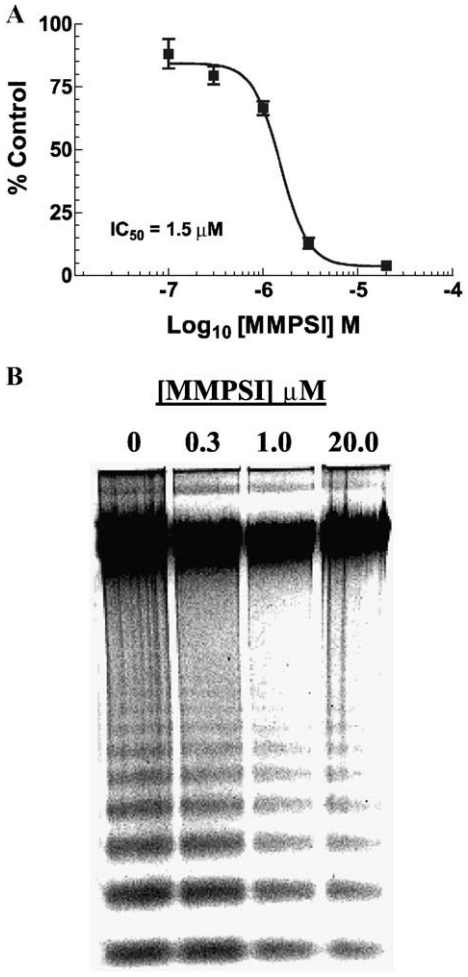

Fig. 8. (A) Effect of MMPSI on apoptosis in adult rabbit cardiomyocytes. Adult rabbit cardiomyocytes were allowed to undergo apoptosis in culture in the presence of various concentrations of MMPSI and then soluble nucleosomes were detected by ELISA, as described in Materials and methods. Data are the mean ± S.E.M., $n = 8$. (B) Effect of MMPSI on apoptosis in adult rabbit cardiomyocytes assessed by DNA laddering. Adult rabbit cardiomyocytes were allowed to undergo apoptosis in culture overnight in the presence of 10 μM MMPSI, as described in Materials and methods. DNA was extracted and separated on a 1.8% agarose gel and visualized with SYBR Green.

The effect of MMPSI on ischemia–reperfusion-induced apoptosis was also evaluated in the H9c2 rat myoblast cell line. After 16 h of simulated ischemia and 2 h of simulated reperfusion, apoptosis was measured by soluble nucleosome ELISA and DNA laddering. Fig. 7A illustrates that MMPSI reduced the detection of soluble nucleosomes in a concentration-dependent manner with an $IC_{50} = 0.5$ μM, and a maximal reduction of 57% at 50 μM. Fig. 7B qualitatively demonstrates a similar reduction in the appearance of DNA laddering elicited by MMPSI. Similar effects of MMPSI on apoptosis were observed using freshly isolated adult rabbit cardiomyocytes (which underwent spontaneous apoptosis during an overnight incubation); MMPSI reduced the detection of soluble nucleosomes by greater than 95% at 20 μM, with an $IC_{50} = 1.5$ μM (Fig. 8A). DNA laddering was similarly reduced by MMPSI, as shown in Fig. 8B.

4. Discussion

Infarct size is a critical determinant of long-term prognosis in patients following myocardial infarction. Prompt reperfusion is the primary means of salvaging myocardium after an ischemic insult, but reperfusion may lead to additional expansion of the infarct (Mocanu et al., 2000). Apoptosis, possibly the predominant form of cell death in early human myocardial infarcts (Veinot et al., 1997), not only contributes to cardiomyocyte loss in the infarcted region (Anversa et al., 1998), but may also be exacerbated by subsequent reperfusion (Black et al., 1998; Gottlieb et al., 1994; Weiland et al., 2000).

The first selective, potent, competitive, and reversible nonpeptidic inhibitors of caspase-3/7 were recently reported (Lee et al., 2000). These isatin sulfonamides achieve selectivity through interaction with the S_2 subsite of the enzyme and do not bind in the primary aspartic acid bind pocket. Of the compounds examined, (*S*)-(+)-5-[1-(2-methoxymethylpyrrolidinyl)sulfonyl]isatin (MMPSI) inhibited caspase-3 activity with a K_i of 60 nM and exhibited 100-fold or greater selectivity vs. all other family members, except caspase-9, for which the selectivity was ~ 30-fold. MMPSI and its analogues were observed to inhibit mouse bone marrow-derived neutrophil apoptosis (Lee et al., 2000), which is caspase-3 dependent (Woo et al., 1998).

Given the reported efficacy and selectivity of MMPSI, we investigated whether this small molecule caspase 3/7 inhibitor would ameliorate myocardial ischemic damage, and corroborate a role for caspase-3/7 in this process. Initially, we used several peptidic caspase inhibitors of differing selectivities to provide a preliminary indication for (or refute) the involvement of caspase-3/7 in myocardial ischemic injury. Both the pan-caspase inhibitor, ZVAD-fmk, and the caspase-3-like inhibitor, DEVD-fmk, reduced infarct size in the isolated rabbit heart, while the caspase-1-like inhibitor, YVAD-cmk, had no effect. To rule out nonselec-

