Cardioprotective effects of ingliforib, a novel glycogen phosphorylase inhibitor


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Tracey, W. Ross, Judith L. Treadway, William P. Magee, Jill C. Sutt, R. Kirk McPherson, Carolyn B. Levy, Donald E. Wilder, Li J. Yu, Yue Chen, Ravi M. Shanker, Alison K. Mutchler, Andrew H. Smith, David M. Flynn, and Delvin R. Knight. Cardioprotective effects of ingliforib, a novel glycogen phosphorylase inhibitor. Am J Physiol Heart Circ Physiol 286: H1177–H1184, 2004. First published November 13, 2003; 10.1152/ajpheart.00652.2003.—Interventions such as glycogen depletion, which limit myocardial anaerobic glycolysis and the associated proton production, can reduce myocardial ischemic injury; thus it was observed that inhibition of glycogen phosphorylation should also be cardioprotective. Therefore, we examined whether the novel glycogen phosphorylase inhibitor 5-Chloro-N-(15,2R)-3-[(3R,4S,3,4-dihydroxy-1-pyrrolidinyl)]-2-hydroxy-3-oxo-1-[(phenylmethyl)propyl]-1H-indole-2-carboxamide (ingliforib; CP-368,296) could reduce infarct size in both in vitro and in vivo rabbit models of ischemia-reperfusion injury (30 min of regional ischemia, followed by 120 min of reperfusion). In Langendorff-perfused hearts, constant perfusion of ingliforib started 30 min before regional ischemia and elicited a concentration-dependent reduction in infarct size; infarct size was reduced by 69% with 10 μM ingliforib. No significant drug-induced changes were observed in either cardiac function (heart rate, left ventricular developed pressure) or coronary flow. In open-chest anesthetized rabbits, a dose of ingliforib (15 mg/kg loading dose; 23 mg·kg−1·h−1 infusion) selected to achieve a free plasma concentration equivalent to an estimated EC50 in the isolated hearts (1.2 μM, 0.55 μg/ml) significantly reduced infarct size by 52%, and reduced plasma glucose and lactate concentrations. Furthermore, myocardial glycogen phosphorylase activity and total glycogen phosphorylase activity were reduced by 65% and 40%, respectively, and glycogen stores were preserved in ingliforib-treated hearts. No significant change was observed in mean arterial pressure or rate-pressure product in the ingliforib group, although heart rate was modestly decreased postischemia. In conclusion, glycogen phosphorylase inhibition with ingliforib markedly reduces myocardial ischemic injury in vitro and in vivo; this may represent a viable approach for both achieving clinical cardioprotection and treating diabetic patients at increased risk of cardiovascular disease.

ischemia; reperfusion; heart; infarct; rabbit

Although the influence of ischemia and reperfusion on cardiac metabolism has been extensively investigated (see Refs. 5, 16, and 24 for reviews), less well defined are the ways in which pharmacological manipulation of cardiac metabolism may be cardioprotective. Under ischemic conditions, myocardial oxidative metabolism is suppressed and glycolysis becomes an important source of ATP generation (32). The increased glycolytic rate in the face of impaired glucose oxidation leads to uncoupling of the two pathways and a buildup of lactate and H+ (4, 16), a process which may continue during reperfusion (22). This accumulation of protons leads to downstream activation of pathways (Na+/H+ exchanger, Na+/Ca2+ exchanger) that result in Ca2+ overload, impaired contractile function, and/or cell death. Therefore, the approaches that are able to improve glycolytic/oxidative coupling by reducing the glycolytic rate could be expected to be cardioprotective.

One possible approach would be to reduce myocardial glycogenolysis and thus restrict a source of substrate for glycolysis. Several studies (3, 7, 28, 30, 42, 43) examining the mechanistic basis of ischemia preconditioning have demonstrated in preconditioned hearts that myocardial glycogen stores are depleted, accompanied by attenuated glycogenolysis and glycolysis, and reduced accumulations of lactate and protons. Moreover, the loss of myocardial protection in preconditioned hearts correlates with the time course of glycogen recovery (43). Experimental manipulations designed to deplete myocardial glycogen before ischemia-reperfusion have also been shown to be cardioprotective (1, 19, 31). Nevertheless, the ability of glycogen to modulate ischemia-reperfusion injury is controversial in that other studies (10, 15, 20, 37) have failed to show either a link between glycogen depletion and ischemic preconditioning, or a cardioprotective benefit of reducing glycogen stores before ischemia and reperfusion.

