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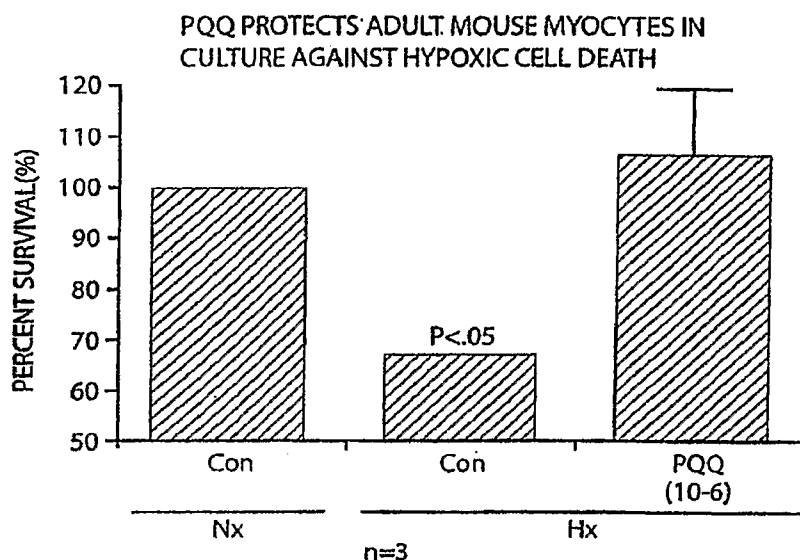
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(54) Title: PYRROLOQUINOLINE QUINONE DRUGS AND METHODS OF USE THEREOF



(57) Abstract: The invention includes compositions comprising substantially purified pyrroloquinoline quinone, that are useful in methods for the treatment and prevention of cardiac injury caused by hypoxia or ischemia. The invention also includes methods for the treatment and prevention of cardiac injury comprising contacting a composition of the invention with a human patient.



For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

PYRROLOQUINOLINE QUINONE DRUGS AND METHODS OF USE THEREOF

BACKGROUND OF THE INVENTION

5 The heart is critically dependent on uninterrupted blood flow for the delivery of oxygen and nutrients and the removal of harmful products of metabolism. Ischemia leads to rapid changes in myocardial metabolism and cellular injury, the extent of the injury being dependent upon the severity of ischemia. Continued ischemia leads to total tissue necrosis in a few hours.

10 Reperfusion, although generally considered beneficial, causes tissue injury by several mechanisms. Clinically, in open heart surgery, heart transplantation, and reversal of heart disease, protection of the myocardium against injury by ischemia-reperfusion is an issue of utmost clinical interest. Further, exacerbation of hypoxic injury after restoration of oxygenation (reoxygenation) by reperfusion is an important mechanism of cellular injury in
15 other types of organ transplantation and in hepatic, intestinal, cerebral, renal, and other ischemic syndromes. Cellular hypoxia and reoxygenation cause ischemia-reperfusion injury in part by generating reactive oxygen species (ROS).

 Since the first isolation of free PQQ from bacteria in the late 1970s, further work has indicated that PQQ is an essential nutrient for vertebrate animals and perhaps belongs to the
20 B group of vitamins (Paz MA, *et al.* The biomedical significance of PQQ. Edited by Davidson VL: Principles and Applications of Quinoproteins, 1992 by Marcel Dekker, Inc. P381-393., Kasahara T, and Kato T. Nature 2003;422:832). Free PQQ has been identified in red blood cells, neutrophils, cerebrospinal fluid, synovial fluid, bile (Gallop PM, *et al.* Connect Tissue Res 1993;29:153-161), and in human milk (Mitchell AE, *et al.* Analytical
25 Biochemistry 1999;269:317-325). Trace amounts of free PQQ have also been detected in spleen, pancreas, lung, brain, heart, intestine, liver, and testis, plasma and urine of humans, and in small intestine, liver, and testis of the rat (Kumazawa T, *et al.* Biochim Biophys Acta 1992;1156:62-66). A PQQ-dependent dehydrogenase enzyme is crucial for the amino acid lysine-degradation pathway in mice. In this reaction PQQ acts as a mammalian redox
30 cofactor (Kasahara, T. and Kato, T., Nature 2003;422:832.). Since PQQ levels in human tissues and body fluids are 5-10 times lower than those found in foods, it is probable that PQQ in human tissues is derived, at least partly, from dietary sources including vegetables

and meat (Kumazawa T, *et al.* Biochem J 1995;307:331-333). When mice are fed a PQQ-deficient diet, they grow slowly, have fragile skin and a reduced immune response, and do not reproduce well. It has been shown that PQQ supplementation can improve reproductive performance, growth, and may modulate indices of neonatal extracellular matrix production and maturation in mice fed chemically defined, but otherwise nutritionally complete diets (Steinberg F, *et al.* Exp Biol Med (Maywood) 2003;228:160-166, Steinberg FM, *et al.* J Nutr 1994;124:744-753). Excessive activation of the *N*-methyl-D-aspartate (NMDA) subtype of the glutamate receptor is critical in the process of neuronal injury in hypoxia/ischemia, and NMDA antagonists can ameliorate neuronal damage in both in vitro and in vivo models of glutamate-mediated neurotoxicity. The results of a previous study demonstrated that PQQ had a protective effect on brain injury in a rodent model of cerebral hypoxia/ischemia and suggested that PQQ could have potential use in the therapy of stroke (Jensen FE, *et al.* Neuroscience 1994;62(2):399-406). Although PQQ has been shown to be effective in an animal model of focal cerebral ischemia and epilepsy, the protective mechanism is not well understood (Zhang Y, and Rosenberg PA. European J Neuroscience 2002;16:1015-1024., Jensen FE, *et al.*).

Only one report investigated the potential cardioprotective effects of PQQ. This study showed that PQQ protected isolated rabbit heart from re-oxygenation injury measured by LDH activity released into the cardiac effluent (Xu F, *et al.* Biochemical Biophysical Research Communications 1993;193:434-439). However, based on this information it could not be determined whether PQQ is an effective agent in reducing infarct size when given either prophylactically (pretreatment) or after the onset of ischemia at the time of reperfusion (treatment).

Herein, we are the first to demonstrate that either pretreatment or treatment with PQQ can significantly reduce myocardial infarct size in an intact rat model of ischemia or ischemia-reperfusion injury.

SUMMARY OF THE INVENTION

The present invention relates to the discovery that myocardial oxidative stress can be prevented or minimized by administration of certain cardioprotective factors, and thus has benefit for treating cardiovascular and other diseases. In particular, it has been found that non-toxic dosages of pyrroloquinoline quinone ("PQQ") drugs are useful as cardioprotective agents, and are therefore valuable in the treatment of a variety of various heart-related

ailments such as ischemia-reperfusion injury, congestive heart failure, cardiac arrest and myocardial infarction such as due to coronary artery blockage, and for cardioprotection. PQQ in particular has been found to modulate myocardial oxidative stress such that myocardial cells (which are the subject of the oxidative stress) are protected from cell death.

5 The compositions and methods of the invention are surprisingly useful for the reduction or elimination of hypoxic/ischemic cardiac injury *in vivo* and *ex vivo*, as well as the prevention and/or treatment of cardiovascular disease in mammals in need thereof, such as humans.

10 In another aspect of the invention, PQQ has been found to modulate, *e.g.*, enhance or maintain the effect of, cardioprotective signaling pathways such as the regulation of the mitochondrial channel mitoK_{ATP}, the nitric oxide-protein kinase C pathway, and the angiotensin-converting enzyme pathway.

15 In another aspect of the invention, the present invention related to treating or preventing myocardial oxidative stress in myocardial cells in a subject by administering an agent that modulates myocardial oxidative stress such that the myocardial cells are protected from cell death.

20 In another aspect of the invention, the present invention related to treating or preventing myocardial hypoxic or ischemic damage in a subject by administering an agent that modulates myocardial hypoxic or ischemic damage such that myocardial cells are protected from cell death.

25 In another aspect of the invention, PQQ has been found to modulate free radical damage caused by myocardial oxidative stress. Free radicals generated by ischemic or hypoxic conditions have been found to be a significant cause of myocardial damage leading to myocardial death. As such, administration of PQQ, administered *in vivo* in non-toxic dosages, is an effective treatment for inhibiting or preventing myocardial oxidative stress free radical damage.

The invention further relates to methods of improving coronary blood flow in a subject by administering to the subject PQQ in a non-toxic amount, such that coronary blood flow is improved.

30 In one aspect, the present invention relates to treating or preventing cardiac injury caused by hypoxia or ischemia in a subject by administering pyrroloquinoline quinone, *e.g.*, in an amount effective to treat or prevent cardiac injury. PQQ is typically administered at a

non-toxic concentration, *e.g.*, between about 1nM and less than 10 μ M, including less than 900 μ M, less than 700 μ M, less than 500 μ M, less than 300 μ M, less than 100 μ M, or less than 50 μ M. In other embodiments, PQQ may be administered at a concentration of about 1 to 10 μ M. In other embodiments, PQQ is administered as a function of the subject's body weight. PQQ may typically be administered at a concentration of between about 1 μ g/kg to 1g/kg of a subject's body weight, including less than 500mg/kg, 250mg/kg, 100mg/kg, 10mg/kg, 5mg/kg, 3mg/kg, 2mg/kg, 1mg/kg, 500 μ g/kg, 250 μ g/kg, 100 μ g/kg, 10 μ g/kg, 5 μ g/kg, 2 μ g/kg or 1 μ g/kg.

The invention further includes cardioprotective agents containing PQQ, *e.g.*, in an amount effective to effect cardioprotection, and a pharmaceutically acceptable carrier. Also included are kits for treating patients at risk of cardiac injury, stroke, or migraine headaches, containing in one or more containers, an effective amount of pyrroloquinoline quinone, a pharmaceutically acceptable carrier, and instructions for use.

In another aspect, the invention relates to treatment or prevention of cardiac injury caused by hypoxia or ischemia *in vivo*, by administration of an NADPH-dependent methemoglobin reductase substrate; and kits for use in treatment or prevention of cardiac injury, including an effective amount of an NADPH-dependent methemoglobin reductase substrate, a pharmaceutically acceptable carrier, and instructions for use. In some embodiments of the invention the NADPH-dependent methemoglobin reductase substrate is purified from erythrocytes, such as mammalian erythrocytes (*e.g.*, human, bovine, or murine) or non-mammalian erythrocytes (*e.g.*, *Rana catesbeiana*).

In yet another aspect, the invention relates to methods for preventing organ damage during organ or tissue transplantation, wherein PQQ is administered to an organ donor prior to and/or concurrent with removal of the organ or tissue; and kits for use in preventing organ damage during organ or tissue transplantation, including an effective amount of pyrroloquinoline quinone, a pharmaceutically acceptable carrier, and instructions for use.

In a further aspect, the invention relates to methods for preventing stroke, *e.g.*, in subjects suffering from heart failure, by administering PQQ in amounts effective to obtain the desired protective effect. The PQQ may be desirably administered, *e.g.*, at concentrations of about of about 1 to 10 μ M. In one embodiment, PQQ can be co-administered with a therapeutically effective amount of tamoxifen for preventing stroke in a subject at risk of suffering a stroke.

The invention includes methods for treating heart failure in a subject by administering PQQ and one or more additional therapeutic compounds. In some embodiments, the additional therapeutic compound may be an anti-platelet drug, anti-coagulant drug and/or an anti-thrombotic drug, or combinations thereof.

5 In another aspect, the invention relates to methods of treating myocardial infarction in a subject by administering PQQ at levels such that the myocardial infarction is decreased or stabilized.

In yet another aspect, the invention relates to methods of preventing migraine headaches in a subject by treating the subject with PQQ. The PQQ may be desirably
10 administered, *e.g.*, at concentrations from about 1 to about 10 μ M.

In yet another aspect, the invention relates to methods of preventing reperfusion injury in a subject suffering from or at risk of hypothermia, by treating the subject with PQQ. The PQQ may be desirably administered, *e.g.*, at concentrations from about 1 to about 10 μ M.

The invention further relates to methods for preventing vascular occlusion following
15 balloon angioplasty in a subject by pre-treating the subject with PQQ. The subject may be also pre-treated with PQQ and one or more additional therapeutic compounds (*e.g.*, coumadin, angiotensin converting enzyme (ACE) inhibitors such as captopril, benazepril, enalapril, fosinopril, lisinopril, quinapril, ramipril, imidapril, peridopril erbumine and trandolapril, and ACE receptor blockers such as losartan, irbesartan, candesartan cilexetil and
20 valsartan). In some embodiments, the additional therapeutic compound may be an anti-platelet drug, anti-coagulant drug and/or an anti-thrombotic drug, or combinations thereof.

In another aspect, the present invention involves a method for preventing or reducing reperfusion injury in a subject suffering from hypothermic injury by administering PQQ to the subject.

25 The invention further relates to a pharmaceutical composition for treating myocardial infarction in a subject in need thereof, including a therapeutically effective dose of pyrroloquinoline quinone and a therapeutically effective dose of metoprolol. In one embodiment of the pharmaceutical composition for treating myocardial infarction, the therapeutically effective dose of pyrroloquinoline quinone is 3 mg/kg. In another
30 embodiment of the pharmaceutical composition for treating myocardial infarction, the therapeutically effective dose of metoprolol is 1 mg/kg.

The invention further relates to a kit for treating or preventing a hypoxia or ischemic-related cardiac injury, comprising in one or more containers pyrroloquinoline quinone, metoprolol, a pharmaceutically acceptable carrier, and instructions for use of said kit.

5 The invention further relates to a method of treating or preventing myocardial oxidative stress in a subject, comprising administering to a subject in need thereof a therapeutically effective dose of pyrroloquinoline quinone and a therapeutically effective dose of metoprolol.

10 The invention further relates to a method of treating or preventing myocardial infarction in a subject, comprising administering to a subject in need thereof a therapeutically effective dose of pyrroloquinoline quinone and a therapeutically effective dose of metoprolol.

The invention further relates to a method of treating or preventing cardiac injury caused by hypoxia or ischemia in a subject, comprising administering to a subject in need thereof a therapeutically effective dose of pyrroloquinoline quinone and a therapeutically effective dose of metoprolol.

15 The invention further provides methods for treating vascular injuries and disorders due to protein nitration by administering to a subject in need thereof a therapeutically effective amount of PQQ alone, or in combination with urate.

20 The invention also provides methods of reducing kidney toxicity associated with PQQ administration by administering to a subject in need thereof a therapeutically effective amount of PQQ in combination with probenecid, cilastatin, or other blockers of transtubular flux.

These and other objects of the present invention will be apparent from the detailed description of the invention provided below.

25 BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a bar graph demonstrating the increase in viable adult cardiac mouse myocytes following hypoxia by pretreatment with PQQ.

Figure 2 is a bar graph showing that PQQ protection is not inhibited by 10 μ M 5-hydroxydecanoic acid, a mitochondrial K_{ATP} channel inhibitor.

30 Figure 3 is a line graph demonstrating that PQQ treatment prior to ischemia preserves left ventricular developed pressure (LVDP).

Figure 4 is a line graph demonstrating that PQQ treatment prior to ischemia preserves left ventricular end-diastolic pressure (LVEDP); left ventricular systolic pressure minus left ventricular end-diastolic pressure).

5 Figure 5 is a line graph demonstrating the effect of PQQ treatment prior to ischemia as measured by the maximum positive first derivative of left ventricular pressure (LVDP).

Figure 6 is a line graph demonstrating the effect of PQQ treatment prior to ischemia as measured by the maximum negative first derivative of left ventricular pressure (LVDP).

Figure 7 is a line graph showing that coronary blood flow is significantly improved by PQQ treatment as compared to control.

10 Figure 8 is a bar graph indicating that 2 minutes of pretreatment with PQQ at several concentrations shown has progressively favorable responses between 10nM and 1μM, but that toxicity occurs at 10μM.

Figure 9 is a bar graph demonstrating the changes in cardiac infarction size after PQQ pre-treatment. There is a progressive reduction in infarction size between 10nM and 1μM, but infarction size is not reduced at 10μM PQQ.

Figure 10 is a schematic showing experimental protocols Model 1 (ischemia 2 hours) and Model 2 (ischemia/reperfusion). Model 2 included two separate sets of experiments; Set 1: (ischemia 17 min/reperfusion 2 hours) and Set 2: (ischemia 30 min/reperfusion 2 hours). The Pretreatment rats received PQQ by i.p. injection before 30 min of ischemia. In the Treatment group PQQ was given by i.v. injection at the onset of reperfusion. Control rats were given an equivalent volume of vehicle at the times indicated. Arrows indicate timing of PQQ administration. i.p. = intraperitoneal; i.v. = intravenous; I = ischemia; LAD = left anterior descending coronary artery.

Figure 11A is a line graph showing left ventricular systolic pressure (LVSP) in model 2 rats during ischemia/reperfusion. Either pretreatment with PQQ (PQQ by i.p. injection before 30 min of ischemia) or treatment with PQQ (PQQ by i.v. injection at the onset of reperfusion) resulted in increased LVSP at 2 hours of reperfusion. B=baseline; I=ischemia; R=reperfusion.

Figure 11B is a line graph showing left ventricular developed pressure (LVDP) in model 2 rats during ischemia/reperfusion. Treatment with PQQ increased LVDP after both 30

min and 2 hours of reperfusion. Pretreatment with PQQ increased LVDP at 2 hours of reperfusion. B=baseline; I=ischemia; R=reperfusion.

Figure 12A is a line graph showing left ventricular (LV) (+)dP/dt in model 2 rats during ischemia/reperfusion. Either pretreatment with PQQ or treatment with PQQ significantly increased LV (+)dP/dt at 2 hours of reperfusion. B=baseline; I=ischemia; R=reperfusion.

Figure 12B is a line graph showing left ventricular (LV) (-)dP/dt in model 2 rats during ischemia/reperfusion. Either pretreatment with PQQ or treatment with PQQ significantly decreased LV (-)dP/dt at 2 hours of reperfusion. B=baseline; I=ischemia; R=reperfusion.

Figure 13 is a bar graph showing myocardial infarct size in *model 1* (ischemia only). Pretreatment with PQQ 20 mg/kg significantly reduced infarct size (Infarct mass/LV mass %). Ischemia was induced by 2 hours of LAD ligation without reperfusion.

Figure 14 is a bar graph showing myocardial infarct size in model 2 (ischemia/reperfusion). In these experiments ischemia was induced by 17 min of LAD occlusion followed by 2 hours of reflow (reperfusion). Pretreatment with PQQ 20 mg/kg significantly reduced infarct size (as measured either by Infarct mass/Risk area % or Infarct mass/LV mass %).

Figure 15 is a bar graph showing myocardial infarct size in additional experiments in Model 2 (ischemia/reperfusion). In these experiments 30 min of ischemia was followed by 2 hours of reperfusion. Either pretreatment with PQQ 15 mg/kg or treatment with PQQ 15 mg/kg significantly reduced infarct size (as measured either by Infarct mass/Risk area % or Infarct mass/LV mass %). P values refer to respective I/R infarct size measurements.

Figure 16 is a line graph showing effects of pretreatment with different doses of PQQ on infarct size in five groups of rats pretreated with the indicated range of PQQ doses by i.p. injection. There was a strong negative relationship between infarct size and the dose of PQQ.

Figure 17A is a bar graph showing average episodes of ventricular fibrillation (VF) per rat in combining data from both model 1 and model 2. Pretreatment with PQQ 15-20 mg/kg significantly decreased average episodes of VF per rat. Analysis was by one-way analysis of variance (ANOVA).

Figure 17B is a bar graph showing the percentage of rats with VF using combined data from model 1 and model 2. Either pretreatment with PQQ 15- 20 mg/kg or treatment with PQQ 15-20 mg/kg significantly decreased the percentage of rats with VF. Analysis was by the Fisher Exact test.

5 Figure 18A is a line graph showing myocardial MDA levels from the anterior segment of the LV subjected to 30 min of LAD occlusion followed by 2 hours of reperfusion. Pretreatment with PQQ 15 mg/kg significantly decreased MDA in the ischemic myocardium. Differences between rats subjected to I/R and treated or not (Control) were significant by two-way analysis of variance. Sham = rats subjected to LAD coronary artery isolation
10 without occlusion for the total study period.

Figure 18B is a line graph showing myocardial MDA levels from the posterior (non-ischemic) segment of the LV. Pretreatment with PQQ 15 mg/kg also decreased MDA in this non-ischemic remote myocardium.

15 Figure 19 is a bar graph showing respiratory control ratios of mitochondria isolated from rat hearts under the following conditions: (i) Controls: 3 hours pentobarbital anesthesia, n = 4, (ii) PQQ treatment: 3 mg/kg, 20 min equilibration period, 30 min ischemia, PQQ injection, 2 hrs reperfusion, n = 5; and (iii) ischemia/reperfusion: 20 min equilibration period, 30 min ischemia followed by 2 hrs reperfusion, n = 3.

Figure 20A is a schematic showing a synthesis schemes of PQQ conjugated PVA.

20 Figure 20B is a schematic showing a PVA unit with a PQQ molecule.

Figure 20C is a schematic showing a PVA molecule with multiple PQQ molecules.

Figure 21 depicts the GPC retention time of PQQ using a fluorescence detector.

Figure 22 depicts the absorption spectrum of PQQ in water.

Figure 23 depicts the GPC spectrum of PQQ conjugated PVA.

25 Figure 24 depicts the GPC spectrum of PVA using a fluorescence detector.

Figure 25 A-C depict the UV absorption spectrum: (A) PQQ conjugated PVA with a retention time of 10.19 minutes (40K molecular weight); (B) PQQ conjugated PVA with retention time of 13.67 minutes (10K molecular weight); (C) PQQ residues.

Figure 26 Based on the integral area of the PQQ's aromatic peaks at 8.45 and 7.25 and the aliphatic peaks at 3.84, 1.93 and 1.50 ppm, the loading level was around 1-1.5 (± 0.4) (PQQ unit per PVA molecule chain. The loading level is approximately 4(± 2) wt % PQQ in the conjugated products.

Figure 27 is a picture of the gross pathology of the kidneys in control mice, in mice treated with PQQ alone, in mice treated with PQQ in combination with Probenecid, and PQQ in combination with PVA.

Figure 28 is a representative micrograph (bovine cells) of nitrotyrosine fluorescence of PMEM immunostained with monoclonal anti-nitrotyrosine showing that anti-nitrotyrosine immunocytochemical specificity and the effect of urate and PQQ on the TNF-induced increase in nitrotyrosine. Micrograph of PMEM immunostained with the same antibody after pre-incubation of the antibody with 3-nitrotyrosine for 30 min at a 10:1 antigen:antibody molar ratio. Confocal histogram analysis of nitrotyrosine fluorescence obtained from both rat and bovine control, urate, PQQ and TNF treated PMEM after 0.5 or 4 hr (N=4, 6 samplings each per treatment). Statistical difference is determined with Kruskal-Wallis One Way ANOVA on Ranks followed by multiple comparisons using Dunn's Method.

* =different from Control Group

=different from respective TNF Group.

Figure 29 A-B. Urate and PQQ prevents the TNF-induced co-localization of nitrotyrosine with β -actin in PMEM. Representative confocal micrographs (bovine cells) of control, PQQ, urate and TNF treated PMEM after 0.5 hr (A) and 4.0 hr (B). Nitrotyrosine has been immunostained with anti-nitrotyrosine and appears as green fluorescence. β -actin has been immunostained with anti- β -actin and appears as red fluorescence. The resultant color change of the combined red and green micrographs appears yellow where co-localization occurs (inset: arrows). A total of 4 preparations were generated for each treatment and time point from both rat and bovine PMEM.

Figure 30. Urate and PQQ prevents TNF-induced increases in albumin clearance rate in PMEM. The albumin clearance response of combined data obtained from rat and bovine PMEM. The treatments are control, urate, PQQ and TNF for 4.0 hr. Statistical difference is determined with Kruskal-Wallis One Way ANOVA on Ranks followed by multiple comparisons using Dunn's Method.

* =different from Control Group;

#=different from TNF Group.

Fig. 31. Effects of treatment with PQQ 10 mg/kg, 3 mg/kg and 1 mg/kg (i.v.) on brain infarct size (31A) and dose response curve (31B). PQQ given immediately before (0 hr Vehicle and PQQ 10 mg groups) and at 3 hours after (3 hr vehicle and PQQ 10 mg groups) ischemia reduces infarct volume significantly ($p < 0.05$; Mann-Whitney test). When given at 3 hours after ischemia, PQQ at 3 mg/kg (3 hr vehicle and PQQ 3 mg groups) but not at 1 mg/kg (3 hr vehicle and PQQ 1 mg groups) reduces infarct volume. There is a significant effect of treatment in 3 mg/kg groups ($p < 0.05$, Mann-Whitney test) but there is not a significant effect in 1 mg/kg groups ($p > 0.05$, Mann-Whitney test).

Fig. 32. Representative sections from normal animal (A); Vehicle - treated animal (B); PQQ 10 mg/kg treated (at 3 hours after ischemia) animal (C); PQQ 3 mg/kg treated animals (D).

Fig. 33. Effects of treatment with PQQ 10 mg/kg, 3 mg/kg and 1 mg/kg (i.v.) on neurobehavioral scores. Treatment with PQQ at 10 mg/kg immediately before (33A) and at 3 hours after (33B) ischemia results in improved neurobehavioral scores at 24, 48 and 72 hours. There is a significant effect of treatment in both 32A and 32B groups ($p < 0.05$; repeated measures ANOVA). Treatment with PQQ at 3 mg/kg 3 hours after ischemia results in improved neurobehavioral scores at 24, 48 and 72 hours. There is a significant effect of treatment given at 3 hours after ischemia in 3 mg/kg groups (4C, $p < 0.05$; repeated measures ANOVA) but there is not a significant effect of treatment given at 3 hours after ischemia in 1 mg/kg groups (33D, $p > 0.05$; repeated measures ANOVA).

Figure 34 depicts the calibration curve of PQQ (31.25-2500 ng/ml) in rat plasma treated with two-step extract and determined with HPLC-fluorescent detector (360/460 nm).

Figure 35 depicts rat plasma PQQ concentrations for rats in Groups A (PQQ alone) and B (PQQ plus Probenecid)

Figure 36 depicts the plasma PQQ concentration-time curve in rats ($n=3$) in Groups A (20 mg PQQ/kg, i.v.) and B (pretreated with 100 mg probenecid/kg, i.p., following 20 mg PQQ/kg, i.v.).

Figure 37 is a graph showing left ventricle systolic pressure (LVSP) in rats at baseline, 15 minutes of occlusion, 30 minutes of occlusion, 30 minutes of reperfusion, 60 minutes of reperfusion and 120 minutes of reperfusion with and without treatment with a combination of 100 mg/kg of probenecid and 2 or 3 mg/kg of PQQ.

Figure 38 is a graph showing left ventricle end diastolic pressure (LVEDP) in rats at baseline, 15 minutes of occlusion, 30 minutes of occlusion, 30 minutes of reperfusion, 60 minutes of reperfusion and 120 minutes of reperfusion with and without treatment with a combination of 100 mg/kg of probenecid and 2 or 3 mg/kg of PQQ.

5 Figure 39 is a graph showing left ventricle developed pressure (LVDP) in rats at baseline, 15 minutes of occlusion, 30 minutes of occlusion, 30 minutes of reperfusion, 60 minutes of reperfusion and 120 minutes of reperfusion with and without treatment with a combination of 100 mg/kg of probenecid and 2 or 3 mg/kg of PQQ.

10 Figure 40 is a graph showing left ventricle maximum positive first derivative (LV +dp/dt) in rats at baseline, 15 minutes of occlusion, 30 minutes of occlusion, 30 minutes of reperfusion, 60 minutes of reperfusion and 120 minutes of reperfusion with and without treatment with a combination of 100 mg/kg of probenecid and 2 or 3 mg/kg of PQQ.

15 Figure 41 is a graph showing left ventricle maximum negative first derivative (LV - dp/dt) in rats at baseline, 15 minutes of occlusion, 30 minutes of occlusion, 30 minutes of reperfusion, 60 minutes of reperfusion and 120 minutes of reperfusion with and without treatment with a combination of 100 mg/kg of probenecid and 2 or 3 mg/kg of PQQ.

Figure 42 is a bar graph showing infarct size percentage in rats with and without treatment with a combination of 100 mg/kg of probenecid and 2 or 3 mg/kg of PQQ.

20 Figure 43 is a bar graph showing infarct size/risk area percentage and infarct size/left ventricle mass in rats with and without treatment with a combination of 100 mg/kg of probenecid and 2 or 3 mg/kg of PQQ.

Figure 44 is a bar graph showing increase in creatine kinase in rats with and without treatment with a combination of 100 mg/kg of probenecid and 2 or 3 mg/kg of PQQ.

25 Figure 45 is the proton NMR spectra of received PQQ and PQQ/PVA conjugate with d_6 -DMSO as solvent labeled at 2.50 ppm.

Figure 46 is the proton H-NMR spectrum of PQQ/PVA conjugate (D₂O as solvent, labeled as internal standard at 4.79 ppm).

Figure 47 is the ATR mode FT-IR spectra of PVA powder.

Figure 48 is the ATR mode FT-IR spectra of PVA powder.

30 Figure 49 is the ATR mode FT-IR spectra of PVA/PQQ conjugate powder.

Figure 50 is the XRD spectra of two PVA powders. (A) PQQ/PVA conjugate. (B) PVA powder. (C) PQQ powder. (D) Steel substrate.

Figure 51 is the standard calibration curve of PQQ (10–1,000 ng/ml) assay with HPLC-FLD.

Figure 52 is the limit of quantity (0.2 ng/20 μ l) of HPLC-FLD method for PQQ assay.

Figure 53 is the limit of detect (0.1 ng/20 μ l) of HPLC-FLD method for PQQ assay.

5 Figure 54 A-JJ show various small molecular weight (SMW) PQQ conjugates.

DETAILED DESCRIPTION OF THE INVENTION

The features and other details of the invention will now be more particularly described with reference to the accompanying drawings and pointed out in the claims. It will
10 be understood that particular embodiments described herein are shown by way of illustration and not as limitations of the invention. The principal features of this invention can be employed in various embodiments without departing from the scope of the invention. All parts and percentages are by weight unless otherwise specified.

Definitions

15 For convenience, certain terms used in the specification, examples, and appended claims are collected here. Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention pertains. However, to the extent that these definitions vary from meanings circulating within the art, the definitions below are to control.

20 “Ischemia” includes the decrease or cessation of blood flow to any organ or tissue of the body. As used herein, the term “ischemia” relates to any ischemic syndrome including, for example, vascular ischemia (*e.g.*, heart and lungs), hepatic ischemia, intestinal ischemia, cerebral ischemia, renal ischemia, and limb ischemia.

“Hypoxia” includes the deficiency in the amount of oxygen reaching body tissues.

25 “Hypoxia or ischemic-related injury” includes, but is not limited to, cardiac injury.

“Reperfusion” includes the restoration of blood flow to an organ or tissue that has had its blood supply cut off, as after a heart attack or stroke.

“Oxidative stress” includes conditions that occur when there is an excess of free radicals, a decrease in antioxidant levels, or both.

30 “Necrosis” includes the death of cells or tissues through injury or disease, particularly in a localized area of the body such as the myocardium.

“Apoptosis” refers to programmed cell death.

“Beta blockers” include agents such as atenolol, metoprolol, and propranolol, which act as competitive antagonists at the adrenergic beta receptors. Such agents also include those more selective for the cardiac (beta-1) receptors which allows for decreased systemic side effects. Beta blockers reduce the symptoms connected with hypertension, cardiac arrhythmias, migraine headaches, and other disorders related to the sympathetic nervous system. Beta blockers also are sometimes given after heart attacks to stabilize the heartbeat. Within the sympathetic nervous system, beta-adrenergic receptors are located mainly in the heart, lungs, kidneys, and blood vessels. Beta blockers compete with the nerve-stimulating hormone epinephrine for these receptor sites and thus interfere with the action of epinephrine, lowering blood pressure and heart rate, stopping arrhythmias, and preventing migraine headaches.

“Cardiac injury” includes any chronic or acute pathological event involving the heart and/or associated tissue (*e.g.*, the pericardium, aorta and other associated blood vessels), including ischemia-reperfusion injury; congestive heart failure; cardiac arrest; myocardial infarction; cardiotoxicity caused by compounds such as drugs (*e.g.*, doxorubicin, herceptin, thioridazine and cisapride); cardiac damage due to parasitic infection (bacteria, fungi, rickettsiae, and viruses, *e.g.*, syphilis, chronic *Trypanosoma cruzi* infection); fulminant cardiac amyloidosis; heart surgery; heart transplantation; and traumatic cardiac injury (*e.g.*, penetrating or blunt cardiac injury, aortic valve rupture).

“Subject” includes living organisms such as humans, monkeys, cows, sheep, horses, pigs, cattle, goats, dogs, cats, mice, rats, cultured cells therefrom, and transgenic species thereof. In a preferred embodiment, the subject is a human. Administration of the compositions of the present invention to a subject to be treated can be carried out using known procedures, at dosages and for periods of time effective to treat the condition in the subject. An effective amount of the therapeutic compound necessary to achieve a therapeutic effect may vary according to factors such as the age, sex, and weight of the subject, and the ability of the therapeutic compound to treat the foreign agents in the subject. Dosage regimens can be adjusted to provide the optimum therapeutic response. For example, several divided doses may be administered daily or the dose may be proportionally reduced as indicated by the exigencies of the therapeutic situation.

“Substantially pure” includes compounds, *e.g.*, drugs, proteins or polypeptides that have been separated from components which naturally accompany it. Typically, a compound is substantially pure when at least 10%, more preferably at least 20%, more preferably at least 50%, more preferably at least 60%, more preferably at least 75%, more preferably at least 90%, and most preferably at least 99% of the total material (by volume, by wet or dry weight, or by mole percent or mole fraction) in a sample is the compound of interest. Purity can be measured by any appropriate method, *e.g.*, in the case of polypeptides by column chromatography, gel electrophoresis or HPLC analysis. A compound, *e.g.*, a protein, is also substantially purified when it is essentially free of naturally associated components or when it is separated from the native contaminants which accompany it in its natural state. Included within the meaning of the term “substantially pure” are compounds, such as proteins or polypeptides, which are homogeneously pure, for example, where at least 95% of the total protein (by volume, by wet or dry weight, or by mole percent or mole fraction) in a sample is the protein or polypeptide of interest.

“Administering” includes routes of administration which allow the compositions of the invention to perform their intended function, *e.g.*, treating or preventing cardiac injury caused by hypoxia or ischemia. A variety of routes of administration are possible including, but not necessarily limited to parenteral (*e.g.*, intravenous, intraarterial, intramuscular, subcutaneous injection), oral (*e.g.*, dietary), topical, nasal, rectal, or via slow releasing microcarriers depending on the disease or condition to be treated. Oral, parenteral and intravenous administration are preferred modes of administration. Formulation of the compound to be administered will vary according to the route of administration selected (*e.g.*, solution, emulsion, gels, aerosols, capsule). An appropriate composition comprising the compound to be administered can be prepared in a physiologically acceptable vehicle or carrier and optional adjuvants and preservatives. For solutions or emulsions, suitable carriers include, for example, aqueous or alcoholic/aqueous solutions, emulsions or suspensions, including saline and buffered media, sterile water, creams, ointments, lotions, oils, pastes and solid carriers. Parenteral vehicles can include sodium chloride solution, Ringer’s dextrose, dextrose and sodium chloride, lactated Ringer’s or fixed oils. Intravenous vehicles can include various additives, preservatives, or fluid, nutrient or electrolyte replenishers (*See generally, Remington’s Pharmaceutical Science*, 16th Edition, Mack, Ed. (1980)).

“Effective amount” includes those amounts of pyrroloquinoline quinone which allow it to perform its intended function, *e.g.*, treating or preventing, partially or totally, cardiac injury caused by hypoxia or ischemia as described herein. The effective amount will depend upon a number of factors, including biological activity, age, body weight, sex, general health, severity of the condition to be treated, as well as appropriate pharmacokinetic properties. For example, dosages of the active substance may be from about 0.01mg/kg/day to about 500mg/kg/day, advantageously from about 0.1mg/kg/day to about 100mg/kg/day. A therapeutically effective amount of the active substance can be administered by an appropriate route in a single dose or multiple doses. Further, the dosages of the active substance can be proportionally increased or decreased as indicated by the exigencies of the therapeutic or prophylactic situation.

“Specific binding” or “specifically binds” includes proteins, such as an antibody which recognizes and binds an pyrroloquinoline quinone or a ligand thereof, but does not substantially recognize or bind other molecules in a sample.

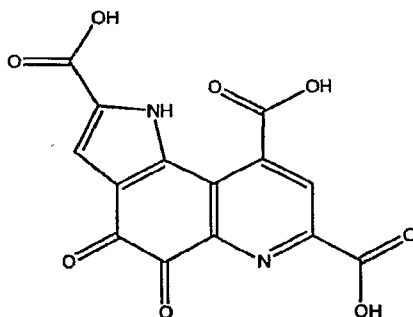
“Pharmaceutically acceptable carrier” includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like which are compatible with the activity of the compound and are physiologically acceptable to the subject. An example of a pharmaceutically acceptable carrier is buffered normal saline (0.15M NaCl). The use of such media and agents for pharmaceutically active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the therapeutic compound, use thereof in the compositions suitable for pharmaceutical administration is contemplated. Supplementary active compounds can also be incorporated into the compositions.

“Pharmaceutically acceptable esters” includes relatively non-toxic, esterified products of therapeutic compounds of the invention. These esters can be prepared *in situ* during the final isolation and purification of the therapeutic compounds or by separately reacting the purified therapeutic compound in its free acid form or hydroxyl with a suitable esterifying agent; either of which are methods known to those skilled in the art. Acids can be converted into esters according to methods well known to one of ordinary skill in the art, *e.g.*, via treatment with an alcohol in the presence of a catalyst.

“Additional ingredients” include, but are not limited to, one or more of the following: excipients; surface active agents; dispersing agents; inert diluents; granulating and disintegrating agents; binding agents; lubricating agents; sweetening agents; flavoring agents; coloring agents; preservatives; physiologically degradable compositions such as gelatin; aqueous vehicles and solvents; oily vehicles and solvents; suspending agents; dispersing or wetting agents; emulsifying agents, demulcents; buffers; salts; thickening agents; fillers; emulsifying agents; antioxidants; antibiotics; antifungal agents; stabilizing agents; and pharmaceutically acceptable polymeric or hydrophobic materials. Other “additional ingredients” which may be included in the pharmaceutical compositions of the invention are known in the art and described, *e.g.*, in *Remington’s Pharmaceutical Sciences*.

“Unit dose” includes a discrete amount of the pharmaceutical composition comprising a predetermined amount of the active ingredient.

Pyrroloquinoline quinone (PQQ) is a water soluble anionic quinone that can transfer electrons catalytically between a variety of reductants and oxidants, and may be part of a soluble electron transport system in eukaryotic cells. PQQ proper is of the general structure



As used herein, “Pyrroloquinoline quinone” or “PQQ” includes any member of the pyrroloquinoline quinone family having chemical similarity, including closely related isomeric and stereoisomeric analogs of PQQ (*See e.g.*, Zhang et al., 1995, *Biochem. Biophys. Res. Commun.* 212: 41-47, 1995), and further includes any PQQ-conjugated polymers (*e.g.*, PQQ-conjugated poly vinyl alcohol). PQQ is also known as methoxatin. PQQ is found in animal tissues and fluids. Without wishing to be bound by theory, PQQ may act in part as a free-radical scavenger, particularly of reactive oxygen species (ROS). As such, PQQ may function as an NADPH-dependent methemoglobin reductase substrate (*See e.g.*, Xu et al., *Proc. Natl. Acad. Sci. USA*, 1992, 89(6):2130-4). Other NADPH-dependent methemoglobin

reductase substrates may function to decrease or eliminate hypoxia or ischemia-related cardiac injury.

Compositions comprising substantially purified pyrroloquinoline quinone may include pyrroloquinoline quinone alone, or in combination with other components such as beta blockers, and compounds which are effective to favorably modulate cardioprotective signaling pathways such as phenylephrine, sphingosine-1-phosphate, or the ganglioside GM-1. Pyrroloquinoline quinone may be substantially purified by any of the methods well known to those skilled in the art. (See, e.g., E. J. Corey and Alfonso Tramontano, J. Am. Chem. Soc., 103, 5599-5600 (1981); J.A. Duine, Review *Ann. Rev. Biochem.* 58, 403 (1989)).

In one embodiment, the invention provides PQQ conjugated to one or more polymers, thereby improving the pharmacokinetic, pharmacodynamics, efficacy, and safety of PQQ for tissue protection. A polymer to which PQQ can be conjugated includes, but is not limited to, polyvinyl alcohol, PEG-NH₂, or any of those polymers disclosed by Example 7 below and Exhibit A (incorporated herein by reference in its entirety).

The pyrroloquinoline quinone of the invention is, in one embodiment, a component of a pharmaceutical composition, which may also comprise buffers, salts, other proteins, and other ingredients acceptable as a pharmaceutical composition. The invention also includes a modified form of pyrroloquinoline quinone, which is capable of preventing or reducing hypoxic/ischemic cardiac injury as described herein.