tive protease inhibition by the fluoromethylketone (fmk) moiety of these compounds, ZFA-fmk was also administered to the hearts, and was found not to significantly affect infarct size. Thus, the peptide inhibitor data suggested caspase-3 and/or caspase-7 (since they share similar substrate specificities) contributed to myocardial ischemic damage in this model. More thorough studies were subsequently performed with MMPSI, which demonstrated this compound inhibited apoptosis in the left ventricle area-at-risk, as shown by a decrease in DNA laddering and a reduction in the presence of soluble nucleosomes. Furthermore, MMPSI reduced infarct size in a concentration-dependent manner, and provided a degree of cardioprotection that compared favorably with ischemic preconditioning (Tracey et al., 1997), indicating caspase activation is an important component of myocardial ischemic injury. Although the assessments of apoptosis in the whole hearts did not distinguish between specific cell types (i.e., cardiomyocytes vs. non-cardiomyocytes), we confirmed the ability of MMPSI to inhibit apoptosis in isolated adult rabbit cardiomyocytes, and the myoblast cell line, H9c2. MMPSI concentration-dependently reduced both "ischemia–reperfusion"-induced apoptosis in the H9c2 cells, and spontaneous apoptosis of adult rabbit cardiomyocytes. Previous studies have demonstrated caspase-3 activation in adult (de Moissac et al., 2000) or neonatal (Bialik et al., 1999) cardiomyocytes, or H9c2 cells (Ekhterae et al., 1999) undergoing apoptosis following exposure to conditions mimicking ischemia.

Interestingly, the K_i we determined for MMPSI against recombinant human caspase-3 was higher (1.6 μM) than that reported by Lee et al. (2000) (60 nM) even though we performed the assay as described. An explanation for this difference is not immediately evident; the IC_{50}s we obtained from the whole cell and Langendorff assays were within three- to eight-fold of the K_i we determined against the recombinant enzyme.

Due to the central role of caspases as mediators of apoptosis, much effort has focused on evaluating whether caspase inhibition will lead to a diminution of myocardial ischemic injury. Using peptidic caspase inhibitors either identical or similar to those used in the present study, several reports have described the cardioprotective outcomes of caspase inhibition, while implicating different caspases in this process. zVAD-fmk reduced infarct size in vivo in rat hearts when administered either before ischemia (Huang et al., 2000; Yaoita et al., 1998) or shortly before reperfusion (Huang et al., 2000); the number of TUNEL positive cells and the appearance of DNA laddering were also reduced by this compound (Yaoita et al., 1998). Interestingly, while several studies have implicated a role for caspase-3 (Black et al., 1998; Condorelli et al., 2001; Holly et al., 1999; Piot et al., 1999) or caspase-7 (Holly et al., 1999) in myocardial ischemic injury, it appears only a recent study examined whether a peptide inhibitor of caspase-3-like proteases would reduce infarct size; although DEVD-cmk did provide cardioprotection, apoptosis was not assessed in this study (Mocanu et al., 2000). Inhibitors of caspase-8 and -9 also reduced infarct size, and the authors reasonably pointed out they could not exclude the possibility that these compounds might inhibit other proteases implicated in myocardial ischemia–reperfusion injury (Mocanu et al., 2000), such as calpains (Iwamoto et al., 1999). We cannot completely exclude this possibility either, except to point out that ZFA-fmk did not affect infarct size in our studies. Furthermore, the isatin sulfonamides such as MMPSI have IC_{50}s $\gg 5$ μM against other cysteine proteases, i.e., cathepsins, and elicit $<20\%$ inhibition of calpain I at 50 μM (Lee et al., 2000).

While not a primary endpoint of our study, we did not observe any significant improvement in left ventricular developed pressure at the end of the ischemic period. Of the two previous studies which reported post-ischemic cardiac function in isolated hearts treated with peptidic caspase inhibitors, one found no change (Okamura et al., 2000), while the other observed a decrease in left ventricular end-diastolic pressure and no change in left ventricular systolic pressure (Yaoita et al., 1998). Mocanu et al. (2000) reported that several caspase inhibitors had no effect on post-ischemic rate–pressure product, although this endpoint is more of a measure of myocardial oxygen consumption. It is unclear why caspase inhibitors do not have a more robust effect on maintaining or improving cardiac function, but the spared myocardium following caspase inhibitor treatment may still be functionally impaired, i.e., stunned.

It was intriguing to note the small percentage of TUNEL positive cells (1.8%) in the ischemic myocardium, and the predominant oncotic vs. apoptotic, changes in cardiomyocyte ultrastructure revealed by electron microscopy (changes which were prevented by MMPSI). While some cautions is appropriate when evaluating single timeframe endpoint determinations of a dynamic process such as apoptosis, these observations suggest that apoptosis per se may have been a relatively minor component of the overall infarct (54% of the left ventricle myocardium at risk). Similar observations have been reported by Ohno et al. (1998), leading us to speculate that while caspase-3/7 inhibitors such as MMPSI and the peptide compounds clearly both reduce infarct size and inhibit apoptosis, these two effects may not necessarily be directly related, i.e., caspase inhibition might also prevent cardiomyocyte loss by impeding an oncotic pathway.

In summary, we have demonstrated for the first time that a small molecule, caspase-3/7-selective inhibitor, MMPSI, can significantly limit ischemia–reperfusion-induced infarct size in the isolated rabbit heart, and reduce apoptosis in both the ischemic myocardium and isolated cardiomyocytes. These observations, combined with data obtained with peptidic caspase inhibitors, support a key role for caspase-3/7 in myocardial ischemic injury. Clinical cardioprotection through caspase 3/7 inhibition may be feasible via a small molecule approach.

Acknowledgements

The authors would like to thank Dr. Delvin Knight for reviewing the manuscript.

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