Given that both glycogenolysis (30, 42) and conversion of glycogen phosphorylase (GP) to the active (a) form (GPa) (42) are reduced in preconditioned hearts, and both GP activity and glycogenolysis are increased during ischemia in nonpreconditioned hearts (8), pharmacological inhibition of GP, and thus glycogenolysis, could be postulated to be cardioprotective. A limitation facing past investigations was the lack of pharmacological tools with which to specifically inhibit GP, although α-1,6-glucosidase glycogen debranching enzyme inhibitors N-hydroxyethyl-1-deoxyojirimycin (miglitol) and N-methyl-1-deoxyojirimycin (MOR-14) have been reported to reduce both myocardial glycogen breakdown and infarct size (2, 29). Nevertheless, the putative cardioprotective benefit of inhibiting GP has not been formally demonstrated. We recently described a novel class of GP inhibitors (12, 27), which bind at a newly discovered allosteric binding site on the enzyme (34). One of

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these inhibitors is 5-Chloro-N-{(1S,2R)-3-[(3R,4S)-3,4-dihydroxy-1-pyrrolidinyl]-2-hydroxy-3-oxo-1-(phenylmethyl)propyl}1H-indole-2-carboxamide (ingliforib; CP-368,296) (13) (Fig. 1), which inhibits the GP isoforms expressed in the myocardium with IC50 values of 352 nM (muscle GP) and 150 nM (brain GP), respectively. Thus, to help further clarify the involvement of glycogenolysis in myocardial ischemia-reperfusion injury, we used this novel compound to investigate whether GP inhibition is cardioprotective in both in vitro and in vivo rabbit models of ischemia-reperfusion injury.

MATERIALS AND METHODS

This investigation conforms to the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, Revised 1996).

In vitro Langendorff preparation. Male New Zealand White rabbits (3 to 4 kg; Covance; Denver, CO) were anesthetized by intravenous administration of pentobarbital sodium (30 mg/kg), followed by intubation and ventilation with 100% O2 with the use of a positive pressure ventilator. A left thoracotomy was performed, the heart exposed, and a snare (2-0 silk) was 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 perfusion (nonrecirculating) with a modified Krebs solution composed of (in mM) 118.5 NaCl, 4.7 KCl, 1.2 MgSO4, 1.2 KH2PO4, 24 NaHCO3, 2.5 CaCl2, and 10 glucose at a constant pressure of 80 mmHg and a temperature of 38.5°C. Perfusate pH was maintained at 7.4 to 7.5 by bubbling with 95% O2–5% CO2. 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 mmHg, and a diastolic pressure ≤10 mmHg. Heart rate (HR), left ventricular (LV) systolic and diastolic pressures, and LV developed pressure (LVDP) were recorded using a PO-NE-MAH Data Acquisition and Archive System (Gould Instrument Systems; Valley View, OH). Total coronary flow (CF) rate was determined using an in-line flow probe (Transonic Systems; Ithaca, NY); CF was normalized for heart weight. Each heart was allowed to equilibrate for 30 min; if stable LV pressures within the parameters outlined above were not observed, the heart was discarded. Pacing was not used unless the heart rate fell <180 beats/min before 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.