The structure of the therapeutic compounds of this invention may include asymmetric carbon atoms. It is to be understood accordingly that the isomers (e.g., enantiomers and diastereomers) arising from such asymmetry are included within the scope of this invention. Such isomers can be obtained in substantially pure form by classical separation techniques and by sterically controlled synthesis. For the purposes of this application, unless expressly noted to the contrary, a therapeutic compound shall be construed to include both the R or S stereoisomers at each chiral center. In certain embodiments, a therapeutic compound of the invention comprises a cation. If the cationic group is hydrogen, H⁺, then the therapeutic compound is considered an acid. If hydrogen is replaced by a metal ion or its equivalent, the therapeutic compound is a salt of the acid. Pharmaceutically acceptable salts of the therapeutic compound are within the scope of the invention, e.g., pharmaceutically acceptable alkali metal (e.g., Li⁺, Na⁺, or K⁺) salts, ammonium cation salts, alkaline earth cation salts

(*e.g.*, Ca^{2+} , Ba^{2+} , Mg^{2+}), higher valency cation salts, or polycationic counter ion salts (*e.g.*, a polyammonium cation). (*See, e.g.*, Berge et al. (1977) "Pharmaceutical Salts", *J. Pharm. Sci.* 66:1-19). It will be appreciated that the stoichiometry of an anionic compound to a salt-forming counter ion (if any) will vary depending on the charge of the anionic portion of the compound (if any) and the charge of the counter ion. Preferred pharmaceutically acceptable salts include a sodium, potassium or calcium salt, but other salts are also contemplated within their pharmaceutically acceptable range.

The invention also relates to methods of treating or preventing myocardial oxidative stress, such as is caused by hypoxia or ischemia, in a subject. This is done by administering to a subject in need thereof a preferably non-toxic amount of an agent such as PQQ which modulates myocardial oxidative stress such that the myocardial cells which are the target of the oxidative stress are protected from cell death. The cell death may be due, *e.g.*, to necrosis or apoptosis.

Cardioprotective signaling pathways are known in the art. These pathways may be targeted for enhancement in patients in need of cardioprotection, by administering, pyrroloquinoline quinone in an amount effective to enhance or maintain the effect of cardioprotective signaling pathway.

Free radicals generated by ischemic or hypoxic conditions have been found to be a significant cause of myocardial damage leading to myocardial death. As such, administration of PQQ, administered in vivo, *e.g.*, in non-toxic dosages, is an effective treatment for inhibiting or preventing myocardial oxidative stress free radical damage, either by PQQ-mediated free radical scavenging, or by inhibition of free radical generation.

Administration of the compounds of the invention may be done where clinically necessary or desirable, *e.g.*, at the onset of reperfusion, or prior to reperfusion.

It has surprisingly also been found that coronary flow may be beneficially improved in a subject, *e.g.*, one suffering from a low blood flow condition, by administering to a subject in need thereof a non-toxic amount of pyrroloquinoline quinone. This is illustrated in the Examples. Coronary flow may be measured by several indicators, such as the left ventricular diastolic pressure ("LVDP") or the left ventricular ("LVEDP"). Measurement of coronary flow, such as by determining LVDP or LVEDP, is within the skill of those in the art.

Cardiac injury caused by hypoxia or ischemia, such as myocardial infarction, may therefore be treated or prevented by administration of pyrroloquinoline quinone, preferably in a non-toxic dosage, *e.g.*, at a concentration of less than about 10 μ M.

Rats were subject to PQQ treatment subject to two different models. In model 1 (shown schematically in Figure 10A), male Sprague-Dawley rats were subjected to 2 hours of left anterior descending (LAD) coronary artery ligation without reperfusion. In model 2 (ischemia-reperfusion, shown in Figure 10B), rats were subjected to 17 or 30 minutes of LAD occlusion and 2 hours of reperfusion with left ventricular (LV) hemodynamic monitoring. PQQ (15-20 mg/kg) was given either 30 min before LAD occlusion by i.p. injection (Pretreatment) or by i.v. injection at the onset of reperfusion (Treatment) to mimic the clinical state in humans. Controls received vehicle (2% NaHCO₃).

In model 1, infarct size (infarct mass/LV mass) after PQQ treatment was smaller than control (PQQ treatment resulted in infarct size of 10.0 $\pm$ 1.5 vs control of 19.1 $\pm$ 2.1%, n=9, P<0.01). In model 2, either pretreatment or treatment with PQQ resulted in reduced infarct size (infarct mass/risk area) (PQQ pretreatment infarct size 18.4 $\pm$ 2.3 and treatment infarct size 25.6 $\pm$ 3.5% vs control of 38.1 $\pm$ 2.6%, P<0.01). PQQ protected against ischemia-induced cardiac dysfunction with higher LV developed pressure, LV (+)dP/dt and lower LV (-)dP/dt after 1-2.

In summary, PQQ had cardioprotective effects in two separate intact rat infarction models consisting either of ischemia or ischemia-reperfusion. PQQ reduced infarct size either when given prior to ischemia or ischemia-reperfusion, or when given at the onset of reperfusion. Moreover, PQQ had beneficial hemodynamic effects as evidenced by increased left ventricle (LV) developed pressure and LV (+)dP/dt at 1-2 hours of reperfusion. Pretreatment with PQQ decreased average episodes of ventricular fibrillation (VF) per rat and the percentage of rats with VF while treatment with PQQ decreased the percentage of rats with VF during ischemia and reperfusion. The dose of PQQ was inversely related to infarct size. PQQ reduced levels of malondialdehyde (MDA), an index of lipid peroxidation, in ischemic myocardium.

Mortality during the ischemia-reperfusion period in the three groups in model 2 tended to decrease after PQQ (Control: 28.6%, Pretreatment: 12.9%, Treatment: 21.7%). However, these results did not reach statistical significance. It should be noted that the study

focused on measurements of infarct size and hemodynamics and was not designed as a mortality trial which would have required a much larger number of animals.

PQQ is also effective when given at the onset of reperfusion. Studies of myocardial tissue levels of malondialdehyde (MDA), a lipid peroxidation product that reacts with thiobarbituric acid have been completed. Ischemia/reperfusion augmented MDA levels and PQQ prevented this increase. The values comparing the PQQ and sham controls after ischemia/reperfusion (I/R) differed by 3-fold. Similar effect was seen in the remote “normal” myocardium.

PQQ given either as pretreatment or as treatment at the onset of reperfusion is highly effective in reducing myocardial infarct size and improving cardiac function in a dose-related manner in rat models of ischemia and ischemia-reperfusion. The malondialdehyde (MDA) results showing that this indicator of lipid peroxidation was reduced by PQQ, suggest that PQQ acts as a free radical scavenger in ischemic myocardium.

While not wishing to be bound by theory, one possible mechanism of PQQ action is that PQQ acts as a free radical scavenger. Recent studies indicate that PQQ functions as a free radical scavenger in addition to acting as a cofactor of quinoprotein enzymes (Urakami T, *et al.* J Nutr Sci Vitaminol (Tokyo) 1997;43:19-33, He K, *et al.* Biochemical Pharmacology 2003;65:67-74). PQQ can act as a neuroprotectant by suppressing peroxynitrite formation (Zhang Y and Rosenberg PA). PQQ was an effective antioxidant protecting mitochondria against oxidative stress-induced lipid peroxidation, protein carbonyl formation and inactivation of the mitochondrial respiratory chain (He K, *et al.*, Miyauchi K, *et al.* Antioxid Redox Signal 1999;1:547-554.). Phagocytic cells, such as monocytes and neutrophils, generate superoxide in response to stimuli. Several inhibitors of redox cycling of PQQ were demonstrated to be blocking agents for superoxide release by both stimulated neutrophils and monocytes. This suggests that PQQ is involved in the respiratory burst of both macrophages and neutrophils (Bishop A, *et al.* Free Radic Bio Med 1995;18:617-620, Bishop A, *et al.* Free Radic Bio Med 1994;17:311-320).

Our results are consistent with the above studies. We found that pretreatment with PQQ significantly decreased myocardial MDA levels in the infarct zone and in the remote “normal” myocardium. Our data indicate that MDA was increased by I/R in this putative normal area and are in accord with reports by others indicating the presence of LV dysfunction in remote myocardium during acute ischemia in humans and animals (Yang Z,

et al. Circulation 2004;109:1161-1167, Kramer CM, *et al.* Circulation 1996; 94:660-666.).

Our observations are consistent with the hypothesis that PQQ reduces lipid peroxidation and inactivates superoxide in both ischemic and non-ischemic myocardium. Therefore, the protective effect of PQQ on ischemia-reperfusion injury can be due to its action as a free radical scavenger. In our study PQQ given either as pretreatment or treatment also reduced the incidence of ventricular fibrillation. Furthermore, PQQ may have a direct antiarrhythmic effect, and may cause a reduction in VF due to its anti-ischemic properties.

The data, shown in Example 5 below, suggest that PQQ reduces lipid peroxidation as well as scavenges superoxide. Further evidence that the protective effect of PQQ on I/R injury is due to its action as a free radical scavenger. The observation that MDA increased after I/R in the putative normal area is consistent with observations reported by others that shows both in humans and in animal models that myocardium remote from the infarct zone exhibits depressed function.

The least dose of PQQ that is effective in reducing infarct size at the time of reperfusion was also determined as shown in Example 5. In addition we have determined the effect of this dose of PQQ on mitochondrial function. For these studies a previously described method of tissue preparation was used. The heart was visually divided longitudinally into an anterior portion comprising the territory perfused by the left anterior descending coronary artery and non-infarcted posterior portion. Rats were treated or not (sham controls) with PQQ after 30 min of ischemia immediately before the onset of 2 hr of reperfusion (I/R).

Treatment at the onset of reperfusion with 1 mg/kg PQQ did not protect either the heart or isolated intact mitochondria from I/R injury. However, treatment with only 3 mg/kg PQQ was highly effective in reducing infarct size by 49% and in restoring mitochondrial respiration. In these experiments hemodynamic results did not differ from those described in previous reports.

Our data also indicate that either prophylactic administration of PQQ in high-risk patients or treatment at the time of an active ischemic episode is of benefit by reducing infarct size and ventricular arrhythmias. Our results also indicate that treatment with PQQ is also effective at the time of reperfusion as occurs with chemical thrombolysis or balloon angioplasty/stenting when these procedures are employed as early treatment of acute myocardial infarction. The absence of depressant effects on systemic hemodynamics in this

study is also encouraging. Further exploration in other models of ischemia-reperfusion injury, where free radical generation is a paramount cause of damage may be desired. Acute toxicity, especially adverse effects on renal function that have been described in rats (Watanabe A, *et al.* Hiroshima J Med Sci 1989; 38:49-51), and the potential benefit of PQQ in humans, if any, remains to be determined.

Thus, PQQ given either as pretreatment before ischemia or as treatment at the onset of reperfusion following ischemia is highly effective in reducing myocardial infarct size and improving cardiac function in a dose-related manner in intact rats. PQQ appears to act as a free radical scavenger in ischemic myocardium.

Metoprolol is a β_1 -selective (cardioselective) adrenoceptor blocking agent. It reduces oxygen demand of the heart, slowing the heart rate and reducing cardiac output at rest and upon exercise; reduces systolic blood pressure, among other things. The drug is available in the United States as the tartrate salt (LOPRESSORTM, Geigy Pharmaceuticals), as 50 mg and 100 mg tablets. The effective daily dose is 100 mg to 450 mg, and LOPRESSORTM is usually dosed as 100 mg given in two daily doses. Metoprolol is also available as 50 mg, 100 mg and 200 mg extended release tablets in the United States as the succinate salt (TOPROL XLTM, Astra Pharmaceutical Products, Inc.), which may be dosed once daily.

PQQ may be coadministered with metoprolol. Results shown in Example 6, below, show that the combined use of PQQ and metoprolol tended to reduce infarct size greater than PQQ or metoprolol alone. In one embodiment, metoprolol is administered in a 1:3 ratio with the dose of PQQ. For example, a 3 mg/kg dose of PQQ is accompanied by a 1 mg/kg dose of metoprolol. In another embodiment, the metoprolol is administered at a daily dose from about 50 mg to about 450 mg combined with a daily dose of PQQ from about 50 mg to about 500 mg per day. Myocardial oxidative stress can be prevented or minimized by administration of a combination of PQQ and metoprolol, and thus has benefit for treating cardiovascular and other diseases. In particular, combinations of PQQ and metoprolol are useful as cardioprotective agents, and are therefore valuable in the treatment of a variety of various heart-related ailments such as ischemia-reperfusion injury, congestive heart failure, cardiac arrest and myocardial infarction such as due to coronary artery blockage, and for cardioprotection. The combinations in particular are useful for modulating myocardial oxidative stress such that myocardial cells (which are the subject of the oxidative stress) are protected from cell death.

The invention encompasses methods of treating or preventing cardiac injury caused by hypoxia or ischemia in a subject, wherein PQQ is administered to a subject in need thereof, such that hypoxia or ischemic-related injury is prevented or decreased. In certain embodiments, the PQQ is administered at a concentration of less than about 10 μ M. In other
5 embodiments, the PQQ is administered at a concentration in the range of about 10nM to about 10 μ M, about 10nM to about 1 μ M, 100nM to about 10 μ M, and 100nM to about 500nM. In still other embodiments of the invention, the PQQ is administered at a concentration such that the concentration of PQQ at the site of cardiac tissue is in the range of 10nM to about 10 μ M. PQQ may also be administered as a function of the subject's body weight. In some
10 embodiments of the invention, PQQ is administered at a concentration of between about 1 μ g/kg to 1g/kg of a subject's body weight, including less than 500mg/kg, 250mg/kg, 100mg/kg, 10mg/kg, 5mg/kg, 3mg/kg, 2mg/kg, 1mg/kg, 500 μ g/kg, 250 μ g/kg, 100 μ g/kg, 10 μ g/kg, 5 μ g/kg, 2 μ g/kg or 1 μ g/kg. In further embodiments of the invention, the PQQ is administered at a non-toxic concentration, which includes concentrations of PQQ which are
15 cytostatic but not cytotoxic, and concentrations which are cytotoxic to cell types other than the intended one or more cell types (*e.g.*, cardiomyocytes). The determination of the cytotoxicity of a known concentration of PQQ to one or more cell types is within the abilities of one of ordinary skill in the art. By way of non-limiting example, toxicity to cultured adult mouse cardiac myocytes is observed at a concentration of 100 μ M PQQ. In some
20 embodiments, PQQ is administered in combination with other compounds, such as anti-platelet drugs, anti-coagulant drugs, and anti-thrombotic drugs.

The cardiac injury that can be treated or prevented by the methods and compositions of the present invention includes all cardiac injury caused or affected by hypoxia and/or ischemia. Such injury includes, but is not limited to, ischemia-reperfusion injury, congestive
25 heart failure, myocardial infarction, cardiotoxicity caused by compounds such as drugs (*e.g.*, doxorubicin), cardiac damage due to parasitic infection, fulminant cardiac amyloidosis, heart surgery, heart transplantation, and traumatic cardiac injury. All or a portion of the heart may be injured, including associated blood vessels and/or tissue, such as the pericardium.

The invention also encompasses a method of treating or preventing cardiac injury
30 caused by hypoxia or ischemia in a subject, by administering to a subject in need thereof an NADPH-dependent methemoglobin reductase substrate, such that said hypoxia or ischemic-

related injury is prevented or decreased. In embodiments of the invention, the NADPH-dependent methemoglobin reductase substrate is purified from erythrocytes, such as mammalian erythrocytes (e.g., human, bovine, or murine) or non-mammalian erythrocytes (e.g., *Rana catesbeiana*). One of ordinary skill in the art will know how to isolate and purify NADPH-dependent methemoglobin reductase substrates with minimal experimentation.

The invention further encompasses a method of preventing organ or tissue damage during organ or tissue transplantation, by administering to a donor pyrroloquinoline quinone prior to or concurrent with removal of said organ or tissue, such that damage caused by reperfusion of said organ or tissue is decreased or prevented. The organ or tissue to be protected from reperfusion injury can include any organ or tissue including, but not limited to, the heart, the lungs, the kidneys, the stomach, the liver, the brain, the eyes, the reproductive organs, and skin tissue. In preferred embodiments, the organ or tissue to be transplanted is the heart or cardiac tissue. The PQQ may also be contacted with the organ or tissue following surgical removal of the organ or tissue from the donor. In some embodiments, the PQQ is added in addition to known organ or tissue preservation solutions, such as University of Wisconsin solution or Celsior solution (See, e.g., Thabut et al., *Am J Respir Crit Care Med*, 2001, 164(7):1204-8; Faenza et al., *Transplantation*, 2001, 72(7):1274-7).

The invention also provides methods for preventing, treating, or reducing organ failure or tissue damage resulting from an ischemic syndrome such as intestinal, hepatic, cerebral, renal, vascular, or limb ischemia by administering to a subject in need thereof a therapeutically effective amount of PQQ, alone or in combination with another biologically active agent, such that the organs or tissues are protected upon reperfusion of the ischemic area. Organs and tissue which can be protected include, but are not limited to, the kidneys, the lungs, the liver, the heart, the stomach, the pancreas, the appendix, the brain, the eyes, the reproductive organs, cardiac tissue, and skin tissue.

The invention also provides methods for preventing, treating, or reducing the symptoms of acute mountain or altitude sickness or high altitude pulmonary edema such as increased pulmonary blood pressure by administering to a subject in need thereof a therapeutically effective amount of tetraiodothyroacetic acid (Tetrac) and/or PQQ. Organs and tissue which can be protected include, but are not limited to, the kidneys, the lungs, the

liver, the heart, the stomach, the pancreas, the appendix, the brain, the eyes, the reproductive organs, cardiac tissue, and skin tissue.

The invention still further encompasses methods of providing neuroprotection by preventing stroke in a subject (*e.g.*, a human) suffering from heart failure, by treating a
5 subject with pyrroloquinoline quinone and a pharmaceutically acceptable carrier (see Example 10). In some embodiments, the pyrroloquinoline quinone is administered to the subject at a concentration of less than about 10 μ M. The PQQ may be administered prior to, or concomitant with, a surgical procedure that may increase the likelihood of a stroke in the patient. In one embodiment, the procedure is balloon angioplasty. Other procedures include
10 coronary artery bypass surgery and valve replacement surgery. The PQQ may be administered prior to, concomitant with, or after anti-thrombogenic agents (*e.g.*, coumadin). In yet another embodiment, neuroprotection can be achieved by administering PQQ prior to, concomitant with, or after a therapeutically effective dose of tamoxifen is administered to a subject at risk for a stroke.

15 The invention also encompasses methods of reducing or preventing headaches in a subject (such as a human), by treating the subject with pyrroloquinoline quinone and a pharmaceutically acceptable carrier. Such headaches include acute and chronic migraine headaches and sinus headaches.

The invention still further encompasses a method of preventing reperfusion injury in a
20 subject (such as a human) suffering from hypothermia, by treating the subject with pyrroloquinoline quinone and a pharmaceutically acceptable carrier. The subject may be treated with PQQ prior to or concomitant with the standard rewarming procedures for treating a person suffering from hypothermia as are generally known in the art.

As noted above, combination therapies of PQQ and metoprolol are part of the
25 invention. The combination therapies of the invention are administered in any suitable fashion to obtain the desired treatment of myocardial infarction in the patient. Substantially simultaneous administration can be accomplished, for example, by administering to the subject a single infusion having a fixed ratio of a PQQ and, metoprolol, or in multiple, single injections. The components of the combination therapies, as noted above, can be
30 administered by the same route or by different routes. For example, a PQQ is administered orally, while the metoprolol is administered intravenously; or all therapeutic agents may be

administered by intravenous injection. The sequence in which the therapeutic agents are administered is not believed to be critical.

PQQ can also be co-administered with nephroprotectants to reduce or prevent renal toxicity. Nephroprotectants suitable for co-administration with PQQ include any compound
5 which is an impedance blocker for transtubular flux, *i.e.*, a compound which impedes transtubular flux of compounds causing nephrotoxicity. Exemplary compounds include probenecid and cilastatin.

Probenecid is currently on the market for use in treating chronic gout and gouty arthritis. It is used to prevent attacks related to gout, not to treat them once they occur.

10 Probenecid acts on the kidneys (inhibiting renal tubular secretions) to help the body eliminate uric acid. It is also used to make certain antibiotics more effective by preventing the body from passing them in the urine.

Renal toxicity at high doses of PQQ alone has been observed in rats (See Example 5). PQQ may be co-administered with probenecid to reduce or prevent renal toxicity. Results
15 from Example 8, below, show that the combined use of PQQ and probenecid tended to reduce kidney toxicity. In one embodiment, PQQ is administered at a ratio between 1:4 and 1:100 with the dose of probenecid. For example, a 25 mg/kg dose of PQQ is accompanied by a 100 mg/kg dose of probenecid or a 1 mg/kg dose of PQQ is accompanied by a 100 mg/kg dose of probenecid, or a 2 mg/kg dose of PQQ is accompanied by a 100 mg/kg dose of
20 probenecid or a 3 mg/kg dose of PQQ is accompanied by a 100 mg/kg dose of probenecid. Kidney toxicity can be prevented or minimized by administration of a combination of PQQ and probenecid. Thus, administering PQQ in combination with probenecid will allow treatment of various indications with PQQ (*e.g.*, cardioprotection) while preventing or minimizing renal toxicity.

25 In another embodiment, the invention provides combination therapies of PQQ and cilastatin for preventing or reducing renal toxicity and/or kidney failure. Cilastatin is a renal dehydropeptidase-I and leukotriene dydiptidase inhibitor. It is typically administered with the antibiotic imipenem to increase its effectiveness by preventing its breakdown by the kidneys.

30 Sequential or substantially simultaneous administration of each therapeutic agent can be effected by any appropriate route including, but not limited to, oral routes, intravenous

routes, intramuscular routes, and direct absorption through mucous membrane tissues. The therapeutic agents can be administered by the same route or by different routes. For example, a first therapeutic agent of the combination selected may be administered by intravenous injection while the other therapeutic agents of the combination may be administered orally.

5 Alternatively, for example, all therapeutic agents may be administered orally or all therapeutic agents may be administered by intravenous injection. The sequence in which the therapeutic agents are administered is not narrowly critical.

For the combination of PQQ with nephroprotectants, the nephroprotectant may be administered prior to, at the same time as, or after, the PQQ. In a preferred embodiment, the
10 nephroprotectant is administered prior to PQQ administration, so that the nephroprotectant will be present in the blood stream to block any potential toxic effect of PQQ. In alternative embodiments, such as acute scenarios when sequential administration is not possible, the nephroprotectant may be administered at the same time as or after the PQQ. One specifically preferred embodiment includes administering 200 mg/kg Probenecid prior to the PQQ
15 administration and 100 mg/kg Probenecid one hour after the PQQ administration.

“Combination therapy” also can embrace the administration of the therapeutic agents as described above in further combination with other biologically active ingredients and non-drug therapies. Where the combination therapy further comprises a non-drug treatment, the non-drug treatment may be conducted at any suitable time so long as a beneficial effect from
20 the co-action of the combination of the therapeutic agents and non-drug treatment is achieved. For example, in appropriate cases, the beneficial effect is still achieved when the non-drug treatment is temporally removed from the administration of the therapeutic agents, perhaps by days or even weeks.

Thus, the compounds of the invention and the other pharmacologically active agent
25 may be administered to a patient simultaneously, sequentially or in combination. If administered sequentially, the time between administrations generally varies from 0.1 to about 48 hours. It will be appreciated that when using a combination of the invention, the compound of the invention and the other pharmacologically active agent may be in the same pharmaceutically acceptable carrier and therefore administered simultaneously. They may be
30 in separate pharmaceutical carriers such as conventional oral dosage forms which are taken

simultaneously. The term "combination" further refers to the case where the compounds are provided in separate dosage forms and are administered sequentially.

The beneficial effect of the combination composition of the invention includes, but is not limited to, pharmacokinetic or pharmacodynamic co-action resulting from the combination of therapeutic agents. In one embodiment, the co-action of the therapeutic agents is additive. In another embodiment, the co-action of the therapeutic agents is synergistic. In another embodiment, the co-action of the therapeutic agents improves the therapeutic regimen of one or both of the agents.

The invention further relates to kits for treating patients suffering a myocardial infarction, comprising a therapeutically effective dose of at least one metoprolol, and a PQQ, either in the same or separate packaging, and instructions for its use. Metoprolol is administered at a dose from about 0.1 mg/kg to about 10 mg/kg. Metoprolol is administered with PQQ at a ratio from about 2:1 to about 1:3. For example, when 1 mg/kg of metoprolol is administered, one proper dose to co-administer is 1 mg/kg of PQQ. Another proper dose of PQQ is 2 mg/kg. Another is 3 mg/kg of PQQ.

To evaluate whether a patient is benefiting from the (treatment), one would examine the patient's symptoms in a quantitative way, by decrease in the frequency of relapses, or increase in the time to sustained progression. In a successful treatment, the patient status will have improved, measurement number or frequency of relapses will have decreased, or the time to sustained progression will have increased.

As for every drug, the dosage is an important part of the success of the treatment and the health of the patient. In every case, in the specified range, the physician has to determine the best dosage for a given patient, according to gender, age, weight, height, pathological state and other parameters.

The pharmaceutical compositions of the present invention contain a therapeutically effective amount of the active agents. The amount of the compound will depend on the patient being treated. The patient's weight, severity of illness, manner of administration and judgment of the prescribing physician should be taken into account in deciding the proper amount. The determination of a therapeutically effective amount of an PQQ or metoprolol is well within the capabilities of one with skill in the art.

In some cases, it may be necessary to use dosages outside of the ranges stated in pharmaceutical packaging insert to treat a patient. Those cases will be apparent to the prescribing physician. Where it is necessary, a physician will also know how and when to interrupt, adjust or terminate treatment in conjunction with a response of a particular patient.

5 The invention encompasses the preparation and use of pharmaceutical compositions comprising a compound useful for the prevention or reduction of hypoxic/ischemic cardiac injury as an active ingredient. Such a pharmaceutical composition may consist of the active ingredient alone, in a form suitable for administration to a subject, or the pharmaceutical composition may comprise the active ingredient and one or more pharmaceutically acceptable carriers, one or more additional ingredients, or some combination of these. The active ingredient may be present in the pharmaceutical composition in the form of a pharmaceutically acceptable ester or salt, such as in combination with a physiologically-acceptable cation or anion, as is well known in the art. Further, the pyrroloquinoline quinone may contain pharmacologically acceptable additives (*e.g.*, carrier, excipient and diluent),
10 stabilizers or components necessary for formulating preparations, which are generally used for pharmaceutical products, as long as it does not adversely affect the efficacy of the preparation, *e.g.*, in decreasing or inhibiting ischemia or reperfusion injury.

 Examples of additives and stabilizers include saccharides such as monosaccharides (*e.g.*, glucose and fructose), disaccharides (*e.g.*, sucrose, lactose and maltose) and sugar
20 alcohols (*e.g.*, mannitol and sorbitol); organic acids such as citric acid, maleic acid and tartaric acid and salts thereof (*e.g.*, sodium salt, potassium salt and calcium salt); amino acids such as glycine, aspartic acid and glutamic acid and salts thereof (*e.g.*, sodium, calcium or potassium salt); surfactants such as polyethylene glycol, polyoxyethylene-polyoxypropylene copolymer and polyoxyethylenesorbitan fatty acid ester; heparin; and albumin.

25 The formulations of the pharmaceutical compositions described herein may be prepared by any method known or hereafter developed in the art of pharmacology. In general, such preparatory methods include the step of bringing the active ingredient into association with a carrier or one or more other accessory ingredients, and then, if necessary or desirable, shaping or packaging the product into a desired single- or multi-dose unit.

30 Although the descriptions of pharmaceutical compositions provided herein are principally directed to pharmaceutical compositions that are suitable for ethical

administration to humans, it will be understood by the skilled artisan that such compositions are generally suitable for administration to animals of all sorts. Modification of pharmaceutical compositions suitable for administration to humans in order to render the compositions suitable for administration to various animals is well understood, and the ordinarily skilled veterinary pharmacologist can design and perform such modification with merely ordinary, if any, experimentation. Subjects to which administration of the pharmaceutical compositions of the invention is contemplated include, but are not limited to, humans and other primates.

Pharmaceutical compositions that are useful in the methods of the invention may be prepared, packaged, or sold in formulations suitable for oral, rectal, vaginal, parenteral, topical, pulmonary, intranasal, buccal, ophthalmic, or another route of administration. The preferred mode is intravenous administration.

The pyrroloquinoline quinone and the above-mentioned ingredients are admixed as appropriate to give powder, granule, tablet, capsule, syrup, injection and the like. Other contemplated formulations include projected nanoparticles, liposomal preparations, resealed erythrocytes containing the active ingredient, and immunologically-based formulations.

A pharmaceutical composition of the invention may be prepared, packaged, or sold in bulk, as a single unit dose, or as a plurality of single unit doses. The amount of the active ingredient is generally equal to the dosage of the active ingredient, which would be administered to a subject, or a convenient fraction of such a dosage such as, for example, one-half or one-third of such a dosage.

The relative amounts of the active ingredient, the pharmaceutically acceptable carrier, and any additional ingredients in a pharmaceutical composition of the invention will vary, depending upon the identity, size, and condition of the subject treated and further depending upon the route by which the composition is to be administered. By way of example, the composition may comprise between 0.1% and 100% (w/w) active ingredient.

In addition to the active ingredient, a pharmaceutical composition of the invention may further comprise one or more additional pharmaceutically active agents.

Particularly contemplated additional agents include anti-emetics and scavengers such as cyanide and cyanate scavengers. Controlled- or sustained-release formulations of a pharmaceutical composition of the invention may be made using conventional technology.

A formulation of a pharmaceutical composition of the invention suitable for oral administration may be prepared, packaged, or sold in the form of a discrete solid dose unit including, but not limited to, a tablet, a hard or soft capsule, a cachet, a troche, or a lozenge, each containing a predetermined amount of the active ingredient. Other formulations suitable for oral administration include, but are not limited to, a powdered or granular formulation, an aqueous or oily suspension, an aqueous or oily solution, or an emulsion.

A tablet comprising the active ingredient may, for example, be made by compressing or molding the active ingredient, optionally with one or more additional ingredients. Compressed tablets may be prepared by compressing, in a suitable device, the active ingredient in a free-flowing form such as a powder or granular preparation, optionally mixed with one or more of a binder, a lubricant, an excipient, a surface active agent, and a dispersing agent. Molded tablets may be made by molding, in a suitable device, a mixture of the active ingredient, a pharmaceutically acceptable carrier, and at least sufficient liquid to moisten the mixture. Pharmaceutically acceptable excipients used in the manufacture of tablets include, but are not limited to, inert diluents, granulating and disintegrating agents, binding agents, and lubricating agents. Known dispersing agents include potato starch and sodium starch glycollate. Known surface active agents include sodium lauryl sulfate. Known diluents include calcium carbonate, sodium carbonate, lactose, microcrystalline cellulose, calcium phosphate, calcium hydrogen phosphate, and sodium phosphate. Known granulating and disintegrating agents include corn starch and alginic acid. Known binding agents include gelatin, acacia, pre-gelatinized maize starch, polyvinylpyrrolidone, and hydroxypropyl methylcellulose. Known lubricating agents include magnesium stearate, stearic acid, silica, and talc.

Tablets may be non-coated or they may be coated using known methods to achieve delayed disintegration in the gastrointestinal tract of a subject, thereby providing sustained release and absorption of the active ingredient. By way of example, a material such as glyceryl monostearate or glyceryl distearate may be used to coat tablets. Further by way of example, tablets may be coated using methods described in, *e.g.*, U.S. Patent Nos. 4,256,108; 4,160,452; and 4,265,874 to form osmotically-controlled release tablets. Tablets may further comprise a sweetening agent, a flavoring agent, a coloring agent, a preservative, or some combination of these in order to provide pharmaceutically elegant and palatable preparation.

Hard capsules comprising the active ingredient may be made using a physiologically degradable composition, such as gelatin. Such hard capsules comprise the active ingredient, and may further comprise additional ingredients including, for example, an inert solid diluent such as calcium carbonate, calcium phosphate, or kaolin.

5 Soft gelatin capsules comprising the active ingredient may be made using a physiologically degradable composition, such as gelatin. Such soft capsules comprise the active ingredient, which may be mixed with water or an oil medium such as peanut oil, liquid paraffin, or olive oil.

10 Liquid formulations of a pharmaceutical composition of the invention which are suitable for oral administration may be prepared, packaged, and sold either in liquid form or in the form of a dry product intended for reconstitution with water or another suitable vehicle prior to use.

Liquid suspensions may be prepared using conventional methods to achieve suspension of the active ingredient in an aqueous or oily vehicle. Aqueous vehicles include, 15 for example, water and isotonic saline. Oily vehicles include, for example, almond oil, oily esters, ethyl alcohol, vegetable oils such as arachis, olive, sesame, or coconut oil, fractionated vegetable oils, and mineral oils such as liquid paraffin. Liquid suspensions may further comprise one or more additional ingredients including, but not limited to, suspending agents, dispersing or wetting agents, emulsifying agents, demulcents, preservatives, buffers, salts, 20 flavorings, coloring agents, and sweetening agents. Oily suspensions may further comprise a thickening agent. Known suspending agents include, but are not limited to, sorbitol syrup, hydrogenated edible fats, sodium alginate, polyvinylpyrrolidone, gum tragacanth, gum acacia, and cellulose derivatives such as sodium carboxymethylcellulose, methylcellulose, hydroxypropylmethylcellulose. Known dispersing or wetting agents include naturally- 25 occurring phosphatides such as lecithin, condensation products of an alkylene oxide with a fatty acid, with a long chain aliphatic alcohol, with a partial ester derived from a fatty acid and a hexitol, or with a partial ester derived from a fatty acid and a hexitol anhydride (*e.g.*, polyoxyethylene stearate, heptadecaethyleneoxycetanol, polyoxyethylene sorbitol monooleate, and polyoxyethylene sorbitan monooleate, respectively). Known emulsifying 30 agents include lecithin and acacia. Known preservatives include methyl, ethyl, or *n*-propyl-para-hydroxybenzoates, ascorbic acid, and sorbic acid. Known sweetening agents include

glycerol, propylene glycol, sorbitol, sucrose, and saccharin. Known thickening agents for oily suspensions include, for example, beeswax, hard paraffin, and cetyl alcohol.

Liquid solutions of the active ingredient in aqueous or oily solvents may be prepared in substantially the same manner as liquid suspensions, the primary difference being that the active ingredient is dissolved, rather than suspended in the solvent. Liquid solutions of the pharmaceutical composition of the invention may comprise each of the components described with regard to liquid suspensions, it being understood that suspending agents will not necessarily aid dissolution of the active ingredient in the solvent. Aqueous solvents include, for example, water and isotonic saline. Oily solvents include, for example, almond oil, oily esters, ethyl alcohol, vegetable oils such as arachis, olive, sesame, or coconut oil, fractionated vegetable oils, and mineral oils such as liquid paraffin.

Powdered and granular formulations of a pharmaceutical preparation of the invention may be prepared using known methods. Such formulations may be administered directly to a subject, used, for example, to form tablets, to fill capsules, or to prepare an aqueous or oily suspension or solution by addition of an aqueous or oily vehicle thereto. Each of these formulations may further comprise one or more of dispersing or wetting agent, a suspending agent, and a preservative. Additional excipients, such as fillers and sweetening, flavoring, or coloring agents, may also be included in these formulations.

A pharmaceutical composition of the invention may also be prepared, packaged, or sold in the form of oil-in-water emulsion or a water-in-oil emulsion. The oily phase may be a vegetable oil such as olive or arachis oil, a mineral oil such as liquid paraffin, or a combination of these. Such compositions may further comprise one or more emulsifying agents such as naturally occurring gums such as gum acacia or gum tragacanth, naturally-occurring phosphatides such as soybean or lecithin phosphatide, esters or partial esters derived from combinations of fatty acids and hexitol anhydrides such as sorbitan monooleate, and condensation products of such partial esters with ethylene oxide such as polyoxyethylene sorbitan monooleate. These emulsions may also contain additional ingredients including, for example, sweetening or flavoring agents.

A pharmaceutical composition of the invention may be prepared, packaged, or sold in a formulation suitable for rectal administration. Such a composition may be in the form of,

for example, a suppository, a retention enema preparation, and a solution for rectal or colonic irrigation.

Suppository formulations may be made by combining the active ingredient with a non-irritating pharmaceutically acceptable excipient which is solid at ordinary room temperature (*i.e.*, about 20°C) and which is liquid at the rectal temperature of the subject (*i.e.*, about 37°C in a healthy human). Suitable pharmaceutically acceptable excipients include, but are not limited to, cocoa butter, polyethylene glycols, and various glycerides. Suppository formulations may further comprise various additional ingredients including, but not limited to, antioxidants and preservatives.

Retention enema preparations or solutions for rectal or colonic irrigation may be made by combining the active ingredient with a pharmaceutically acceptable liquid carrier. As is well known in the art, enema preparations may be administered using, and may be packaged within, a delivery device adapted to the rectal anatomy of the subject. Enema preparations may further comprise various additional ingredients including, but not limited to, antioxidants and preservatives.

A pharmaceutical composition of the invention may be prepared, packaged, or sold in a formulation suitable for vaginal administration. Such a composition may be in the form of, for example, a suppository, an impregnated or coated vaginally-insertable material such as a tampon, a douche preparation, a gel or cream or solution for vaginal irrigation.

Methods for impregnating or coating a material with a chemical composition are known in the art, and include, but are not limited to methods of depositing or binding a chemical composition onto a surface, methods of incorporating a chemical composition into the structure of a material during the synthesis of the material (*i.e.*, such as with a physiologically degradable material), and methods of absorbing an aqueous or oily solution or suspension into an absorbent material, with or without subsequent drying.

Douche preparations or solutions for vaginal irrigation may be made by combining the active ingredient with a pharmaceutically acceptable liquid carrier. As is well known in the art, douche preparations may be administered using, and may be packaged within, a delivery device adapted to the vaginal anatomy of the subject.

Douche preparations may further comprise various additional ingredients including, but not limited to, antioxidants, antibiotics, antifungal agents, and preservatives.

Additional delivery methods for administration of compounds include a drug delivery device, such as that described in U.S. Patent No. 5,928,195.

5 As used herein, "parenteral administration" of a pharmaceutical composition includes any route of administration characterized by physical breaching of a tissue of a subject and administration of the pharmaceutical composition through the breach in the tissue. Parenteral administration thus includes, but is not limited to, administration of a pharmaceutical composition by injection of the composition, by application of the composition through a surgical incision, by application of the composition through a tissue-penetrating non-surgical wound, and the like. In particular, parenteral administration is contemplated to include, but is not limited to, subcutaneous, intraperitoneal, intramuscular, intrasternal injection, and kidney dialytic infusion techniques.

15 Formulations of a pharmaceutical composition suitable for parenteral administration comprise the active ingredient combined with a pharmaceutically acceptable carrier, such as sterile water or sterile isotonic saline. Such formulations may be prepared, packaged, or sold in a form suitable for bolus administration or for continuous administration. Injectable formulations may be prepared, packaged, or sold in unit dosage form, such as in ampules or in multi-dose containers containing a preservative. Formulations for parenteral administration include, but are not limited to, suspensions, solutions, emulsions in oily or aqueous vehicles, pastes, and implantable sustained-release or biodegradable formulations. Such formulations may further comprise one or more additional ingredients including, but not limited to, suspending, stabilizing, or dispersing agents. In one embodiment of a formulation for parenteral administration, the active ingredient is provided in dry (*i.e.*, powder or granular) form for reconstitution with a suitable vehicle (*e.g.*, sterile pyrogen-free water) prior to parenteral administration of the reconstituted composition.

25 The pharmaceutical compositions may be prepared, packaged, or sold in the form of a sterile injectable aqueous or oily suspension or solution. This suspension or solution may be formulated according to the known art, and may comprise, in addition to the active ingredient, additional ingredients such as the dispersing agents, wetting agents, or suspending agents described herein. Such sterile injectable formulations may be prepared using a non-

toxic parenterally-acceptable diluent or solvent, such as water or 1,3-butane diol, for example. Other acceptable diluents and solvents include, but are not limited to, Ringer's solution, isotonic sodium chloride solution, and fixed oils such as synthetic mono- or diglycerides. Other parentally-administrable formulations that are useful include those, which comprise the active ingredient in microcrystalline form, in a liposomal preparation, or as a component of a biodegradable polymer systems. Compositions for sustained release or implantation may comprise pharmaceutically acceptable polymeric or hydrophobic materials such as an emulsion, an ion exchange resin, a sparingly soluble polymer, or a sparingly soluble salt.

Formulations suitable for topical administration include, but are not limited to, liquid or semi-liquid preparations such as liniments, lotions, oil-in-water or water-in-oil emulsions such as creams, ointments or pastes, and solutions or suspensions. Topically-administrable formulations may, for example, comprise from about 1% to about 10% (w/w) active ingredient, although the concentration of the active ingredient may be as high as the solubility limit of the active ingredient in the solvent. Formulations for topical administration may further comprise one or more of the additional ingredients described herein.

A pharmaceutical composition of the invention may be prepared, packaged, or sold in a formulation suitable for pulmonary administration via the buccal cavity. Such a formulation may comprise dry particles which comprise the active ingredient and which have a diameter in the range from about 0.5 to about 7 nanometers, and preferably from about 1 to about 6 nanometers. Such compositions are conveniently in the form of dry powders for administration using a device comprising a dry powder reservoir to which a stream of propellant may be directed to disperse the powder or using a self-propelling solvent/powder-dispensing container such as a device comprising the active ingredient dissolved or suspended in a low-boiling propellant in a sealed container. Preferably, such powders comprise particles wherein at least 98% of the particles by weight have a diameter greater than 0.5 nanometers and at least 95% of the particles by number have a diameter less than 7 nanometers. More preferably, at least 95% of the particles by weight have a diameter greater than 1 nanometer and at least 90% of the particles by number have a diameter less than 6 nanometers. Dry powder compositions preferably include a solid fine powder diluent such as sugar and are conveniently provided in a unit dose form.

