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Protection Against Ischemia and Reperfusion Injury

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(54) **PROTECTION AGAINST ISCHEMIA AND REPERFUSION INJURY**

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(51) **Int. Cl.**^{7} **A61K 38/00**
(52) **U.S. Cl.** **514/13**; 530/326; 435/114; 424/278.1
(58) **Field of Search** 530/324, 326; 514/2, 13; 435/114, 187; 604/19; 424/278.1

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(57) **ABSTRACT**

A compound and method for using compound-D SEQ ID NO:1 to reduce injury associated with ischemia and reperfusion of mammalian organs such as the heart. The compound may be administered as part of a preconditioning strategy which reduces the extent of injury and improves organ function following cessation and restoration of blood flow. The compound may be used in preparation for planned ischemia or in a prophylactic manner in anticipation of further ischemic events.

12 Claims, 6 Drawing Sheets

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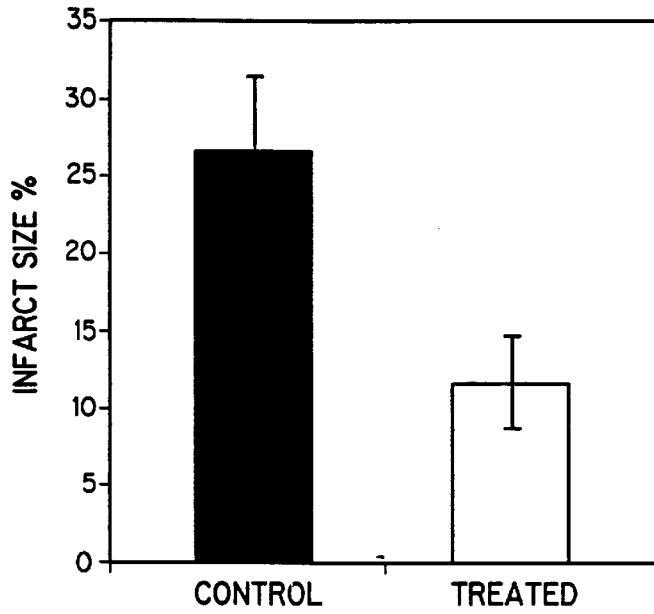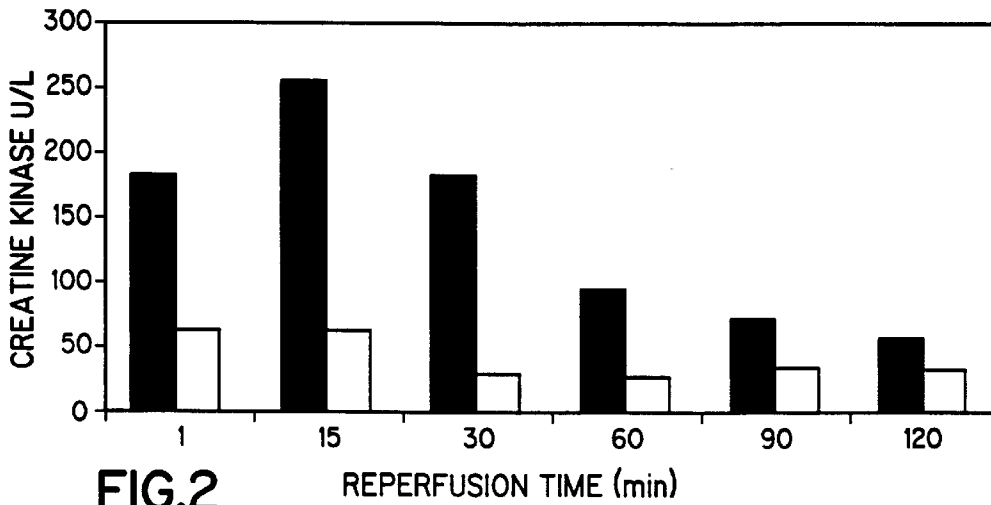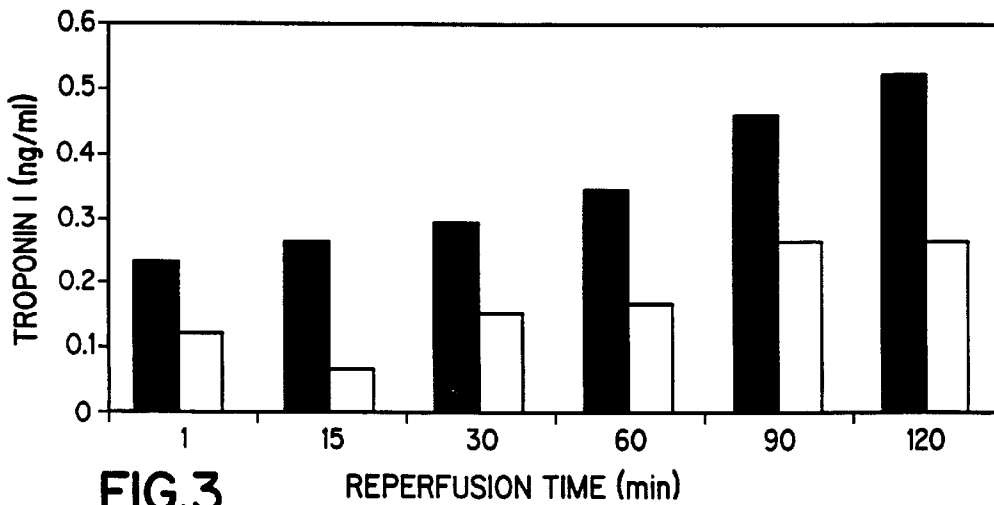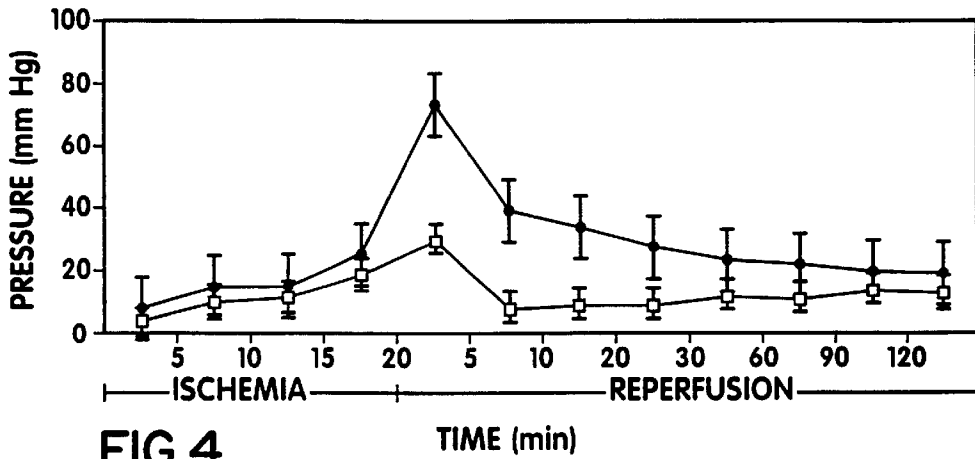
* cited by examiner

FIG.1

FIG.2   REPERFUSION TIME (min)