Langendorff experimental protocols. After a 30-min equilibration period, a constant perfusion with ingliforib was initiated, and continued for the duration of the experiment. Thirty minutes after drug perfusion was started, a 30-min period of regional ischemia was produced by tightening the snare around the branch of the coronary artery. At the end of the ischemic period, the snare was released, and the heart was reperfused for an additional 120 min. Myocardial ischemia was confirmed by regional cyanosis and ST segment elevation; reperfusion was confirmed by reactive hyperemia and rapid decline of the ST elevation. At the end of either the ischemic period or reperfusion period, each rabbit was euthanized with an intravenous overdose of pentobarbital sodium (100 mg/kg). The heart was quickly excised and prepared for measurement of the amount of activity and glycogen content, or mounted on a Langendorff apparatus and perfused with physiological saline at 38.5°C for subsequent determination of infarct size.

Determination of infarct size. After completion of each experiment (in vitro or in vivo) and with the heart suspended and perfused on the Langendorff apparatus, the coronary artery snare was retightened, 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 (AAR; nonlabeled) in the LV 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 noninfarcted (stained) from infarcted (nonstained) LV tissue. The infarct area (IA) and the AAR were calculated for each slice of LV using video-captured images and image analysis software (model ETC3000, Engineering Technology Center; Mystic, CT), followed by adding the values for each tissue slice to obtain the total IA and total AAR for each heart. To normalize the infarct area for differences in the AAR between hearts, the infarct size was expressed as the ratio of IA versus AAR (%IA/AAR).

Determination of drug concentrations in plasma and protein binding. Quantitation of ingliforib was accomplished with the use of a liquid chromatography/tandem mass spectrometry (LC/MS/MS) instrument (model API3000, PE-Scieix; Toronto, Canada). An aliquot (10 μl) of plasma or tissue homogenate (0.2 g/ml in 10 mM sodium phosphate buffer at pH 7.4) was precipitated using 200 μl of methanol-acetonitrile (1:1). After centrifugation, an aliquot (40 μl) of supernatant was diluted with 200 μl of methanol-acetonitrile (1:1), and the diluted sample (5 μl) was injected onto a Phenomenex 40×2 mm 5 μm C18 column maintained at 37°C with a run time of ~3 min. The analyte was eluted at 0.5 ml/min flow rate with a linear gradient program consisting of methanol (pump A, 5–95% ramping) and 10 mM ammonium acetate (pump B, 5–95% ramping) produced by two Shimadzu LC-10ADVP binary pumps and a 10-μl static mixer. The column effluent was analyzed using a TurboIonSpray source at 500°C of a PE-Scieix API-3000 triple quadrupole mass spectrometer. Ingliforib was detected at m/z 456.2 → 193.0 at a retention time of 1.65 min. The calibration curve was prepared by analysis using a blood gas analyzer (model 248, Bayer Diagnostics; Norwood, MA). The ventilator was adjusted as needed to maintain blood pH and PCO2 within normal physiological ranges for rabbits. The heart was exposed through a left thoracotomy at the fourth intercostal space and a 2-0 silk suture was placed around a prominent branch of the left coronary artery. Lead II ECG was measured using an ECG amplifier (Gould) connected to surface ECG electrodes. Arterial pressure was measured using a calibrated strain gauge transducer (Spectromed; Oxnard, CA) connected to the arterial catheter. Heart rate, arterial mean pressure (MAP) were derived using the PO-NE-MAH Data Acquisition and Archive System. Rate-pressure product (RPP) was calculated as the product of HR and MAP. RPP has been previously used as an index of myocardial O2 consumption in this model (17).

In vivo experimental protocols. At least 1 h after surgery, when arterial pressure, HR, and RPP had stabilized for at least 30 min (baseline), the rabbits received a bolus of either 15.4 mg/kg of ingliforib or vehicle (administered in 15 s), followed by a constant infusion of 23.1 mg·kg−1·h−1 ingliforib or vehicle at the same dose volume for a total of 3.5 h. Sixty minutes after starting the infusion, regional ischemia was produced by tightening the coronary artery snare for 30 min. The snare was released, and the heart was reperfused for an additional 120 min. Myocardial ischemia was confirmed by regional cyanosis and ST segment elevation; reperfusion was confirmed by reactive hyperemia and rapid decline of the ST elevation. At the end of either the ischemic period or reperfusion period, each rabbit was euthanized with an intravenous overdose of pentobarbital sodium (100 mg/kg). The heart was quickly excised and prepared for measurement of the presence of activity and glycogen content, or mounted on a Langendorff apparatus and perfused with physiological saline at 38.5°C for subsequent determination of infarct size.