Low boiling propellants generally include liquid propellants having a boiling point of below 65°F at atmospheric pressure. Generally the propellant may constitute 50 to 99.9% (w/w) of the composition, and the active ingredient may constitute 0.1 to 20% (w/w) of the composition. The propellant may further comprise additional ingredients such as a liquid
5 non-ionic or solid anionic surfactant or a solid diluent (preferably having a particle size of the same order as particles comprising the active ingredient).

Pharmaceutical compositions of the invention formulated for pulmonary delivery may also provide the active ingredient in the form of droplets of a solution or suspension. Such formulations may be prepared, packaged, or sold as aqueous or dilute alcoholic solutions or
10 suspensions, optionally sterile, comprising the active ingredient, and may conveniently be administered using any nebulization or atomization device. Such formulations may further comprise one or more additional ingredients including, but not limited to, a flavoring agent such as saccharin sodium, a volatile oil, a buffering agent, a surface active agent, or a
15 preservative such as methylhydroxybenzoate. The droplets provided by this route of administration preferably have an average diameter in the range from about 0.1 to about 200 nanometers.

The formulations described herein as being useful for pulmonary delivery are also useful for intranasal delivery of a pharmaceutical composition of the invention.

Another formulation suitable for intranasal administration is a coarse powder
20 comprising the active ingredient and having an average particle from about 0.2 to 500 micrometers. Such a formulation is administered in the manner in which snuff is taken *i.e.*, by rapid inhalation through the nasal passage from a container of the powder held close to the nose.

Formulations suitable for nasal administration may, for example, comprise from about
25 as little as 0.1% (w/w) and as much as 100% (w/w) of the active ingredient, and may further comprise one or more of the additional ingredients described herein.

A pharmaceutical composition of the invention may be prepared, packaged, or sold in a formulation suitable for buccal administration. Such formulations may, for example, be in the form of tablets or lozenges made using conventional methods, and may, for example, 0.1
30 to 20% (w/w) active ingredient, the balance comprising an orally dissolvable or degradable composition and, optionally, one or more of the additional ingredients described herein.

Alternately, formulations suitable for buccal administration may comprise a powder or an aerosolized or atomized solution or suspension comprising the active ingredient. Such powdered, aerosolized, or aerosolized formulations, when dispersed, preferably have an average particle or droplet size in the range from about 0.1 to about 200 nanometers, and may
5 further comprise one or more of the additional ingredients described herein.

A pharmaceutical composition of the invention may be prepared, packaged, or sold in a formulation suitable for ophthalmic administration. Such formulations may, for example, be in the form of eye drops including, for example, a 0.1-1.0% (w/w) solution or suspension of the active ingredient in an aqueous or oily liquid carrier. Such drops may further comprise
10 buffering agents, salts, or one or more other of the additional ingredients described herein. Other ophthalmically-administrable formulations that are useful include those, which comprise the active ingredient in microcrystalline form or in a liposomal preparation.

The mixture of pyrroloquinoline quinone and pharmacologically acceptable additives is preferably prepared as a lyophilized product, and dissolved when in use. Such preparation
15 can be prepared into a solution containing about 0.01-100.0 mg/ml of pyrroloquinoline quinone, by dissolving same in distilled water for injection or sterile purified water. More preferably, it is adjusted to have a physiologically isotonic salt concentration and a physiologically desirable pH value (pH 6-8).

While the dose is appropriately determined depending on symptom, body weight, sex,
20 animal species and the like, it is generally assumed that treatment options holding the blood concentration at about 1 μ M will be preferred. This plasma concentration may be achieved through administration of one to several doses a day. When pyrroloquinoline quinone is to be administered to a subject, 0.1ng to 10mg/kg body weight (e.g., 1ng to 1mg/kg body weight) of pyrroloquinoline quinone can be given intravenously.

The compound may be administered to an animal as frequently as several times daily,
25 or it may be administered less frequently, such as once a day, once a week, once every two weeks, once a month, or even less frequently, such as once every several months or even once a year or less. The frequency of the dose will be readily apparent to the skilled artisan and will depend upon any number of factors, such as, but not limited to, the type and severity
30 of the disease being treated, the type and age of the animal, etc.

EXAMPLES

These Examples are provided for the purpose of illustration only and the invention should in no way be construed as being limited to these Examples, but rather should be construed to encompass any and all variations which become evident as a result of the teaching provided herein.

Example 1. *In vitro* studies of PQQ preservation of cardiac myocyte viability.

An *in vitro* model of cultured adult cardiac mouse myocytes was developed to study cardioprotection by PQQ. These cells are viable in culture for up to 48 hours at a physiologic pH and consist of >90% rod-shaped cells. These cells can be used readily for determination of cell viability by trypan blue exclusion, and for biochemical, immunochemical, and molecular studies. In this model, approximately 35% of the cells die when exposed to 0% oxygen in a hypoxia chamber for 2-3 hours. As shown in Figure 1, 1 μ M PQQ added 1 hour before subjecting the cells to severe hypoxia (0% oxygen for 2-3 hours) produces a significant increase in the proportion of viable cells as indicated by trypan blue exclusion. A higher concentration of PQQ (100 μ M) is highly toxic under normoxic conditions as evidenced by 100% cell death. Figure 2 demonstrates that 1 μ M PQQ protection against hypoxia-induced cell death is not inhibited by 10 μ M 5-hydroxydecanoic acid, a mitochondrial K_{ATP} channel inhibitor. Without wishing to be bound by theory, these data suggest that PQQ does not exert cardioprotection by opening mitochondrial K_{ATP} channels.

Example 2. *Ex vivo* studies of PQQ preservation of cardiac function.

Ex vivo studies were performed using an isolated mouse heart preparation employing the Langendorff technique. In this approach, the heart is removed and mounted on a perfusion apparatus in which drugs can be given via an aortic cannula. The heart is paced at a constant rate, and left ventricular developed pressure [LVDP; left ventricular systolic pressure minus left ventricular end-diastolic pressure], left ventricular end-diastolic pressure [LVEDP], and the maximum positive and negative first derivatives of left ventricular pressure [$+dP/dt_{max}$ and $-dP/dt_{max}$] are recorded. The heart is equilibrated for 20 min. After drug or vehicle is infused, the heart is subjected to 20 min of ischemia [coronary flow completely stopped] followed by 30 min of reperfusion. Coronary sinus flow as a reflection of coronary blood flow is also measured. This protocol leads to severe myocardial injury as measured by hemodynamic parameters.

As seen in Figure 3, 100 nM PQQ infused for only 2 minutes prior to complete cessation of coronary blood flow produces significant preservation of LVDP Baseline 1. VDP averages 60 mmHg. Similar results are obtained with LVEDP [Figure 4] Baseline LVEDP averages 8 mm Hg. (Note that an increase in LVEDP represents an adverse response). As expected, the data for + and -dP/dtmax track the LVDP results [Figures 5 and 6]. Similarly, coronary flow is significantly improved by PQQ pretreatment compared to control [Figure 7].

In Figure 8, it is shown that 2 min of pretreatment with PQQ at the concentrations shown has progressively favorable responses between 10nM and 1µM, but that toxicity occurs at 10µM. Of major interest is that 100nM PQQ given at the time of onset of reperfusion (PQQ tre.) is equivalent to pretreatment. Therefore, PQQ is useful in both pretreatment, e.g., in cardiac or other surgical procedures, and after symptoms occur, e.g., in the acute critical cardiac events.

Figure 9 shows the results of experiments of infarct size measurements after PQQ pretreatment. As indicated, there is a progressive reduction in infarct size between 10 nM and 1µM, paralleling the hemodynamic data. Consistent with the latter, infarct size is not reduced at 10µM PQQ.

Example 3. PQQ preservation of oxidatively stressed cells.

Cultured cardiac myocytes are subjected to oxidative stress by *in vitro* administration of H₂O₂. Two studies are done, one in which PQQ is added in concentrations between 10nM and less than 10µM to cardiac myocytes, after which H₂O₂ is added. In the other study, cardiac myocytes are subjected to insult *in vitro* administration of H₂O₂ for two hours, after which PQQ is added in concentrations between 10nM and less than 10µM. In both studies, PQQ is found to be protective.

Example 4. Use of PQQ for prevention/reduction of oxidative stress *in vivo*.

Male Sprague-Dawley rats were randomly treated with pyrroloquinoline quinone (PQQ) either before ischemia or ischemia-reperfusion. PQQ (15-20 mg/kg) was given 30 min before left anterior descending coronary artery (LAD) occlusion by intraperitoneal injection (pretreatment) or at the onset of reperfusion by intravenous injection (treatment). Rats were subjected to 17 or 30 min of LAD occlusion and 2 hours of reperfusion with left ventricle (LV) hemodynamic monitoring. PQQ given either as pretreatment or treatment decreased infarct size in these rat models. PQQ protected against ischemia-induced cardiac

dysfunction with higher LV developed pressure, LV (+)dP/dt and lower LV (-)dP/dt after 1-2 hour of reperfusion. There were fewer episodes of ventricular fibrillation (VF) in PQQ treated rats. Myocardial malondialdehyde (MDA), an indicator of lipid peroxidation, was reduced by PQQ. Thus, PQQ given either as pretreatment or as treatment at the onset of reperfusion is highly effective in reducing myocardial infarct size and improving cardiac function in a dose-related manner in rat models of ischemia and ischemia-reperfusion. The MDA results suggest that PQQ acts as a free radical scavenger in ischemic myocardium.

Statistical analysis.

All results are presented as mean $\pm$ SEM. The two treatment groups (pretreatment and treatment) were compared with the normal control group using one-way analysis of variance (ANOVA) with the regression equation for multiple group comparisons. Differences in mortality during the occlusion and reperfusion period among the three groups were assessed by the Chi-square test. The percentages of rats with VF were assessed by the Fisher Exact test. All computations were done using the general linear model procedure in Minitab, version 7.2 (Minitab Statistical Software) or Primer of Biostatistics: The program, version 3.03 (McGraw-Hill). Statistical significance was set at $p < 0.05$.

Models of ischemia and ischemia- reperfusion.

PQQ was dissolved in vehicle (2% NaHCO₃). The volume given either intraperitoneally (i.p.) or intravenously (i.v.) was one ml. All controls were treated with one ml of vehicle. In model 1, PQQ at 20 mg/kg was given i.p. 30 min before 2 hours of ischemia induced by LAD ligation. In model 2, PQQ at 15 mg/kg was given i.p. 30 min before either 17 or 30 min of ischemia followed by 2 hours of reperfusion (pretreatment). In other model 2 experiments, PQQ at 15 mg/kg was given at the onset of reperfusion by i.v. bolus injection via the femoral vein (treatment). These protocols are summarized in Figure 10.

After induction of anesthesia (ketamine 80 mg/kg, xylazine 4 mg/kg body weight intraperitoneally), a tracheostomy was performed and the animal was ventilated on a Harvard Rodent Respirator (Model 683, Harvard Apparatus). *Model 1* rats were subjected to 2 hours of proximal left anterior descending (LAD) coronary artery ligation without reperfusion. *Model 2* employed ischemia-reperfusion as previously described (Sievers RE, *et al.* Magn Reson Med 1989; 10:172-81). In this model, a reversible coronary artery snare occluder was placed around the proximal LAD coronary artery through a midline sternotomy. Rats were

then subjected to 17 or 30 minutes of LAD occlusion and 120 minutes of reflow. In addition, model 2 rats had hemodynamic measurements recorded. A 4F Millar catheter was inserted through the right carotid artery into the left ventricle (LV). After 20 min of equilibration, heart rate (HR), systolic pressure (LVSP), end diastolic pressure (LVEDP), LV (+)dP/dt max, and LV (-)dP/dt max were monitored using a MacLab/4S (Milford, MA). LV developed pressure (LVDP) was calculated by subtracting LVEDP from LVSP.

Body weights among the three groups of rats in both model 1 and model 2, sets 1 and 2 did not differ (values for Control, Pretreatment, and Treatment groups were: 320 ± 16 , 321 ± 22 , and 306 ± 18 gm, respectively; $p=0.799$ by analysis of variance (ANOVA)).

There were no significant differences in heart rate, LVSP, LVEDP, LV (+)dP/dt, and LV (-)dP/dt among control, pretreatment and treatment groups in model 2 at baseline. Whether given as pretreatment or treatment, PQQ protected against ischemia-induced cardiac dysfunction with higher LVSP, LVDP, LV (+)dP/dt and lower LV (-) dP/dt after 1-2 hours of reperfusion (Figures 11A-B; 12A-B).

Infarct size.

Infarct size was measured as described previously (Sievers RE, *et al.* Magn Reson Med 1989; 10:172-81, Zhu B-Q, *et al.* J Am Coll Cardiol 1997; 30:1878-85). In model 1, hearts were excised at the end of the 2 hour ischemic period. The sections were then incubated in a 1% solution of triphenyltetrazolium chloride (TTC) for 10 to 15 min until viable myocardium was stained brick red.

In model 2, after 2 hours of reperfusion, the LAD was reoccluded, and phthalocyanin dye (Engelhard Cooperation, Louisville, KY) was injected into the LV cavity, allowing normally perfused myocardium to stain blue. The heart was then excised, rinsed of excess dye and sliced transversely from apex to base into 2-mm-thick sections. The sections were incubated in TTC as described above. Infarcted myocardium fails to stain with TTC. The tissue sections were then fixed in a 10% formalin solution and weighed. Color digital images of both sides of each transverse slice were obtained using a videocamera (Leica DC 300 F) connected to a microscope (Stereo Zoom 6 Photo, Leica). The regions showing blue-stained (nonischemic), red-stained (ischemic but noninfarcted) and unstained (infarcted) tissue were outlined on each color image and measured using NIH Image 1.59 (National Institutes of Health, Bethesda, MD) in a blinded fashion. On each side, the fraction of the LV area representing infarct-related tissue (average of two images) was multiplied by the weight of

that section to determine the absolute weight of infarct-related tissue. The infarct size for each heart was expressed as:

$$\text{Infarct size/LV mass (\%)} = \frac{\sum \text{Infarct weight in each slice}}{\text{Total LV weight}} \times 100\%,$$

$$\text{Risk area/LV mass (\%)} = \frac{\text{Total weight of non-blue-stained section}}{\text{Total LV weight}} \times 100\%,$$

Infarct size as a percentage of risk area was then calculated as

$$\frac{\sum \text{Infarct weight in each slice}}{\sum \text{Risk area weight of each slice}} \times 100\%$$

In the ischemic model (*model 1*), infarct size (infarct mass/LV mass, without phthalocyanin blue dye injected) after PQQ was smaller than Control (Figure 13). In the first set of experiments in model 2, ischemia was for 17 min followed by 2 hours of reperfusion, infarct size (infarct mass/risk area, infarct mass/LV mass) was reduced by pretreatment with PQQ 20 mg/kg (Figure 14). In the second set of model 2 experiments, ischemia was for 30 min followed by 2 hours of reperfusion. Infarct size after either Pretreatment or Treatment with PQQ 15 mg/kg was smaller than Control (Figure 15).

Figure 16 shows that the dose of PQQ given as Pretreatment was inversely related to infarct size. In these experiments, 17 min of ischemia was followed by 2 hours of reperfusion. **Ventricular fibrillation (VF).**

Electrocardiograms (ECGs, lead II) were obtained by inserting subcutaneous needle electrodes into the limbs. The ECG was monitored during ischemia and reperfusion and episodes of paroxysmal VF were recorded. The number episodes of VF per rat and the percentage of rats with VF in each group were calculated. No rat received antiarrhythmics before or during occlusion and reperfusion. Episodes of VF were successfully treated by rapidly striking the exposed myocardium with the thumb and index finger of one hand.

Pretreatment with PQQ at 15 or 20 mg/kg decreased average episodes of VF per rat (Figure 17A). Either Pretreatment or Treatment with PQQ at 15 or 20 mg/kg decreased the percentage of rats with VF (Figure 17B).

In separate additional experiments in which malondialdehyde was measured (see below) there were no episodes of VF in either the Sham or PQQ groups (n=5 each). However, VF in the I/R group (30 min of ischemia followed by 2 hours reperfusion) averaged 1.8 ± 0.4 episodes/rat and was reduced by Pretreatment with PQQ 15 mg/kg to 0.2 ± 0.2 episodes/rat (n=5 each group, $P < 0.001$ by two-way ANOVA with a significant interaction between PQQ and I/R $P = 0.002$).

Myocardial malondialdehyde (MDA) measurement.

Myocardial tissue MDA, a lipid peroxidation product that reacts with thiobarbituric acid, was determined spectrophotometrically at an absorbance of 532 nm. The concentration of the samples was calculated using an extinction coefficient of 1.56×10^5 /M cm and the results were expressed as nmol/g wet weight heart (Ohkawa H, *et al.* Analytical Biochemistry 1979;95:351-358., Moritz F, *et al.* Cardiovascular Research 2003;59:834-843).

For measurements of myocardial tissue MDA, an indicator of lipid peroxidation, a different method of tissue preparation was used. The heart was divided visually from apex to base into an anterior portion comprising the territory perfused by the LAD and the remaining non-ischemic portion. Rats were pretreated or not (Sham controls) with 15 mg/kg PQQ and subjected to 30 min of ischemia and 2 hours of reperfusion. In these additional experiments hemodynamic results did not differ from those described above. As can be seen in Figure 18A, I/R augmented MDA levels and PQQ prevented this increase. PQQ and Control values after I/R differed by 3-fold (Figure 18A). A similar effect was seen in the remote "normal" myocardium (Figure 18B). When given at the onset of reperfusion, PQQ 15 mg/kg also reduced MDA levels in ischemic myocardium from 176 ± 16 to 123 ± 17 nmol/g (n=8, $P < 0.05$).

Example 5. PQQ Restores Mitochondrial Respiration at Low Doses and is Cardioprotective in *In Vivo* Models of Ischemia/Reperfusion Injury.

Adult male rats underwent 30 min of left anterior descending coronary artery (LAD) occlusion and 2 hrs of reperfusion. To assess the potential benefits of reperfusion therapy in humans, PQQ was given by i.v. injection at doses of 1 and 3 mg/kg bodyweight at the onset of reperfusion. After removal, the hearts were divided from apex to base into an anterior part comprising the LAD-perfused territory and a posterior segment, and mitochondria were isolated. Mitochondrial respiration of each myocardial segment was measured and compared

to that of mitochondria isolated from preconditioned (without PQQ) and sham operated hearts.

Treatment with 1 mg/kg PQQ did not protect mitochondria from I/R injury. However, treatment with 3 mg/kg PQQ was highly effective in restoring mitochondrial respiration. The respiratory control and ADP-to-oxygen consumption ratios (RCR: 8.0 ± 0.5 , ADP/O: 4.5), and state 3 respiration rates (RR: 41 nmol O atom/min/mg protein, $n = 5$) of the ischemic areas matched those of the shams (RCR: 8.2 ± 0.3 , ADP/O: 3.7, RR = 43 nmol O atom/min/mg protein), while the RCR values of hearts preconditioned without PQQ were surprisingly 20% lower. Respiratory responses from mitochondria of ischemic, untreated hearts were reduced by 50-100%. Electron micrographs of PQQ-treated tissue and mitochondria did not reveal the morphology typical of myocardial damage. PQQ also reduced infarct size and myocardial malondialdehyde (MDA) tissue levels by 49% and 61%, respectively.

Low-dose, 3 mg/kg PQQ given at reperfusion very effectively restores mitochondrial respiration inhibited by ischemia and reduces oxidative damage to mitochondria and infarct size in I/R injury. PQQ may exert its cardioprotective function as a lipid peroxidation inhibitor or radical scavenger. Thus, PQQ treatment may emerge as a powerful therapy of acute ischemic syndromes.

The respiratory control and ADP-to-oxygen consumption ratios (RCR: 8.0 ± 0.5 , ADP/O: 4.5), and state 3 respiration rates (RR: 41 nmol O atom/min/mg protein, $n = 5$) of the ischemic areas matched those of the shams, while the RCR values of hearts preconditioned without PQQ were surprisingly 20% lower. Respiratory responses from mitochondria of ischemic, untreated hearts were reduced by 50-100%. RCR data are shown graphically in Figure 19. Electron micrographs of PQQ-treated tissue and mitochondria did not reveal the morphology typical of myocardial damage. Thus, low-dose 3 mg/kg PQQ given at reperfusion very effectively restores mitochondrial respiration inhibited by ischemia and reduces oxidative damage to mitochondria and infarct size in I/R injury.

Toxicity studies were also carried out. We found no renal or hepatic toxicity at doses of either 3 mg/kg or 10 mg/kg (see Table 1). As noted above, PQQ 3 mg/kg given at the time of reperfusion appears to be an effective cardioprotective dose. At 15 mg/kg, 7/8 animals showed no renal or hepatic toxicity, but one rat excluded from the data presented in Table 1 did develop uremia at 4 days and was dead at 10 days. Rats that received 20 mg/kg had

received 10 mg/kg two weeks previously for a cumulative dose of 30 mg/kg. As indicated in Table 1, all of these animals developed uremia and were dead at 10 days. All laboratory studies were performed in a blinded fashion by the clinical laboratory at the San Francisco VA Medical Center. Note that baseline levels of some measures, such as albumin, are lower
5 in rats than in humans, while others, such as creatine kinase (CK), are higher.

Table 1

POQ	BUN	CRE	NA	K	CL	CO2	TP	ALB	ALP	AST	ALT	CK	BW(g)
3 mg/kg (n=4)													
Baseline	16.5±1.0	0.57±0.03	140±0.9	5.2±0.6	103±0.4	27±1.4	5.8±0.1	1.7±0.04	256±6.1	82±1.3	52±5	873±240	357.5±7.5
4 days	16.3±1.1	0.38±0.03	141±0.7	5.9±0.3	104±0.7	25±0.8	5.8±0.1	1.5±0.08	206±5.5#	100±19	58±2	775±258	377.5±7.8
10 days	14.3±0.3	0.35±0.03	139±0.5	4.8±0.5	104±1.0	27±0.3	6.0±0.2	1.6±0.03	215±15	83±10	62±3	712±325	395±8.9#
10 mg/kg (n=6)													
Baseline	16.3±1.3	0.38±0.05	135±1.9	8.2±2	101±1.0	25±2	5.4±0.2	1.7±0.03	305±24	79±21	43±1	1113±831	350±11
4 days	17.3±0.8	0.38±0.03	135±2.1	11.6±2	101±0.3	24±2	6.0±0.1	1.7±0.07	305±29	95±22	60±2	902±441	368.7±10
10 days	14.8±0.5	0.38±0.03	138±1.0	5.5±0.7	102±0.7	27±1	6.0±0.1	1.6±0.04	273±33	85±22	50±2	2240±1450	387.6±11
15 mg/kg (n=7)&													
Baseline	17.9±0.5	0.34±0.02	140.4±0.5	4.7±0.2	100.8±0.9	29.4±0.6	5.9±0.1	1.7±0.07	365±48	81.4±6.7	50±3	507±109	334±16
4 days	17.4±1.5	0.44±0.05	140.6±0.8	4.8±0.5	101.1±0.7	29.6±0.5	5.9±0.1	1.5±0.08	293±46	77.3±8.0	56±5	640±353	364±18
10 days	16.0±1.0	0.38±0.02	140.3±0.7	5.5±0.8	100.5±1.0	30.2±0.5	6.08±0.1	1.6±0.1	294±39	70.0±4.9	50±3	726±209	370±20
20 mg/kg (n=4)@													
Baseline	18.5±1.0	0.36±0.03	139±0.7	5.2±0.4	102±0.8	28±2	6.0±0.1	1.6±0.03	211±9.5	97±15	55±3	872±443	402.5±7.8
4 days	390±2.6**	9.7±0.7**	129±4.3	11.8±0.6**	88±1.0#	6±1**	4.8±0.1*	1.1±0.04**	124±5.8*	173±16#	54±2	1331±851	377.5±12
10 days	Died												

(** P<0.001, *P<0.01, #P<0.05 vs Baseline)
 (& One rat (not included) developed severe uremia (BUN441, CRE10.8) @ 4 days and was dead at 10 days)
 (# These rats received 20 mg/kg at 2 weeks after receiving 10 mg/kg)

Example 6. Studies of PQQ and metoprolol's effect on cardiac function.

Pretreatment or treatment with 15 mg/kg of PQQ reduced infarct size and improved cardiac function in a rat model of ischemia/reperfusion (I/R). The beta-blocker metoprolol is used as standard treatment in patients with acute myocardial infarction. Accordingly, a
5 experiments were conducted to study the combined treatment of myocardial infarction with metoprolol and low-dose PQQ compared to each drug alone. To determine mechanisms of cardioprotection, changes were measured in mitochondrial function and lipid peroxidation.

Intact male rats were subjected to 30 min of left anterior descending coronary artery occlusion and 2 hours of reperfusion with left ventricular hemodynamic monitoring. In
10 preliminary experiments metoprolol at a dose of 1 mg/kg was found to be optimal in this system for infarct size reduction. Accordingly, metoprolol (1 mg/kg) and/or PQQ (3 mg/kg) was given by femoral vein injection at the onset of reperfusion to mimic clinical treatment. In separate experiments after ischemia/reperfusion, the mitochondrial respiratory control and ADP-to-oxygen consumption ratios (RCR) of the ischemic and non-ischemic myocardium
15 were measured, as well as levels of malondialdehyde (MDA), an index of lipid peroxidation.

Results indicate that either treatment with metoprolol or PQQ reduced myocardial infarct size (infarct mass/risk area). The combined use of these agents tended to further reduce infarct size. Metoprolol and/or PQQ also protected against ischemia-induced left ventricular (LV) dysfunction after 1-2 hours of reperfusion. Thus, LV developed pressure was increased
20 and LV end-diastolic pressure was decreased. Metoprolol and/or PQQ also reduced CK release. Mitochondrial RCR in ischemic and non-ischemic myocardium were enhanced primarily by PQQ, and less so by metoprolol. PQQ decreased MDA in ischemic and non-ischemic myocardium. These results are summarized in Table 2, below.

These experiments suggest that PQQ and metoprolol are effective in treating myocardial
25 infarction, but the combination of PQQ and metoprolol may be more effective than either agent alone. For mitochondrial protection, PQQ is superior. It should be noted that in a recent large study of 45,852 patients with acute myocardial infarction randomized to metoprolol or placebo, the incidence of cardiogenic shock was about 30% higher in the metoprolol group (Collins R, *et al.* COMMIT/CCS-2; Placebo-controlled trial of early metoprolol in 46,000 acute
30 myocardial infarction patients. Late-breaking trials presented at the American College of Cardiology Annual Scientific Session 2005. March 6-9, 2005. Orlando, Fla.). This may be due at least in part to the inability of metoprolol to restore mitochondrial function.

Table 2		Infarct size (%)	LVEDP (mmHg)	LV Developed Pressure (mmHg)	Mitochondria (RCR) Ischemic	Mitochondria (RCR) Non-ischemic	MDA (nmol/g) Ischemic	MDA (nmol/g) Non-ischemic
Groups	(Change in CK U/L)							
I/R (n=9)	39.9±4.2 (1443±220)	11.8±2.1	78.3±6.1	3.0±0.5	5.7±0.4	316±88	237±61	
MP (n=6)	26.5±3.3** (1290±389)	4.7±3.0*	87.7±7.7	5.0±0.7**	7.2±0.5**	283±36	263±17	
PQQ (n=12)	24.3±2.7** (358±129*)	1.9±1**	89.1±5.8	7.8±0.3**	8.0±0.3**	99±14**	118±30*	
MP+PQQ (n=9)	18.8±1.1** (497±141*)	3.0±1.7**	105±2**	4.5±0.5*	6.5±0.3	260±9	232±9	
Sham (n=7)	0	7.4±0.9	100±6	7.7±0.3**	7.9±0.2**	238±26	206±19	
Sham+PQQ (n=6)	0	4.6±2.4*	99±6	9.2±0.5**	9.5±0.5**	138±15*	128±12*	

Change in CK = CK at end of reperfusion minus baseline value. MP = Metoprolol.

*P<0.05, **P<0.01 vs Ischemia/Reperfusion (I/R)

Example 7: Synthesis of PQQ Conjugated Polyvinyl Alcohol and PQQ-conjugated polymers

5 PQQ was first activated, and then reacted with polymer to obtain the PQQ conjugated polymer. Different molecular weight poly (vinyl alcohol) (PVA) from 9k-100K were tested in this application. PVA is a polymer of great interest because of its many desirable characteristics, specifically for various pharmaceutical and biomedical application.

10 The synthesis procedure is shown in Figure 20A. This is a two-step reaction. At the first step, the PQQ reacted with the dehydration agent (e.g. DCC or CDI) to obtain an active intermediate. At the second step, the active PQQ reacted with PVA, the ester bond was formed and PQQ was chemically bonded to the PVA main chain.

The design of experiment is shown in Table 3. The PQQ loading level from 1-10%, the reaction temperature was controlled at 0, 25 and 50 °C.

15

Table 3 Design of PQQ Conjugated PVA Synthesis

		Temp. (°C)		
Loading		0	25	50
DCC method	1% wt	√	√	√
	5% wt	√	√	√
	10% wt			
CDI method	1% wt	√	√	√
	5% wt	√	√	√
	10% wt			

* The experiment was performed in DMF solution. Three molecular weight PVAs were tested (M.W. ~ 10K, 40K, and 100K).

The synthesis and purification procedure:

1. PQQ was dissolved in N,N-dimethylformamide (DMF) solution at controlled temperature (0, 25, and 50°C).
2. Dehydrating agent (DCC or CDI) was added into the solution to form activated PQQ immediate.
3. PVA was added into the solution, and the reaction was kept for twenty hours.
4. The solution was transferred into a dialysis tube (COMW 4,000), and dialysis was done in de-ion water for 2 days. The water was changed three times a day.
5. After dialysis, the solution was concentrated under vacuum and dried in vacuum oven at 50°C.

H-NMR analysis

The proton NMR spectra of received PQQ and PQQ/PVA conjugate are shown in Figure 45 and Figure 46. Two peaks were shown at 8.58 and 7.19 ppm, which were assigned to the two aromatic protons shown in the PQQ. The d_6 -DMSO peaks appeared at 2.50 ppm, and the peak from water residue was shown at 3.31 ppm. The purity was calculated by comparing the proton integral area, which was $>95 \pm 5\%$ for the two resources. The PQQ/PVA conjugate's NMR spectrum clearly showed the formation of conjugating bond. In the low field, two peaks at 8.58 and 7.19 ppm was from PQQ and the multiple peaks from 4–1 ppm in the high field were from the PVA.

FT-IR spectra of PQQ and PVA

The ATR mode FT-IR spectra of PVA, PQQ, and PQQ/PVA conjugated are shown in Error! Reference source not found.47, 48, and 49. The characteristic peak near $3,000\text{ cm}^{-1}$ is from the hydroxyl group in PVA. In the PQQ/PVA conjugate, this peak is evidently decreased due to conjugating reaction. Furthermore, a strong peak at $1,720\text{ cm}^{-1}$ was formed as a result of new ester bonds.

XRD Spectrum of PVA Powder

The X-ray diffraction was performed on Shimadzu XRD-6000. The crystallinity degree will have a big effect on the PQQ release kinetics. The crystallinity degree is decided by many

factors (e.g. the molecular weight and the molecular weight poly-dispersity, the heat history, etc). The received PVA (MW: 10K) showed similar crystallinity degree, which is around 32–34%, with a main peak at 19-20 (2θ), as shown in **Error! Reference source not found.**50. When PQQ reacted with PVA to form PQQ/PVA conjugate, the products showed only an amorphous peak. This conjugating reaction totally changed the microscopic structure.

Quantitative Assay of PQQ by HPLC

A standard calibration curve was shown in Figure 51 which can detect the PQQ amount as low as 0.1 ng/20 µl. The limit of quality and the limit of detection are 0.2 ng/20 µl and 0.1 ng/20 µl, respectively; this means that when the PQQ amount is >0.1 ng/20 µl, PQQ can be effectively detected (Figures 52 and 53). When the PQQ amount is >0.2 ng/20 µl, the amount can be known by using the calibration curve.

Verification of PQQ Conjugated PVA System by GPC

During the dialysis, the solution was checked for the wash-out PQQ by UV lamp. After two-day dialysis, there is no detectable amount of PQQ. The PQQ conjugated PVA solution was concentrated by rotation vapor machine, and the final composite was dried in oven.

To verify the binding of PQQ to polymer, GPC analysis was performed. Figure 21 shows the PQQ GPC spectrum, using fluorescence detector. A sharp peak appeared at 14.90 min, which is the retention of pure PQQ in this operation's conditions. The structure of the peak was verified by the UV absorption spectrum as shown in Figure 22

When PVA was detected by fluorescence detector, the intensity was very weak compared to that of PQQ. This is because PVA showed almost no fluorescence.

The GPC spectrum of PQQ conjugated PVA is shown in Figure 23, in which three peaks were shown at 10.19 minutes, 13.67 minutes and a overlap peak at 14.90 minutes. From the calculation, the molecular weight was around 40K, 10K and low molecular weight molecules. Because it can be detected by fluorescence detector, all the molecules contained PQQ. Further verification was done by examining the each peak's UV absorption spectrum. Generally, they are the same with minor difference due to the new ester bond formation in the molecules (Figure 24).

Various analysis method can be used for this PQQ-conjugated PVA product. Proton NMR test's have been performed to cross-exam the binding of PQQ with PVA, except for the GPC.

PQQ-PVA conjugates can also include 20 wt % of PQQ and 80 wt % of PVA. In other words, every 30 repeating PVA units contains one PQQ molecule, and every PVA molecule contains about 6 to about 16 PQQ molecules, and preferably about 7 to about 8 PQQ molecules. See Figures 20 B and C. The PQQ/PVA conjugates have been synthesized through PQQ reaction with PVA in DMF solution using CDI as dehydrate agent. The general loading level is approximately 20 wt %. The pure products were obtained after hydrolysis and lyophilization. The purity was verified by HPLC. The conjugate products were characterized by various methods.

PQQ conjugated polymers

Additional PQQ-conjugated polymers can be synthesized as disclosed below and in Figures 54 A-JJ. These compounds offer a great variety of candidates to control the PQQ release. PEG-NH₂ is one of the candidates. Different molecular weights (from several thousand to 20K) and with one or two amines groups at the end of main chain are commercially available. The amide bond will be expected to have a longer release time compared with the ester bond in PQQ-conjugated PVA system.

PQQ-CONJUGATED POLYMERS: TETHERED & NON-TETHERED MASKING

1. Conjugation with Polymer of Neutral Charge and Least Atomic Volume
2. Conjugation with Polymer of Least/Minimum Lipophilicity
3. Conjugation with Polymer of Mid-Level Lipophilicity
4. Conjugation with Polymer of High Lipophilicity
5. Conjugation with Polymer of High Hydrophilicity
6. Conjugation with Polymer of Amphoteric Nature
7. Conjugation with Polymer of Basic Properties
8. Conjugation with Polymer of Border-line Acidic Properties
9. Conjugation with Polymer of Short Length -For Increased Bio Adsorption
10. Conjugation with Polymer of Long Backbone Length- For

Suppressed-Bio Adsorption

11. Conjugation with Short Chain Aliphatic Alcoholic Moieties (<C6)
12. Conjugation with Long Chain Aliphatic Moieties (>C6-C14)
13. Conjugation with Extra Long Chain Aliphatic Moieties (>C14)
- 5 14. Conjugation with Telechelic and Non-Telechelic Polymers
15. Conjugation with Aromatic Non-Bioactive Alcohols
16. Conjugation with Small Amines (Non-Hydrolysable Product)
17. Conjugation with Medium Sized Amines
18. Conjugation with Large Sized Amine (Long Half-life)
- 10 19. Conjugation with Swellable/Hydrogellic Polymers
20. Conjugation with Thermo-sensitive Polymer
21. Conjugation with Electroactive Polymers
22. Conjugation with Time-Resolved-Linker Mediated Polymer
23. pH Responsive Polymer Conjugation
- 15 24. Photo-Reactive Polymer Conjugation
25. Surface & Matrix Assisted Adsorbable Polymer Conjugation
26. Kinetic Polymer Conjugation
27. Conjugation with Bio-permeable Polymers
28. Cellular Uptake Polymer Conjugation
- 20 29. Thermodynamic equilibrium (to & fro) Polymer Conjugation
30. Conjugation of Homo & Hetero Polymer of Isotactic,
Syndiotactic and Atactic Nature

PQQ release kinetics *in vitro*

- 25 The PQQ release kinetics can be tested *in vitro* using human plasma or pure esterases, which offer results for the future clinical research. The established HPLC method can also be used for this study.

Example 8: PQQ in Combination with Probenecid Reduces Kidney Toxicity

30 Methods:

PQQ/Probenecid/ PQQ Analogs- rats

Dates of conduct: 1/11-1/13/2005

Dose PQQ: 25 mg/kg

Dose PQQ analogs: based upon PQQ equivalents (25 mg/kg), not by total weight.

5 Dose Probenecid: 100 mg/kg IP (5 ml/kg of 20 mg/ml solution)

Formulations:

PQQ:

10 made up in 2% NaHCO₃ immediately prior to use:

1.) 2 grams NaHCO₃ qs 100 ml DD H₂O = 2% NaHCO₃

2.) 75 mg PQQ plus 15 ml 2% NaHCO₃ = 5 mg PQQ/ml

Probenecid:

15 1.) 600 mg probenecid weighed out

2.) add to 27 ml DD H₂O

3.) 4-5 drops 19.1 N NaOH

4.) Stir

5.) pH to 7.4 with 1.0 N KH₂PO₄

20 (1.36 grams potassium phosphate monobasic qs 10 ml with DD H₂O)

(requires 0.5 to 1.2 ml)

6.) qs 30 ml with DD H₂O

fit immediately prior to use.

PQQ Analogs: 10 mg/ml solutions in saline. N.B. that analog 81 did not go into solution

25 100%

Experimental: 5 female SD/group

30 Group 1: Controls – no treatment

Group 2: 5 ml PQQ/kg IV

Group 3: 5 ml Probenecid/kg IP; 5 ml PQQ IV 30 min later, 6.0 hr later repeat
probenecid

Group 4: PVA-PQQ-80, 1.9 mg PQQ/ml, 13.2 ml/kg

Group 5: PVA-PQQ-81, 2.2 mg PQQ/ml, 11.3 ml/kg

48 hr later sacrifice, draw blood for BUN, creatinine, serum phosphorous; remove
5 kidneys for weights and histopathology.