FIG.3   REPERFUSION TIME (min)

FIG.4
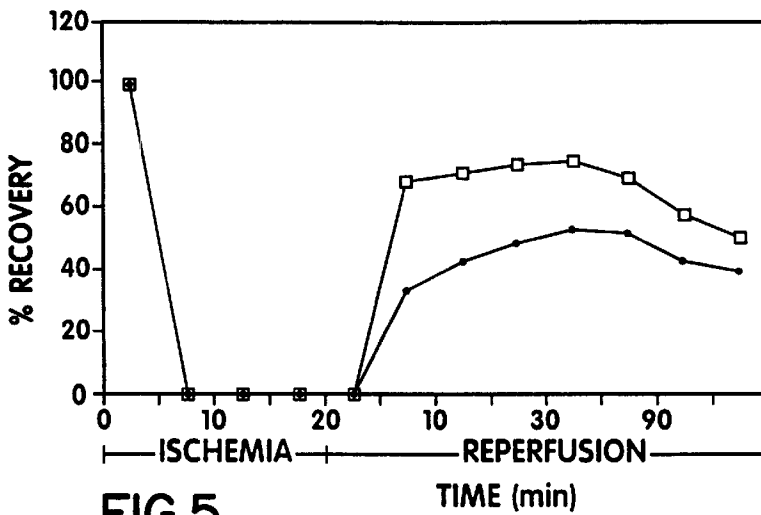
FIG.5

FIG.6

FIG.7

FIG.8

FIG.9
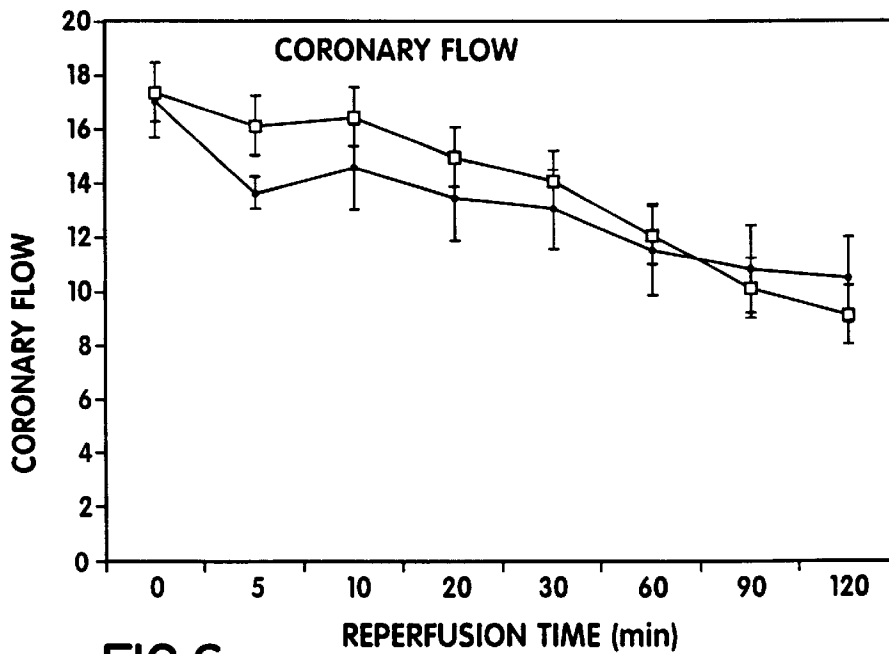