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addition of authentic standard (ingliforib) to the control plasma or control tissue homogenate, at concentrations of 0.05 to 50 µg/ml for the plasma and 0.1 to 50 µg/ml for the tissue (6 to 7 concentrations per standard curve). The standards were processed as the unknowns described above. The standard curve was obtained by fitting linear least-squares regression analysis from the peak area of ingliforib with 1/(concentration)^2 weighting. The acceptance criterion for the analysis was that all standards used in the curve were ±20% absolute deviation from the normal value. The absolute tissue-to-plasma concentration ratio was found to be ~1.5 in heart and ~2.7 in liver after a 2-h infusion (at steady state).

Plasma protein binding was determined by a 96-well equilibrium dialysis apparatus. Spectra-pro number 2 membranes with molecular weight cutoff of 12–14 kDa were used for the study and were conditioned for 15 min in deionized water, 15 min in 30% ethanol, and 30 min in sodium phosphate buffer (pH 7.4; 100 mM). Fresh rabbit plasma was obtained from control animals on the day of the study. Plasma samples were spiked with ingliforib to achieve a concentration of 1 µg/ml; 150-µl aliquots (n = 6) were loaded into the 96-well equilibrium dialysis apparatus and dialyzed against 150 µl of sodium phosphate buffer. Equilibrium was achieved by incubating the 96-well equilibrium dialysis apparatus in a 37°C shaking water bath at 155 rpm for 5 h. At the end of the dialysis period, 10 µl of the dialyzed plasma and 90 µl of the buffer were transferred to HPLC vials containing 100 µl of methanol-acetonitrile (1:1). Control buffer (90 µl) was added to the vial containing the dialyzed plasma sample, and 10 µl of control plasma was added to the vial containing the buffer sample. The vials were vortexed and centrifuged, and the supernatant was assayed by the LC/MS/MS assay described above. The plasma unbound fraction (fu) was estimated by the ratio of drug concentration in the buffer sample to the drug concentration in the plasma sample. (The mean fu for ingliforib was 0.036 ± 0.002 in rabbit plasma).

Determination of plasma glucose and lactate concentrations. Blood samples were collected in heparinized tubes, followed by centrifugation and collection of the plasma. Plasma glucose and lactate concentrations were determined as the region not cleared of blood by the saline perfusion and was dissected free of the remainder of the heart. In hearts in which infarct size was determined, the right ventricular free wall was used for determining GP activity. Myocardial samples were frozen in liquid nitrogen, and stored at −80°C until analysis. Approximately 25 mg of frozen tissue were added to 16 × 100 mm glass test tubes, followed by the addition of 1.5 ml of 30% KOH. The tubes were heated in a 60°C oven for 30 min and repeatedly agitated. Two milliliters of 100% ethanol and 250 µl of saturated sodium sulfate were added to each sample. The tubes were heated for 3 min at 90°C, and then placed on ice for 15 min. Samples were centrifuged at 4°C, 3,200 g for 5 min. After aspiration of the supernatants, the pellets were dried for 60 min in a 60°C oven. The pellets were then hydrolyzed in 1 ml of 5N HCl for 1 h in a 60°C oven. Samples were cooled at room temperature and neutralized with 1 ml of 5N NaOH and 3 ml of deionized water. For each sample, 2 ml of anthrone reagent (200 mg of anthrone (Sigma) in 100 ml H2SO4) were added to a 16 × 100 mm test tube, followed by the addition of 1 ml of neutralized heart hydrolysate or 1 ml of glucose standard. Tubes were vortexed and heated at 90°C for 15 min; samples were then immediately cooled at 4°C. Two hundred microliters were transferred in duplicate to a 96-well plate, which was read at 620 nm in a SpectroMax Plus microplate reader (Molecular Devices; Sunnyvale, CA).