Rat Body Weights Female

10 *Treatment Group: 1 Control (No Treatment)*

Body Weights (Grams)

	<u>ID#</u>	<u>Day 1</u>	<u>Day 2</u>	<u>Day 3</u>
	1	241	240	244
15	2	258	258	264
	3	244	237	249
	4	249	246	249
	5	264	258	256
20	Average:	251	248	252
	S. D.	10	10	8

Treatment Group: 2 25 mg PQQ/kg x 1 iv

25

Body Weights (Grams)

	<u>ID#</u>	<u>Day 1</u>	<u>Day 2</u>	<u>Day 3</u>
	6	256	241	243
	7	255	244	249
30	8	259	263	253
	9	265	246	248
	10	268	235	262
35	Average:	261	246	251
	S. D.	6	10	7

*Treatment Group: 3 100 mg Probenecid/kg x 1 IP; at 30 min., 25 mg PQQ/kg x 1
iv; at 6 hr., 100 mg Probenecid/kg x 1 IP; at 12 hr., 100 mg Probenecid/kg x 1 IP*

40

Body Weights (Grams)

<u>ID#</u>	<u>Day 1</u>	<u>Day 2</u>	<u>Day 3</u>
11	234	208	217
12	218	194	205

	13	222	203	202
	14	219	196	208
	15	232	208	218
5	Average:	<u>225</u>	<u>202</u>	<u>210</u>
	S. D.	7	7	7

10 *Treatment Group: 4 25 mg PQQ (PVA-PQQ-80)/kg x 1 iv*

Body Weights (Grams)

	<u>ID#</u>	<u>Day 1</u>	<u>Day 2</u>	<u>Day 3</u>
	16	152	145	152
15	17	162	156	162
	18	180	171	180
	19	166	156	162
	20	167	163	171
20	Average:	<u>165</u>	<u>158</u>	<u>165</u>
	S. D.	10	10	11

25 *Treatment Group: 5 25 mg PQQ (PVA-PQQ-81)/kg x 1 iv*

Body Weights (Grams)

	<u>ID#</u>	<u>Day 1</u>	<u>Day 2</u>	<u>Day 3</u>
	21	182	175	187
30	22	193	179	188
	23	170	163	167
	25	158	151	160
35	Average:	<u>176</u>	<u>167</u>	<u>176</u>
	S. D.	15	13	14

Serum Chemistry

Day: 3

Group#		1	Treatment Group 1: Control (No Treatment)		
5			Serum Proteins	Miscellaneous	
	Rodent No	Sex	Bun	Creat	Phos
10			mg/dL	mg/dL	mg/dL
	1	Female	17	0.5	5.60
	2	Female	15	0.5	6.20
	3	Female	18	0.4	6.90
	4	Female	13	0.5	7.10
	5	Female	12	0.5	6.00
15	Average		15	0.5	6.36
	S. D.:		3	0.0	0.63

Day: 3

Group#		2	Treatment Group 2: 25 mg PQQ/kg x 1 iv		
20			Serum Proteins	Miscellaneous	
	Rodent No	Sex	Bun	Creat	Phos
25			mg/dL	mg/dL	mg/dL
	6	Female	166	6.8	14.90
	7	Female	60	1.6	5.60
	8	Female	194	5.5	15.40
	9	Female	196	6.4	16.70
	10	Female	178	5.9	13.80
	Average		159	5.2	13.28
	S. D.:		57	2.1	4.42

Day: 3

Group# 5 Treatment Group 5: 25 mg PQQ (PVA-PQQ-81)/kg x 1 iv

			Serum Proteins	Miscellaneous	
	Rodent No	Sex	Bun	Creat	Phos
5			mg/dL	mg/dL	mg/dL
	21	Female	15	0.4	8.30
	22	Female	15	0.4	9.30
	23	Female	19	0.4	8.10
	25	Female	17	0.4	9.80
10		Average	17	0.4	8.88
		S. D.:	2	0.0	0.81

15 **Kidney Weights**

Day: 3

Treatment Group 1: Control (No Treatment)

Sex	Weight
	g
Female	0.9272
Female	0.9024
Female	0.9934
Female	0.9313
Female	0.9365
Average	0.9382
S. D.:	0.0335

Day: 3

Treatment Group 2: 25 mg PQQ/kg x 1 iv

Sex	Weight
	g
Female	1.1320
Female	1.3325
Female	1.2329
Female	1.3518
Female	1.0893
Average	1.2277
S. D.:	0.1170

Day: 3

5 Treatment Group 3: 100 mg Probenecid/kg x 1 IP; at 30 min., 25 mg PQQ/kg x 1 iv; at 6 hr., 100 mg Probenecid/kg x 1 IP; at 12 hr., 100 mg Probenecid/kg x 1 IP

	Sex	Weight g
10	Female	1.0022
	Female	0.9151
	Female	1.1557
	Female	1.0084
	Female	1.1350
15	Average	1.0433
	S. D.:	0.1005

Day: 3

20

Treatment Group 4: 25 mg PQQ (PVA-PQQ-80)/kg x 1 iv

	Sex	Weight g
25	Female	0.6610
	Female	0.8192
	Female	0.7262
	Female	0.7205
	Female	0.7566
30	Average	0.7367
	S. D.:	0.0577

Day: 3

35

Treatment Group 5: 25 mg PQQ (PVA-PQQ-81)/kg x 1 iv

	Sex	Weight g
40	Female	0.7509
	Female	0.6611
	Female	0.9336
	Female	0.7508
	Average	0.7741
45	S. D.:	0.1144

Tissue Weights Organ Weight to Body Weight Ratios

(Tissue Weight at Sacrifice/Last Body Weight Taken)

	Group#	1	Treatment:	Control (No Treatment)
	Sacrificed Day	3		
5	Rodent No	Sex	Body Weight	Kidney
				% of Body Weight
	1	Female	264	0.3512
	1	Female	249	0.3624
10	1	Female	249	0.3990
	1	Female	256	0.3638
	1	Female	244	0.3838
	Average:		252	0.3720
	S. D.:		8	0.0191
15				
	Group#	2	Treatment:	25 mg PQQ/kg x 1 iv
	Sacrificed Day	3		
20	Rodent No	Sex	Body Weight	Kidney
				% of Body Weight
	2	Female	243	0.4658
	2	Female	249	0.5351
	2	Female	253	0.4873
25	2	Female	248	0.5451
	2	Female	262	0.4158
	Average:		251	0.4898
	S. D.:		7	0.0529
30				
	Group#	3	Treatment:	100 mg Probenecid/kg x 1 IP; at 30 min., 25 mg PQQ/kg x 1 iv; at 6 hr., 100 mg Probenecid/kg x 1 IP; at 12 hr., 100 mg Probenecid/kg x 1 IP
35	Sacrificed Day	3		
	Rodent No	Sex	Body Weight	Kidney
				% of Body Weight
40	3	Female	202	0.4961
	3	Female	205	0.4464
	3	Female	208	0.5556
	3	Female	218	0.4626

3	Female	217	0.5230
Average:		210	0.4968
S. D.:		7	0.0443

5 Group 4 Treatment: 25 mg PQQ (PVA-PQQ-80)/kg x 1 iv

Sacrificed Day 3

	Rodent No	Sex	Body Weight	Kidney
				% of Body Weight
10	4	Female	152	0.4349
	4	Female	162	0.5057
	4	Female	180	0.4034
	4	Female	162	0.4448
	4	Female	171	0.4425
15	Average:		165	0.4462
	S. D.:		11	0.0371

Group# 5 Treatment: 25 mg PQQ (PVA-PQQ-81)/kg x 1 iv

20 Sacrificed Day 3

	Rodent No	Sex	Body Weight	Kidney
				% of Body Weight
	5	Female	160	0.4693
25	5	Female	187	0.3535
	5	Female	188	0.4966
	5	Female	167	0.4496
	Average:		176	0.4423
	S. D.:		14	0.0622

30

Dunnett Test**Parameter: BUN – Day 3****P-Value: <0.0001**

Contrast	Difference	Dunnett 95% CI	
Group 2 v Group 1	143.80000	99.38383	to 188.21617
Group 3 v Group 1	14.60000	-29.81617	to 59.01617
Group 4 v Group 1	-3.20000	-47.61617	to 41.21617
Group 5 v Group 1	1.50000	-45.61047	to 48.61047

(significant)

Parameter: Creatinine – Day 3**P-Value: <0.0001**

Contrast	Difference	Dunnett 95% CI	
Group 2 v Group 1	4.76000	3.13111	to 6.38889
Group 3 v Group 1	0.32000	-1.30889	to 1.94889
Group 4 v Group 1	-0.14000	-1.76889	to 1.48889
Group 5 v Group 1	-0.08000	-1.80770	to 1.64770

(significant)

Parameter: Phosphorous – Day 3**P-Value: 0.0008**

Contrast	Difference	Dunnett 95% CI	
Group 2 v Group 1	6.92000	3.35919	to 10.48081
Group 3 v Group 1	1.24000	-2.32081	to 4.80081
Group 4 v Group 1	2.80000	-0.76081	to 6.36081
Group 5 v Group 1	2.51500	-1.26181	to 6.29181

(significant)

Parameter: Kidney Weight- Day 3**P-Value: <0.0001**

Contrast	Difference	Dunnett 95% CI	
Group 2 v Group 1	0.28954	0.13897	to 0.44011
Group 3 v Group 1	0.10512	-0.04545	to 0.25569
Group 4 v Group 1	-0.20146	-0.35203	to -0.05089
Group 5 v Group 1	-0.16406	-0.32377	to -0.00435

(significant)

(significant)

(significant)

Parameter: Kidney Weight to Body Weight Ratio- Day 3**P-Value: 0.0022**

Contrast	Difference	Dunnett 95% CI	
Group 2 v Group 1	0.11778	0.04293	to 0.19263
Group 3 v Group 1	0.12470	0.04985	to 0.19955
Group 4 v Group 1	0.07422	-0.00063	to 0.14907
Group 5 v Group 1	0.07021	-0.00918	to 0.14960

(significant)

(significant)

Experimental Design:

- Group# 1 Control (no Treatment)
- Group# 2 25 mg PQQ/kg x 1 iv
- Group# 3 100 mg Probenecid/kg x 1 IP; at 30 min, 25 mg PQQ/kg x 1 iv; at 6 hr, 100 mg Probenecid/kg x 1 IP; at 12 hr, 100 mg Probenecid/kg x 1 IP
- Group# 4 25 mg PQQ (PVA-PQQ-80)/kg x 1 iv
- Group# 5 25 mg PQQ (PVA-PQQ-81)/kg x 1 iv

Conclusion:

Figure 27 shows that PQQ administered alone versus control was significantly different. However, PQQ (or analogs 80 and 81) in combination with probenecid was not significantly different in comparison to controls. Thus, PQQ administered in combination with probenecid is useful for reducing kidney toxicity.

Example 9: PQQ Prevents Actin Nitration and TNF-induced Barrier Dysfunction in an Endothelial Cell Monolayer

Small pulmonary arteries are the major determinants of pulmonary artery pressure and vascular resistance. Their endothelium modulates pulmonary resistance, remodeling, and blood fluidity. The effect of PQQ on pulmonary microvessel endothelial cell cultures was studied to determine the benefits of use of PQQ for treating vascular injuries and vascular injury related disorders.

Materials/Reagents:

All reagents were obtained from Sigma Chemical Company (St. Louis, MO) unless otherwise noted.

Pulmonary Microvessel Endothelial Cell Culture

Rat lung microvessel endothelial cells (RLMVEC) and Bovine lung microvessel endothelial cells (BLMVEC) were obtained at 4th passage (Vec Technologies, Rensselaer, NY). The preparations were identified by Vec Technologies as pure populations by : (i) the characteristic "cobblestone" appearance as assessed by phase contrast microscopy, (ii) the presence of Factor VIII-related antigen (indirect immunofluorescence), (iii) the uptake of acylated low-density lipoproteins and (iv) the absence of smooth muscle actin (indirect immunofluorescence). For all studies, both RLMVEC and BLMVEC were cultured from 4 to 12 passages in culture medium containing either Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Grand Island, NY) supplemented with 20% fetal bovine serum (Hyclone, Hyclone Laboratories, Logan, Utah), 15 µg/ml Endothelial Cell Growth Supplement (Upstate Biotechnology, Lake Placid, New York) and 1% non-essential amino acids (Gibco-BRL) for BLMVEC and MCDB-131 complete media containing 10% fetal bovine serum (VEC Technologies) for RLMVEC. Both cell lines were maintained in 5% CO₂ plus humidified air at 37°C. A confluent pulmonary microvessel endothelial cell monolayer (PMEM) was reached within two to three population doublings which took 3 to 4 days.

Treatments

TNF Treatment: Highly purified recombinant human TNF α from *Escherichia coli* (Calbiochem-Novabiochem, La Jolla, CA) in a stock solution of 10 μ g/ml was used. The endotoxin level was less than 0.1 ng/ μ g of TNF α as determined by standard limulus assay. We previously showed that boiling TNF α for 0.75 h blocks the effect of TNF in our system (14) which indicates no endotoxin contamination. PMEM were treated with TNF α at 100 ng/ml, since dose response studies indicate this dose consistently induces a permeability increase.

Anti- ONOO⁻ agent: The ONOO⁻ inhibitor used was Urate (5 FM) and PQQ (1 μ M). We have previously shown that Urate scavenges TNF-induced ONOO⁻ and has no effect on cell viability in endothelium (30). PQQ is a putative vitamin and superoxide anion radical scavenger. Cells were either treated with urate or PQQ alone or co-treated with urate, PQQ and TNF.

Treatment Medium: For all studies, incubation of PMEM with TNF, PQQ, urate and all corresponding controls were performed with phenol-free DMEM (pf-DMEM, Gibco BRL) supplemented with 10% FBS to avoid a potential antioxidant effect of phenol.

Assay of Endothelial Permeability

Nucleopore Track-Etch Polycarbonate Membranes (13 mm diameter, 0.8 mm pore size; Corning Costar, Cambridge, MA) were coated with gelatin (type B from bovine skin; Sigma) mounted on modified Boyden chemotaxis chambers (9 mm inner diameter; Adaps, Dedham, MA) with MF cement no. 1 (Millipore, Bedford, MA), and sterilized by ultraviolet light for 12.0-24.0 h. as previously described (8, 16). Either BLMVEC or RLMVEC (1.5×10^5 in 0.50 ml of DMEM) were plated on the gelatinized membranes and allowed to reach confluence within 3-5 days (37°C, 5% CO₂).

The experimental apparatus for the study of transendothelial transport in the absence of hydrostatic and oncotic pressure gradients has been described (16). In brief, the system consists of two compartments separated by a microporous polycarbonate membrane lined with the endothelial cell monolayer as described above. The luminal (upper) compartment (0.7 ml) was suspended in the abluminal (lower) compartment (25 ml). The lower compartment was stirred continuously for complete mixing. The entire system was kept in a water bath at a constant

temperature of 37° C. The fluid height in both compartments was the same to eliminate convective flux.

Endothelial permeability was characterized by the clearance rate of Evans Blue-labeled albumin using our adaptation (16) of the original technique described by Patterson et al (29). A buffer solution containing Hanks' Balanced Salt Solution (HBSS, Gibco-BRL) containing 0.5 % bovine serum albumin and 20 mM-(2-hydroxyethyl) piperazine-N'-2-ethanesulfonic acid (HEPES) buffer was used on both sides of the monolayer. The luminal compartment buffer was labeled with a final concentration of 0.057% Evans Blue dye in a volume of 700µl. The absorbance of free Evans Blue in the luminal and abluminal compartments was always less than 1% of the total absorbance of Evans Blue in the buffer. At the beginning of each study a luminal compartment sample was diluted 1:100 to determine the initial absorbance of that compartment. Abluminal compartment samples (300 ml) were taken every 5 min for 60 min. The absorbance of the samples was measured in a SpectraMax Plus microplate spectrophotometer (Molecular Devices, Sunnyvale, California) at 620nm. The clearance rate of Evans blue-labeled albumin was determined by least squares linear regression between 10 and 60 min for the control and experimental groups.

Immunofluorescence and Confocal Microscopy

Cell preparation and antibody treatment: Either RLMVEC or BLMVEC (1 x 10⁴/0.20 ml of culture medium) were plated on 18 mm cover slips inside a 35 mm culture dish, incubated at 37°C for 2 hr to allow attachment, and then grown to confluence in an additional 2 ml of culture medium (16). The PMEM were treated as indicated, washed with Dulbecco's Phosphate Buffered Saline (DPBS, Gibco BRL), fixed with 3.7% formaldehyde solution at room temperature (RT) for 20 min. and then permeabilized with 1% Triton X-100 in DPBS at RT for 5 min. The cells were washed with DPBS, and then blocked in 10% normal goat serum (NGS, Gibco BRL) at RT for 1 hour. PMEM were incubated with mouse monoclonal anti-nitrotyrosine antibody (clone 1A6, Upstate) at a 1:1000 dilution in 10% NGS at RT for 1 hr then washed sufficiently. The secondary antibody, Alexa Fluor 488 labeled goat anti-mouse IgG (Molecular Probes, Eugene, OR) was added at a 1:1000 dilution in 10% NGS, and incubated at RT for 1 hr and then washed sufficiently. Total β-actin was stained with mouse monoclonal anti-β-actin antibody (clone AC74), followed by Alexa Fluor 568 labeled goat anti-mouse IgG (Molecular Probes).

The quantification strategy for the fluorescent images is as follows. PMEM were visualized and quantified with confocal microscopy using the Leica Confocal System TCS SP2 (Leica Microsystems Inc., Exton, PA). There were four separate studies with four treatment groups and two treatment times per study. All fields were selected by random movement of the microscope stage to another area within an intact endothelial monolayer. Six entire fields per treatment group were analyzed with one image per field. All treatment groups were normalized for fluorescent intensity by initially adjusting the settings for noise, brightness and contrast, as determined by the slide with the maximum fluorescence (16).

Specificity of the anti-nitrotyrosine antibody was confirmed by antibody-antigen competition. A 10:1 molar ratio of nitrotyrosine antigen to nitrotyrosine antibody was pre-incubated in 10% NGS for 30 min at 37° C before application to PMEM. The cover slips were mounted on clean glass slides with Permafluor mounting media (Thermo Shandon, Pittsburgh PA). The PMEM were visualized with a Spot RT color camera (Diagnostic Instruments, Inc., Sterling Heights, MI) mounted on an Olympus IX70 inverted microscope (Olympus America, Inc., Melville, NY) equipped for phase, light, and fluorescence detection. Images for illustration were captured at 100x magnification with an exposure time of 8 sec and downloaded into Spot RT imaging software (Diagnostic Instruments, Inc) (16).

Statistics

A one way analysis of variance (ANOVA) was used to compare values among the treatments. If significance among treatments was noted, a post-hoc multiple comparison test was done with a Bonferroni (parametric-equal variance) or a Duncan (non-parametric-unequal variance) test to determine significant differences among the groups (37). A log10 transform was performed to smooth the data when appropriate. Each PMEM well and flask represents a single experiment. All data are reported as mean $\pm$ SEM. Significance was at $p < 0.05$. There are 5-10 samples per group in all studies.

Conclusion

We tested the hypothesis that tumor necrosis factor- α (TNF- α) induces a peroxynitrite (ONOO⁻) dependent increase in permeability of pulmonary microvessel endothelial monolayers (PMEM) that is associated with generation of nitrated β -actin (NO₂- β -actin). The permeability of PMEM was assessed by the clearance rate of Evans Blue labeled albumin. The cellular compartmentalization of NO₂- β -actin was displayed by showing confocal localization of

nitrotyrosine-immunofluorescence with β -actin-immunofluorescence. Incubation of PMEM with TNF (100 ng/ml) for 0.5 hr and 4.0 hr resulted in increases in permeability to albumin. There was an increase in the confocal localization of nitrotyrosine-immunofluorescence with β -actin-immunofluorescence at 0.5 hr. The TNF-induced increase in the confocal localization of nitrotyrosine-immunofluorescence with β -actin-immunofluorescence and permeability were prevented by the anti- ONOO⁻ agents urate (5 μ M) and PQQ (1 μ M). The data indicate that TNF induces an ONOO⁻- dependent barrier dysfunction which is associated with the generation of NO₂- β -actin.

Our studies further show that PQQ prevents (i) the TNF-induced increase in nitrotyrosine, (ii) co-localization of nitrotyrosine with β -actin, and (iii) the increase in permeability of pulmonary microvessel endothelial monolayers. Accordingly, PQQ prevents TNF-induced ONOO⁻ dependent, endothelial cell dysfunction. Therefore, the development of strategies using PQQ and urate provide novel directions for therapy of vascular injuries and vascular injury related disorders.

Example 10: Neuroprotection by PQQ

Pyrroloquinoline quinone (PQQ) is a free, water soluble, anionic compound that is a redox cycling planar orthoquinone which has potential free radical scavenging properties. PQQ dependent enzymes such as methyl alcohol and alcohol dehydrogenases bind PQQ as a prosthetic group and also contain cytochrome c that accepts electrons and donates them to ubiquinone which functions as an electron carrier in the mitochondrial respiratory chain.

PQQ has been demonstrated to depress N-methyl-D-aspartate (NMDA) induced electrical responses and is neuroprotective *in vitro* against NMDA-mediated neurotoxic injury. Jensen et al. (Neuroscience 62 (1994) 399-406) showed that PQQ given intraperitoneally at 30 minutes prior to hypoxia reduces infarct sizes without causing neurobehavioral side effects in an *in vivo* cerebral hypoxia/ischemia (bilateral carotid ligation in combination with hypoxia) model in 7-day-old rat pups. However, no prior studies have been performed to determine whether PQQ given *systemically* can improve neurobehavioral outcome and salvage infarcted brain resulting from a focal cerebral ischemia model in adult animals. Therefore, the effectiveness of PQQ in producing neuroprotection as assessed by neurobehavioral measures and infarct size measurement following 2 hours of reversible middle cerebral artery occlusion (rMCAo) in adult rats was evaluated. The dose response curve for PQQ on infarct volume was also characterized.

Materials and Methods

Animal Model

All animal procedures were in accordance with the Guidelines for Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee. Male Sprague-Dawley rats (300 to 350 g, Taconic, Germantown, NY) were anesthetized with isoflurane in a sealed chamber, after 50 mg/kg atropine sulfate (Sigma, St. Louis, MO) had been given intramuscularly. They were then tracheally intubated and mechanically ventilated with 2.0% isoflurane in 30% O₂/balance N₂. Blood gas analysis verified that PaCO₂ was between 30 and 45 mm Hg, and PaO₂ was above 90 mm Hg. Body temperature was monitored with a rectal probe and maintained between 37.0 °C and 37.5 °C with a heating pad. Temporalis muscle temperature was used to reflect brain temperature and was maintained between 36.0 °C and 37.0 °C with a heating lamp. One femoral artery was cannulated for pressure monitoring and blood gas sampling.

Reversible middle cerebral artery occlusion was performed as described by Longa et al. (Stroke 20 (1989) 84-91), as used previously in our laboratory. A 4-0 nylon intraluminal suture was introduced into the right internal carotid artery (ICA) via the external carotid artery (ECA). The common carotid artery and ICA were temporarily clipped and the suture placed into ECA stump and threaded into the ICA and gently advanced ~20 mm until resistance was felt. The suture was left in place for 2 hours and then withdrawn. PQQ (10 mg/kg, Sigma, St. Louis, MO) was dissolved in phosphate-buffered saline (10 mM solution) and a volume of 1 ml injected into the jugular vein to deliver a dose of 10, 3 or 1 mg/kg immediately prior to initiation of ischemia or 3 hours later. Vehicle-treated controls received an equal volume of phosphate buffered saline. The investigator was blinded as to whether an animal was treated with vehicle or PQQ injection. Body and brain temperature were maintained throughout the experiment until the animal was completely recovered from anesthesia and returned to its cage. After 72 hours animals were sacrificed and the brains examined.

Neurobehavioral Deficit Scoring

Neurobehavioral deficit scoring was based on the 18 point scale described by Garcia et al. (Stroke 26 (1995) 627-634). Neurological status was scored in each rat daily for 3 days, starting 24 hours after the ischemia. Each subject was examined in the late afternoon to avoid any effect of circadian rhythm. The investigator evaluating neurobehavioral deficits was blinded as to whether vehicle or PQQ was administered. The neurobehavioral scale consisted

of the following six tests: 1) spontaneous activity (0 to 3 points); 2) symmetry in the movement of four limbs (0 to 3 points); 3) forepaw outstretching (0 to 3 points); 4) climbing (1 to 3 points); 5) body proprioception (1 to 3 points); and 6) Response to vibrissae touch (1 to 3 points). The score given to each rat at the completion of the evaluation is the summation of all six individual test scores. The minimum neurological score is 3 and the maximum is 18.

Measurement of infarct volume

Infarct volume was assessed using 2,3,5-triphenyltetrazolium chloride (TTC) (Sigma, St. Louis, MO) staining, as used previously in our laboratory (Neuroreport 11 (2000) 2675-2679). Seventy two hrs after ischemia, rats were injected with 120 mg of pentobarbital. The brain was then removed, and cut into 2 mm sections. The slices were placed in a petri dish containing 2% TTC for 30 minutes, and periodically agitated to insure that no slices were resting on the bottom, and then put into 10% formaldehyde. Lesion volumes were calculated from summed, measured areas (SigmaScan Pro, SPSS software) of unstained tissue in mm² multiplied by 2 mm slice thickness.

Statistical Analysis

Statistical assessment of neurobehavioral score was by repeated measures ANOVA (Statistica, StatSoft Inc.). For the assessment of infarct volumes, comparisons were made between treatment groups and the corresponding vehicle groups. The nonparametric Mann-Whitney test was used for assessing the non-normally distributed volumes. Differences were considered statistically significant at $P < 0.05$.

Results

Neuroprotection by PQQ at 10 mg/kg

PQQ was first studied at a dose of 10 mg/kg based on previous report by Jensen et al. (Neuroscience 62 (1994) 399-406). Infarct volume was 319 mm³ (SD: 96.2; n=7) in vehicle - treated animals and was significantly less at 50 mm³ (SD: 39; n=8) in the animals given 10 mg/kg PQQ immediately before the onset of ischemia ($p < 0.01$; Mann-Whitney test). Infarct volume was 362 mm³ (SD: 110; n=5) in vehicle - treated animals and was also significantly less at 67 mm³ (SD: 53; n=8) in the animals given PQQ 3 hours after the onset of ischemia ($p < 0.05$; Mann-Whitney test). These data are shown in Fig. 31A and Fig. 32. Behavioral scores were also better in the PQQ - treated groups compared to the corresponding vehicle - treated controls when PQQ was given immediately before the onset of ischemia and 3 hours after the onset of ischemia, as shown in Fig. 33A and Fig. 33B.

Neuroprotection by PQQ at 3 mg/kg and 1 mg/kg

Since PQQ at 10 mg/kg given at 3 hours post initiation of ischemia appeared to be as effective as its administration simultaneously with ischemia, the effect of different doses at 3 hours after initiation of ischemia was tested, since 3 hours post initiation of ischemia provides an utilizable therapeutic window for treatment (Stroke 30 (1999) 2752-2758). When PQQ was given at 3 mg/kg at 3 hours after the onset of ischemia, infarct volume was 406 mm³ (SD: 114; n=10) in the vehicle - treated animals and was significantly less at 120 mm³ (SD: 47; n=8) in the PQQ - treated animals (p<0.01; Mann-Whitney test; Fig. 2A, Fig. 3). At this dose, behavioral scores were also better in the PQQ group compared to the vehicle groups (Fig. 33C). A dose response curve is shown in Fig. 31B.

When PQQ was given at 1 mg/kg 3 hours after ischemia, infarct volume was 361 mm³ (SD: 132; n=6) in vehicle - treated animals and there was no significant difference at 328 mm³ (SD: 112; n=6) in PQQ - treated animals (p>0.05; Mann-Whitney test; Fig. 2A). Behavioral scores were also not significantly different in the PQQ - treated animals compared to the vehicle - treated animals (Fig. 33D).

Figure 32 shows 4 representative slides from normal sham control, vehicle treated, PQQ 10 mg/kg treated and 3 mg/kg treated animals.

Discussion

The present study is the first that examines neuroprotection of PQQ assessed by both infarct volume and neurobehavioral outcome in the widely used model of focal reversible middle cerebral ischemia/reperfusion in adult rats. The data demonstrate that PQQ is effective in producing behavioral and infarct volume neuroprotection when given either prior to ischemia or 1 hour after reperfusion; and the neuroprotection provided by PQQ is dose related.

Several properties of PQQ could be involved in the neuroprotection. First, PQQ may suppress peroxynitrite formation. The neurotoxicity of nitric oxide in ischemic stroke has been suggested to depend upon its conversion to peroxynitrite. As a free radical scavenger and a cofactor for quinoprotein enzymes, PQQ may suppress peroxynitrite formation. Secondly, PQQ may oxidize the NMDA receptor redox site. Pathological activation of NMDA receptors has been implicated in various CNS disorders including ischemia. Third, PQQ may function as an effective antioxidant in protecting mitochondrial lipid and protein, and has been shown to protect mitochondrial functions from oxidative damage.

In summary, we have found that PQQ reduces infarct size and improves behavioral scores when given as a single dose 3 hours after initiation of 2 hours of rMCAo. Under these conditions PQQ is effective at 3 mg/kg and 10 mg/kg but not at 1 mg/kg. Thus, PQQ, which acts as an essential nutrient, antioxidant and redox modulator in a variety of systems, produces an effective neuroprotection and represents a new class of agents with potential use in the therapy of adult stroke.

Example 11: Pharmacokinetics of PQQ in Rats

A determination of PQQ concentration in rat plasma over time was undertaken to assess the reaction to PQQ, with and without probenecid. Group A (Rats 1-3) was administered 20 mg PQQ/kg, i.v. Group B (Rats 4-6) was administered 100 mg probenecid/kg, i.p., followed by 20 mg PQQ/kg, i.v., 30 minutes later. Blood from the rats in both Groups A and B was collected at 0, 5, and 30 minutes after dosage, and 1, 2, 4, and 6 hours after dosage.

Sample Preparations:

Rat Blood: 100 µl rat blood + 60 µl heparinized saline, centrifuged; 60 µl plasma was quantitatively pipetted to test tube and frozen at -80° C until analysis.

Calibration Samples: Rat plasma was diluted with saline (80:60, v/v), with which a set of calibration curve sample was prepared by spiking PQQ standard ranging from 31.25 to 2500 ng/ml rat plasma. See Figure 34.

Results:

Results of the PQQ concentration in rat plasma for each of the rats in Groups A and B appears in Figure 35 and in Table 4 below. The rat plasma samples were prepared with two-step extraction and diluted 2-100 times prior to HPLC assay.

Table 4.

Rat plasma PQQ concentration ($\mu\text{g/ml}$)					
Time	Rat-1	Rat-2	Rat-3	Mean	SD
5 min	27.50	37.66	25.46	30.21	6.53
30 min	10.33	19.98	18.50	16.27	5.20
1 h	10.65	11.47	14.92	12.35	2.26
2 h	6.34	6.46	9.89	7.56	2.01
4 h	2.73	4.74	6.39	4.62	1.83
6 h	2.73	3.14	3.55	3.14	0.41
Time	Rat-4	Rat-5	Rat-6	Mean	SD
5 min	42.89	33.60	39.68	38.72	4.72
30 min	18.30	19.02	18.64	18.65	0.36
1 h	14.01	10.22	12.69	12.31	1.92
2 h	7.18	5.05	6.87	6.37	1.15
4 h	4.24	1.67	2.55	2.82	1.31
6 h	2.57	0.94	1.14	1.55	0.89

Figure 36 illustrates a comparison of the plasma PQQ concentration time curve of the mean values for each time point in Groups A and B.

Example 12. Use of PQQ and Probenecid for prevention/reduction of oxidative stress *in vivo*.

Male Sprague-Dawley rats were randomly treated with pyrroloquinoline quinone (PQQ), probenecid or both either before ischemia or ischemia-reperfusion. PQQ (1-3 mg/kg) and/or probenecid (100 mg/kg) were given 30 min before left anterior descending coronary artery (LAD) occlusion by intraperitoneal injection (pretreatment) or at the onset of reperfusion by intravenous injection (treatment). Rats were subjected to 15 or 30 min of LAD occlusion and 30 minutes, 1 hour or 2 hours of reperfusion with left ventricle (LV) hemodynamic monitoring. PQQ combined with probenecid decreased infarct size in these rat models. PQQ combined with probenecid protected against ischemia-induced cardiac dysfunction with higher

LV systolic pressure, LV developed pressure, LV (+)dP/dt and lower LV (-)dP/dt after 30 minutes to 2 hours of reperfusion. Creatine kinase (CK) production was reduced by PQQ combined with probenecid. Thus, PQQ combined with probenecid is highly effective in reducing myocardial infarct size and improving cardiac function in a dose-related manner in rat models of ischemia and ischemia-reperfusion.

Statistical analysis.

All results are presented as mean $\pm$ SEM. The two treatment groups (pretreatment and treatment) were compared with the normal control group using one-way analysis of variance (ANOVA) with the regression equation for multiple group comparisons. Differences in mortality during the occlusion and reperfusion period among the three groups were assessed by the Chi-square test. The percentages of rats with VF were assessed by the Fisher Exact test. All computations were done using the general linear model procedure in Minitab, version 7.2 (Minitab Statistical Software) or Primer of Biostatistics: The program, version 3.03 (McGraw-Hill). Statistical significance was set at $p < 0.05$.

Models of ischemia and ischemia- reperfusion.

PQQ was dissolved in vehicle (2% NaHCO_3). The volume given either intraperitoneally (i.p.) or intravenously (i.v.) was one ml. All controls were treated with one ml of vehicle. PQQ at 1-3 mg/kg was given i.p. 30 minutes before either 15 or 30 min of ischemia followed by 30 minutes, 1 hour or 2 hours of reperfusion.

600 mg of probenecid was dissolved in 27 ml of dd H_2O . 4-5 drops of 19.1 N NaOH were added, and the pH was adjusted to 7.4 with 1.0 N KH_2PO_4 . Probenecid was given at 100 mg/kg 30 min before either 15 or 30 min of ischemia followed by 30 minutes, 1 hour or 2 hours of reperfusion.

After induction of anesthesia (ketamine 80 mg/kg, xylazine 4 mg/kg body weight intraperitoneally), a tracheostomy was performed and the animal was ventilated on a Harvard Rodent Respirator (Model 683, Harvard Apparatus). Infarct size measurement rats were subjected to 2 hours of proximal left anterior descending (LAD) coronary artery ligation without reperfusion. The ischemia followed by reflow model employed ischemia-reperfusion as previously described (Sievers RE, *et al.* Magn Reson Med 1989; 10:172-81). In this model, a reversible coronary artery snare occluder was placed around the proximal LAD coronary artery through a midline sternotomy. Rats were then subjected to 15 or 30 minutes of LAD occlusion and 30, 60 or 120 minutes of reflow. In addition, these rats had hemodynamic measurements recorded. A 4F Millar catheter was inserted through the right carotid artery into

the left ventricle (LV). After 20 min of equilibration, heart rate (HR), systolic pressure (LVSP), end diastolic pressure (LVEDP), LV (+)dP/dt max, and LV (-)dP/dt max were monitored using a MacLab/4S (Milford, MA). LV developed pressure (LVDP) was calculated by subtracting LVEDP from LVSP.

- 5 There were no significant differences in heart rate, LVSP, LVEDP, LV (+)dP/dt, and LV (-)dP/dt among control, pretreatment and treatment groups at baseline. Whether given as pretreatment or treatment, PQQ combined with probenecid protected against ischemia-induced cardiac dysfunction with higher LVSP, LV (+)dP/dt and lower LV (-) dP/dt after 30 minutes, 1 hour and 2 hours of reperfusion, as shown in Tables 5-9. (* P<0.05 vs. prior published I/R
- 10 (control) data by ANOVA with Student-Newman-Keuls test. Zhu et al., Journal of Cardiovascular Pharmacology and Therapeutics; 11(2):119-128 (2006)) and Figures 37-41.

Table 5. LV Systolic Pressure (mmHg)

Groups	Baseline	Occlude 15 min	Occlude 30 min	Reflow 30 min	Reflow 1 h	Reflow 2 h
Control (I/R) (probenecid 100 mg/kg ip) (n=2)	109	102	99	97	97	93
Prior Data Control(I/R) (n=9) No Probenecid	113±6	107±6	100±6	99±5	99±7	90±5
Prior Data PQQ 3 mg/kg (n=12) No Probenecid	91±3	89±3	84±3	95±4	89±5	91±7
Probenecid+PQQ 3 mg/kg (n=5)	108±4	102±2	107±4	117±5	117±5	118±5*
Probenecid+PQQ 2 mg/kg (n=5)	109±5	98±10	102±8	111±5	116±6	112±6*
Probenecid+PQQ 1.5 mg/kg (n=4)	100±4	91±5	89±6	105±3	107±4	109±2*
Probenecid+PQQ 1 mg/kg (n=2)	113	105	100	113	108	108
Probenecid 100 mg/kg iv (n=2)	93	107	102	112	114	116

Table 6. LV End-Diastolic Pressure (mmHg)

Groups	Baseline	Occlude 15 min	Occlude 30 min	Reflow 30 min	Reflow 1 h	Reflow 2 h
Control (I/R) (probenecid 100 mg/kg) (n=2)	9	7	6	4	-3	-3
Prior Data Control(I/R) (n=9) No Probenecid	3±1	9±2	11±2	13±4	11±2	12±2
Prior Data PQQ 3 mg/kg (n=12) No Probenecid	1.3±0.7	4.7±2	4.4±2	3.4±1	1.3±1*	1.9±1*
Probenecid+PQQ 3 mg/kg (n=5)	8±2	14±4	16±4	14±4	12±2	13±2
Probenecid+PQQ 2 mg/kg (n=5)	5±2	12±3	12±3	10±2	11±1	7±2
Probenecid+PQQ 1.5 mg/kg (n=4)	0±0.8	6±4	3±1	0.5±1	0.6±1	0.5±2*
Probenecid+PQQ 1 mg/kg (n=2)	3	7	8	4	4	2
Probenecid 100 mg/kg iv (n=2)	-3	3	3	1.5	1	1

Table 7. LV Developed Pressure (mmHg)

Groups	Baseline	Occlude 15 min	Occlude 30 min	Reflow 30 min	Reflow 1 h	Reflow 2 h
Control (I/R) (probenecid 100 mg/kg) (n=2)	100	95	93	97	100	94
Prior Data Control(I/R) (n=9) No Probenecid	110±6	98±5	95±4	86±5	88±6	78±6
Prior Data PQQ 3 mg/kg (n=12) No Probenecid	90±3	84±3	80±3	92±4	88±4	89±6
Probenecid+PQQ 3 mg/kg (n=5)	100±4	88±5	90±6	100±5	105±6	105±6*
Probenecid+PQQ	104±5	85±8	89±4	101±3	105±5	105±5*

2 mg/kg (n=5)						
Probenecid+PQQ 1.5 mg/kg (n=4)	100±4	85±7	87±5	105±3	106±3	109±2*
Probenecid+PQQ 1 mg/kg (n=2)	110	98	92	109	104	107
Probenecid 100 mg/kg iv (n=2)	96	104	99	111	114	116

Table 8. LV (+) dP/dt (mmHg/sec)

Groups	Baseline	Occlude 15 min	Occlude 30 min	Reflow 30 min	Reflow 1 h	Reflow 2 h
Control (I/R) (probenecid 100 mg/kg) (n=2)	4300	4400	4500	4800	5250	4950
Prior Data Control(I/R) (n=9) No Probenecid	5289±370	4678±578	4856±397	4133±458	4378±431	3975±443
Prior Data PQQ 3 mg/kg (n=12) No Probenecid	4850±682	4975±633	4300±129	5275±293	4600±392	5225±272*
Probenecid+PQQ 3mg/kg (n=5)	5460±346	4720±314	4720±473	5560±421	5800±465	5640±385*
Probenecid+PQQ 2 mg/kg (n=5)	5620±395	4500±590	4720±557	5480±256	5440±279	5320±314*
Probenecid+PQQ 1.5 mg/kg (n=4)	5050±337	4750±412	4450±330	5800±294	5725±214	5900±123*
Probenecid+PQQ 1 mg/kg (n=2)	5300	5000	4800	5100	5700	5300
Probenecid 100 mg/kg iv (n=2)	4650	5750	5200	5900	6150	6300

5

Table 9. LV (-) dP/dt (mmHg/sec)

Groups	Baseline	Occlude 15 min	Occlude 30 min	Reflow 30 min	Reflow 1 h	Reflow 2 h
---------------	-----------------	---------------------------	---------------------------	--------------------------	-----------------------	-----------------------

Control (I/R) (probenecid 100 mg/kg) (n=2)	3450	3500	3550	3950	4400	4200
Prior Data Control(I/R) (n=9) No Probenecid	3867±304	3589±432	3600±301	3400±340	3600±403	3050±401
Prior Data PQQ 3 mg/kg (n=12) No Probenecid	3425±392	3600±502	3150±96	4150±150	3650±341	4350±240*
Probenecid+PQQ 3mg/kg (n=5)	3740±331	3440±254	3600±212	4180±229	4440±232	4440±308*
Probenecid+PQQ 2 mg/kg (n=5)	4000±341	3520±445	3520±344	4200±158	4280±314	4300±286*
Probenecid+PQQ 1.5 mg/kg (n=4)	3725±246	3400±392	3250±352	4375±266	4450±287	4625±214*
Probenecid+PQQ 1 mg/kg (n=2)	4050	4000	3950	4400	4000	4250
Probenecid 100 mg/kg iv (n=2)	3500	4200	4050	4550	4750	4800

Infarct size.

Using the ischemia/reperfusion rat model, rats were subjected to 17 or 30 minutes of left anterior descending coronary artery ligation and 2 hours of reperfusion and infarct size was measured as described previously (Sievers RE, *et al.* Magn Reson Med 1989; 10:172-81, Zhu B-Q, *et al.* J Am Coll Cardiol 1997; 30:1878-85). Hearts were excised at the end of the 2 hour ischemic period. The sections were then incubated in a 1% solution of triphenyltetrazolium chloride (TTC) for 10 to 15 min until viable myocardium was stained brick red.

In *model 2*, after 2 hours of reperfusion, the LAD was reoccluded, and phthalocyanin dye (Engelhard Cooperation, Louisville, KY) was injected into the LV cavity, allowing normally perfused myocardium to stain blue. The heart was then excised, rinsed of excess dye and sliced transversely from apex to base into 2-mm-thick sections. The sections were incubated in TTC as described above. Infarcted myocardium fails to stain with TTC. The tissue sections were then fixed in a 10% formalin solution and weighed. Color digital images of both sides of each transverse slice were obtained using a videocamera (Leica DC 300 F) connected to a microscope (Stereo Zoom 6 Photo, Leica). The regions showing blue-stained

(nonischemic), red-stained (ischemic but noninfarcted) and unstained (infarcted) tissue were outlined on each color image and measured using NIH Image 1.59 (National Institutes of Health, Bethesda, MD) in a blinded fashion. On each side, the fraction of the LV area representing infarct-related tissue (average of two images) was multiplied by the weight of that section to determine the absolute weight of infarct-related tissue. The infarct size for each heart was expressed as:

$$\text{Infarct size/LV mass (\%)} = \frac{\sum \text{Infarct weight in each slice}}{\text{Total LV weight}} \times 100\%,$$

$$\text{Risk area/LV mass (\%)} = \frac{\text{Total weight of non-blue-stained section}}{\text{Total LV weight}} \times 100\%,$$

Infarct size as a percentage of risk area was then calculated as

$$\frac{\sum \text{Infarct weight in each slice}}{\sum \text{Risk area weight of each slice}} \times 100\%$$

In the ischemic model (*model 1*), infarct size (infarct mass/LV mass, without phthalocyanin blue dye injected) after PQQ was smaller than Control (Figure 13). In the first set of experiments in model 2, ischemia was for 17 min followed by 2 hours of reperfusion, infarct size (infarct mass/risk area, infarct mass/LV mass) was reduced by pretreatment with PQQ 20 mg/kg (Figure 14). In the second set of model 2 experiments, ischemia was for 30 min followed by 2 hours of reperfusion. Infarct size after either Pretreatment or Treatment with PQQ 15 mg/kg was smaller than Control (Figure 15).

Figures 42 and 43 show that the combination of PQQ and probenecid decreased infarct size both as a percent of risk area and of left ventricle mass. Figure 44 shows that the combination of PQQ and probenecid lessened the increase of creatine kinase. Thus, the combination of PQQ and creatine kinase are effective in prevention and reduction of oxidative stress *in vivo* especially related to cardiac pathologies. Tables 10-15 below more fully illustrate the toxicity of the administration of PQQ.