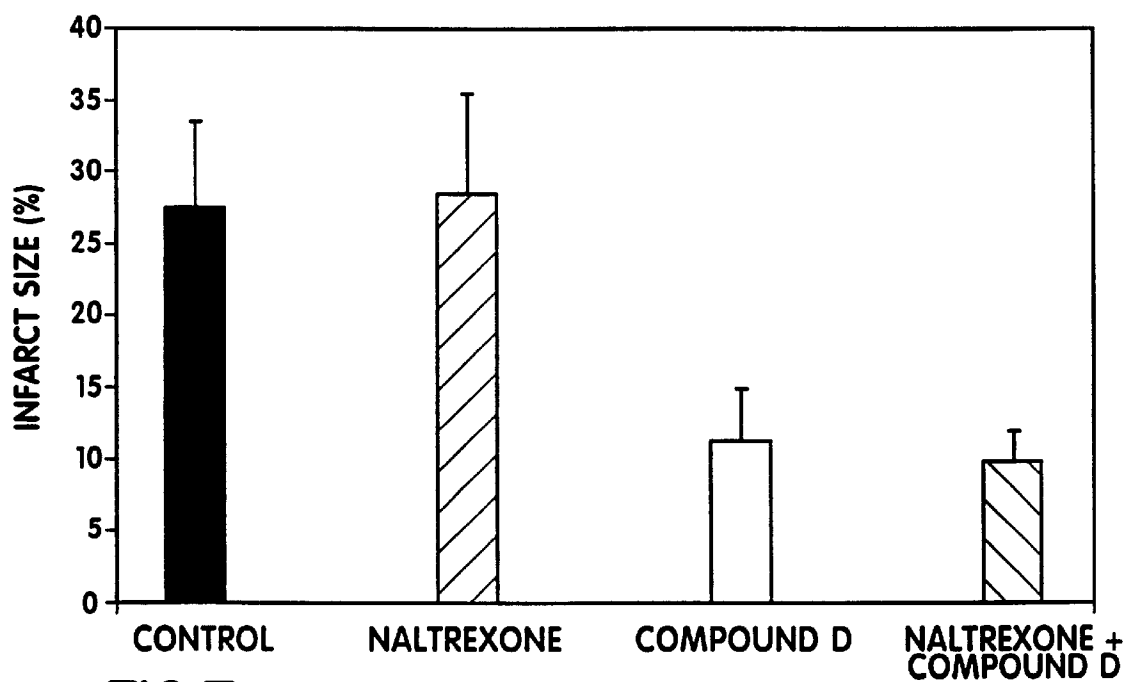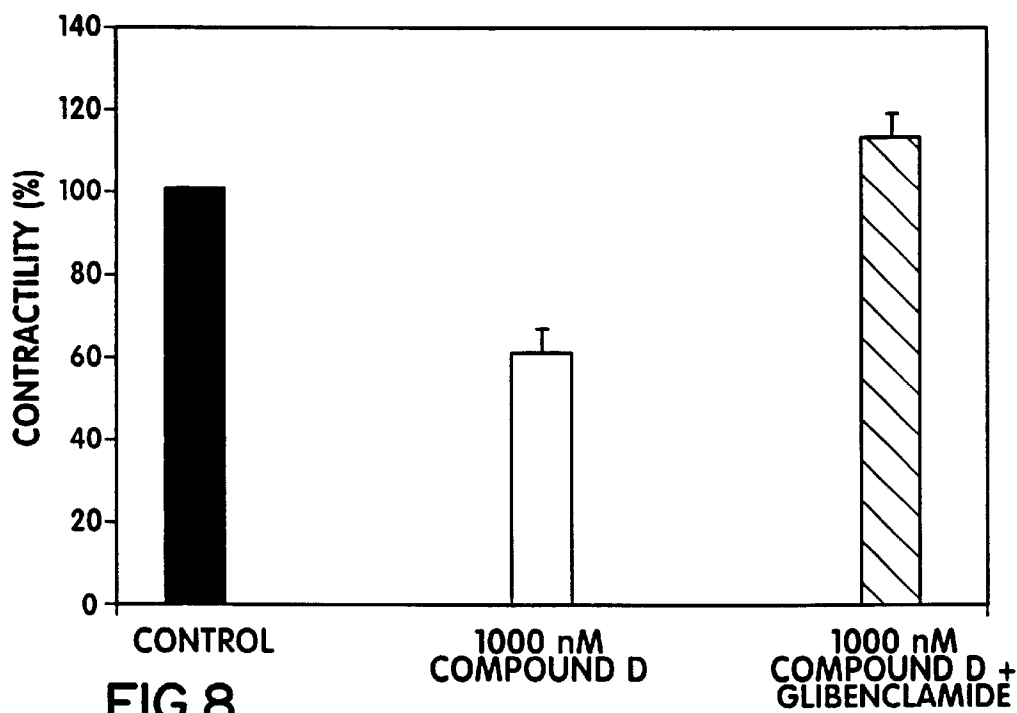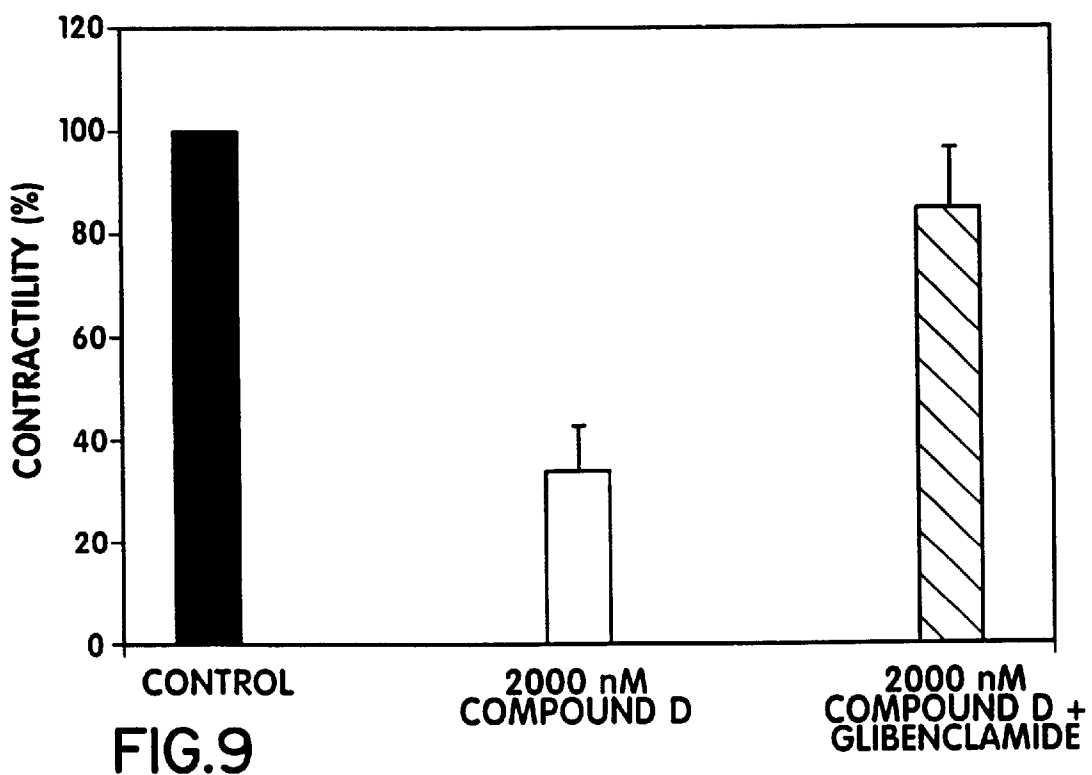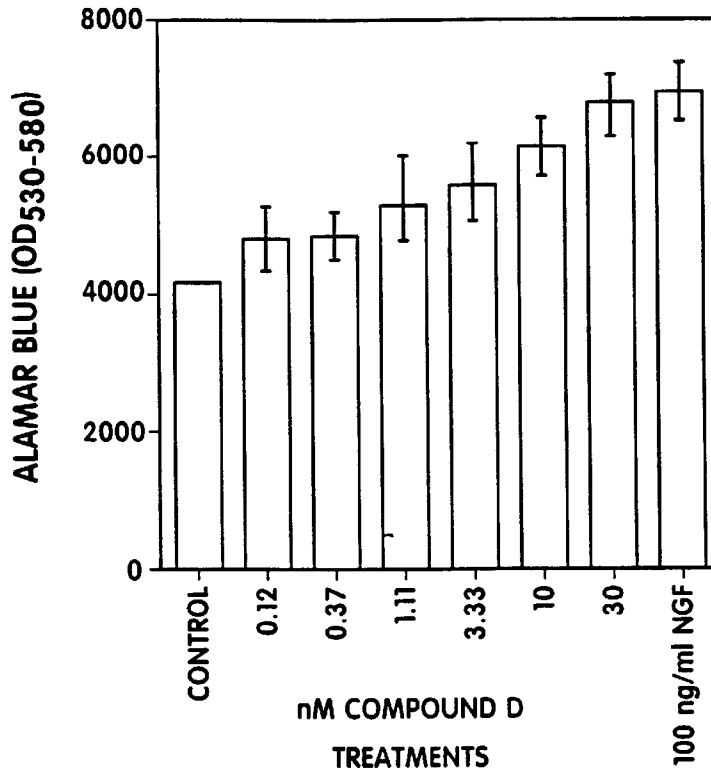
FIG. 10