Data expression and analysis. Data are expressed as the means ± SE. Between group comparisons of in vitro and in vivo AAR expressed as a percentage of LV areas (%AAR/LV) were compared using ANOVA. Temporal comparisons in vivo hemodynamic parameters, plasma glucose concentrations, and plasma lactate concentrations between ingliforib and vehicle control were performed using ANOVA with repeated measures. In vitro hemodynamic, glycogen content, and GP activity comparisons were performed by t-test, whereas in vitro and in vivo %IA/AAR values were compared using a Mann-Whitney test; a Bonferroni correction was applied to multiple comparisons. A P value of <0.05 was considered statistically significant.

Drugs and drug preparation. The synthesis of (ingliforib; CP-368,296) has been reported (13) and was performed at Pfizer Global Research and Development (Groton, CT). Drug administered to the isolated hearts was dissolved in DMSO and diluted in buffer; the final DMSO concentration was <0.1%, which had no effect on infarct size (39). For the in vivo studies, ingliforib was dissolved in 25% sulfobutylether 7-β-cyclodextrin sodium (Capsitol, Cydex; Overland Park, KS) in 0.01 M phosphate-buffered saline at a concentration of 13 mg/ml.
GLYCOGEN PHOSPHORYLASE INHIBITOR REDUCES ISCHEMIC INJURY

RESULTS

In the isolated rabbit hearts, baseline HR, CF, and LVDP values for each of the treatment groups were similar before the regional ischemia and are shown in Table 1. LVDP and CF were significantly ($P < 0.05)$ reduced in all groups by occlusion of the coronary artery, confirming that ischemia was achieved in all groups. In anesthetized rabbits, baseline HR, MAP, and RPP were similar between vehicle control and ingliforib-treated groups (Fig. 2). Administration of ingliforib did not significantly affect HR, LVDP, or CF in the isolated hearts (Table 1), nor did this compound affect MAP or RPP in vivo (Fig. 2, B and C). A modest reduction in HR of the ingliforib group versus the vehicle control group ($P < 0.05)$ was observed during the reperfusion period in the in vivo studies (Fig. 2A).

Ingliforib elicited a concentration-dependent reduction in infarct size in the isolated rabbit hearts (Fig. 3). The maximum reduction in infarct size achieved with 10 $\mu$M ingliforib was 69% (control, 52 ± 2% IA/AAR; 10 $\mu$M ingliforib, 16 ± 2% IA/AAR, $P < 0.05$). %AAR/LV for the ingliforib treatment groups did not differ significantly ($P \geq 0.05$) from that of the control group (33 ± 2%). In anesthetized rabbits, a dose of ingliforib was selected to achieve free drug plasma concentrations comparable to an EC50 concentration (1.2 $\mu$M, 0.55 $\mu$g/ml) estimated from the isolated heart experiments. This dose of ingliforib (15 mg/kg loading dose; 23 mg$\cdot$kg$^{-1}$$\cdot$h$^{-1}$ infusion) provided a plasma concentration of 21.0 ± 1.4 $\mu$g/ml just before the regional ischemia; ingliforib is 96.5% protein bound, yielding a free drug plasma concentration of 0.7 $\mu$g/ml (1.5 $\mu$M). At this dose, infarct size was significantly reduced by 52% in vivo (Fig. 4) (vehicle control: 65 ± 3% IA/AAR; ingliforib: 31 ± 4% IA/AAR, $P < 0.05$); the %AAR/LV did not differ ($P > 0.05$) between these groups (control: 41 ± 5%; ingliforib: 42 ± 4%).