Table 10. 72 hr serum chemistry

Treatment: Controls

Rat # BUN Creatinine

5	1	15	0.4
	2	13	0.5
	3	13	0.5
	4	18	0.5
	5	13	0.4
10	Treatment: 25 mg PQQ/kg		
	Rat #	BUN	Creatinine
	16	306	7.8
	17	278	7.3
15	18	132	4.7
	19	263	9.4
	20	210	6.5

Treatment: 200 mg Prob./kg, 1 min later 25 mg PQQ/kg; 1 hr later 100 mg Prob./kg

Rat # BUN Creatinine

20	31	17	0.4
	32	14	0.4
	33	19	0.4
	34	17	0.5
	35	19	0.4

25

Table 11

72 hr BUN Creatinine

Group #1 Controls

30	1.)	17	0.4
	2.)	13	0.4
	3.)	16	0.5
	4.)	17	0.5
	5.)	18	0.4

35

Group #2 25 mg PQQ/kg

40	6.)	262	7.3
	7.)	176	6.5
	8.)	278	8.3
	9.)	died	
	10.)	243	7.2

Group #3 100 mg Probenecid/kg; 25 mg PQQ/kg

45	11.)	27	0.8
	12.)	450	7.8
	13.)	23	0.6
	14.)	97	2.2
	15.)	31	0.8

Group #4 200 mg Probenecid/kg; 25 mg PQQ/kg

5	16.)	18	0.4
	17.)	25	0.6
	18.)	29	0.8
	19.)	24	0.5
	20.)	20	0.4

Table 12

	BUN 72 hr	BUN Day 8	BUN Day 15	Day 8 weight as % Day 0 Weight	Day 15 weight as % Day 0 Weight	
10	Untreated controls					
	1.)	12	12	18	110	223
	2.)	16	10	17	113	225
15	3.)	14	10	17	111	190
	4.)	12	12	20	112	206
	5.)	14	12	18	111	184
	Probenecid controls (100 mg/kg, 2 min, 1 hr, 2 hr)					
	6.)	14	8	12	114	196
20	7.)	14	12	ND	110	183
	8.)	14	12	19	115	215
	9.)	10	10	20	114	180
	10.)	10	8	17	111	205
	PQQ (25 mg/kg)					
25	11.)	192	Died			
	12.)		Died			
	13.)	246	Died			
	14.)	162	108	43	85	177
	15.)	134	20	19	96	208
30	PQQ (25 mg/kg) + probenecid (100 mg/kg, 2 min prior to PQQ treatment)					
	16.)	24	10	19	112	214
	17.)	26	12	16	108	214
	18.)	22	10	18	111	215
	19.)	38	16	24	111	210
35	20.)	200	Died			
	PQQ (25 mg/kg) + probenecid (100 mg/kg, 2 min prior to PQQ treatment, 1 hr, 2hr).					
	21.)	26	10	14	107	190
	22.)	24	12	17	109	200
	23.)	26	12	19	113	212
40	24.)	26	12	20	110	196
	25.)	26	10	21	112	186
	PQQ (25 mg/kg) + probenecid (100 mg/kg IP, 30 min prior to PQQ treatment).					
	26.)	130	18	23	101	200
	27.)	26	10	19	106	204
45	28.)	26	16	20	104	196
	29.)	28	14	19	113	215
	30.)	34	10	16	107	192
	PQQ (15 mg/kg)					
	31.)	36	14	17	102	182

	32.)	40	14	19	101	203
	33.)	44	14	21	102	211
	34.)	52	20	23	108	186
	35.)	38	14	21	106	202
5	PQQ (15 mg/kg) + probenecid (100 mg/kg, 2 min prior to PQQ treatment)					
	36.)	22	12	16	107	202
	37.)	24	10	13	106	188
	38.)	24	16	14	107	198
	39.)	26	12	15	106	181
10	40.)	26	14	13	111	200

Table 13

		<u>BUN</u>				<u>CREAT.</u>			
		24 hr	48 hr	day 8	day 14	24 hr	48 hr	day 8	day 14
15	<u>RAT #9/1/06</u>	9/3/06	9/7/06	9/14/06		9/1/06	9/3/06	9/7/06	9/14/06
	Saline controls								
	1	14.0	14.0	14.0	16.0	0.2	0.4	0.2	0.4
	2	12.0	12.0	12.0	15.0	0.2	0.4	0.2	0.5
20	3	14.0	14.0	16.0	18.0	0.2	0.4	0.4	0.5
	4	18.0	16.0	16.0	19.0	0.4	0.4	0.4	0.4
	5	18.0	14.0	14.0	18.0	0.2	0.4	0.4	0.4
	100 mg Probenecid/kg								
	6	12.0	14.0	10.0	16.0	0.2	0.4	0.4	0.4
25	7	16.0	16.0	16.0	18.0	0.2	0.4	0.4	0.4
	8	14.0	12.0	12.0	17.0	0.2	0.4	0.4	0.4
	9	12.0	14.0	16.0	16.0	0.2	0.4	0.4	0.4
	10	16.0	14.0	12.0	17.0	0.2	0.4	0.4	0.4
	10 mg PQQ/kg								
30	11	10.0	12.0	10.0	14.0	0.2	0.4	0.2	0.4
	12	12.0	14.0	14.0	16.0	0.4	0.4	0.4	0.4
	13	19.0	14.0	16.0	18.0	0.2	0.4	0.4	0.4
	14	16.0	12.0	10.0	17.0	0.4	0.4	0.4	0.4
	15	12.0	12.0	10.0	18.0	0.2	0.4	0.2	0.4
35	20 mg PQQ/kg								
	16	26.0	46.0	16.0	19.0	1.0	1.2	0.4	0.4
	17	30.0	44.0	18.0	19.0	1.0	1.0	0.4	0.5
	18	26.0	56.0	18.0	20.0	1.0	1.4	0.4	0.5
	19	46.0	58.0	20.0	20.0	1.4	1.4	0.4	0.5
40	20	26.0	90.0	20.0	16.0	1.0	2.0	0.4	0.4
	40 mg PQQ/kg								
	21	224.0				4.8			
	22	174.0				3.2			
	23	128.0	196.0	52.0	37.0		5.4	1.0	0.7
45	24						5.0		
	25	124.0	228.0	188.0	34.0	2.8	5.2	2.4	0.7
	100 mg Probenecid/kg, 10 mg PQQ/kg								
	26	20.0	14.0	14.0	16.0	0.4	0.4	0.4	0.4
	27	16.0	14.0	12.0	13.0	0.4	0.4	0.4	0.4
50	28	14.0	14.0	14.0	15.0	0.4	0.4	0.4	0.4

	29	22.0	16.0	14.0	14.0	0.6	0.4	0.4	0.4
	30	14.0	12.0	14.0	16.0	0.4	0.4	0.4	0.4
	100 mg Probenecid/kg, 20 mg PQQ/kg								
5	31	28.0	28.0	18.0	18.0	0.8	0.6	0.6	0.4
	32	24.0	20.0	14.0	17.0	0.8	0.6	0.4	0.5
	33	26.0	54.0	14.0	15.0	1.0	1.2	0.4	0.4
	34	32.0	30.0	18.0	16.0	1.0	0.6	0.4	0.4
	35	28.0	30.0	20.0	17.0	0.8	0.6	0.4	0.4
	100 mg Probenecid/kg, 40 mg PQQ/kg								
10	36	238.0				4.8			
	37								
	38	182.0	460.0			4.0	12.0		
	39	244.0							
15	40	118.0	230.0			2.8	5.6		

Table 14. PathologySaline Controls

1-5 Normal Histological Structure

100 mg probenecid/kg

6-10 Normal Histological Structure

10 mg PQQ/kg

11-15 Normal Histological Structure

20 mg PQQ/kg

16-20 Slightly dilated tubules

40 mg/PQQ/kg

21 Very severe cortical tubular epithelium lesions

Total tubular epithelial necrosis (proximal and distal tubular epithelium)

Glomeruli and collecting ducts spared

22 as #21

23 Mild lesion, 5% necrotic tubules

24 as #21

25 as #21

100 mg probenecid/kg, 10 mg PQQ/kg

26-30 Normal histological; structure

100 mg probenecid/kg, 20 mg PQQ/kg

31-35 Slightly dilated tubules, as 16-20

100 mg probenecid/kg, 40 mg PQQ/kg

36-40 Essentially as #21

The toxicology studies done with administration of PQQ with and without Probenecid in the preliminary dog studies are shown in Table 15 below.

Table 15

	<u>Day 1</u>	<u>Day2</u>	<u>Day3</u>
BUN	13	36	122
Creatinine	0.9	2.2	8.8
5 Phosphorus	6.0	5.3	13.6
AST	54	104	378
ALT	47	41	136
Amylase	479	628	1196

10 Dog 1475: 5 mm Probenecid infusion (200 mg/kg); 5 mm PQQ infusion (7.5 mg/kg); one hour later 5 mm Probenecid infusion (100 mg/kg).

	<u>Day1</u>	<u>Day2</u>	<u>Day3</u>	<u>Day4</u>	<u>Day7</u>
BUN	28	25	22	22	18
15 Creatinine	1.2	1.2	1.3	1.2	0.9
Phosphorus	5.5	5.3	5.3	4.5	5.4
AST	48	52	51	42	56
ALT	35	44	46	39	53
20 Amylase	435	414	422	433	507

25 Dog 1357: No infusional toxicities; demonstrated severe vomiting days 2 and 3; sacrificed day 3 as dose appeared to be well above the MTD; clinical impression that it would not last another day.

30 Dog 1475: Vomiting and vomituration for 1 hr after treatment (infusional toxicities); appeared completely normal thereafter.

The results of the treatment with Probenecid in Rats for Ischemia/Reperfusion are shown below. Note that the first injection is Probenecid and PQQ or NS after 30 min of ischemia. The second injection is Probenecid after 1 hour of reperfusion. The P value is from a two-sample t test, when PQQ groups vs. Control group.

35 **Table 16: Results of Treatment With Probenecid (200mg/kg)+PQQ (1mg/kg)+Probenecid (100mg/kg) in a Rat Model of Ischemia/Reperfusion**

Groups	Rats code	Infarct/Risk (%)	Infarct/LV (%)	Risk/LV (%)
Control	PQP3	48.0	34.8	72.4
	PQP4	44.9	33.7	75.0
	Prob1	49.3	36.4	73.9
	Prob2	47.6	31.6	66.3
	Prob3	55.4	44.2	79.8
	Prob4	53.6	37.5	69.9
	PQP5	45.0	37.5	67.9
	PQP12	53.3	37.8	71.0
	PQP13	34.1	22.4	65.6

	PQP14	54.6	41.8	76.5
	M±SE	48.6±2.0	35.8±1.9	71.8±1.5
PQQ1mg/kg	PQP1	30.2	23.0	76.3
	PQP6	33.6	25.7	76.6
	PQP7	29.5	22.1	74.9
	PQP8	29.6	20.9	70.8
	PQP9	39.9	28.2	70.6
	PQP10	26.9	18.9	70.3
	PQP11	39.8	24.9	62.6
	PQP15	29.5	21.4	72.6
	PQP16	24.9	15.6	62.6
	PQP17	39.7	28.8	72.4
	M±SE	32.4±1.8	23.0±1.3	71.0±1.6
	P value	0.0001	0.0001	0.69
PQQ1.5mg/kg	PQP2	32.2	23.1	71.6
	PQP18	35.2	27.5	78.1
	PQP19	43.7	33.3	76.2
	PQP20	36.7	25.3	68.9
	PQP21	31.1	21.7	69.6
	PQP22	41.5	27.3	65.9
	PQP23	24.1	15.9	66.1
	M±SE	34.9±2.5	24.9±2.1	70.9±1.8
	P value	0.0011	0.0018	0.70

EQUIVALENTS

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, numerous equivalents to the specific procedures described herein. Such equivalents are considered to be within the scope of the present invention and are covered
5 by the following claims. Various substitutions, alterations, and modifications may be made to the invention without departing from the spirit and scope of the invention as defined by the claims. Other aspects, advantages, and modifications are within the scope of the invention. The contents of all references, issued patents, and published patent applications cited throughout this application are hereby incorporated by reference.

CLAIMS

What is claimed is:

1. A method for preventing, treating, or reducing an ischemia-reperfusion injury, the method comprising administering to a subject in need thereof a therapeutically effective amount of pyrroloquinoline quinone thereby protecting the organs and tissues of said subject from reperfusion injury caused by the ischemic insult.
2. The method of claim 1, wherein said ischemia-reperfusion injury is cardiac injury.
3. The method of claim 1, wherein said pyrroloquinoline quinone is administered in an amount between 1 mg/kg and 10 mg/kg of the subject's body weight.
4. The method of claim 3, wherein the pyrroloquinoline quinone is administered in an amount of about 3 mg/kg of the subject's body weight.
5. The method of claim 1, wherein said pyrroloquinoline quinone is administered to the subject prior to the ischemic insult.
6. The method of claim 1, wherein said pyrroloquinoline quinone is administered to the subject after the ischemic insult.
7. The method of claim 1, wherein said pyrroloquinoline quinone is administered to the subject prior to, at, or after the onset of reperfusion.
8. The method of claim 1, wherein said ischemia-reperfusion injury is stroke.
9. The method of claim 8, wherein said stroke is the result of a heart attack.
10. The method of claim 8, wherein the method further comprises administering to the subject in need thereof a therapeutically effective amount of tamoxifen.
11. The method of claim 7, wherein said pyrroloquinoline quinone is administered in an amount between 1 mg/kg and 10 mg/kg of the subject's body weight.
12. The method of claim 9, wherein said pyrroloquinoline quinone is administered to the subject prior to the ischemic insult.
13. The method of claim 9, wherein said pyrroloquinoline quinone is administered to the subject after the ischemic insult.
14. The method of claim 12, wherein said pyrroloquinoline quinone is administered to the subject prior to, at, or after the onset of reperfusion.

15. The method of claim 1, wherein said ischemia-reperfusion injury is renal injury.
16. The method of claim 15, wherein said renal injury is the result of intestinal ischemia or a gastrointestinal tract disorder.
17. The method of claim 15, wherein said pyrroloquinoline quinone is administered in an amount between 1 mg/kg and 10 mg/kg of the subject's body weight.
18. The method of claim 15, wherein said pyrroloquinoline quinone is administered to the subject prior to the ischemic insult.
19. The method of claim 15, wherein said pyrroloquinoline quinone is administered to the subject after the ischemic insult.
20. The method of claim 15, wherein said pyrroloquinoline quinone is administered to the subject prior to, at, or after the onset of reperfusion.
21. A pharmaceutical composition for treating ischemia-reperfusion injury in a subject in need thereof, comprising a therapeutically effective amount of pyrroloquinoline quinone conjugated to one or more polymers.
22. The pharmaceutical composition of claim 21, wherein said polymer is polyvinyl alcohol.
23. The pharmaceutical composition of claim 21, wherein said polymer is PEG-NH₂.
24. A pharmaceutical composition for treating ischemia-reperfusion injury in a subject in need thereof, comprising a therapeutically effective amount of pyrroloquinoline quinone and a nephroprotectant.
25. The pharmaceutical composition of claim 24, wherein said nephroprotectant is probenecid.
26. The pharmaceutical composition of claim 24, wherein the therapeutically effective dose of pyrroloquinoline quinone is between 1 mg/kg and 10 mg/kg, and wherein the therapeutically effective dose of probenecid is between 100 mg/kg and 200 mg/kg.
27. The pharmaceutical composition of claim 24, wherein said nephroprotectant is cilastatin.

28. The pharmaceutical composition of claim 24, wherein the pyrroloquinoline quinone is conjugated to one or more polymers.
29. The method of claim 1, wherein said ischemia-reperfusion injury is stroke damage.
30. The method of claim 1, wherein said ischemia-reperfusion injury occurs in the limbs.
31. The method of claim 30, wherein the limb injury is the result of diabetes, or diabetes related disorders.
32. The method of claim 1, wherein said ischemia-reperfusion injury occurs in the lungs.
33. A pharmaceutical composition for treating ischemia-reperfusion injury in a subject in need thereof, comprising a therapeutically effective dose of pyrroloquinoline quinone and metoprolol.
34. The pharmaceutical composition of claim 33, wherein the therapeutically effective dose of pyrroloquinoline quinone is between 1 mg/kg and 10 mg/kg.
35. The pharmaceutical composition of claim 34, wherein the therapeutically effective dose of pyrroloquinoline quinone is 3 mg/kg.
36. The pharmaceutical composition of claim 35, wherein the therapeutically effective dose of metoprolol is 1 mg/kg.
37. The pharmaceutical composition of claim 33, wherein said ischemia-reperfusion injury is myocardial infarction.
38. A kit for treating or preventing an injury resulting from an ischemic insult, comprising in one or more containers, a therapeutically effective dose of pyrroloquinoline quinone, a pharmaceutically acceptable carrier, instructions for use of said kit, and optionally, one or more compounds selected from the group consisting of: metoprolol, probenecid, and cilastatin.
39. The kit of claim 38, wherein said pyrroloquinoline quinone is conjugated to one or more polymers.

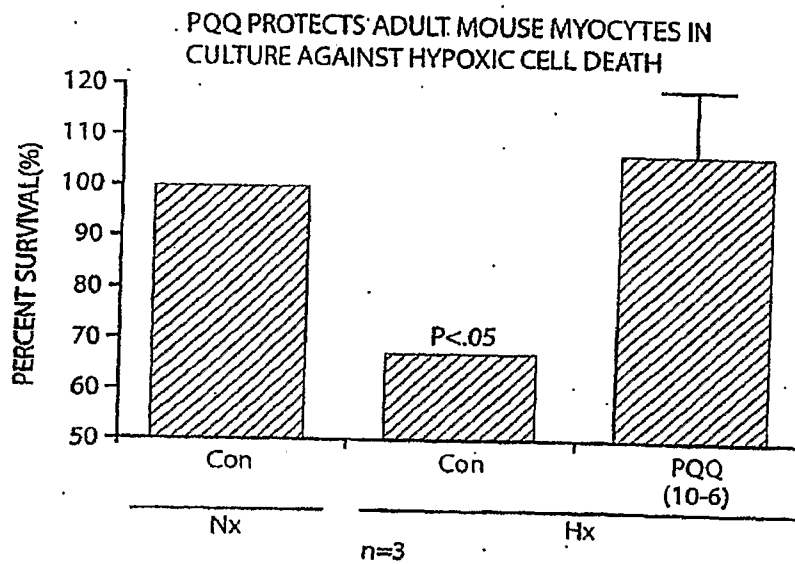


Fig. 1

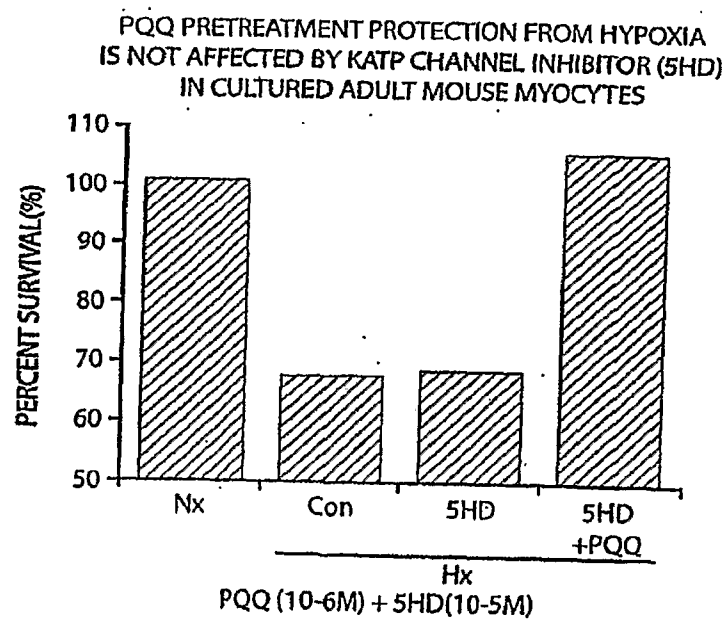


Fig. 2

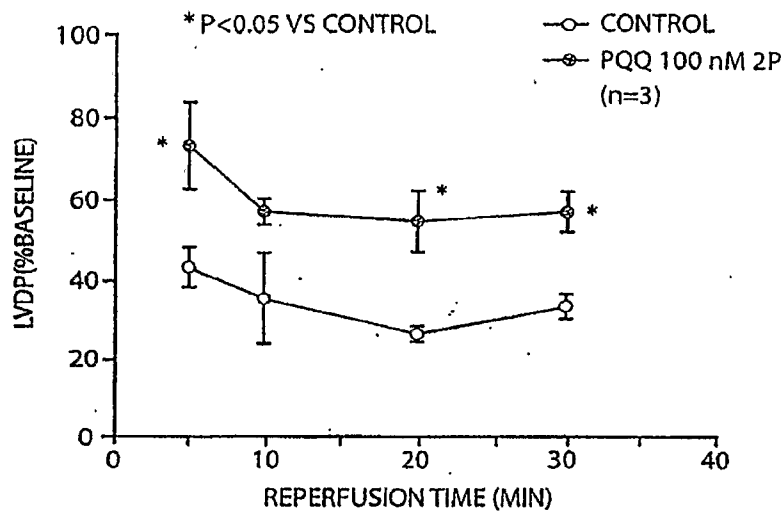


Fig. 3

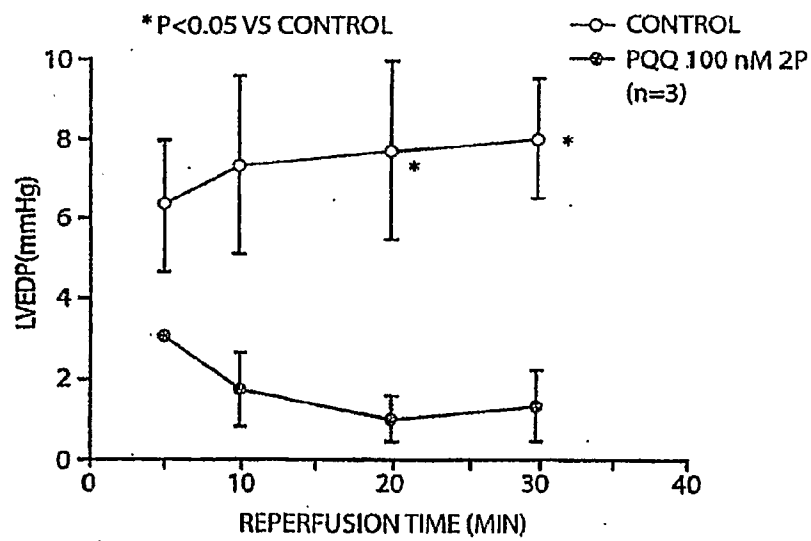


Fig. 4

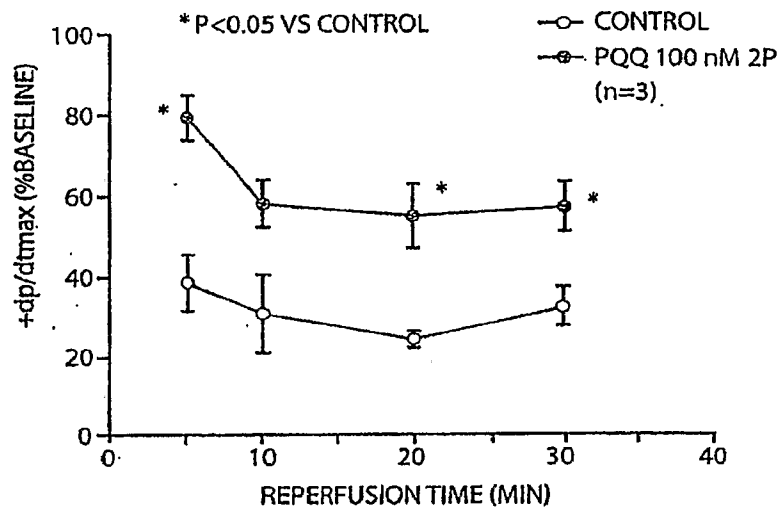


Fig. 5

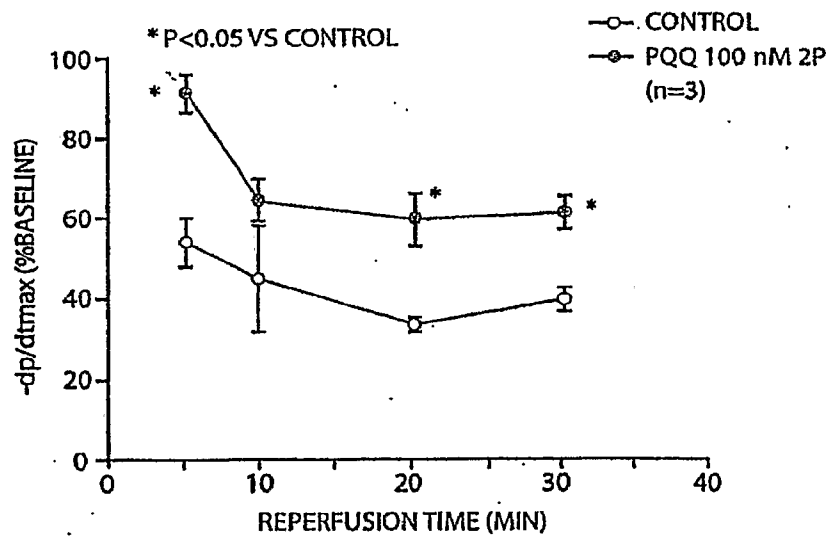


Fig. 6

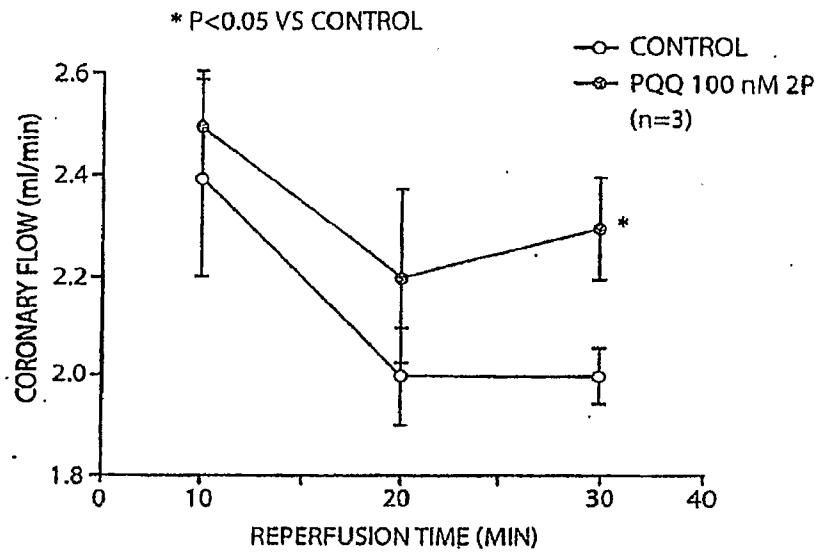


Fig. 7

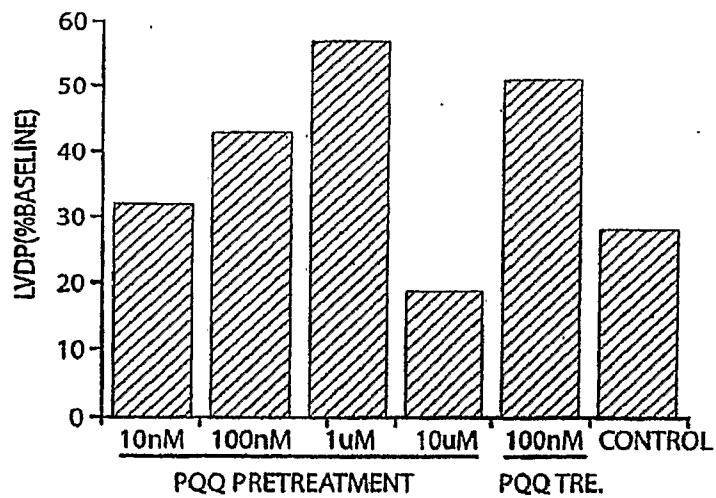


Fig. 8

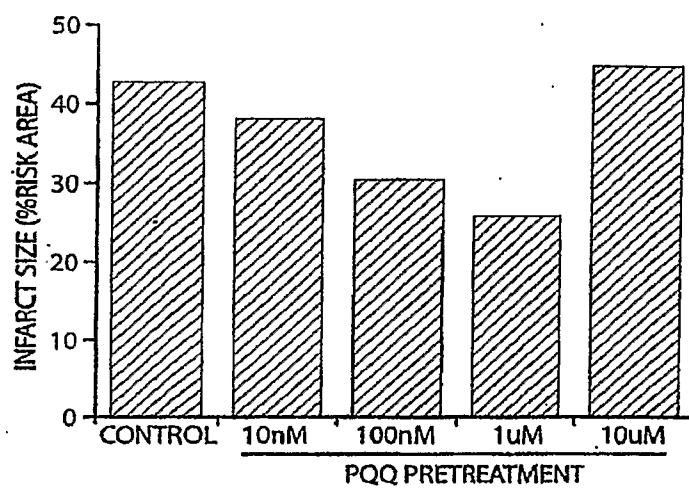


Fig. 9

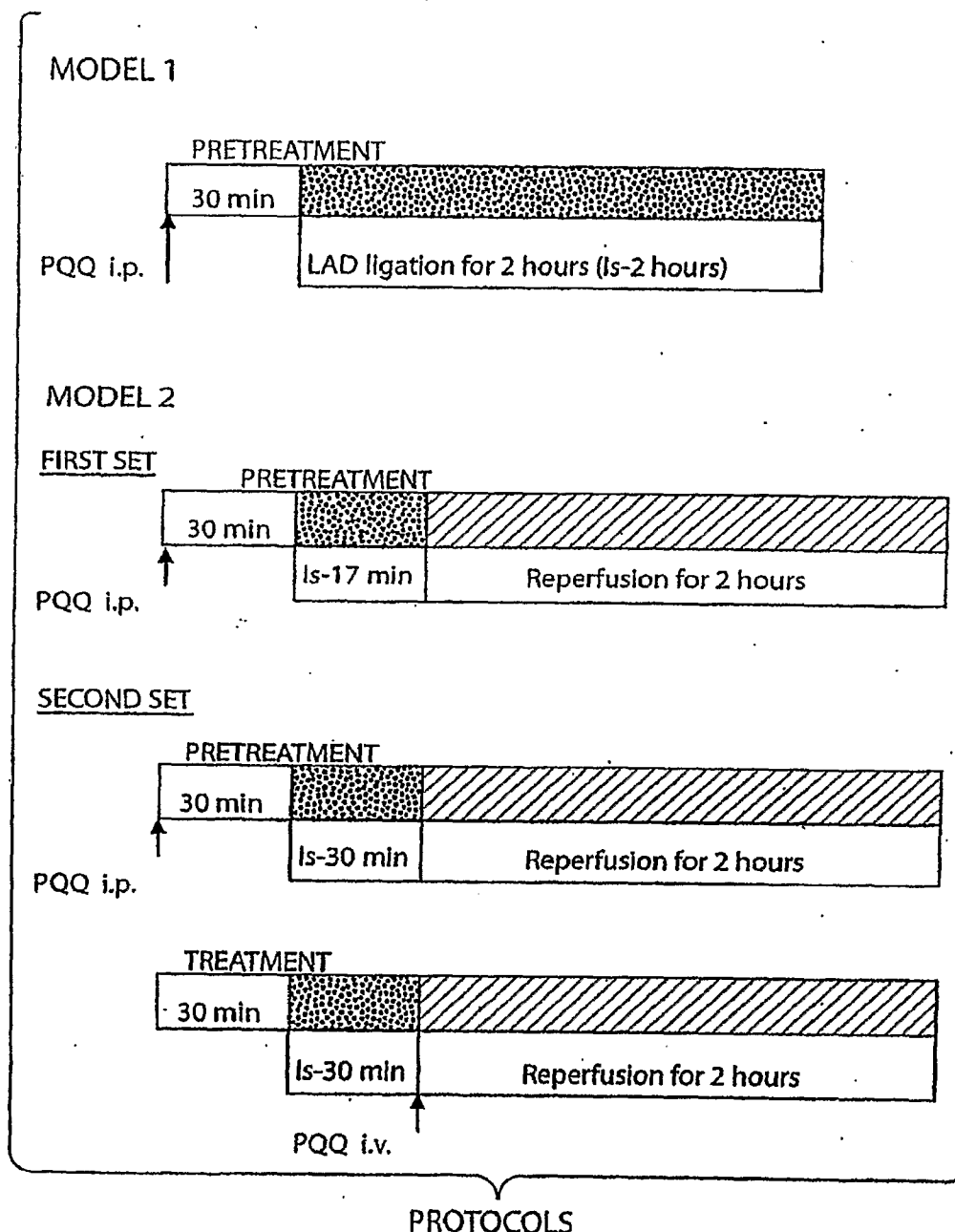


Fig. 10.