FIG. 11

FIG. 12

FIG. 13

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PROTECTION AGAINST ISCHEMIA AND REPERFUSION INJURY

This application claims the benefit of U.S. Application Ser. No. 60/238,989, filed on Oct. 10, 2000.

FIELD OF THE INVENTION

The invention relates to compounds protective against ischemia and reperfusion injury, particularly in the myocardium, and their use.

BACKGROUND

Tissues deprived of blood and oxygen undergo ischemic necrosis or infarction with possible irreversible organ damage. In some circumstances, however, such as during cardiac surgery, it is desirable to interrupt the normal myocardial contractions (cardioplegia) and actually induce ischemia. Such elective or obligatory ischemia occurs in the presence of safeguards such as cardioplegia-induced cardiac arrest and hypothermia. While these safeguards provide considerable myocardial protection, alteration of myocardial energetics (stunning) and poor postoperative ventricular function still remain significant problems.

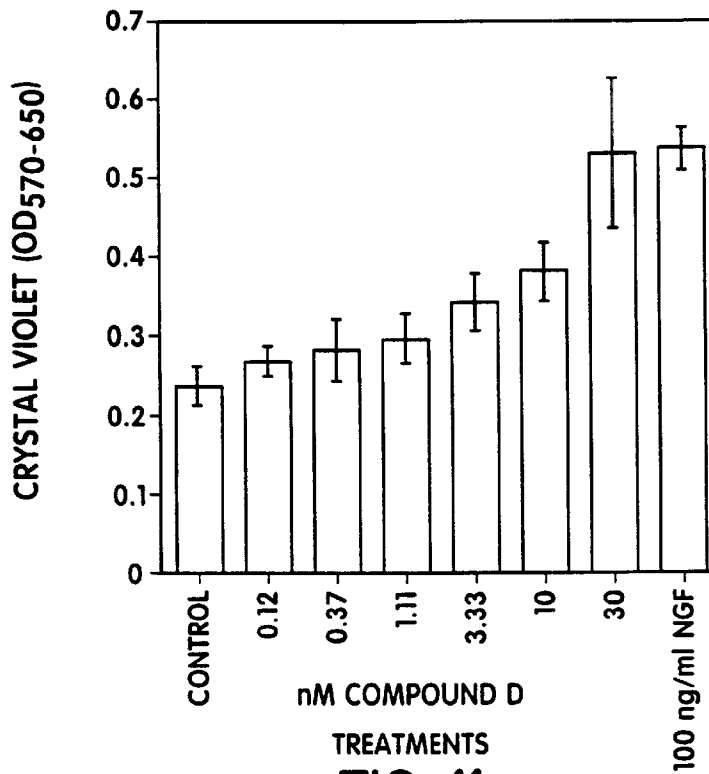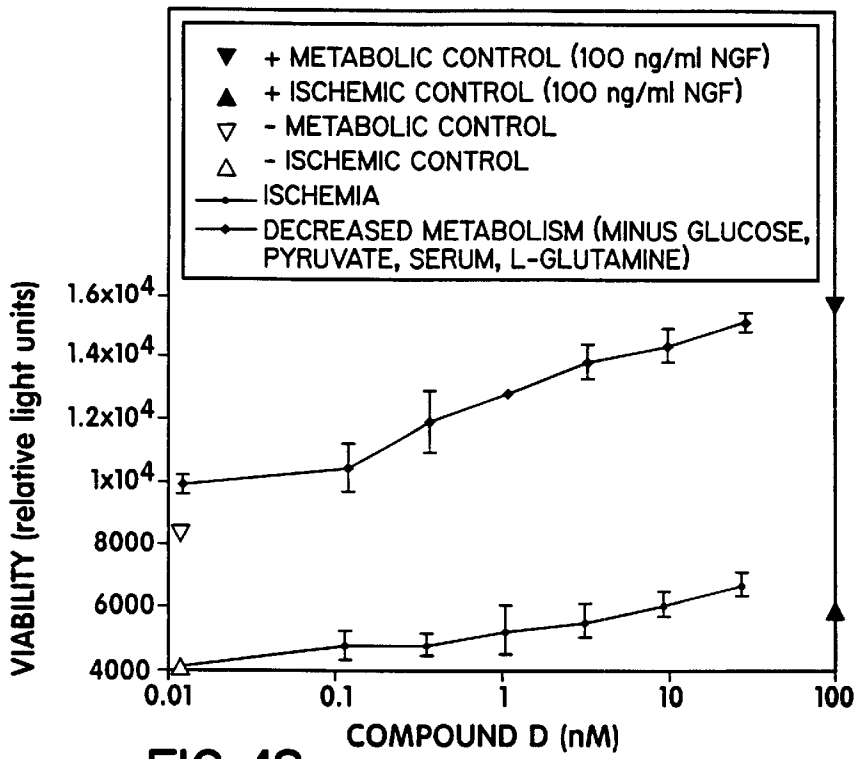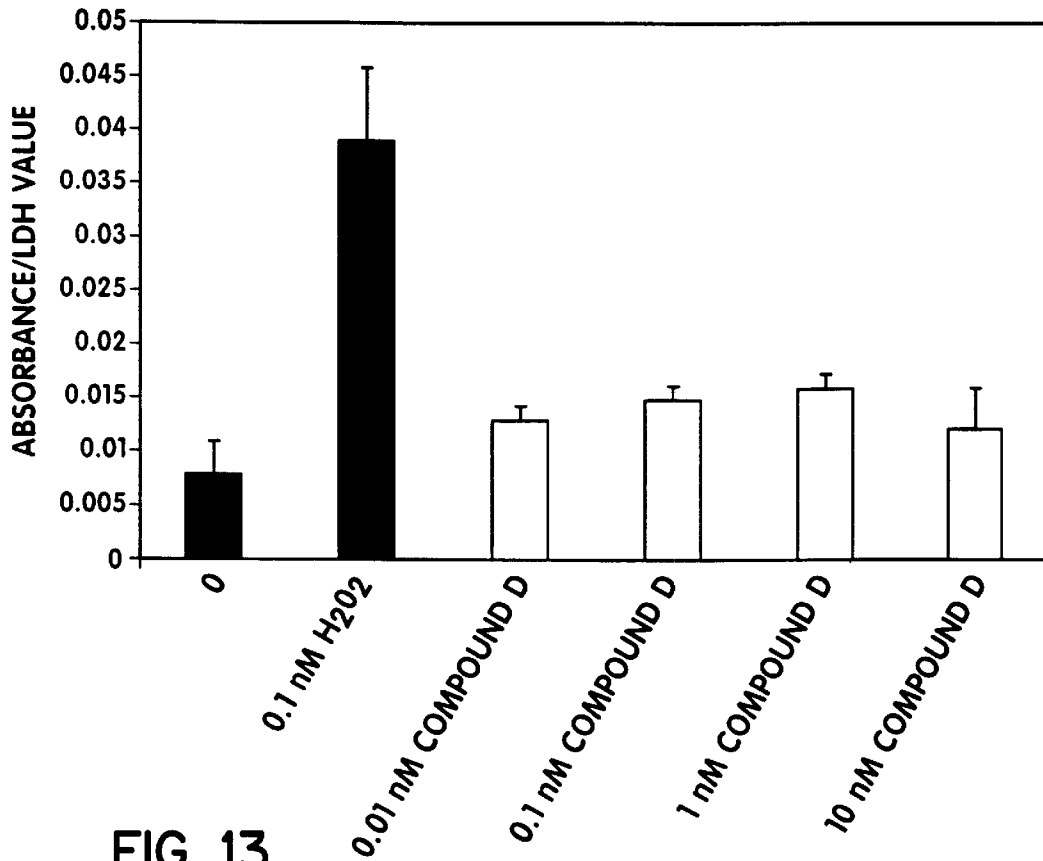Once the flow of blood and oxygen is restored to the organ or tissue (reperfusion), the organ does not immediately return to the normal preischemic state. Reperfused postischemic non-necrotic myocardium is poorly contractile and has reduced concentrations of high energy nucleotides, depressed subcellular organelle function and membrane damage that resolves only slowly. Although reperfusion restores oxygen and reverses ischemia, repletion of high energy nucleotides such as adenosine triphosphate (ATP) and reversal of ischemic membrane damage is slow, and contractile function may be profoundly depressed for a long period. Just minutes of ischemia causes loss of myocardial systolic wall thickening for hours. Longer periods of reversible ischemia may depress contractility for days. Studies confirm that, despite restoration of myocardial flow and a quick recovery of myocardial oxygen consumption (MVO_2) following ischemia, there is only very slow recovery of myocardial contractile function. The problems are exacerbated in high risk patients, such as those with poor preoperative ventricular function, recent myocardial infarction or left ventricular hypertrophy. These same problems also occur during organ storage for cardiac transplant, under which there are time constraints due to the limits of myocardial preservation.