GP activity was significantly ($P < 0.05$) inhibited in the myocardium from the ingliforib-treated animals (Fig. 5). At the end of the 30-min period of regional ischemia, GPs and total GP activity were reduced by 65% and 40%, respectively, in the ischemic myocardium, and 41% and 33%, respectively, in the nonischemic myocardium (Fig. 5). In addition, the ingliforib-dependent GP activity inhibition was significantly ($P < 0.05$) greater in the ischemic versus nonischemic myocardium. GPs and total GP activity were similar in the ischemic and nonischemic myocardium from the vehicle-treated animals (Fig. 5). Inhibition of GP activity by ingliforib was also verified in hearts in which infarct size was determined by measuring GP activity in the right ventricle; GPs and total GP activity were reduced by 83% (vehicle: 4,164 ± 699 dpm/mg tissue, ingliforib: 666 ± 115 dpm/mg tissue; $n = 8$) and 63% (vehicle: 7,044 ± 1,003 dpm/mg tissue, ingliforib: 2,622 ± 247; $n = 8$), respectively, at the end of the reperfusion period.

To establish inhibition of glycogenolysis by ingliforib, glycogen content in the ischemic and nonischemic myocardium from vehicle- and ingliforib-treated anesthetized rabbits was measured at the end of the 30-min period of regional ischemia. Myocardial glycogen stores were significantly ($P < 0.05$) reduced in the ischemic versus nonischemic myocardium, whereas ingliforib treatment significantly ($P < 0.05$) preserved glycogen content in the ischemic myocardium (Fig. 6).

Systemic GP inhibition by the cardioprotective dose of ingliforib was assessed by measuring plasma glucose and lactate concentrations. Baseline plasma glucose and lactate concentrations were comparable in vehicle and ingliforib-treated groups (Fig. 7). In vehicle control animals, a rise in plasma glucose and lactate concentrations were observed during the ischemic period, which peaked at the end of the ischemia and remained elevated during the subsequent reperfusion. Ingliforib significantly ($P < 0.05$) blunted the rise in both glucose and lactate plasma concentrations (Fig. 7).

DISCUSSION

Under the anaerobic conditions of myocardial ischemia, the heart relies primarily on glycolysis for ATP generation (32). Myocardial glycogen and glycogenolysis are important sources of glycolytic substrate, particularly when coronary flow is limited (5) and exogenous glucose delivery to the heart is reduced. However, because oxidation is impaired during ischemia, glycolysis and oxidation become uncoupled, leading to a buildup of lactate and H$^+$ (4, 16). This deleterious process can continue during reperfusion because glycolysis and fatty acid oxidation (on which the heart primarily depends for its energy demands) recover quickly and may exceed preischemic rates (21, 25, 36). Consequently, glucose oxidation remains markedly depressed, glycolytic/oxidative uncoupling continues, and the myocardial lactate/proton load persists (24, 25, 35). When we consider these observations, it follows that inhibition of glycogenolysis would be cardioprotective due to a reduction in glycolytic substrate and improved glycolytic/oxidative coupling; indeed, studies (30, 42) in preconditioned hearts have demonstrated glycogenolysis is significantly attenuated. Weiss et al. (42) established this decrease in glycogenolysis (likely due to the reduced conversion of GP to the “a” or “active” form during early ischemia) resulted in a diminished glycolytic rate and decreased accumulation of lactate and H$^+$. Conversely, GP activity and glycogenolysis have been reported to increase markedly during global low-flow ischemia in nonpreconditioned hearts (8).

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

<table>
<thead>
<tr>
<th>Group</th>
<th>$n$</th>
<th>Preischemia HR, beats/min</th>
<th>Preischemia CF, ml/min$^{-1}$g$^{-1}$</th>
<th>Preischemia LVDP, mmHg</th>
<th>End Ischemia HR, beats/min</th>
<th>End Ischemia CF, ml/min$^{-1}$g$^{-1}$</th>
<th>End Ischemia LVDP, mmHg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>8</td>
<td>213 ± 10</td>
<td>7.1 ± 0.4</td>
<td>101 ± 2</td>
<td>203 ± 11</td>
<td>4.9 ± 0.3*</td>
<td>64 ± 5*</td>
</tr>
<tr>
<td>Ingliforib (0.1 $\mu$M)</td>
<td>6</td>
<td>208 ± 11</td>
<td>6.5 ± 0.2</td>
<td>99 ± 4</td>
<td>199 ± 9</td>
<td>4.6 ± 0.4*</td>
<td>65 ± 7*</td>
</tr>
<tr>
<td>Ingliforib (1 $\mu$M)</td>
<td>6</td>
<td>207 ± 7</td>
<td>7.2 ± 0.3</td>
<td>95 ± 3</td>
<td>194 ± 6</td>
<td>4.7 ± 0.2*</td>
<td>55 ± 6*</td>
</tr>
<tr>
<td>Ingliforib (10 $\mu$M)</td>
<td>10</td>
<td>203 ± 3</td>
<td>7.0 ± 0.3</td>
<td>93 ± 3</td>
<td>200 ± 5</td>
<td>5.2 ± 0.2*</td>
<td>50 ± 5*</td>
</tr>
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</table>