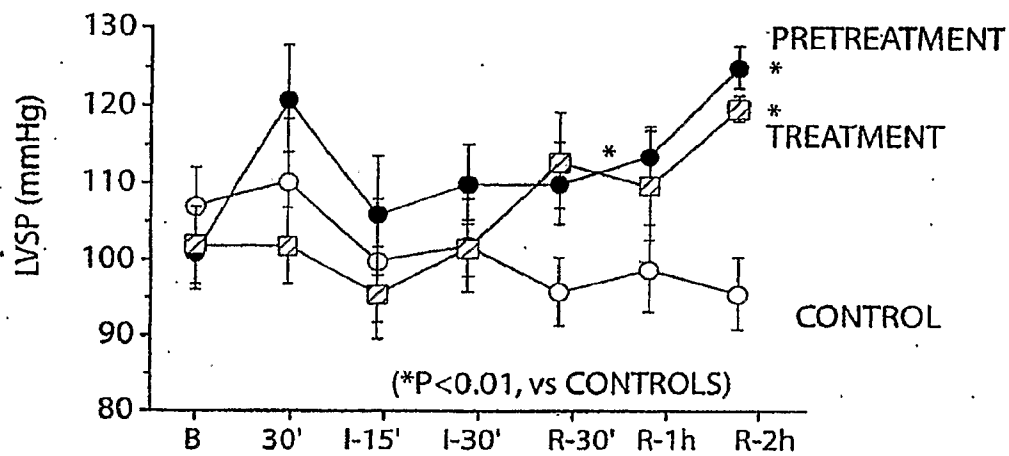


Fig. 11A

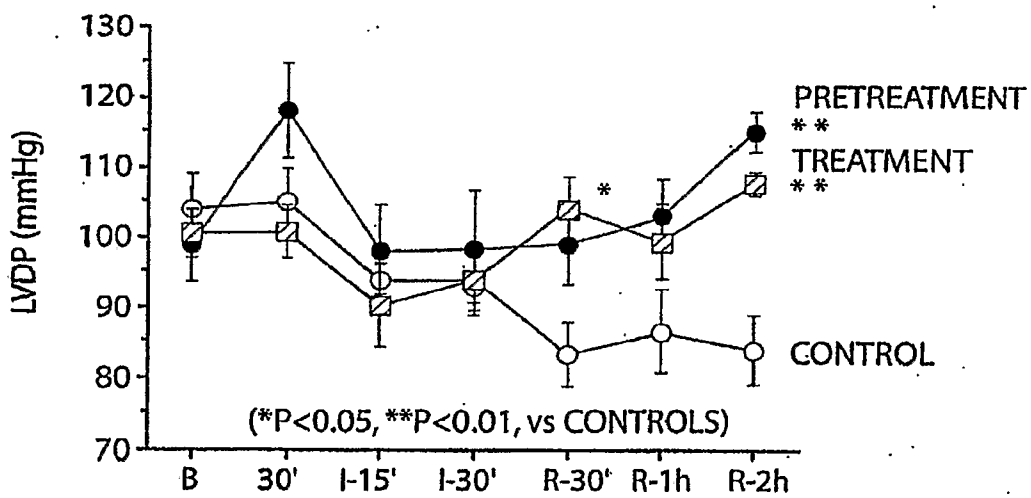


Fig. 11B

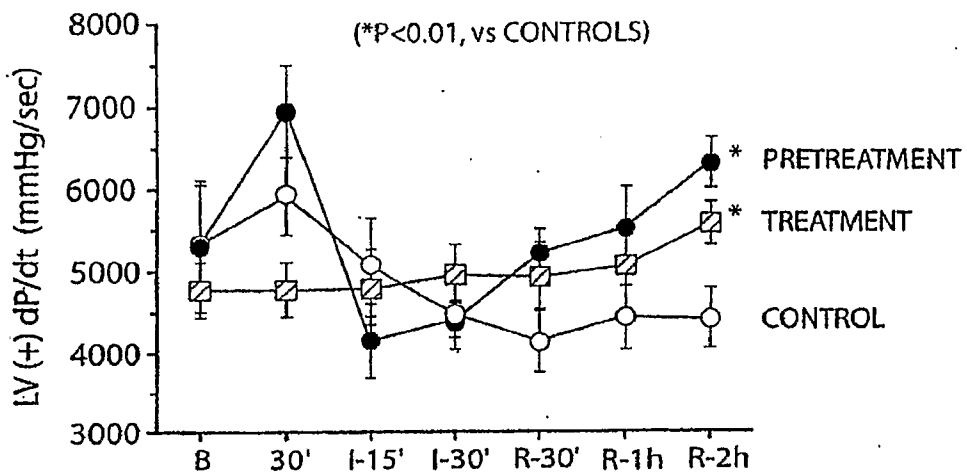


Fig. 12A

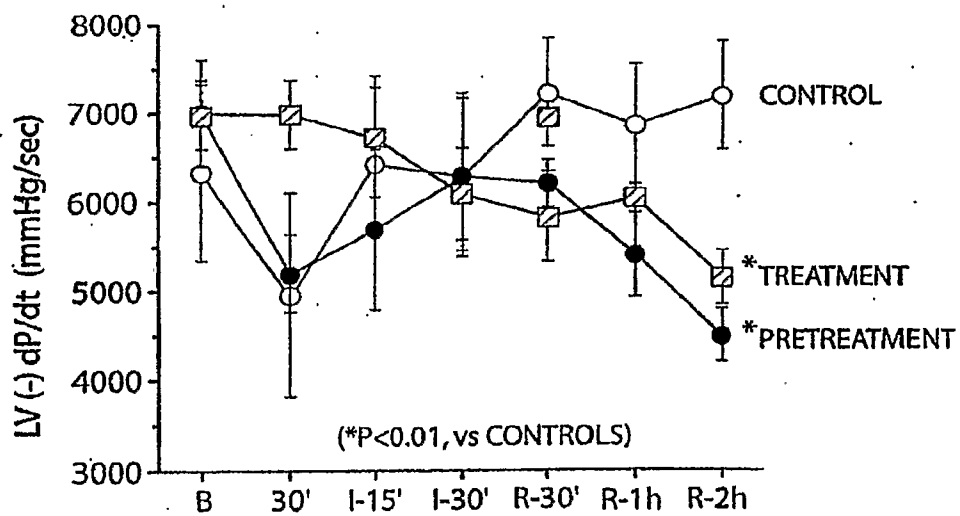


Fig. 12B

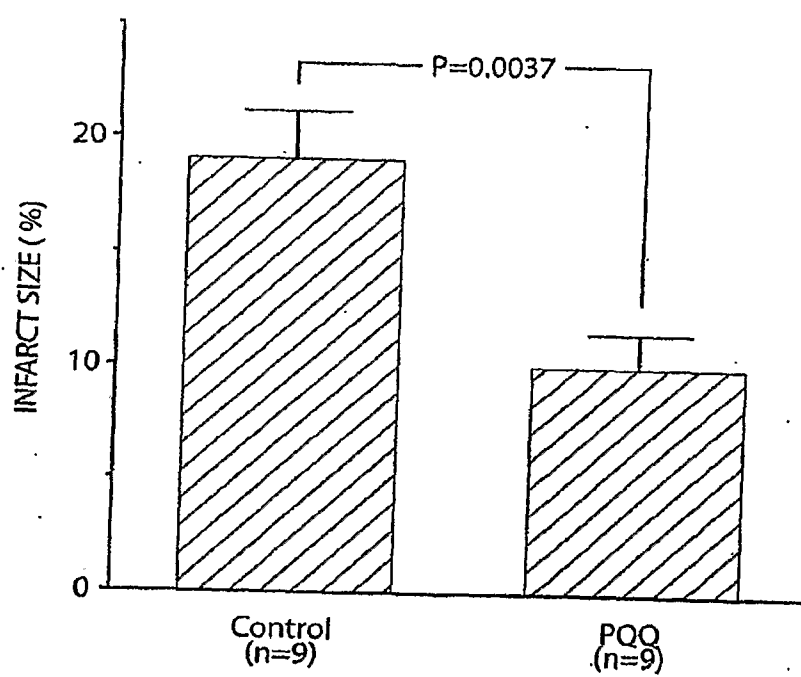


Fig. 13

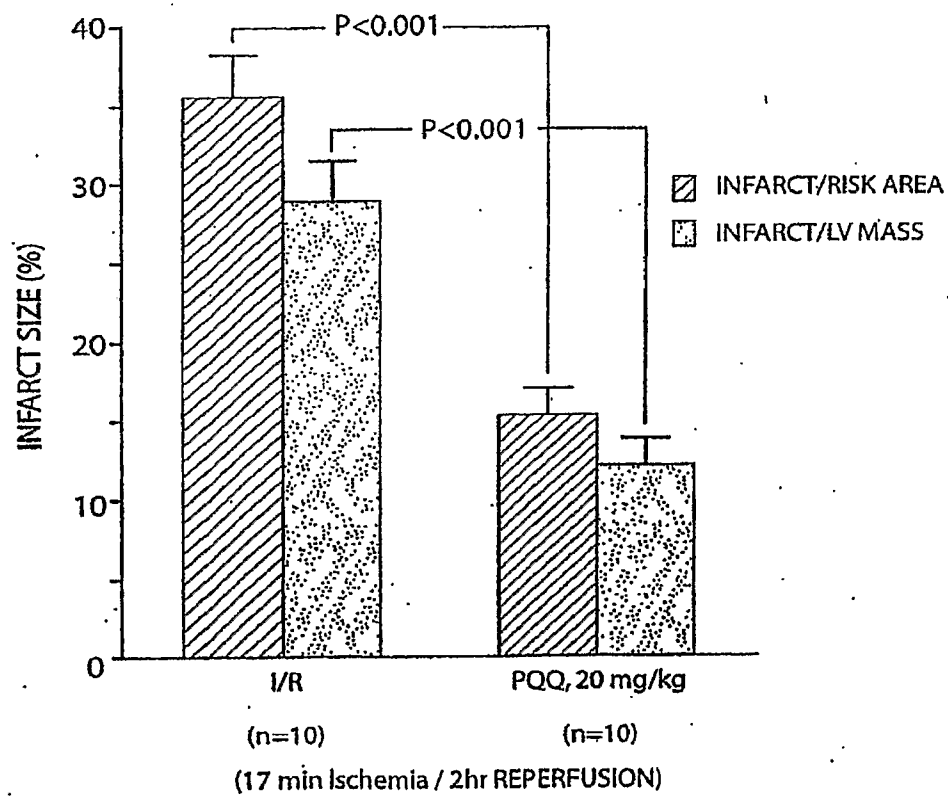


Fig. 14

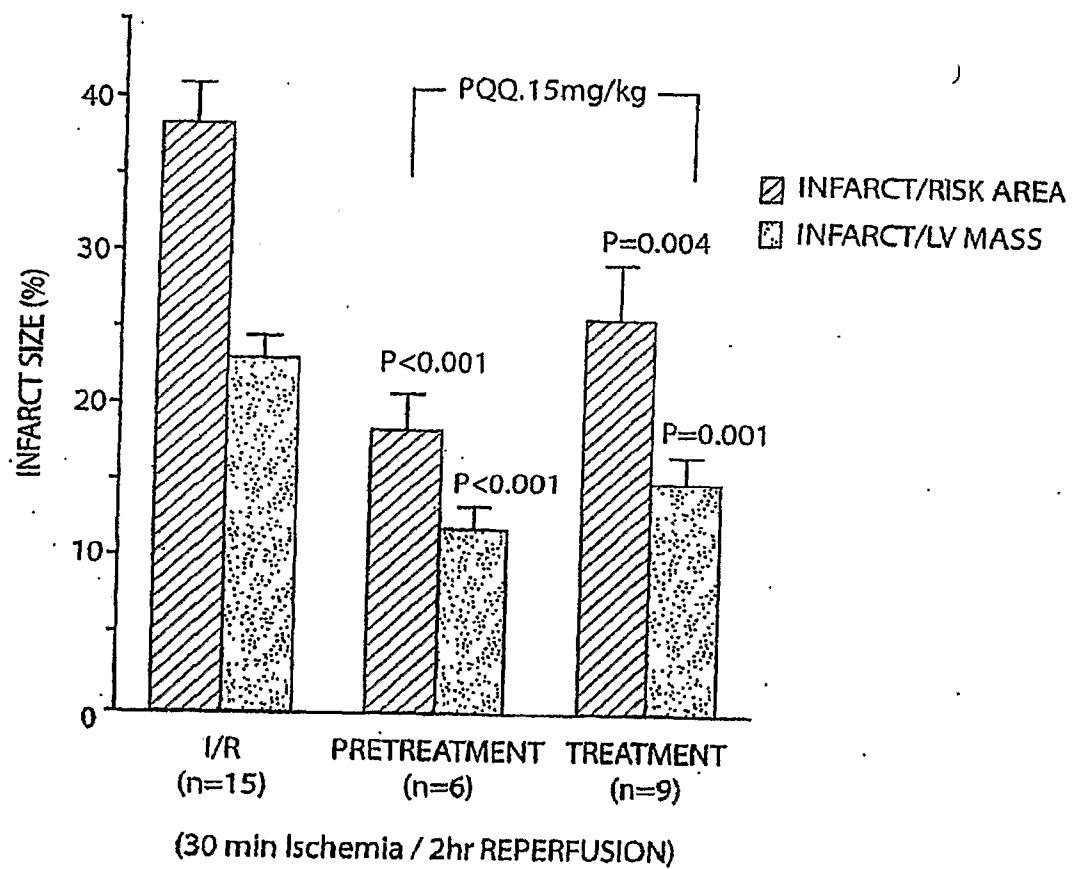


Fig. 15

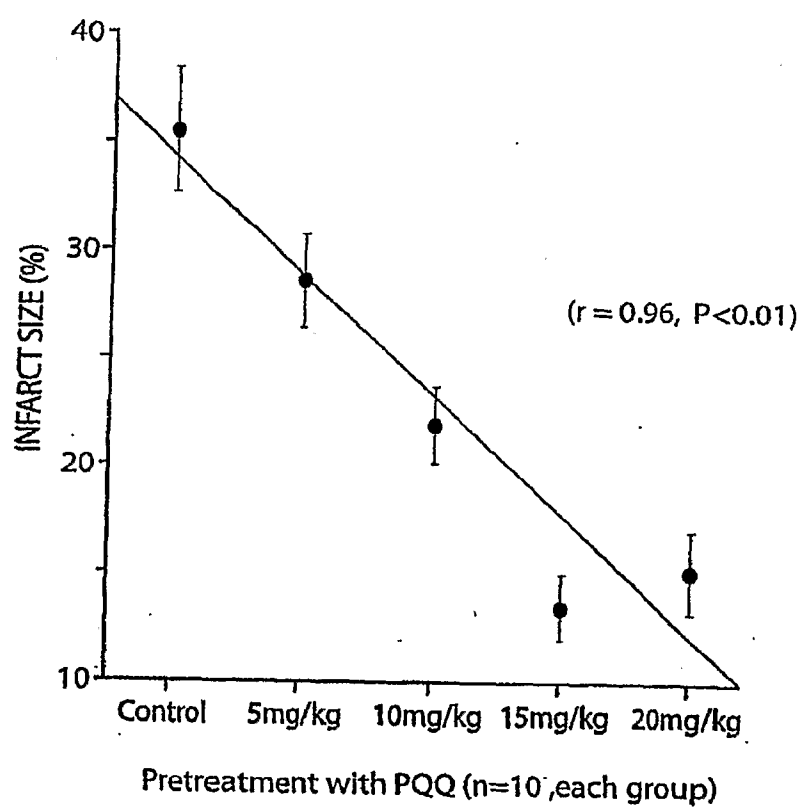


Fig. 16

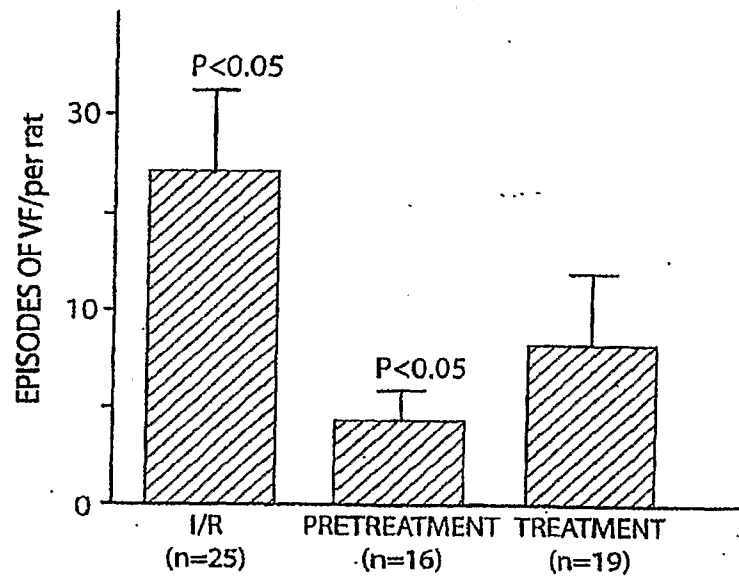


Fig. 17A

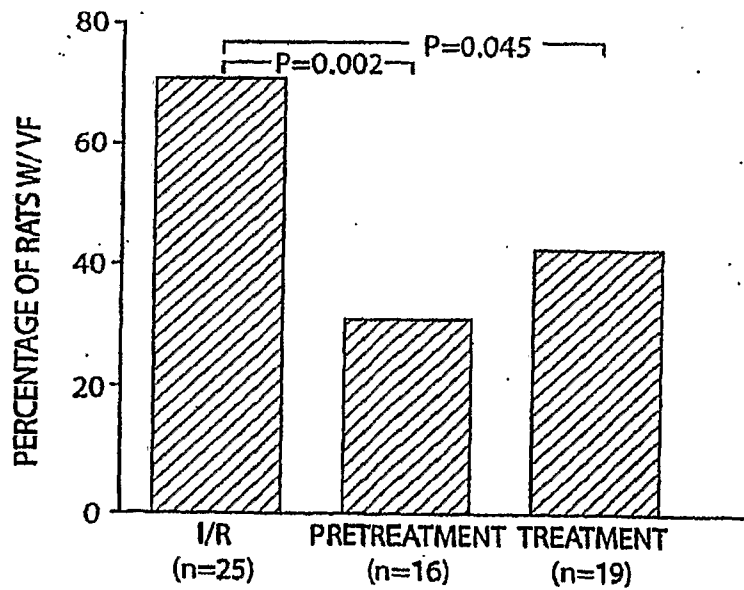


Fig. 17B

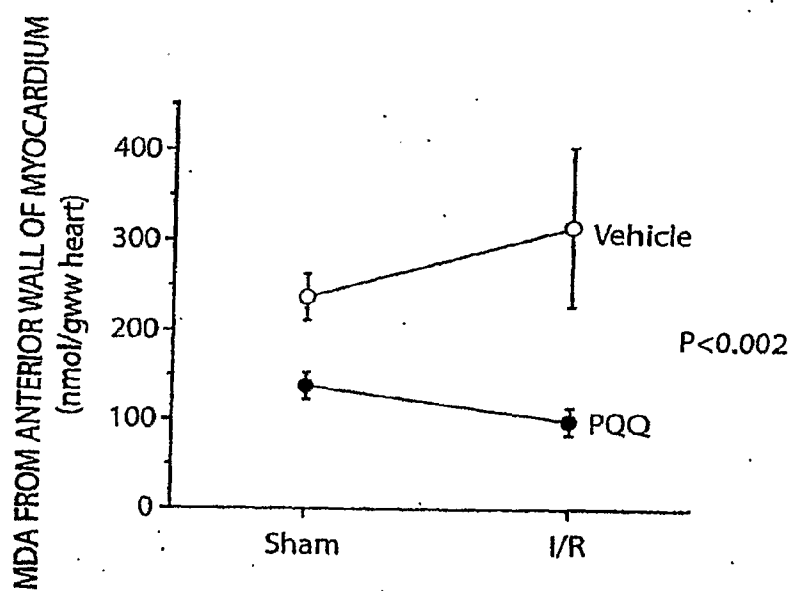


Fig. 18A

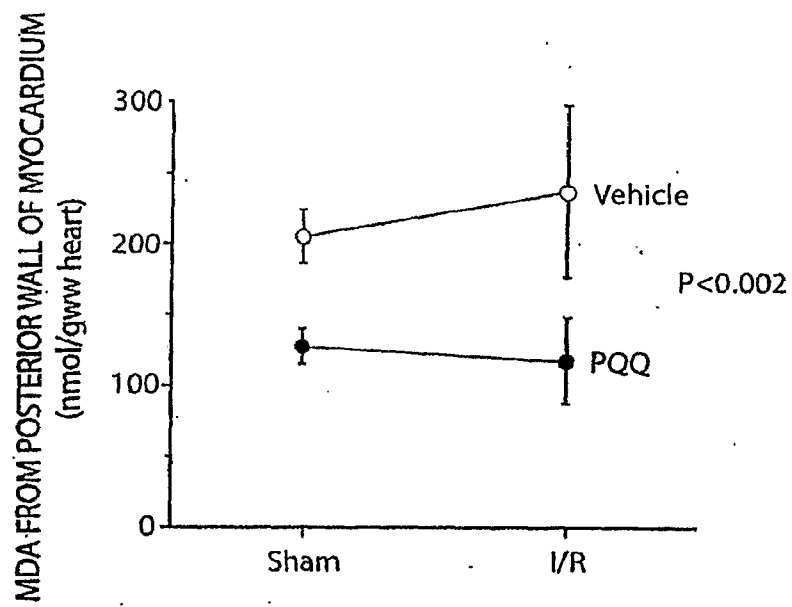


Fig. 18B

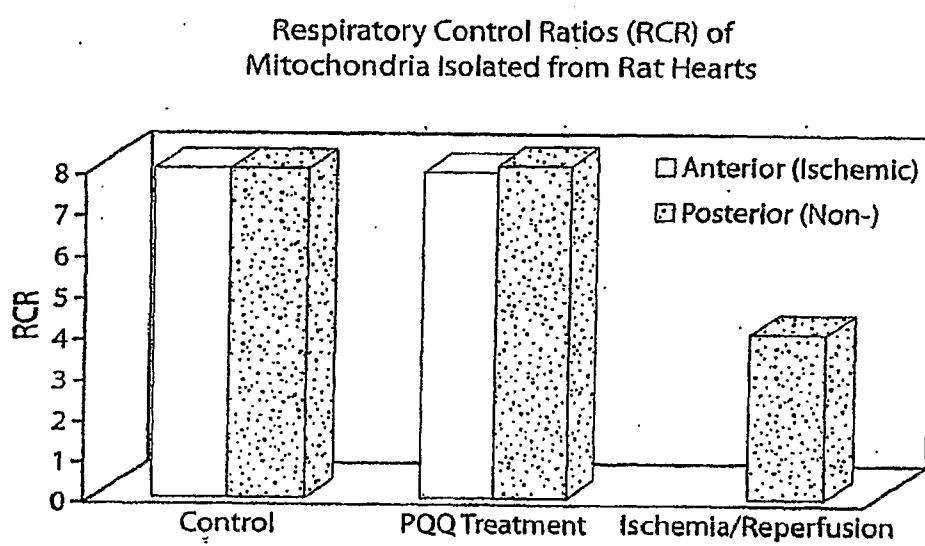


Fig. 19

Fig. 20 A

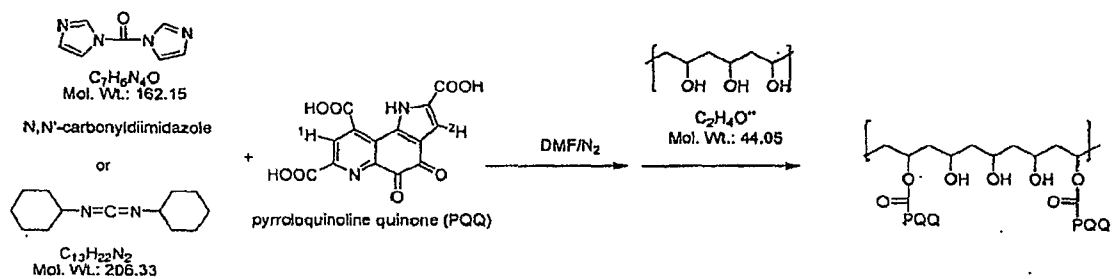


Fig. 20 B

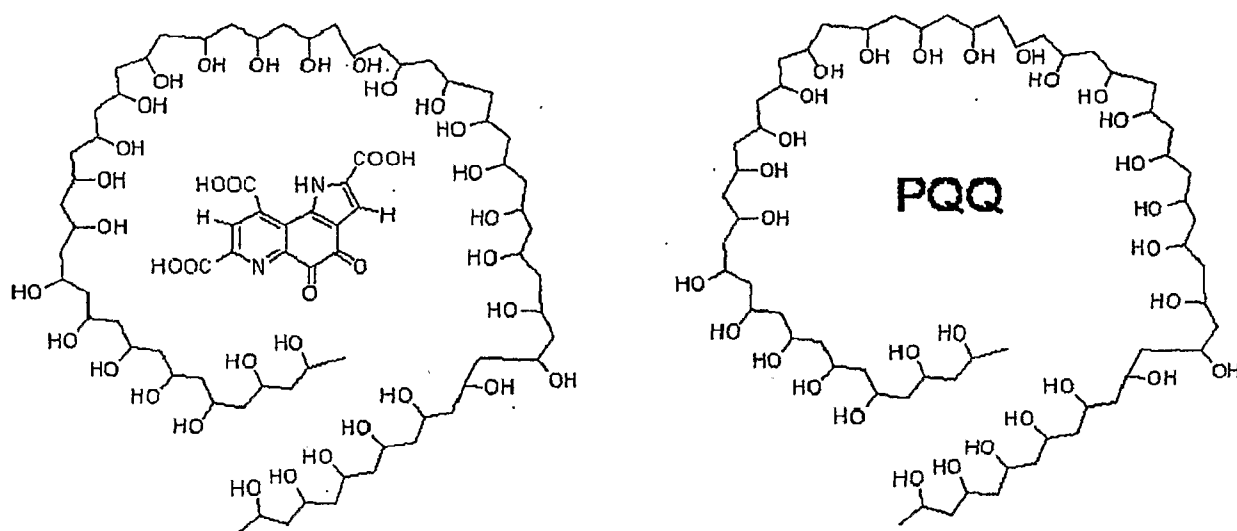


Fig. 20 C

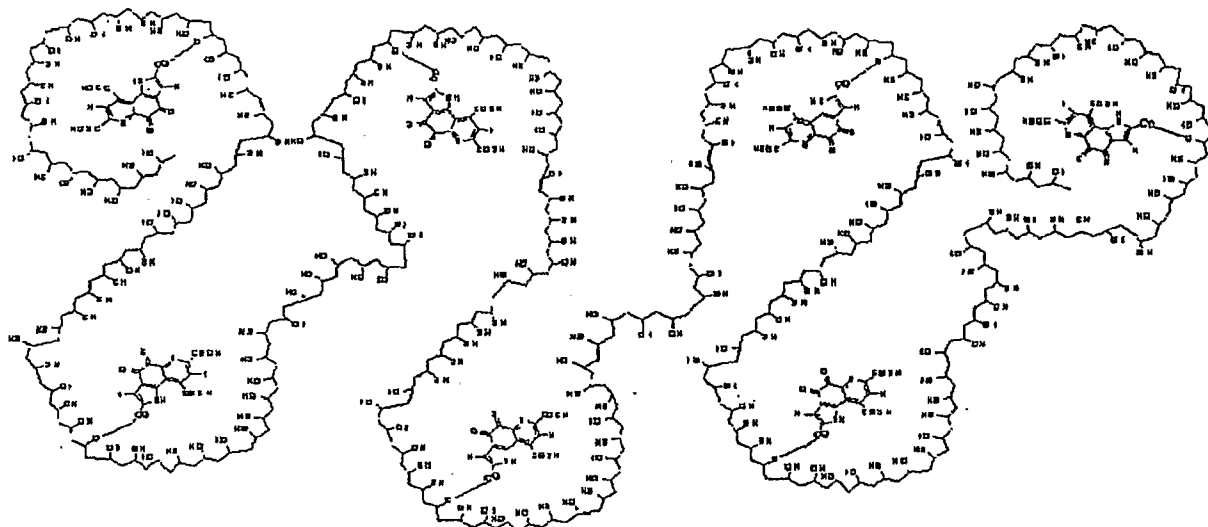


Fig. 21

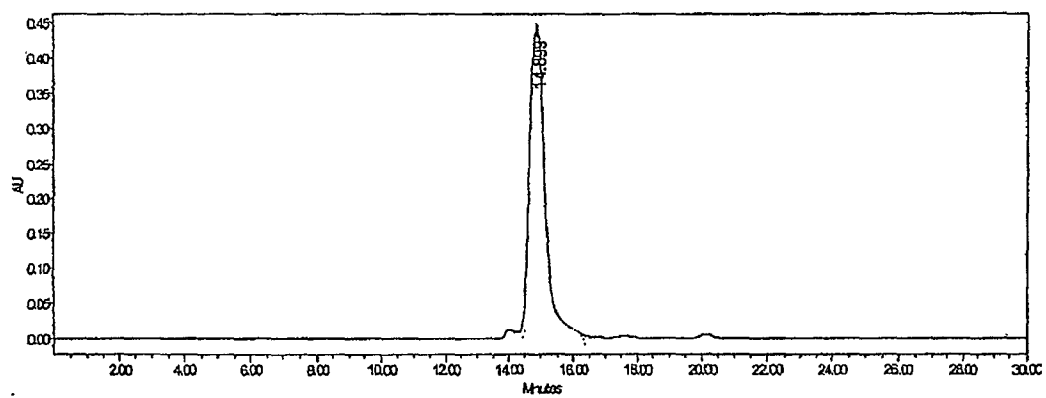


Fig. 22

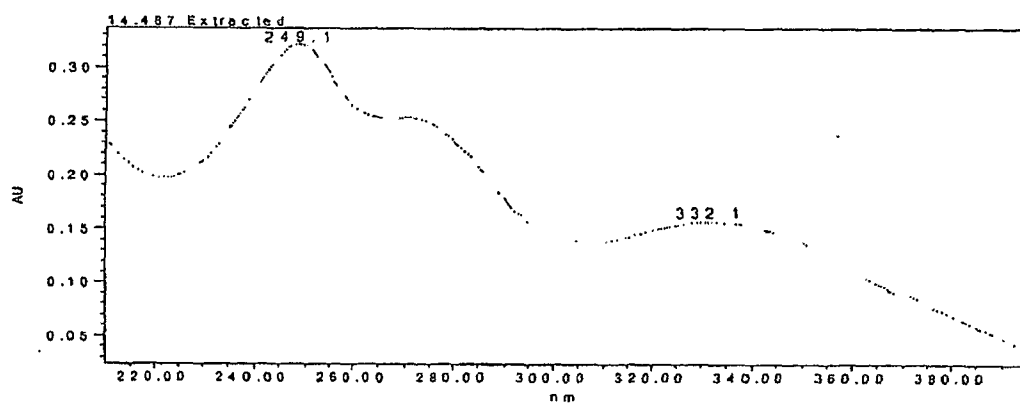


Fig. 23

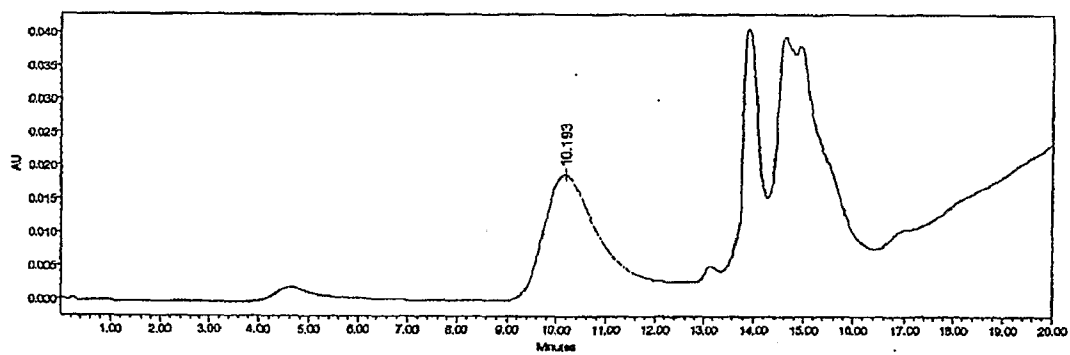
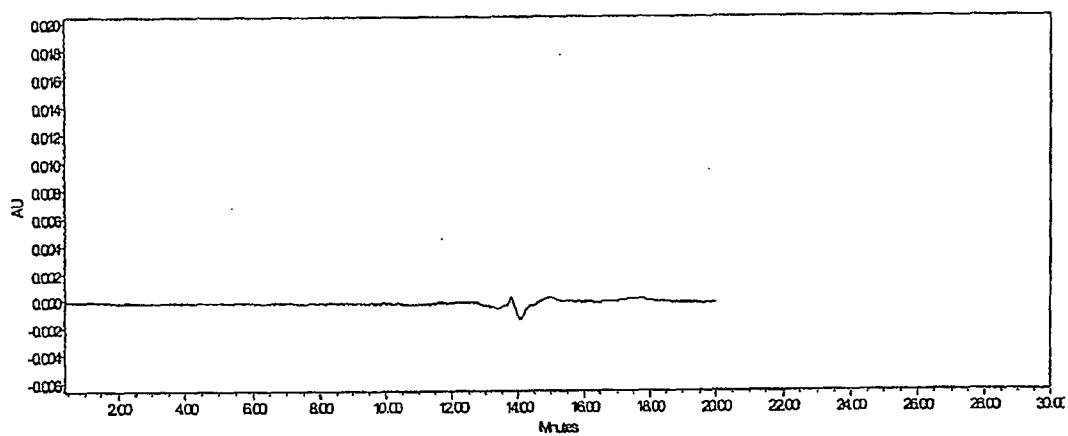
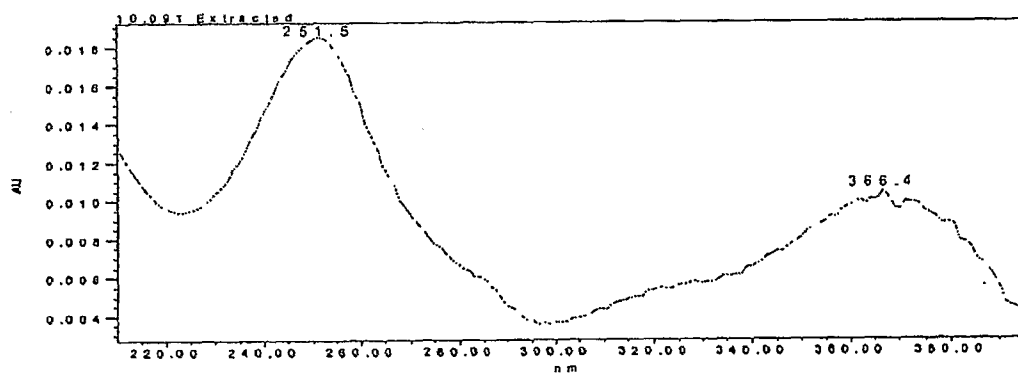


Fig. 24**Fig. 25**

A.



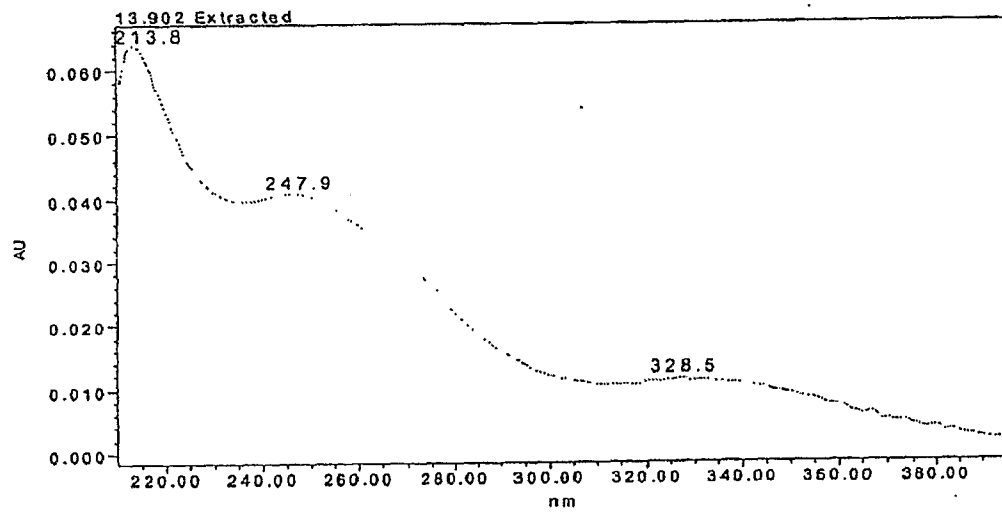
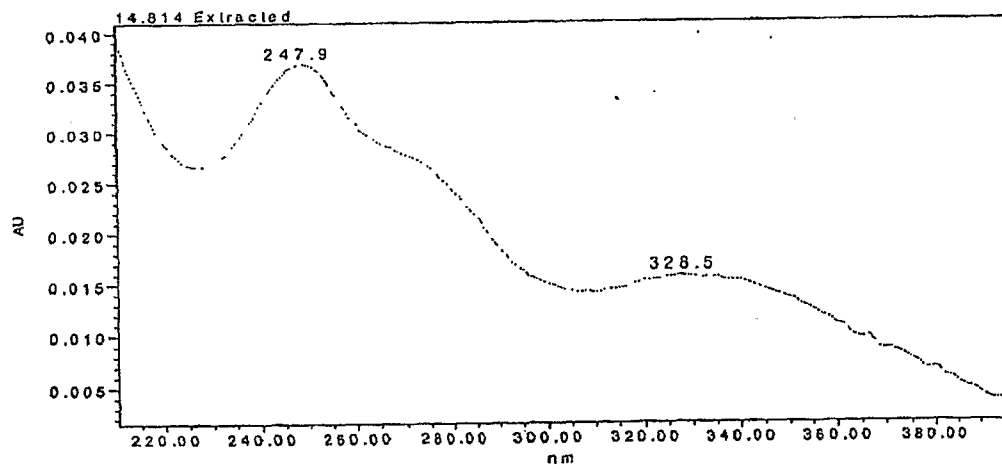
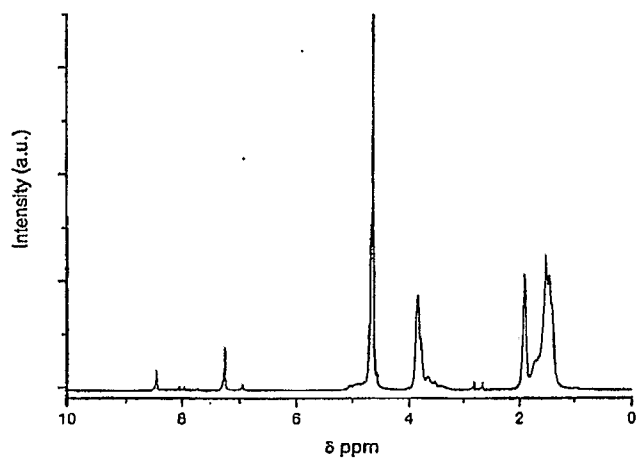
B.**C.**

Fig. 26

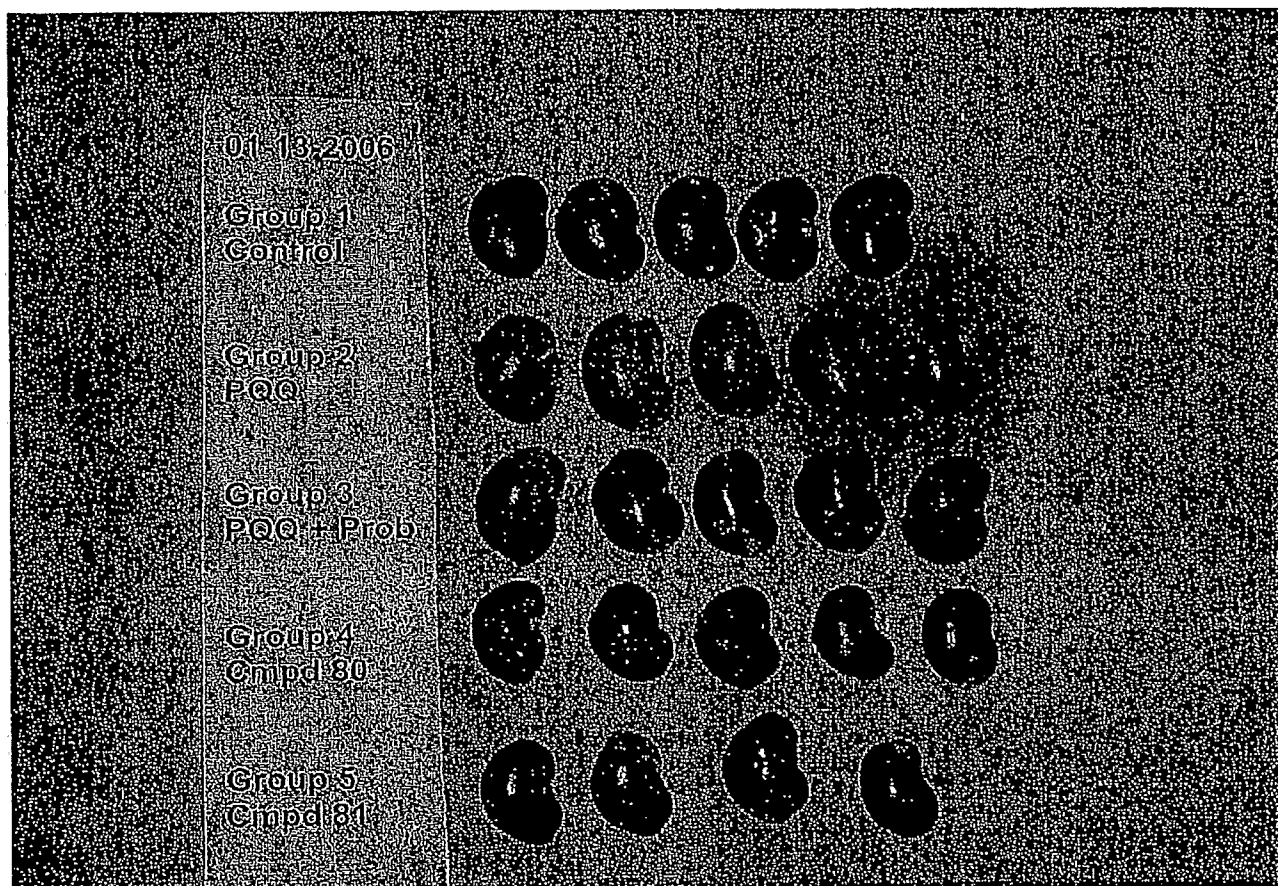


Figure 27

Figure 28

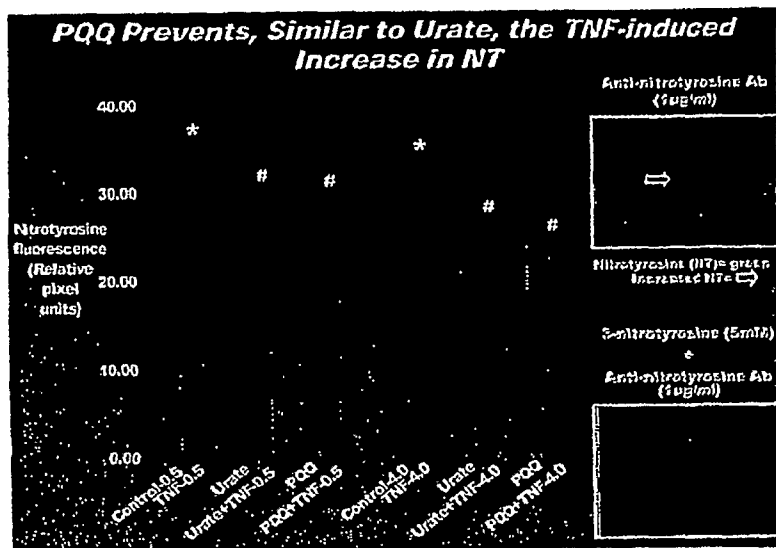


Figure 29

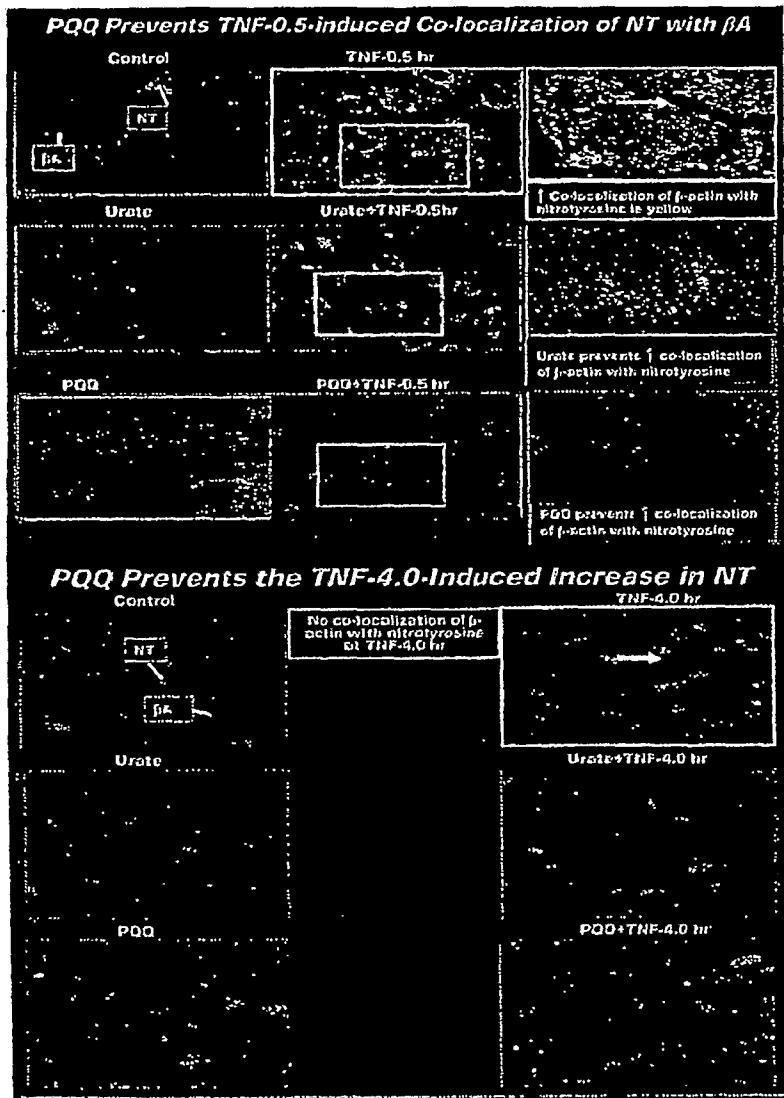


Figure 30

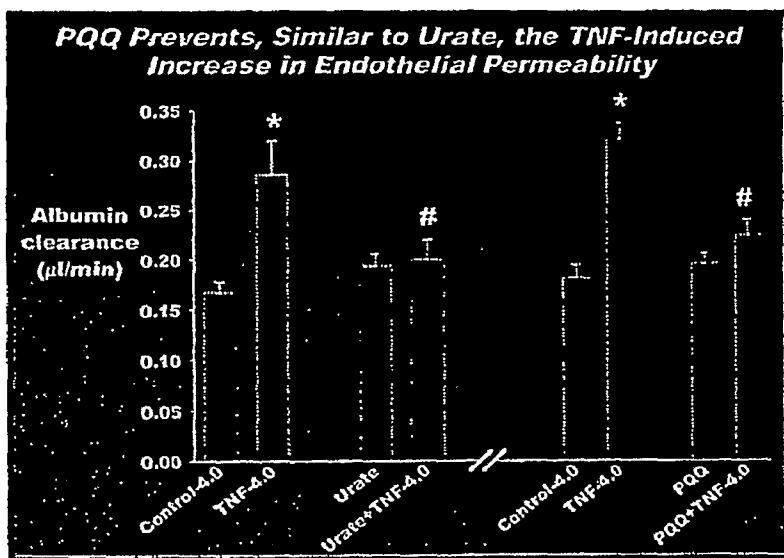


Fig. 31A

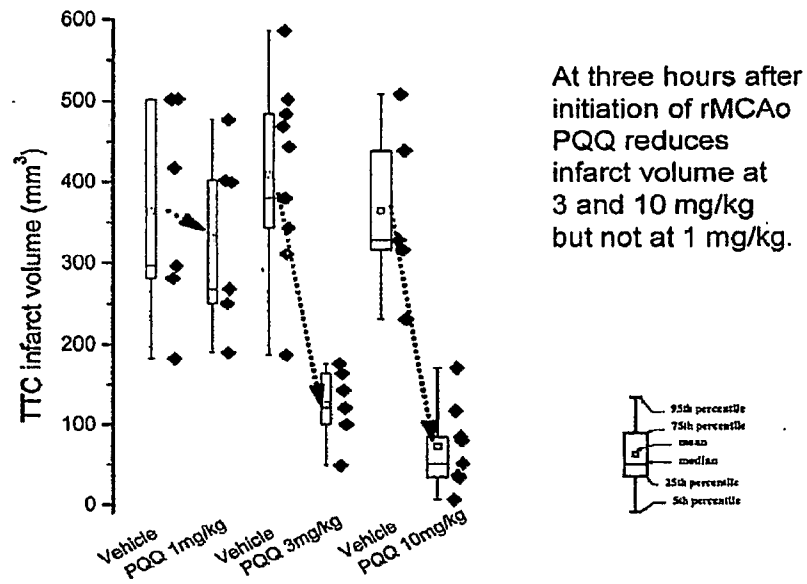


Fig. 31B

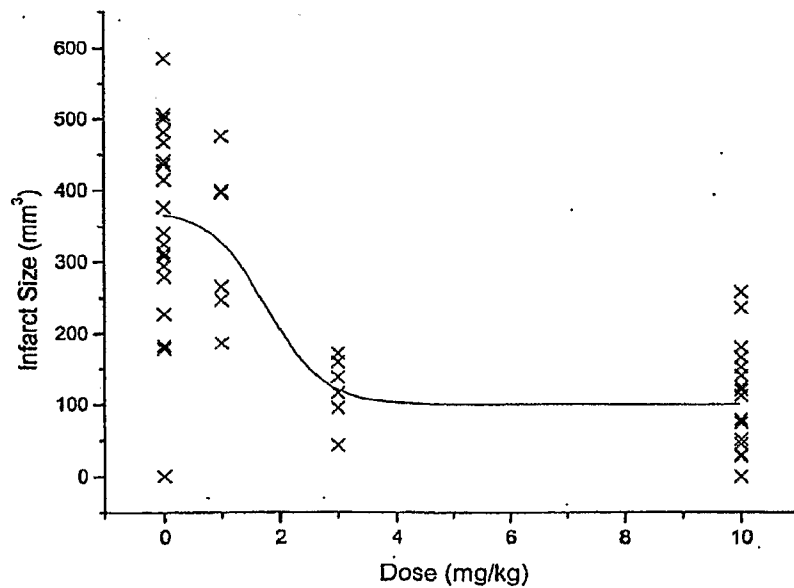
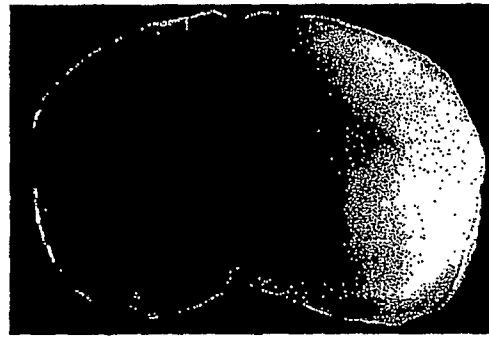


Fig. 32



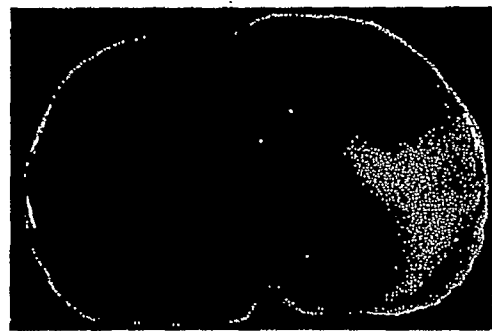
A



B

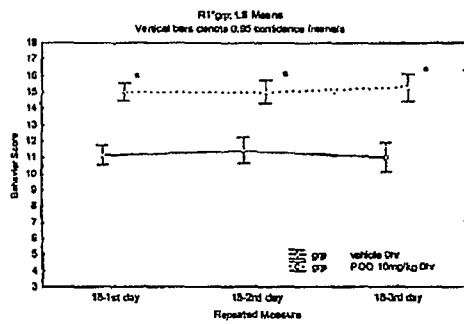


C



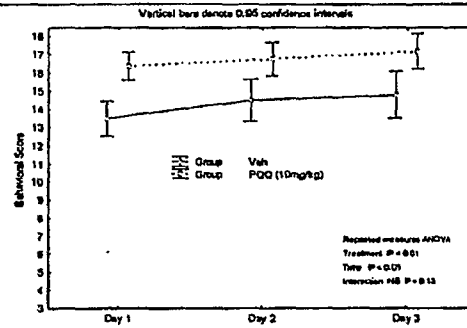
D

Fig. 33



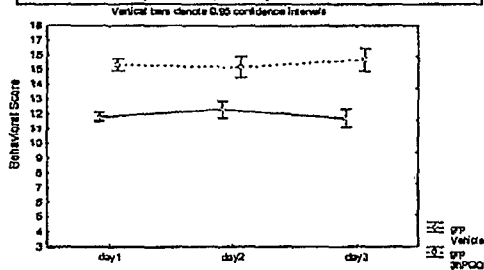
A

Treatment with PQQ (10 mg/kg) three hours after rMCAo results in improved neurobehavioral scores at 24, 48 and 72 hours after rMCAo. Blue squares are vehicle controls and red are PQQ treated. There is a significant effect of treatment by repeated measure ANOVA.