Postischemic dysfunction may be due to a variety of factors. Oxygen free radicals may play a role, as generation of free radicals in stunned myocardium has been demonstrated and free radical scavengers have been shown to attenuate contractile dysfunction. Impaired intracellular calcium handling and calcium overload during early reperfusion may contribute to postischemic dysfunction; while calcium infusions enhance contractility in both normal and postischemic myocardium, ischemia as short as a few minutes produces an impairment in sarcoplasmic reticulum calcium transport and a shift of the calcium ATPase activity. Postischemic myocardium is also associated with reduced concentrations of myocardial high-energy phosphates and adenine nucleotides, as obligatory reduction in myocardial ATP content during ischemia occurs as myocytes utilize ATP for maintenance of cellular integrity. Since ATP is essential for myocardial contraction and relaxation, ATP depletion may have detrimental effects upon postischemic myocardial functional recovery.

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The high volume of cardiac-related surgeries, both elective and emergency procedures and including cardiac transplants, lead to the above-described problems. Thus, methods and agents to provide protection against myocardial ischemia and to avoid post ischemic dysfunction are needed.

SUMMARY OF THE INVENTION

The invention is directed to an agent and a method of using the agent to reduce the injury associated with ischemia and reperfusion of organs such as the heart. The compound is Tyr-D-Leu-Phe-Ala-Asp-Val-Ala-Ser-Thr-Ile-Gly-Asp-Phe-Phe-His-Ser-Ile-NH_2 (SEQ ID NO:1), hereinafter referred to as compound-D. Administration of compound-D SEQ ID NO:1, particularly prior to an ischemic event, reduces tissue necrosis and preserves organ function.

In one embodiment, a method of protecting against ischemia and reperfusion injury in a mammal is disclosed. An effective concentration of compound-D SEQ ID NO:1 is administered to the mammal in a pharmaceutically acceptable formulation prior to the onset of ischemia, for example, 24 hours prior to ischemia. In other embodiments, compound-D SEQ ID NO:1 is administered substantially concurrent with the onset of ischemia, during an ischemic episode, or post-ischemia. The formulation may be administered parenterally at an amount in the range of about 1–20 mg/kg of body weight.

The invention is also directed to a method to prevent damage to an isolated organ, for example, a heart for transplant. The isolated organ is exposed to a preservative solution containing an effective amount of compound-D SEQ ID NO:1. The concentration of compound-D SEQ ID NO:1 in the preservative solution for a heart is about 100 μM.

The invention is additionally directed to a method for reducing effects of an ischemic episode in a mammal by administering an effective concentration of compound-D SEQ ID NO:1 in a pharmaceutically acceptable carrier. Administration is prior to or substantially concurrently with the onset of ischemia, or one hour post cerebral ischemia.

The invention is further directed to a composition that protects a mammalian organ from injury. The composition contains compound-D SEQ ID NO:1. Compound-D may be naturally occurring or may be synthesized.

The invention is also directed to an organ preservative solution that contains compound-D at a concentration effective to protect the organ, such as a heart, from ischemic injury.

These and other advantages of the invention will be apparent in light of the following drawings and detailed description.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a histogram showing myocardial infarction size in control and treated animals.

FIG. 2 is a histogram showing post-ischemic release of creatine kinase in control and treated animals.

FIG. 3 is a histogram showing post-ischemic release of troponin I in control and treated animals.

FIG. 4 is a graph showing end diastolic pressure in the left ventricle of control and treated animals.

FIG. 5 is a graph showing functional recovery in control and treated animals.

FIG. 6 is a graph showing coronary flow in control and treated animals.

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FIG. **7** is a histogram showing the effect of naltrexone on infarct size in animals pretreated with compound-D SEQ ID NO:1.

FIG. **8** is a histogram showing the effect of glibenclamide on vas deferens contractility in animals treated with 1,000 nM compound-D SEQ ID NO:1.

FIG. **9** is a histogram showing the effect of glibenclamide on vas deferens contractility in animals treated with 2,000 nM compound-D SEQ ID NO: 1.

FIG. **10** is a histogram showing the effect of compound-D SEQ ID NO:1 on cell metabolic activity.

FIG. **11** is a histogram showing the effect of compound-D SEQ ID NO:1 on cell viability.

FIG. **12** is a graph showing cell viability and metabolic activity with increasing concentrations of compound-D SEQ ID NO:1.

FIG. **13** is a histogram showing the effect of compound-D SEQ ID NO:1 on cells exposed to oxidative damage.

DETAILED DESCRIPTION

The invention is directed to a compound that has a salutary effect on cardiac function following ischemia, and methods of using the compound. The compound may be administered directly to an individual, and is particularly effective when administered 24 h prior to the onset of ischemia. This may occur, for example, prior to scheduled cardiac surgery. The compound may also be included in a preservative solution for an isolated organ, such as a heart or liver being maintained viable for transplant.

The compound is a peptide having the sequence Tyr-D-Leu-Phe-Ala-Asp-Val-Ala-Ser-Thr-Ile-Gly-Asp-Phe-Phe-His-Ser-Ile-NH_2 SEQ ID NO: 1, hereinafter referred to compound-D. The peptide may be produced by a number of methods, such as using an automated peptide synthesizer, through recombinant molecular techniques, or isolated from a naturally occurring source, as is known to one skilled in the art. Compound-D SEQ ID NO:1 has a molecular weight of 1,902 daltons. Compound-D SEQ ID NO:1 is insoluble in water or saline, but may be solubilized by adding 100 μM of a solution comprised of ethanol, propylene glycol, and 1 N NaOH in a 1:1:1 ratio, with sterile physiological saline then used to obtain the appropriate concentration. The initial alkaline pH is adjusted to 7.4 with 1 N HCl.

Compound-D SEQ ID NO:1 that has been solubilized may be administered by parenteral means, for example, by intravenous injection. In one embodiment, administration of compound-D SEQ ID NO:1 is at the time of induced ischemia, fut may also be added during or even after an ischemic event. For administration into a mammal, a dose of about 1–20 milligrams per kilogram (mg/kg) is useful. For administration into a tissue or organ preservation solution, a concentration of about 100 μM is useful.

Compound-D SEQ ID NO:1 may be administered directly into a mammal, either alone or in combination with other substances. Alternatively, it may be added as a component of a solution used to maintain the viability of isolated organs, such as an additive to cardioplegia and other organ preservation solutions. In one embodiment, compound-D SEQ ID NO:1 is coadministered as an adjuvant with other compounds or strategies that are designed to protect organs from ischemia. As an example, compound-D SEQ ID NO:1 may be administered with agents that affect nitric oxide (NO) synthase, such as arginine hydrochloride. Arginine hydrochloride is known to prevent the decline in cardiac function following an ischemic episode.

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The following description demonstrates use and efficacy of compound-D SEQ ID NO:1 in a variety of systems.