Values are means ± SE; $n$, no. of rabbit hearts. HR, heart rate; CF, total coronary flow; LVDP, left ventricular developed pressure. *$P < 0.05$ vs. preocclusion values.
To formally establish that pharmacological inhibition of GP is cardioprotective, we used a novel GP inhibitor, ingliforib, in well-established models of myocardial ischemia-reperfusion injury. Ingliforib inhibits the myocardial GP isoforms (muscle and brain) with IC$_{50}$s of 352 and 150 nM, respectively, and is also a potent inhibitor of the liver isoform (IC$_{50}$ of 52 nM). By using this compound, we have demonstrated for the first time that inhibition of myocardial GP provides significant protection from myocardial ischemia-reperfusion injury. The cardioprotection afforded by ingliforib in the isolated rabbit heart was concentration dependent; 10 $\mu$M ingliforib reduced infarct size by 69%, which is similar to the efficacy of other cardioprotec-

Fig. 2. Effect of ingliforib and vehicle control on heart rate (HR) (A), mean arterial pressure (MAP) (B), and rate-pressure product (RPP) (C) in anesthetized rabbits. Ingliforib or vehicle was administered by constant infusion beginning 60 min before the regional ischemia and continued for the duration of the experiment, as described in MATERIALS AND METHODS. Ingliforib had no significant effect on MAP or RPP, although a modest ($P < 0.05$) decrease in HR vs. vehicle control was observed during the reperfusion period. Data are the means ± SE for each group ($n = 8$). $^* P < 0.05$, significantly different from vehicle.

Fig. 3. Effect of ingliforib on the percentage of infarct area normalized for area-at-risk (%IA/AAR) in isolated rabbit hearts. Ingliforib was constantly perfused through the hearts beginning 30 min before regional ischemia and continued for the duration of the experiment, as described in MATERIALS AND METHODS. IA and AAR were determined by image analysis. Data from each heart are presented, along with the means ± SE for each group ($n = 6$–10). $^* P < 0.05$, significantly different from control.

Fig. 4. Effect of in vivo administration of ingliforib on %IA/AAR in anesthetized rabbits. Ingliforib or vehicle was administered by constant infusion beginning 60 min before the regional ischemia and continued for the duration of the experiment as described in MATERIALS AND METHODS. IA and AAR were determined by image analysis. Data from each heart are presented, along with the means ± SE for each group ($n = 8$). $^* P < 0.05$, significantly different from vehicle.
tive agents we have characterized in this model (e.g., adenosine A1 receptor agonists, Na+/H+ exchanger inhibitors, Na+/Ca2+ exchanger inhibitors, aldose reductase inhibitors) (18, 26, 40, 41). In vivo studies, which were designed to target a free plasma concentration equivalent to the EC50 we estimated from the isolated heart studies, resulted in a 52% reduction in infarct size. In addition, GP inhibition was confirmed in vivo, both within the heart and systemically. GP activity (total and GPa) was significantly blunted by ingliforib in the ischemic and nonischemic myocardium, and glycogen stores preserved in the ischemic myocardium. Systemically, plasma glucose and lactate concentrations were significantly lowered by ingliforib treatment. However, the in vivo cardioprotective efficacy of ingliforib was independent of systemic GP inhibition because ingliforib reduced infarct size in vitro, and equivalent drug exposure in vitro and in vivo produced similar reductions in infarct size. It was noteworthy that neither in the isolated heart, nor in vivo, were any significant unwanted cardiovascular effects observed, i.e., changes in cardiac function, coronary flow, or mean arterial blood pressure. In vivo, heart rate was minimally reduced in the ingliforib-treated group; whereas this could be viewed as a trend toward reducing myocardial oxygen consumption, a significant drop in RPP was not observed. Our results show that partial (65–83%) inhibition of cardiac GP was associated with reduced infarct size in the absence of other untoward effects on cardiac function. Whether complete inhibition of cardiac GP in the ischemic myocardium would produce a similar profile, or would lead to untoward effects due to energy substrate deprivation, remains to be determined.