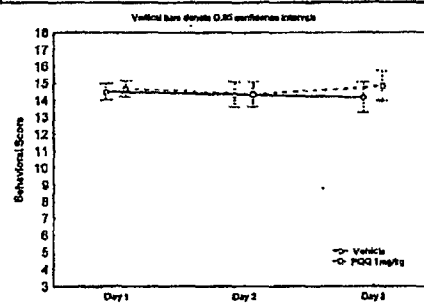
B

Treatment with PQQ (3 mg/kg) at 3 hrs after initiation of ischemia results in improved neurobehavioral scores at 24, 48 and 72 hours after rMCAo. Blue squares are vehicle controls and red are PQQ treated. There is a significant effect of treatment by repeated measure ANOVA and post hoc comparisons by Newman Keuls



C

Treatment with PQQ (1 mg/kg) three hours after rMCAo doesn't result in improved neurobehavioral scores at 24, 48 and 72 hours after rMCAo. Blue squares are vehicle controls and red are PQQ treated. There was no significant effect of treatment by repeated measure ANOVA.



D

Fig. 34

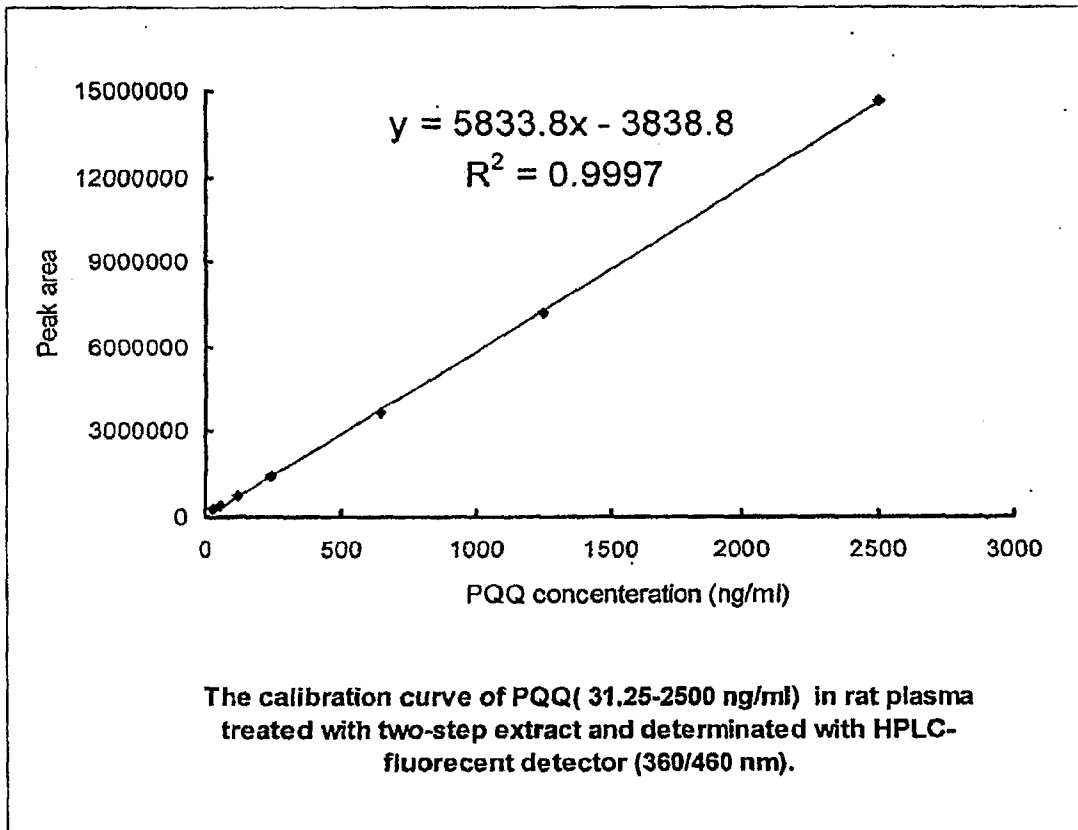


Fig. 35

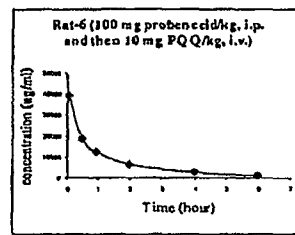
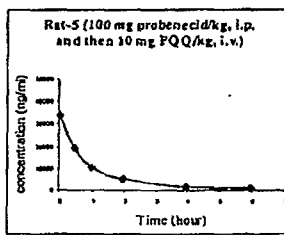
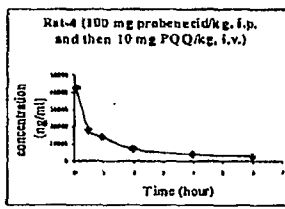
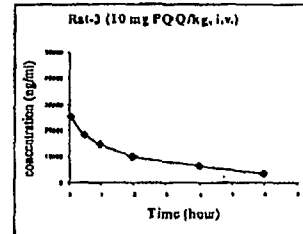
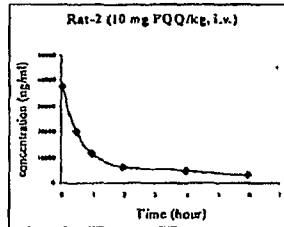
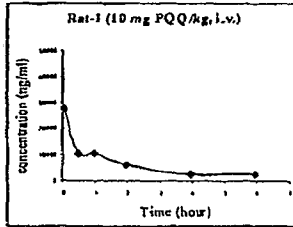
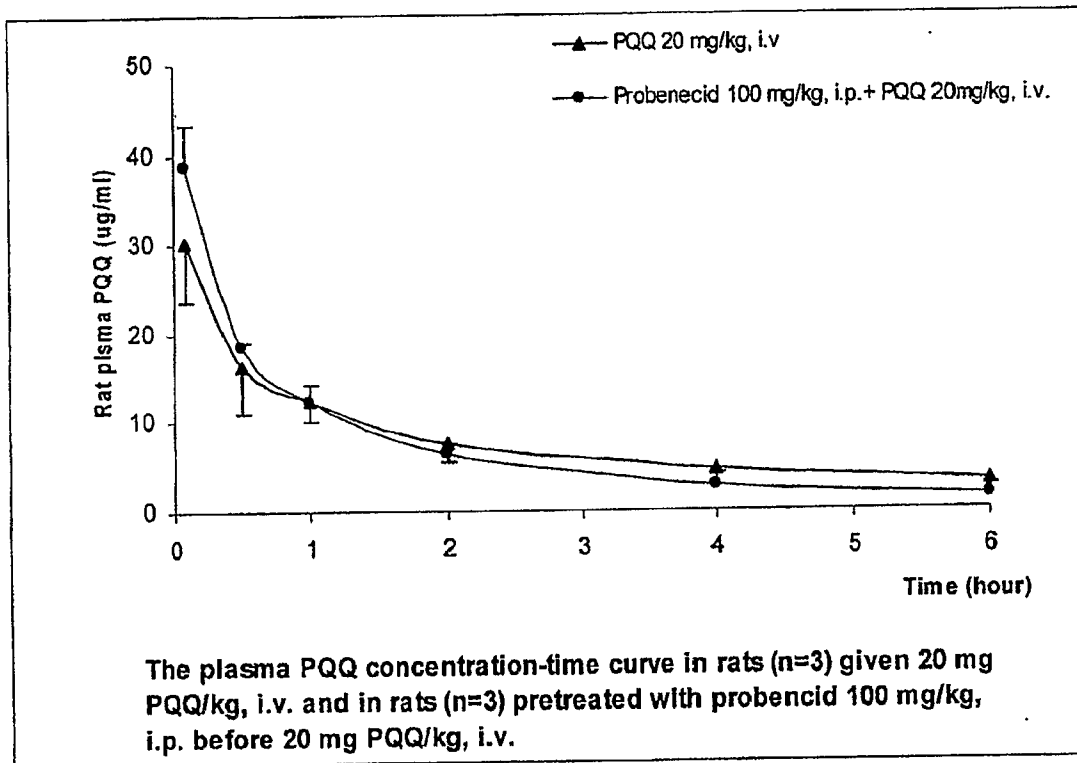
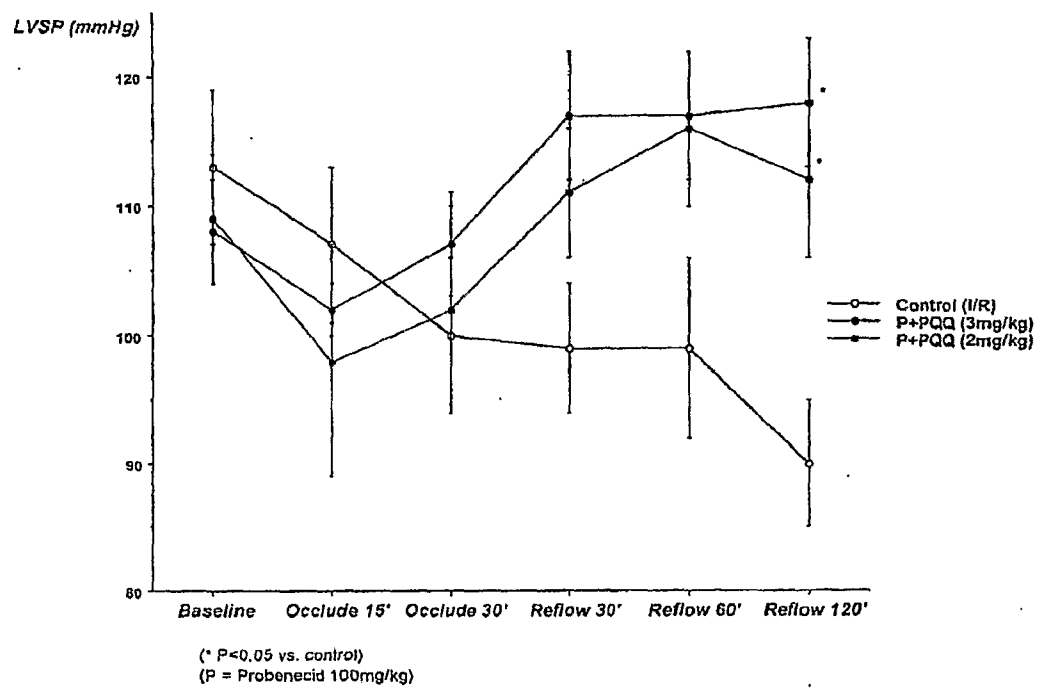
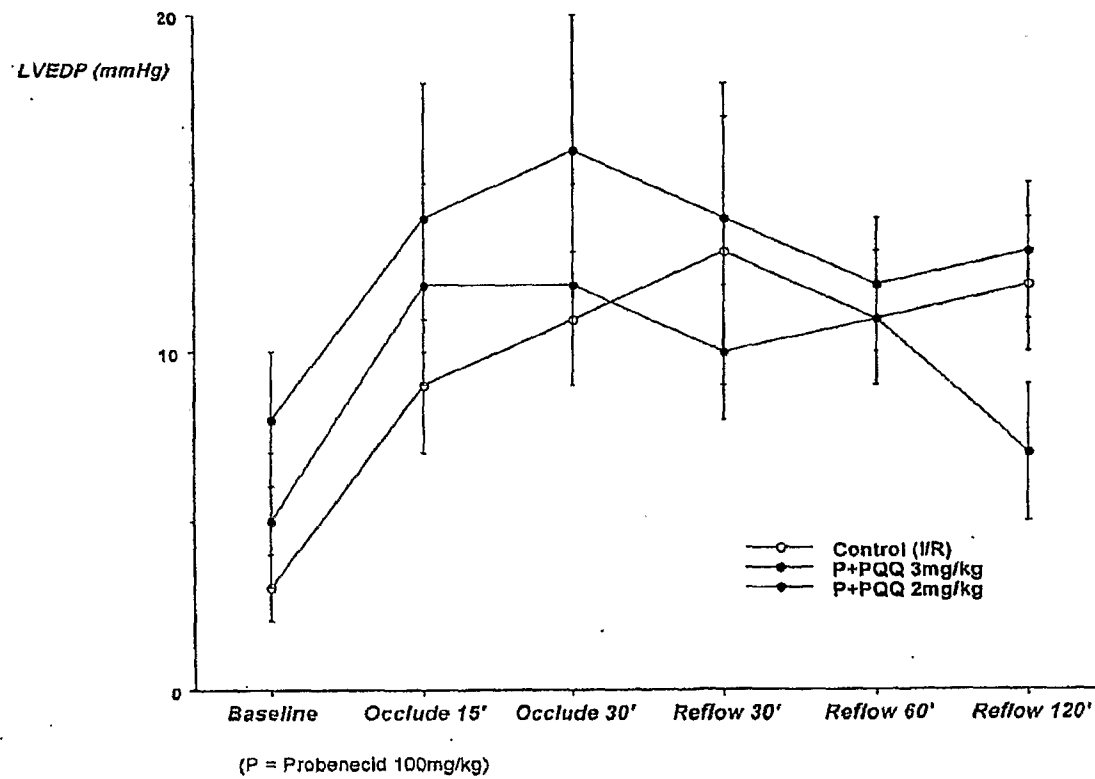
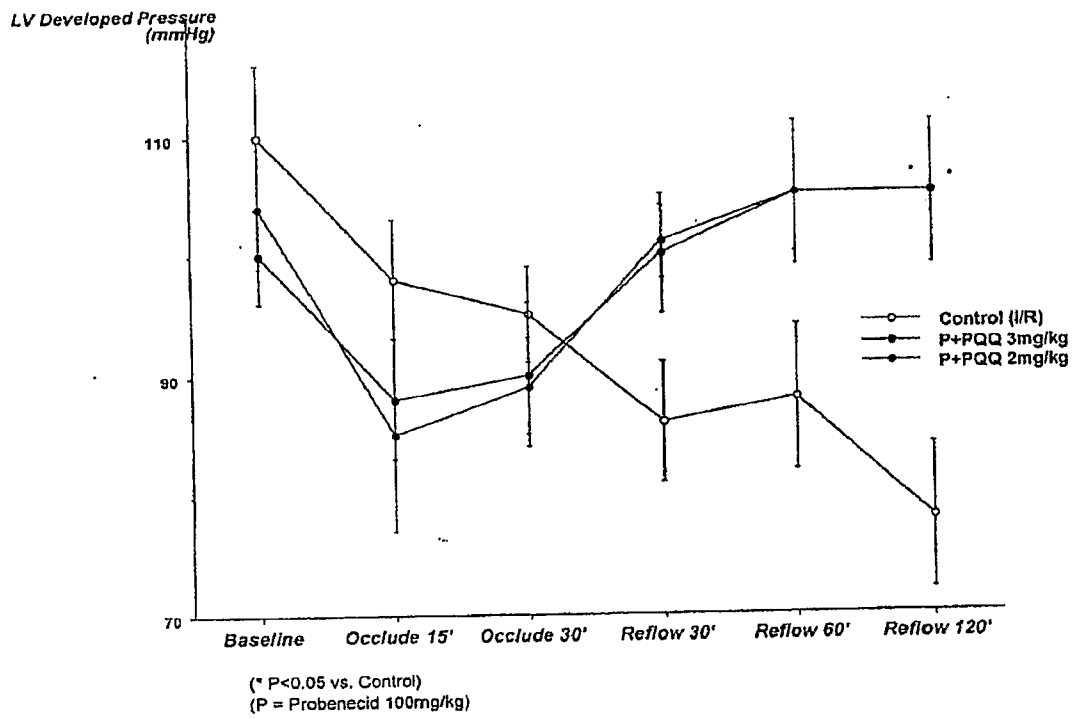
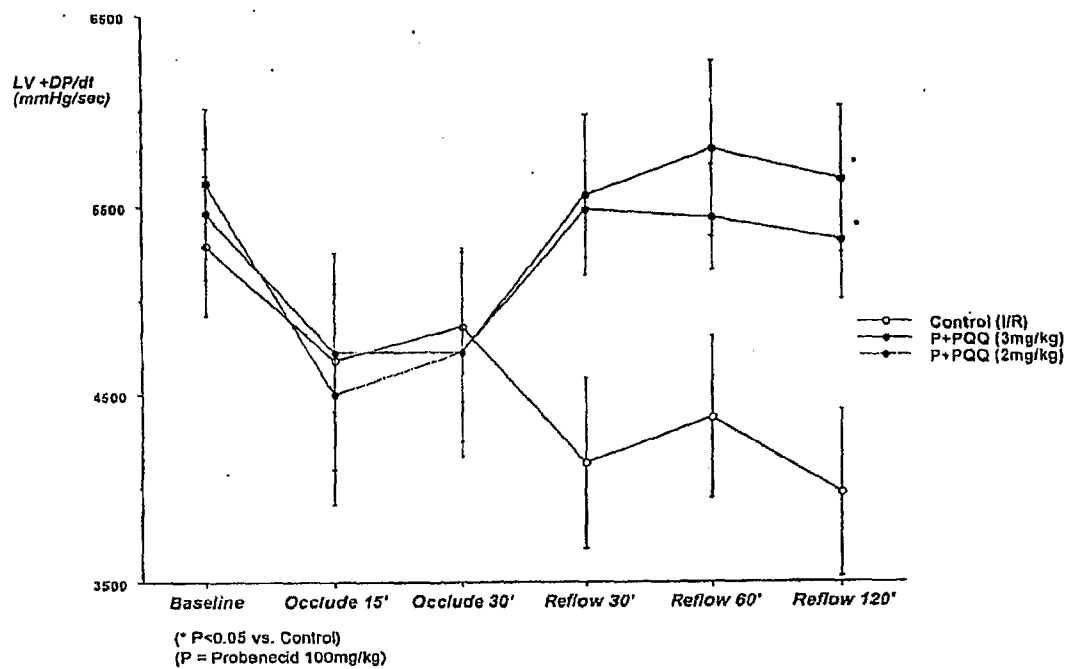


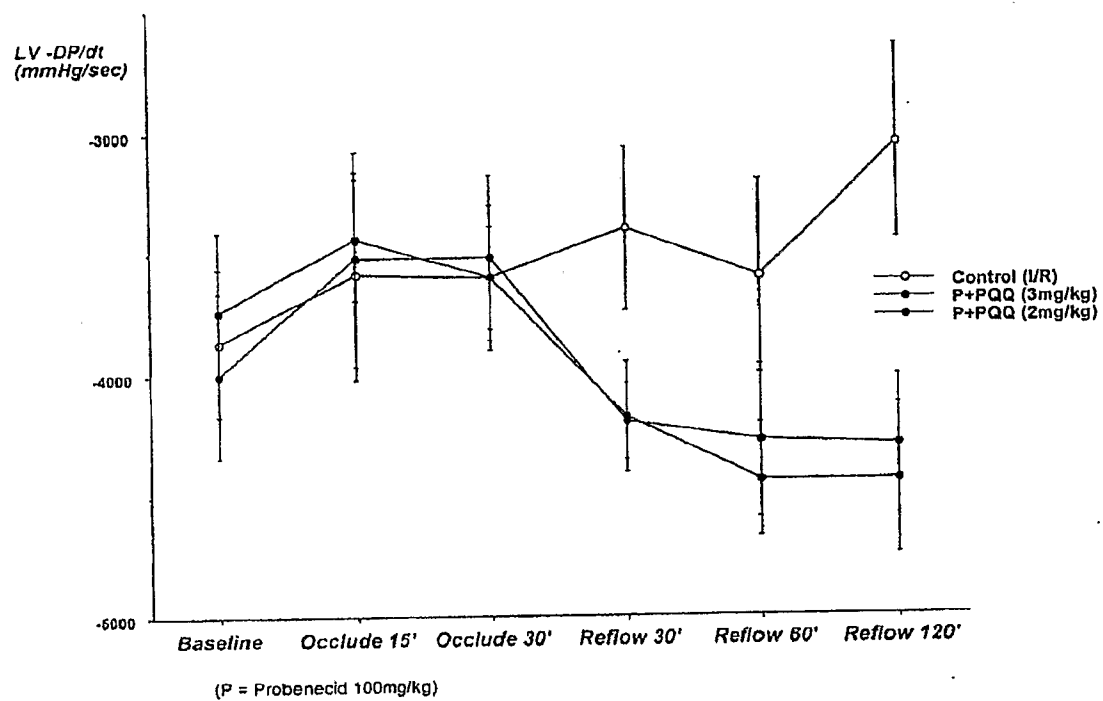
Fig. 36

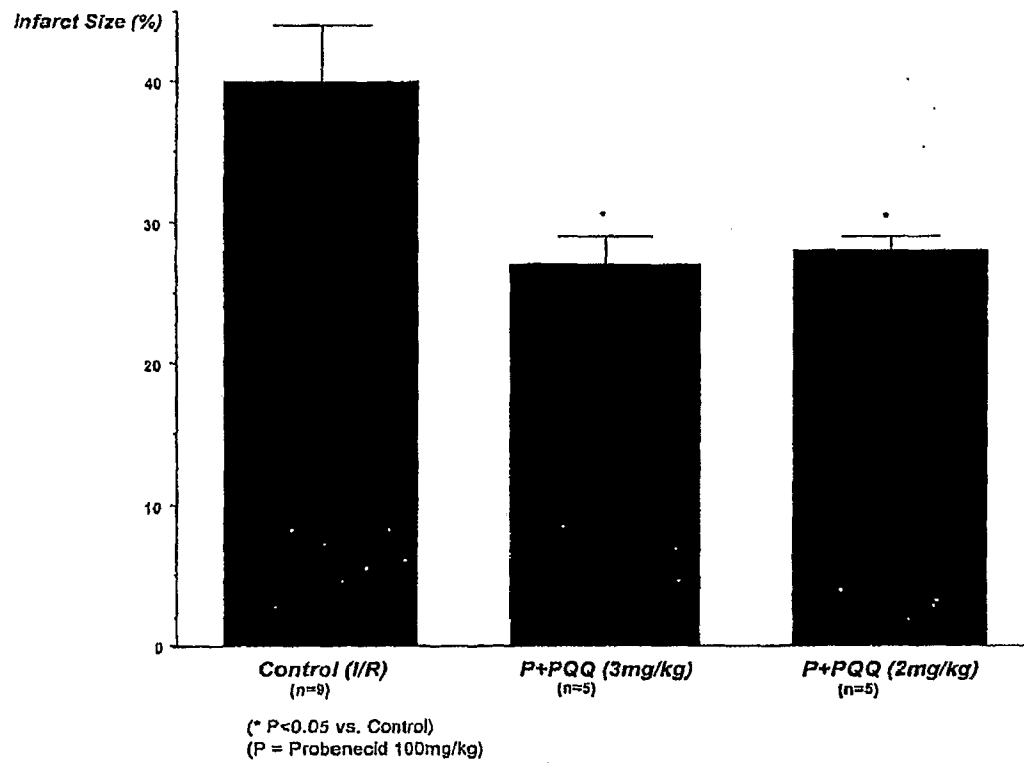
**Figure 37**

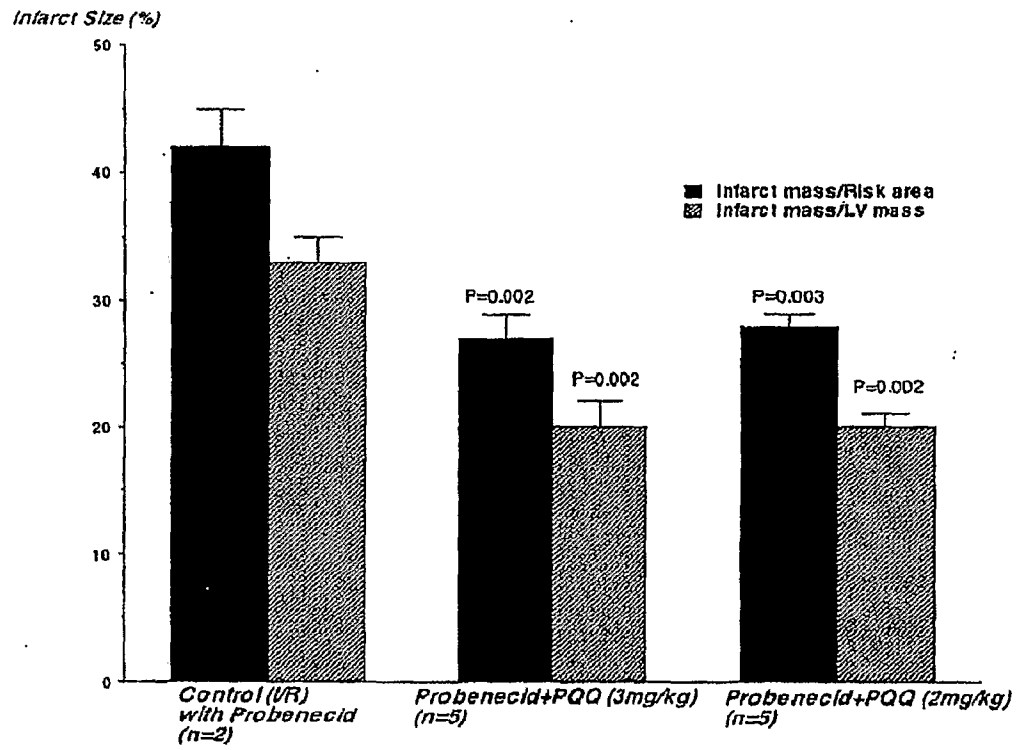
**Figure 38**

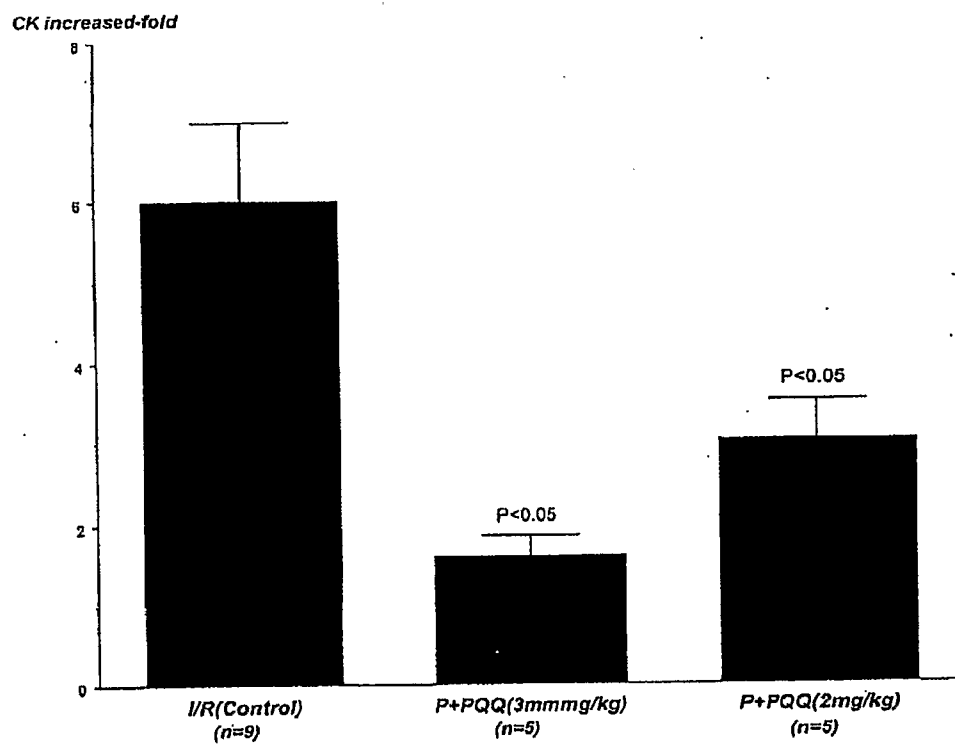
**Figure 39**

**Figure 40**

Figure 41

**Figure 42**

**Figure 43**

**Figure 44**

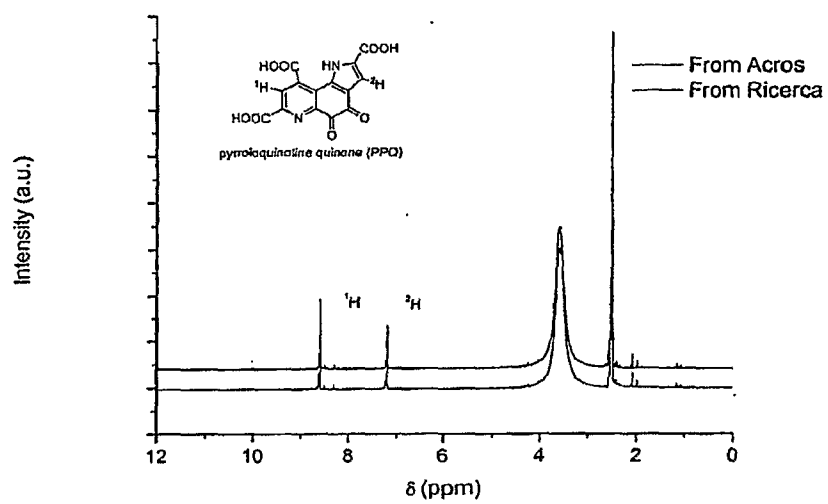
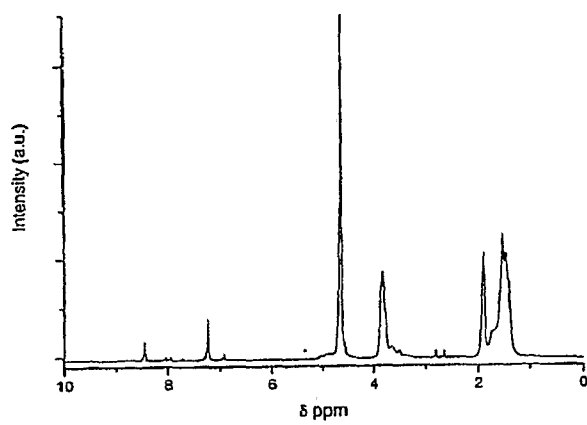
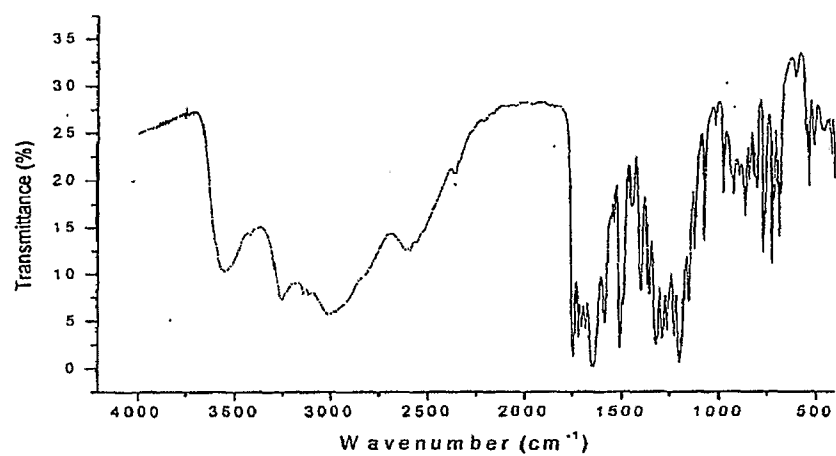