Perfused Heart

Compound-D SEQ ID NO:1, at a dose of 2 mg/kg and solubilized as described above, was administered by tail vein injection into rats weighing between about 350–400 g (number of animals (n)=6). Control rats (n=6) were injected in the same manner with an equal volume of 0.9% NaCl. After 24 h, the hearts from both treated and control animals were excised and perfused in a modified Langendorff perfusion apparatus at 37° C. using oxygenated Krebs-Henseleit buffer as the perfusate, as known to one skilled in the art. Coronary perfusion pressure was maintained at 700 mm Hg by regulating coronary flow. All hearts were paced at 5.5 Hz (300 beats per minute, bpm) except during ischemia. A saline-filled balloon was inserted in the left ventricle to measure developed pressure (DP) and end-diastolic pressure (EDP) in mm Hg. After 15 min equilibration, both groups were subjected to 20 min zero-flow global ischemia, and then were reperfused for 120 min.

Left ventricles were isolated and divided into three segments along their short axis, stained with triphenyltetrazolium chloride, and stored in formalin. Infarct size was measured on digitized images and expressed as a percentage of myocardium. Data were expressed as mean plus or minus standard error of the mean (±SEM) and were analyzed using a paired Student's t-Test. Confidence limits were established at 95%.

The results are shown in FIG. **1**, which is a histogram of the infarct size in hearts from both animals receiving only saline (control, solid bar) and in animals treated with compound-D SEQ ID NO:1 at 2.0 mg/kg (treated, open bar) 24 h prior to 20 min ischemia and 120 min reperfusion. Pretreatment of animals with compound-D SEQ ID NO:1 significantly decreased the percent of infarct size (p<0.05). Control rats had a mean infarct size of 27±5%, while rats pretreated with compound-D had a reduced mean infarct size of 12±3%.

Compound-D SEQ ID NO:1 pretreatment also significantly decreased total creatine kinase (CK) and the cardiac form of troponin I (cTn-1) values following 20 min ischemia, as shown in FIGS. **2** and **3**,respectively. CK is an enzyme that is normally found in muscles such as the heart (that is, in myocytes), but is released during severe muscle trauma such as occurs in a myocardial infarction. Thus, measurement of CK is an indicator of the degree of muscle damage or trauma. Similarly, cTn-I is a protein associated specifically with cardiac muscle, and an increase in cTn-1 indicates myocardial damage.

FIG. **2** is a histogram showing CK released during reperfusion of isolated rat hearts after 20 min ischemia in rats treated 24 h prior to ischemia with 2.0 mg/kg compound-D SEQ ID NO:1. The solid bars represent control animals (n=6) and the open bars represent treated animals (n=6). FIG. **3** is a histogram showing cTn-I released during reperfusion of isolated rat hearts after 20 min ischemia in rats treated 24 h prior to ischemia with 2.0 mg/kg compound-D SEQ ID NO:1. The solid bars represent control animals (n=6) and the open bars represent treated animals (n=6). At each time point during reperfusion where samples were collected for CK and cTn-1 analysis (1, 15, 30, 60, 90 and 120 min reperfusion), CK levels in control rats were higher than CK levels in treated rats, and Tn-1 levels in control rats were higher than Tn-1 levels in treated rats. This indicated that pretreatment with compound-D SEQ ID NO:1 decreased the damage to the myocardium, as compared to animals that did not receive compound-D SEQ ID NO:1.

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Compound-D SEQ ID NO:1 pretreatment also resulted in improved postischemic ventricular function. FIG. **4** is a graph of end diastolic pressure (EDP) in mm/Hg in the left ventricle during reperfusion of isolated rat hearts after 20 min ischemia in rats treated 24 h prior to ischemia with 2.0 mg/kg compound-D SEQ ID NO:1. Open squares are from treated animals, and solid circles are from control animals. FIG. **5** is a graph showing percent of functional recovery during reperfusion of isolated rat hearts after 20 min ischemia in rats treated 24 h prior to ischemia with 2.0 mg/kg compound-D SEQ ID NO:1. Open squares are from treated animals, and solid circles are from control animals. Differences in recovery of developed pressure (DP) of compound-D SEQ ID NO:1 treated hearts remained statistically significant to 90 min following initiation of reperfusion, as shown in FIG. **4**, and in end diastolic pressure to 20 min following initiation of reperfusion, as shown in FIG. **5**. Left ventricular functional recovery (% recovery of baseline preischemic developed pressure during reperfusion) was markedly improved in treated animals (open squares) compared to control animals (solid circles) to 90 min of reperfusion. As shown in FIG. **6**, coronary flow was not significantly different between control animals (solid circles) and compound-D SEQ ID NO:1 treated animals (open squares).

These results show that in a normoxic, isolated perfused rat heart preparation, administration of compound-D can confer cardioprotection when administered 24 h prior to planned ischemia. The salutary effects on the post-ischemic myocardium include reduced infarct size, decreased release of both creatine kinase and cardiospecific troponin I, and improved ventricular performance.

FIG. **7** shows the effect of naltrexone, a universal opioid antagonist, on infarct size. Groups of rats were pretreated 24 h prior to ischemia with either compound-D SEQ ID NO:1 at a dose of 2.0 mg/kg (n=6), naltrexone at a dose of 3 mg/kg (n=6), or a combination of compound-D SEQ ID NO:1 at a dose of 2.0 mg/kg and naltrexone at a dose of 3 mg/kg (n=6). Control rats (n=6) received vehicle (1:1:1 solution of 1N NaOH:propyleneglycol:ethanol, readjusted to 7.4 with HCl) only. After 24 h pretreatment, isolated rat hearts were subjected to 20 min ischemia followed by 120 min reperfusion.

As shown in FIG. **7**, these data indicate that naltrexone administered in combination with compound-D SEQ ID NO:1 did not block the cardioprotective effects of compound-D SEQ ID NO:1. In fact, the data support a synergistic cardioprotective effect from administration of a combination of compound-D SEQ ID NO:1 and naltrexone. Administration of compound-D SEQ ID NO:1 reduced infarct size from about 27% in the control group (solid bar) to about 13% in the compound-D SEQ ID NO:1 treated group (open bar) (p=0.05). The combined preischemic administration of compound-D SEQ ID NO:1 and naltrexone (downward hatched bar) reduced infarct size to about 10% when compared to the control group (p=0.05). Additionally, these data demonstrate that compound-D SEQ ID NO:1 does not operate via delta opioid receptors, since naltrexone, an opioid receptor antagonist, did not prevent compound-D SEQ ID NO:1 activity in reducing infarct size (about 14.5% versus about 13%, respectively).

Mouse Vas Deferens

Utilizing a mouse vas deferens (m.v.d.) assay, compound-D SEQ ID NO:1,it was demonstrated that opioid receptor binding requires activation and/or opening of adenosine triphosphate-sensitive potassium (KATP) chan-

nels. Glibenclamide, a K_{ATP} channel blocker, blocked the inhibition of contraction of mouse vas deferens induced by compound-D SEQ ID NO:1. With reference to FIG. **8**, the effect of administration of 1000 nM compound-D SEQ ID NO:1 alone (open bar) versus 1000 nM compound-D SEQ ID NO:1 with 6000 nM glibenclamide (hatched bar) on contractility of mouse vas deferens (m.v.d.) (n=6) is shown. Glibenclamide restored m.v.d. contractions which compound-D SEQ ID NO:1 had suppressed. FIG. **8** shows that 1000 nM compound-D SEQ ID NO:1 suppressed contractility to above 100%. FIG. **8** also shows that differences between control contractions and 1000 nM compound-D SEQ ID NO:1, as well as control versus 6000 nM glibenclamide, and 1000 nM compound-D SEQ ID NO:1 versus 6000 nM glibenclamide are statistically significant. FIG. **9** shows the effect of administration of 2000 nM compound-D alone (open bars) versus 2000 nM compound-D SEQ ID NO:1 with 6000 nM glibenclamide (hatched bar) on contractility of m.v.d. (n=2).