Our data support earlier studies in which α-1,6-glucosidase glycogen debranching enzyme inhibitors (miglitol, MOR-14) preserved myocardial glycogen content, attenuated lactate accumulation and reduced infarct size (2, 29). The demonstration that ingliforib has similar effects on myocardial glycogen content and infarct size further underscores the significance of inhibiting glycogenolysis for ameliorating myocardial ischemia-reperfusion injury, while validating GP as a cardioprotect-

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**Fig. 5.** Effect of ingliforib on myocardial glycogen phosphorylase a (GPa) and total glycogen phosphorylase (GP) activity at the end of 30 min of regional ischemia. Ingliforib or vehicle was administered by constant infusion to anesthetized rabbits, beginning 60 min before regional ischemia. The ischemic and nonischemic myocardium was harvested and glycogen phosphorylase activity determined as described in MATERIALS AND METHODS. Data are means ± SE for each group (n = 7). *P < 0.05, significantly different from vehicle.

**Fig. 6.** Effect of ingliforib on myocardial glycogen stores after 30 min of regional ischemia. Ingliforib or vehicle was administered by constant infusion to anesthetized rabbits, beginning 60 min before the regional ischemia. The ischemic and nonischemic myocardium was harvested and glycogen content determined as described in MATERIALS AND METHODS. Data are means ± SE for each group (n = 7). NSD, not significantly different. *P < 0.05, significantly different.

**Fig. 7.** Effect of ingliforib and vehicle control on plasma glucose (A) and plasma lactate (B) in anesthetized rabbits. Ingliforib or vehicle was administered by constant infusion beginning 60 min before the regional ischemia and continued for the duration of the experiment as described in MATERIALS AND METHODS. Blood samples were obtained at the indicated time points and glucose and lactate concentrations determined. Data are the means ± SE for each group (n = 8). *P < 0.05, significantly different from vehicle.
tive molecular target. Moreover, the in vivo efficacy of ingliforib and lack of adverse effects suggests that GP inhibition may be a viable therapeutic approach for achieving clinical cardioprotection. As a pharmacological tool, ingliforib should facilitate further study of the role of GP in the physiology/pathophysiology of the heart and other organs.

Although these studies focused on the response of normal hearts to ischemia-reperfusion injury, GP inhibitors are being investigated for the treatment of diabetes (ingliforib reduced plasma glucose and lactate in our normal rabbits, and reduces plasma glucose in diabetic models (D. J. Hoover, E. M. Gibbs, and J. L. Treadway, unpublished observations)). Moreover, diabetic patients have an increased risk for developing cardiovascular complications, including myocardial infarction (11, 14). Although controversial (6, 33), the almost complete reliance of the diabetic heart on fatty acid metabolism and minimal glycogen stores, and provides significant cardioprotection from ischemia-reperfusion injury. The benefit resulting from GP inhibition may ultimately be due to a reduction in myocardial glycolysis, an improvement in glycolytic/oxidative coupling, and a reduction in intracellular proton load. Moreover, the cardioprotection is achieved without eliciting undesirable changes in cardiac function or hemodynamics. Thus GP inhibition may represent an attractive target for clinical cardioprotection and for treating diabetic patients at increased risk for cardiovascular complications.

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DISCLOSURES

All of the authors are employees of Pfizer and have financial interests in the company.

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