These results demonstrate that the effect of compound-D SEQ ID NO:1 requires opening and/or activation of K_{ATP} channels since glibenclamide, a potassium channel blocker, effectively blocked the smooth muscle inhibition of contraction induced by compound-D SEQ ID NO:1 in the m.v.d. assay. The possibility therefore exists that administration of compound-D SEQ ID NO:1 and interventions directly targeted at opening K_{ATP} channels by drugs such as nicorandil or diazoxide, or anesthetics such as isoflurane, could be effective approaches for pharmacologically duplicating both short term and long term (second window) ischemic preconditioning. Thus, preinfusions of compound-D SEQ ID NO:1 may provide extended protection to the ischemic myocardium in a variety of clinical scenarios.

Compound-D, SEQ ID NO:1 has also been shown to be effective in reducing ischemic effects when administered 1 h after an episode of induced cerebral ischemia. A solution of 100 μL of either vehicle alone (control) or compound-D (treated) at a concentration of 1 mg/ml was administered either 1 h before cerebral ischemia or 1 h after cerebral ischemia (n=6 in each group). In animals receiving compound-D before ischemia, there were no significant differences in infarct volume (control 91±10 mm^3 versus treated 89±11 mm^3) or neurological score (control group 1.93±0.29 versus treated group 1.95±0.33). However, when compound-D was administered 1 h post ischemia, there were significant difference in both the infarct volume (control group 90±13 mm^3 versus treated group 56±9 mm^3) and the neurological score (control group 1.91±0.27 versus treated group 1.42±0.28) in rats receiving compound-D SEQ ID NO:1 versus the control group. The mean arterial blood pressure, pO_2,pCO_2, pH and cerebral blood flow showed no significant differences in control versus treated groups when compound-D SEQ ID NO:1 was administered either pre- or post-cerebral ischemia (data not shown). These results suggest a therapeutic role for compound-D SEQ ID NO:1 in mitigating the effects of a cerebral ischemic episode that is in progress or that has occurred.

Cultured Cell Lines

The rat pheochromocytoma cell line, P012 (ATOC; CRL-1721) was cultured in growth medium, RPMI 1640 medium supplemented with 10 mM HEPES (N-2-hydroxyethyl-piperazine-N'-(2'-ethanesulfonic acid)), 1 mM sodium pyruvate, 2 mM L-glutamine (GIBCO BRL), 10% horse serum (HS; Sigma), and 5% fetal bovine serum (FBS; JRH). Ten to twelve days prior to assay, PC12 cells were induced with 50 ng/ml recombinant human beta nerve growth factor (NGF; R&D Systems) to the neuronal phenotype on col-

lagen I coated tissue culture dishes. Two days prior to assay, cells were plated on collagen IV coated 48-well tissue culture plates without NGF at $3-5\times10^5$ cells per well.

For hypoxic/ischemic conditions, the medium was changed to either Dulbecco's Modified Eagle Medium (DMEM; GIBCO BRL) without glucose, or DMEM with 5 mM glucose. Both were supplemented with 10 mM HEPES containing compound-D SEQ ID NO:1 at various concentrations. Cells were placed in an environmental chamber (Plas-Labs) maintained at 37° C. and perfused with either a gas mixture of 85% nitrogen, 10% hydrogen and 5% carbon dioxide to induce anoxic conditions, or a mixture of air and 5% carbon dioxide to induce normoxic conditions. After four hours, plates were removed from the chambers and medium was changed to growth medium with compound-D SEQ ID NO:1 at appropriate concentrations. Cells were returned to the air-5% CO_2 incubator until the next day.

Alamar Blue (AccuMed International Companies), containing a fluorometric/colorimetric oxidation/reduction indicator, was added to the assay medium in an amount equal to 10% of the assay volume. Cells were returned to the incubator for two to four hours. Fluorescence of each well was measured at an excitation wavelength of 530 nm and an emission wavelength of 580 nm in a CytoFluor Multi-well Plate Reader (PerSeptive Biosystems). The average fluorescence readings from three wells were used as a relative measurement of the metabolic activity of the cells.

Viability was assessed in the same wells using a crystal violet staining technique. Cells were washed twice with phosphate buffered saline (PBS). Cells were lysed and stained with 0.5% crystal violet in 20% methanol. Fifty microliters of this solution was left in the wells for five minutes. The plates were then washed under slow running water about five times or until the water ran clear. Excess water was removed by inverting and tapping the plates on an absorbent surface. Stained cells were solublized with at least 200 μl/well of a solution of 0.1% sodium citrate in 50% ethanol. After ten minutes, 50 μl of solublized stain was transferred to a 96-well microtiter plate and read at an absorbance of 570 nm and subtracted from reference wavelength of 650 nm. The average readings from three wells were used as a relative measurement of the viability of the cells.

As shown in FIG. 10, cell metabolic activity as assessed by Alamar Blue uptake by PC12 cells was increased with increasing concentrations of compound-D SEQ ID NO:1.

As shown in FIG. 11, cell viability as assessed by crystal violet uptake of PC12 cells was increased with increasing concentrations of compound-D SEQ ID NO:1.

As shown in FIG. 12, NGF differentiated cells under ischemic conditions and treated with increasing concentrations of compound-D SEQ ID NO:1 (bottom line) showed increasing viability, with the highest concentration of compound-D SEQ ID NO:1 showing cell viability slightly exceeding the positive control. Also as shown in FIG. 12, NGF differentiated cells under suboptimal metabolic conditions and treated with increasing concentrations of compound-D SEQ ID NO:1 (top line) showed increasing cell viability.

Oxidative damage has been suggested to play a critical role in a number of cardiovascular diseases including atherosclerosis, fetal arrhythmia, aortic stenosis, cardiac hypertrophy, or in valvular diseases where prolonged cardiac ischemia is a factor. During reperfusion, reactive oxygen species including hydrogen peroxide (H_2O_2) and hydroxyl radicals are found in the myocardium. These oxidants can be derived from various sources such as activated neutrophils, xanthine oxidase, mitochondrial respiration, and arachidonic acid metabolism. The resulting

oxidative damage has been shown to cause cell death in many experimental systems. (Chen, Q. M., Bahl, J. J., Archives of Biochemistry and Biophysics, Vol. 373, No. 1, 242–248, Jan. 1, 2000).

The rat myoblast cell line H9C2 was used to measure myocardial cell necrosis. H9C2 cells were plated onto 96 well plates and grown to confluency in DMEM +10% FBS. Plates were washed one time with assay buffer consisting of phenol red free DMEM +2.5% FBS supplemented with nonessential amino acids, sodium pyruvate and HEPES buffer. All test reagents were made using the assay buffer.

Compound-D SEQ ID NO:1 was diluted in assay buffer to 2×final concentrations, ranging from 20 nM to 0.2 nM. H_2O_2 stock (30%) was diluted to 2×final concentration (0.2 mM) in assay buffer.

Immediately following the wash step, 50 μl 2×compound-D SEQ ID NO:1 at varying concentrations or assay buffer only (control) was added to quadruplicate wells of a 96 well plate. Cells were incubated at 37° C. under 5% CO_2 for thirty minutes.

Following the pre-incubation, 50 μl of 2×(0.2 mM) H_2O_2 was added to all wells except the controls (media only, no treatment). The final H_2O_2 concentration was 0.1 mM. The plate was again incubated at 37° C. for four hours.

The cell supernatants were immediately assayed for lactate dehydrogenase (LDH) release using the CytoTox 96 Non-Radioactive Cytotoxicity Assay (Promega, catalogue #G 1780), following the manufacturer's assay procedures. LDH is a stable cytosolic enzyme that is released upon cell lysis. It is used to measure either cell mediated cytoxicity or cytotoxicity mediated by chemicals or other agents, and provides a sensitive marker of cell damage and necrosis. Briefly, 50 μl supernatant samples from each well were transferred to another 96 well plate. Assay media only and total cell lysates were included as controls. Substrate mix (50 μl) was added to all wells and the plate was covered and incubated in the dark for thirty minutes at room temperature. Stop solution (50 μl) was added to each well and the absorbance at 490 nM was recorded. Assay media only absorbance values were subtracted to correct for any LDH activity that may be present in the assay buffer.

As shown in FIG. 13, cell necrosis due to oxidative damage was decreased in the presence of compound-D SEQ ID NO:1.

In summary, a compound that is administered to a mammal to reduce the injury associated with ischemia and reperfusion of organs such as the heart is disclosed. The compound is Tyr-D-Leu-Phe-Ala-Asp-Val-Ala-Ser-Thr-Ile-Gly-Asp-Phe-Phe-His-Ser-Ile-NH₂ SEQ ID NO:1. Administration prior to an ischemic event reduces tissue necrosis and preserves organ function, as measured by standard physiological indices of organ function. The compound may be administered in a time frame from 24 h up to 15 min prior to planned ischemia, or even during an ischemic period.

It should be understood that the embodiments of the present invention shown and described in the specification are only preferred embodiments of the inventors who are skilled in the art and thus are not limiting in any way. For example, compound-D SEQ ID NO:1 may be used as a protective agent for organs other than the heart. Organs isolated for transplant such as kidney, liver, lung, cornea, etc. may have enhanced viability after exposure to compound-D SEQ ID NO:1. Similarly, compound-D SEQ ID NO:1 may be administered to individuals in which ischemia to organs other than the heart has occurred or will occur. Therefore various changes, modifications or alterations to these embodiments may be made or resorted to without departing from the spirit of the invention and the scope of the following claims.

SEQUENCE LISTING

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<213> ORGANISM: XYZ
<220> FEATURE:
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<222> LOCATION: (0)...(0)
<221> NAME/KEY: MOD_RES
<222> LOCATION: (1)...(0)
<223> OTHER INFORMATION: Xaa = D-Leu

<400> SEQUENCE: 1

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 1               5                   10                  15

Ile
```

What is claimed is:

1. A method of protecting against ischemia and reperfusion injury in a human comprising administering an effective amount of compound-D (SEQ ID NO:1) to the human in a pharmaceutically acceptable formulation.

2. The method of claim 1 wherein administration is prior to onset of ischemia.

3. The method of claim 2 wherein administration is about 24 hours prior to onset of ischemia.

4. The method of claim 1 wherein compound-D SEQ ID NO:1 is administered as a solution.

5. The method of claim 1 wherein compound-D SEQ ID NO:1 is administered parenterally.

6. The method of claim 1 wherein compound-D (SEQ ID NO:1) is administered at an amount of about 2 mg/kg of body weight of the human.

7. The method of claim 1 wherein compound-D SEQ ID NO:1 is administered to protect against myocardial ischemia.

8. The method of claim 1 wherein the formulation further includes arginine hydrochloride.

9. A method for reducing effects of ischemia in a human comprising administering an effective amount of compound-D (SEQ ID NO:1) in a pharmaceutically acceptable carrier to the human.

10. The method of claim 19 wherein compound-D SEQ ID NO:1 is administered prior to ischemia.

11. The method of claim 9 wherein compound-D (SEQ ID NO:1) is administered concurrently with onset of ischemia.

12. The method of claim 9 wherein compound-D (SEQ ID NO:1) is administered at least one hour after onset of ischemia.

* * * * *

UNITED STATES PATENT AND TRADEMARK OFFICE
CERTIFICATE OF CORRECTION

PATENT NO.   : 6,645,938 B2
DATED   : November 11, 2003
INVENTOR(S) : Oeltgen et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Title page,
Item [56], **References Cited**, OTHER PUBLICATIONS, reference "Bolling et al.," "Delta opioid agonist/antagonist activity and ischemia tolerance," should be -- Delta opioid agonist/antagonist activity and ischemic tolerance, --
"Schultz et al.," "Morphone mimics the cardioprotective effect..." should be -- Morphine mimics the cardioprotective effect.. --
Two references are omitted -- Bolling et al., "Use of "natural" hibernation induction triggers for myocardial protection", annals Thorac. Surg.:623-627, 1997 and Bolling et al., "Hibernation triggers and myocardial protection", Circulation 98:11220-11223, 1998 --

Column 4,
Line 42, "FIGS. 2 and 3,respetively" should be -- FIGS. 2 and 3, respectively for high flavor --

Column 5,
Line 65, "SEQ ID NO:1,it was..." should be -- SEQ ID NO:1, it was ... --
Line 67, "(KATP)" should be -- (K_{ATP}) --

Column 6,
Line 51, "pO_2,pCO_2,pH and..." should be -- pO_2, pCO_2, pH and... --
Line 59, "PO12" should be -- PC12 --

UNITED STATES PATENT AND TRADEMARK OFFICE
CERTIFICATE OF CORRECTION

PATENT NO.   : 6,645,938 B2
DATED   : November 11, 2003
INVENTOR(S) : Oeltgen et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 9,
Line 20, in the Sequence Listing, "Ile," should be -- Ile NH_2 --
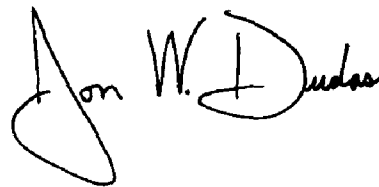Lines 32 and 34, "compound-D SEQ ID NO:1..." should be -- compound-D (SEQ ID NO:1)... --

Column 10,
Line 24, "compound-D SEQ ID NO:1..." should be -- compound-D (SEQ ID NO:1)... --
Line 32, "The method of claim 19 wherein..." should be -- The method of claim 9 wherein... --

Signed and Sealed this

Sixth Day of July, 2004

JON W. DUDAS
Acting Director of the United States Patent and Trademark Office