Figure 45

**Figure 46**

**Figure 47**

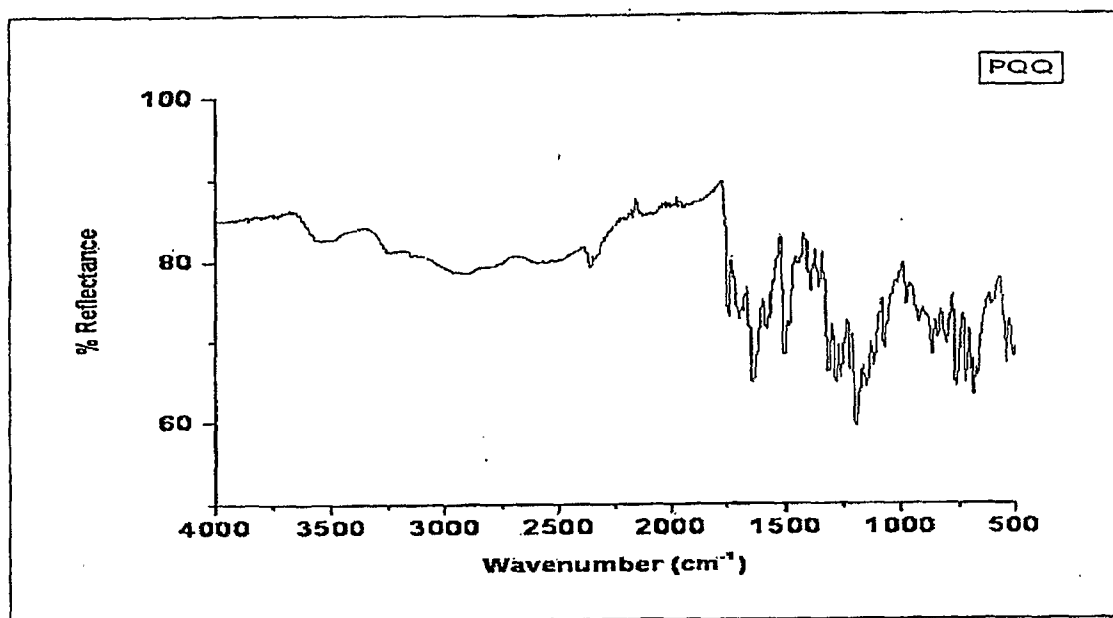


Figure 48

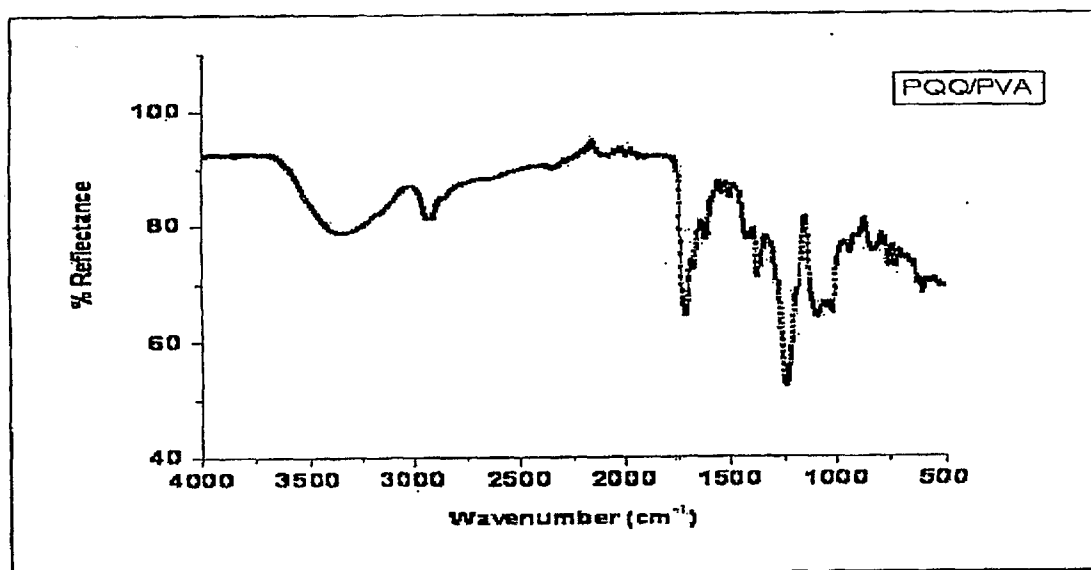


Figure 49

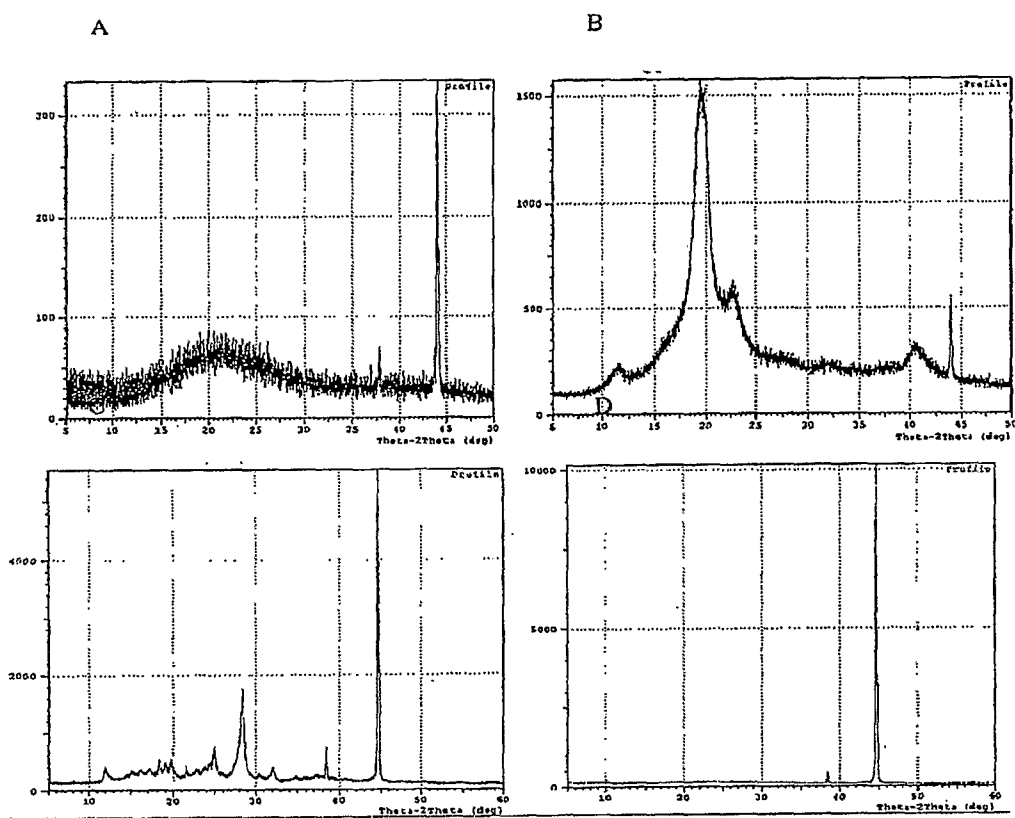


Figure 50

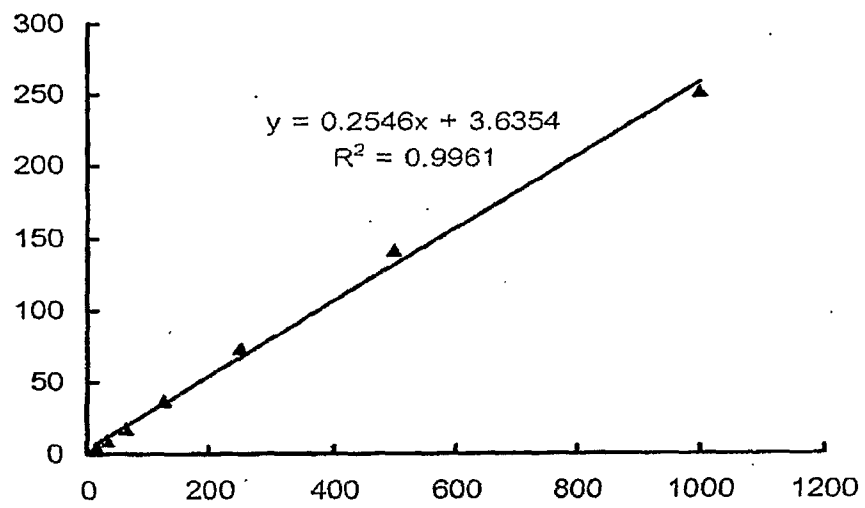


Figure 51

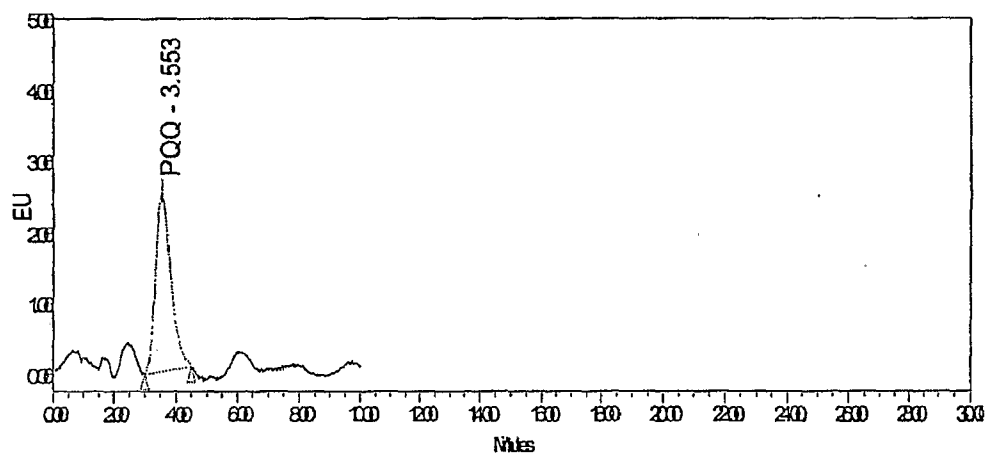


Figure 52

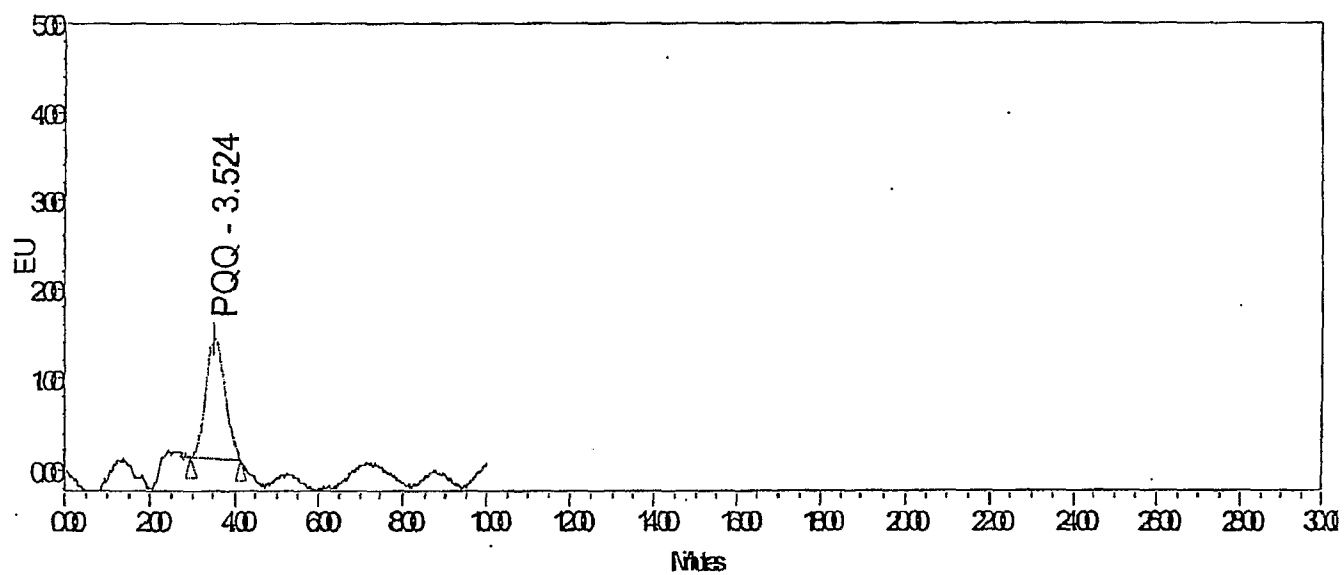
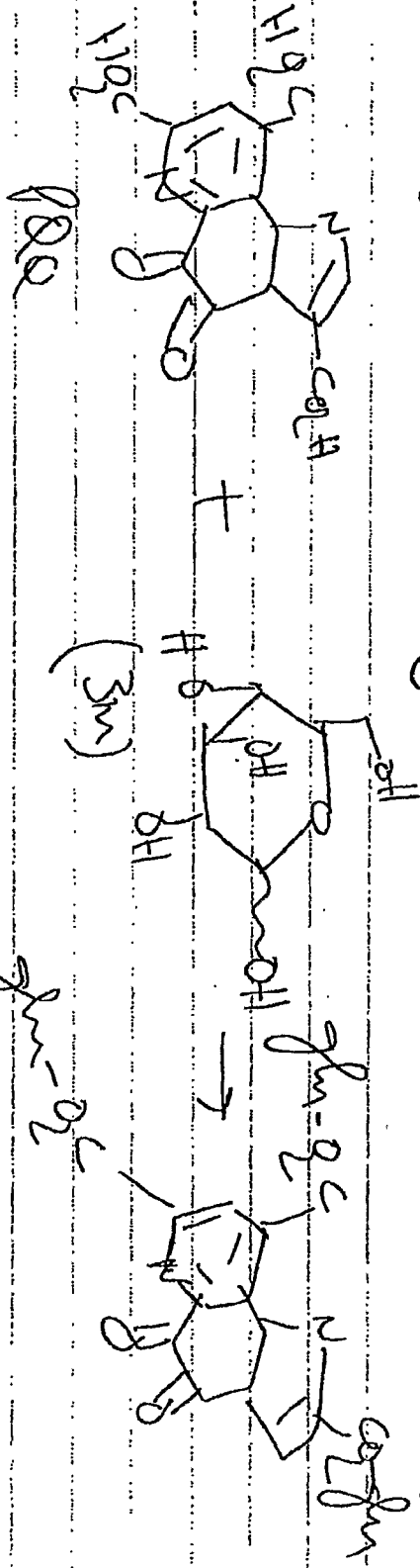


Figure 53

54A

Small Molar Weight (SM) FOD Sugars

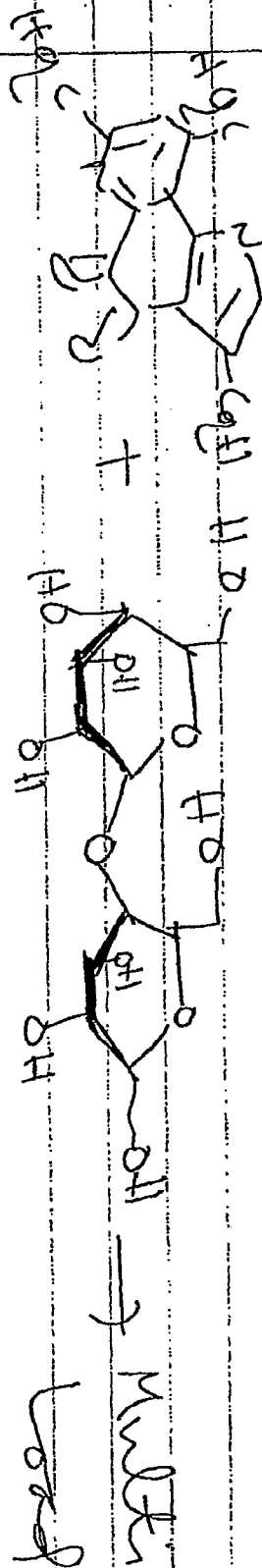
① Glucose Capping: (Other Monosaccharide also)



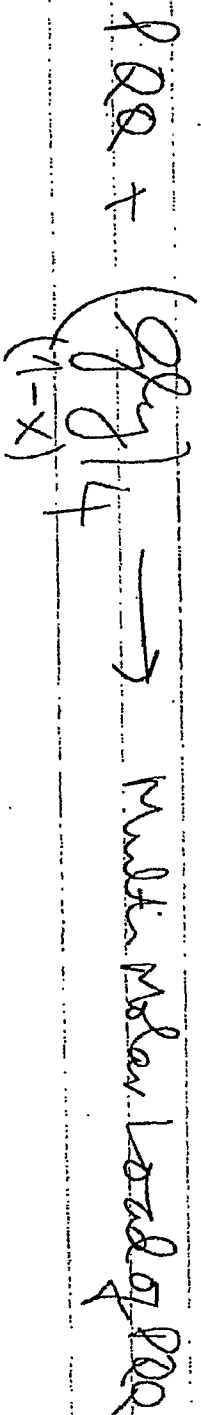
glu-O-C
glu = glucose (D)

54B

② Maltose & other disaccharide Capping of PQQ

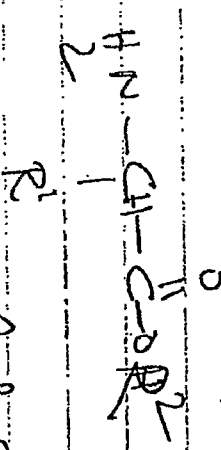
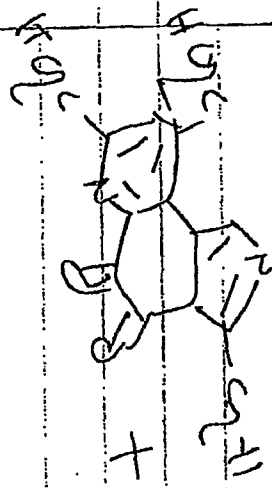


③ In & Tetrasaccharide Capping



54C

① Amino Acids & Derivatives Capping



Amino Acid (AA)

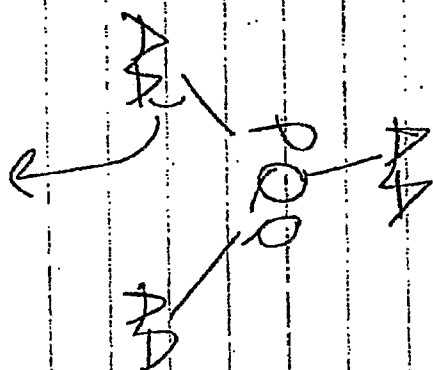
R¹ = Amino Acid Side chain

R¹ = H, Glycine

R¹ = CH₃, Alanine & ...

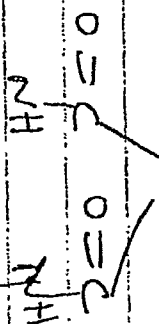
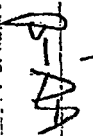
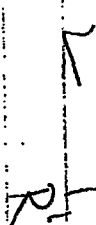
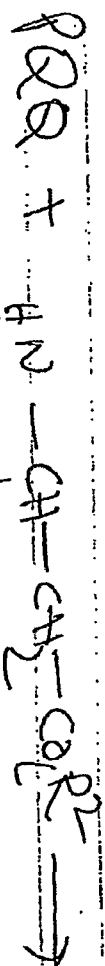
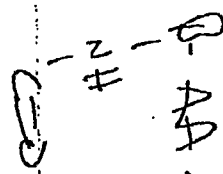
Amino Acid Derivatives of COOH

β-amino acids also

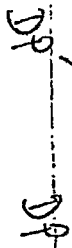
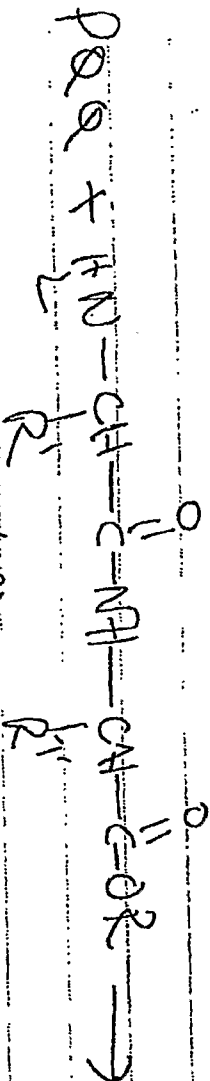


Linked by amide bond

54D

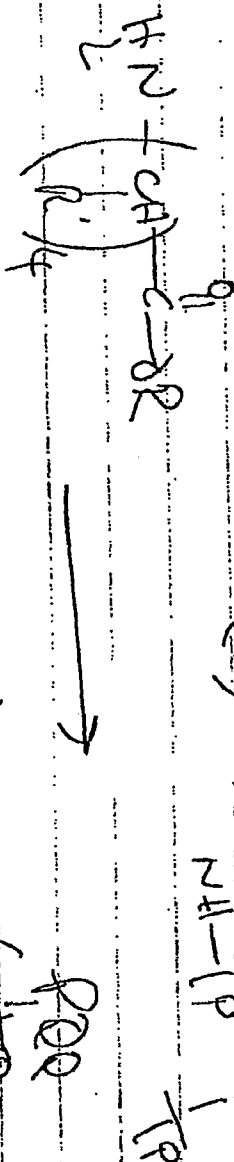
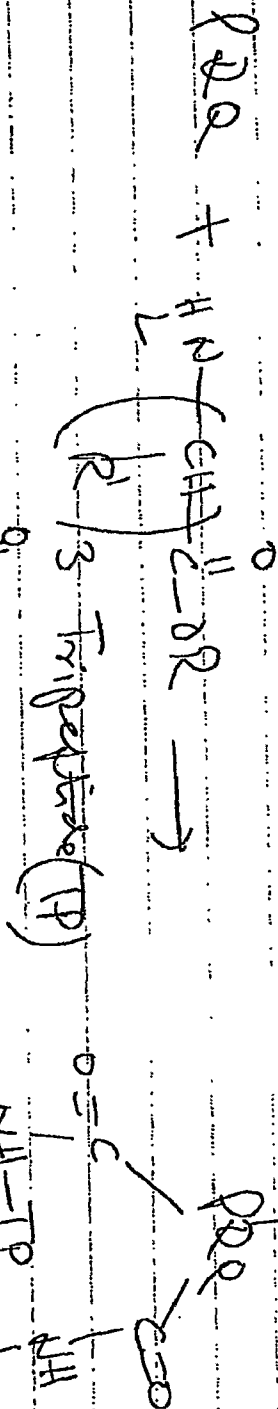


5) ~~2~~, ~~Tripeptide & Tetrapeptide~~ ~~approx PQQ~~

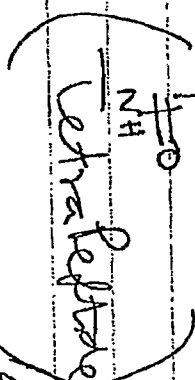


Through linkage

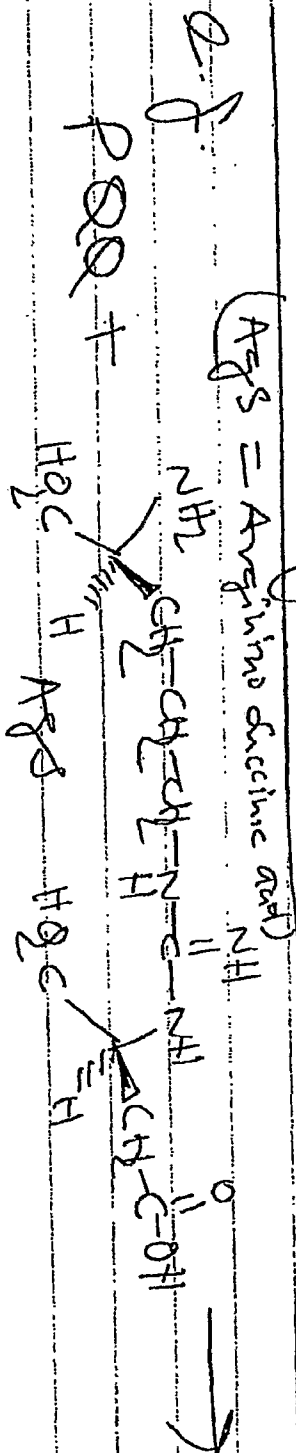
54E



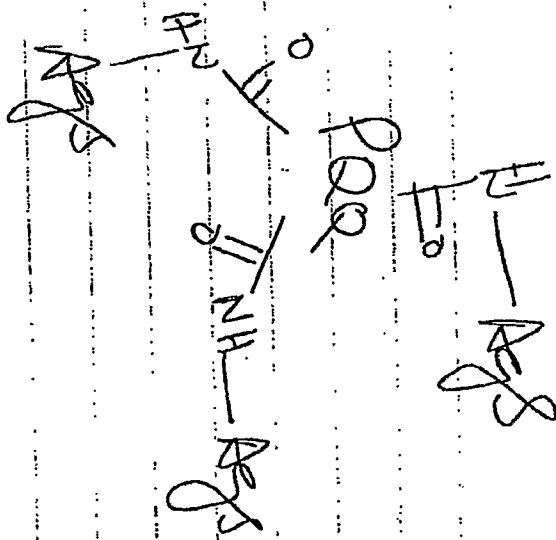
Tetrapeptide (TetP)



⑥ Amino Acid, Organic Acid (AAs)



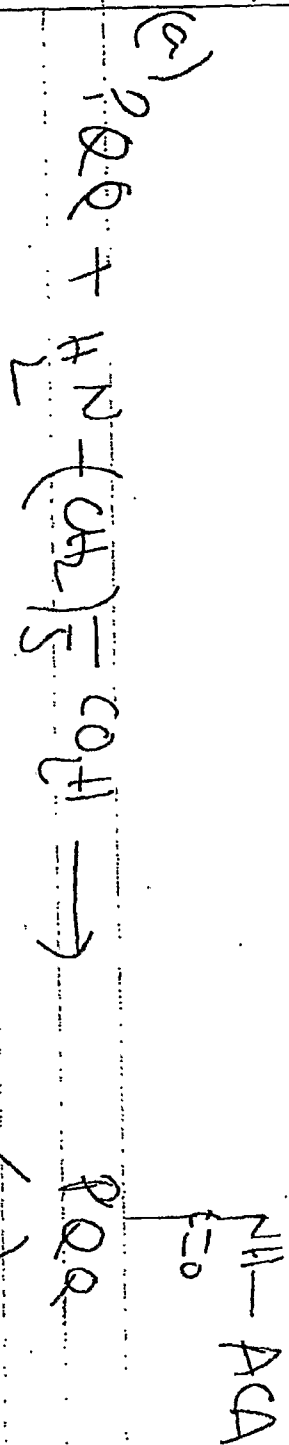
54F



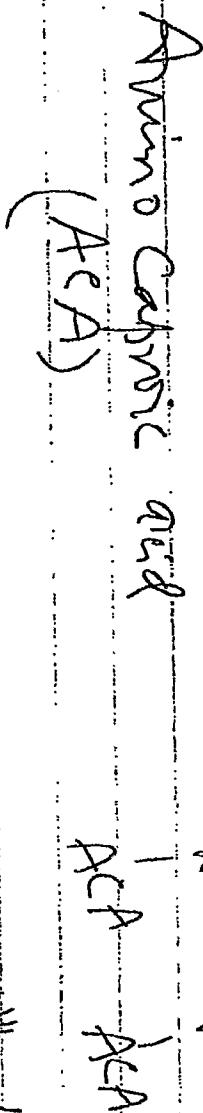
⑦ Lipid (Low MW) capped PO₂

e.g. ε-aminocaproic acid (ACA)

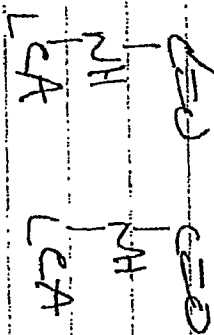
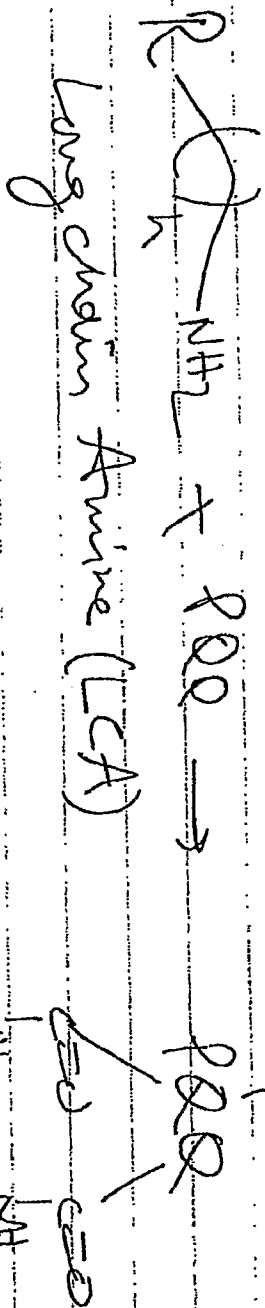
546



(Variable methylene chain)



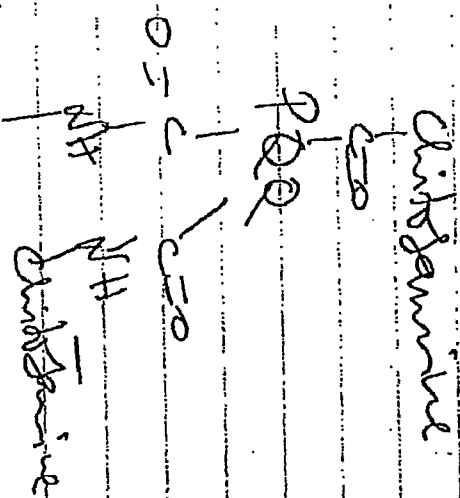
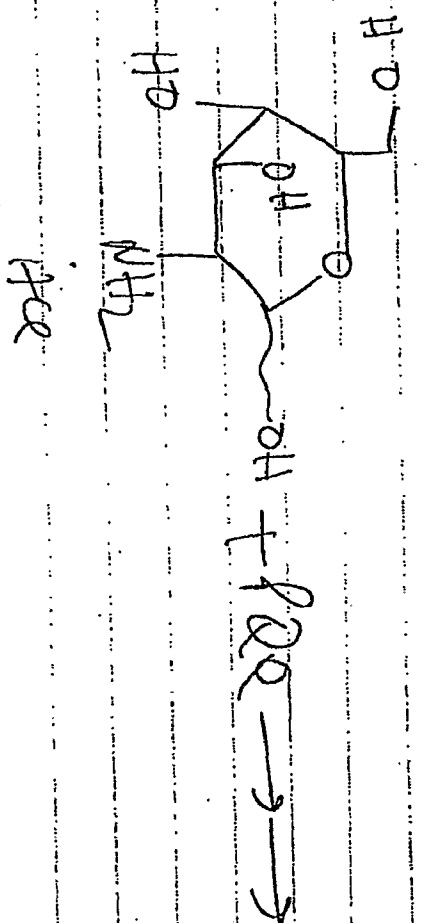
(v)



54I

(10) Glycosamine Based Capping of PQQ:

(5) Chitosamine (Mono Secondary Base)

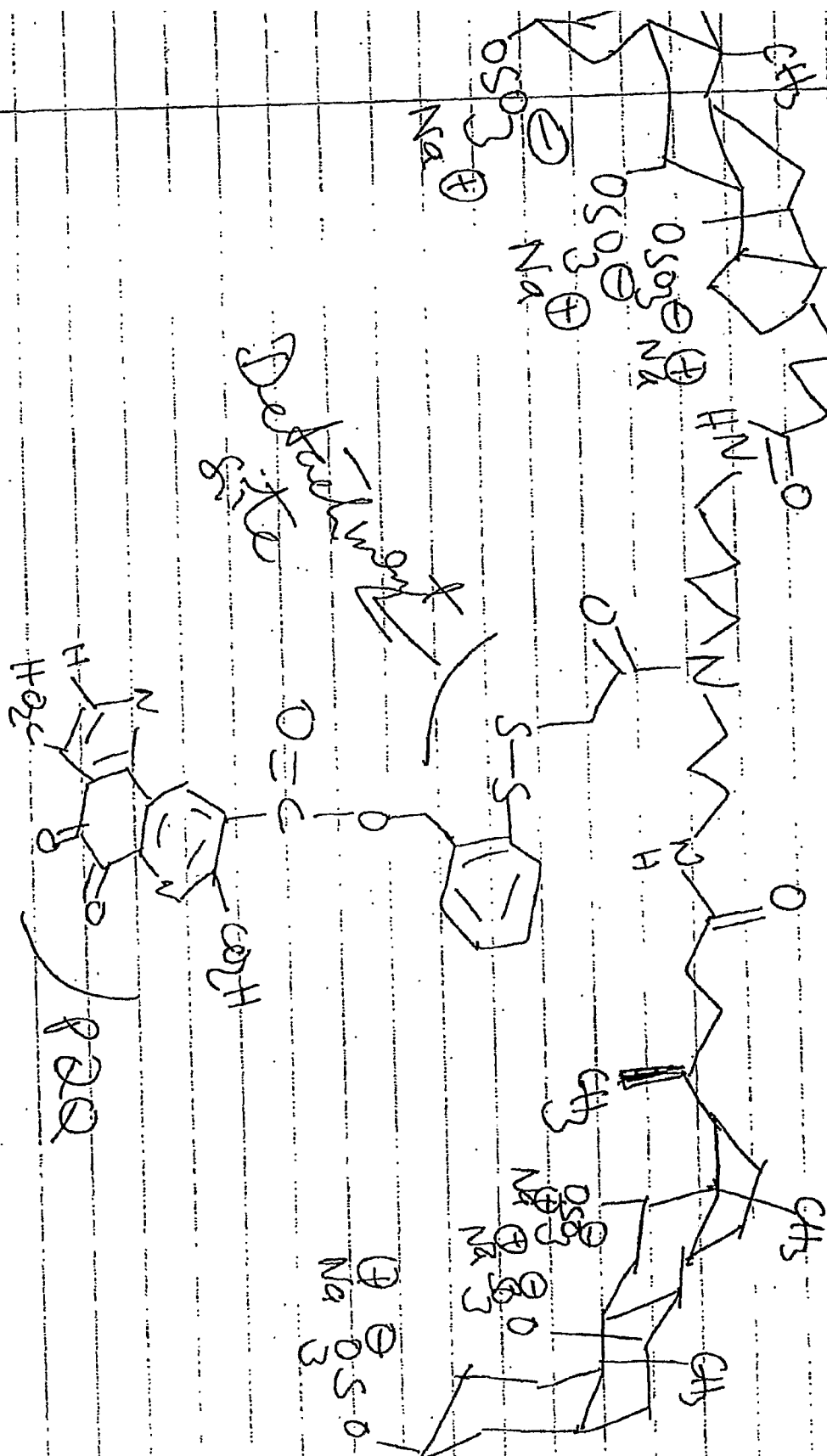


(10) Di, Tri & Tetra saccharide Based Cap:

Chitosamine

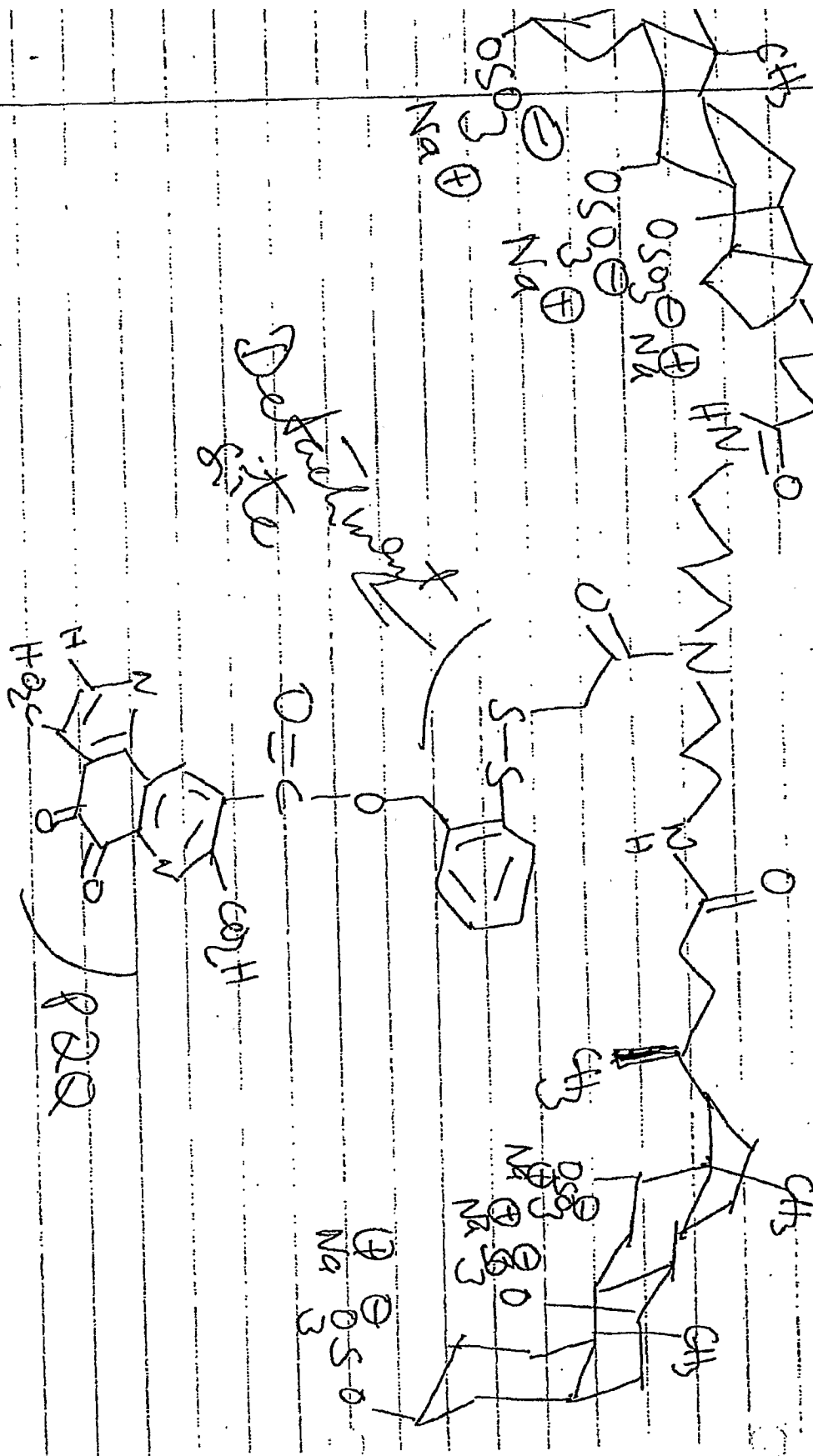
545

Sulfated Steroid



54K

Sulfated Steroid

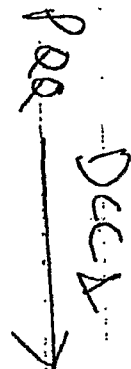


542



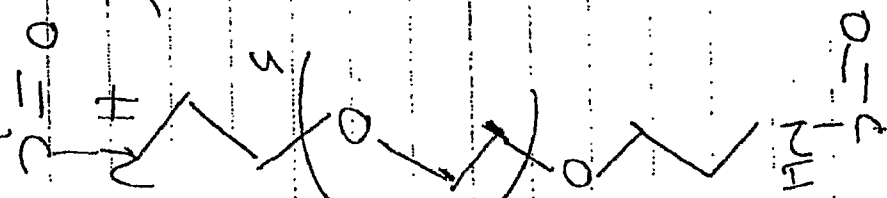
PEG-1 lignine

(mm - 3400)

$$(n \sim 90 \text{ qubits})$$


Post

(Hydroxy Ben
trazole)


$$\text{Inside } \sqrt{\text{leaf} - (0 \pm 2)}$$

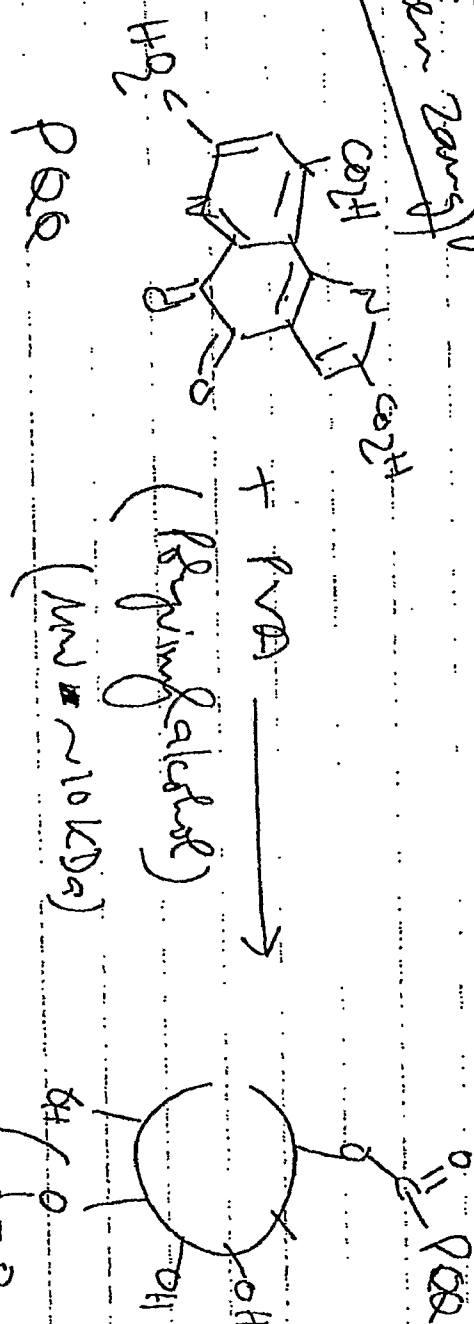
Pa-Pis living Pa

5410

PDQ - Polymer conjugation: 9/14/05
9/13/2005

Work in Progress:

(Hsinfen 2005)

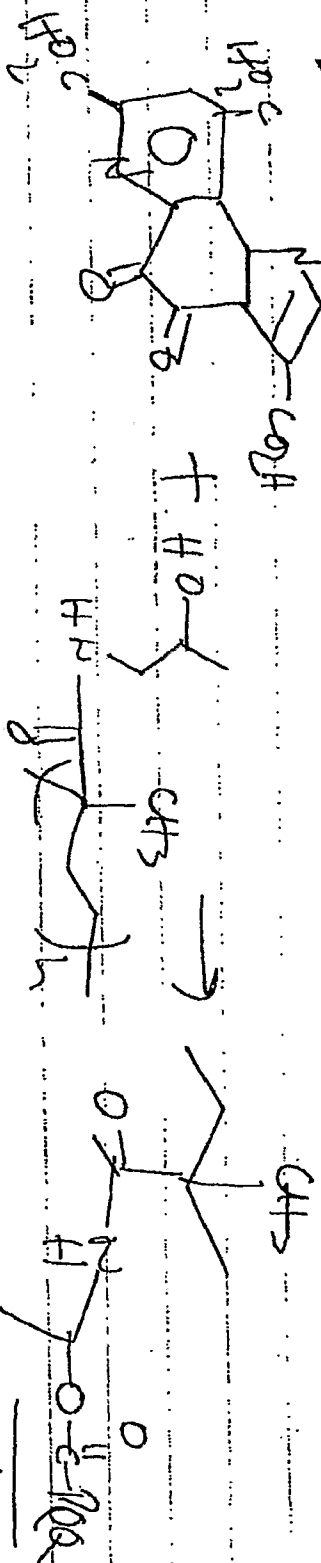


Site of linkage (via ester linkage by carboxylic end).

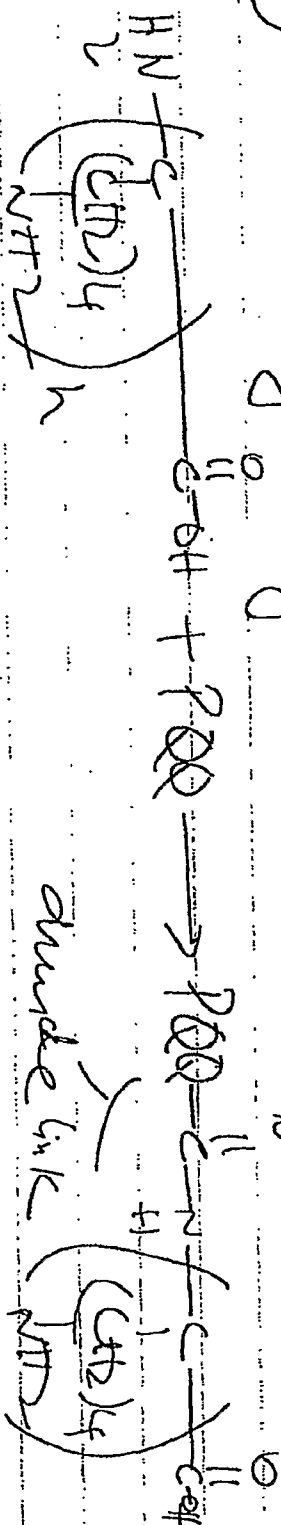
540

(3) PQQ-P(HDMA)

[P(HDMA): Poly (N-Hydroxy Pivaloyl)methyl acrylamide]

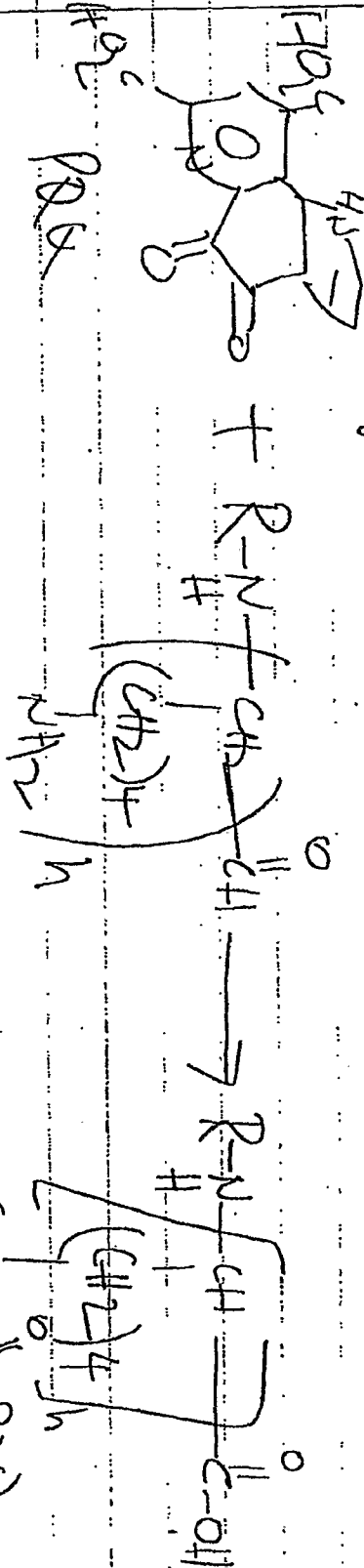


(4) PQQ-Poly-L-lysine (Mono Serin)

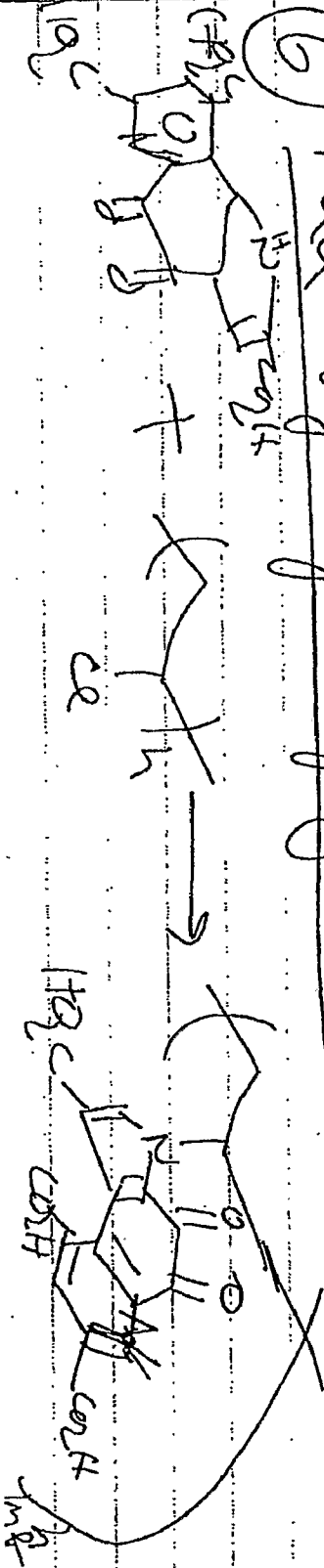


510

⑤ Paa - Pgy + lysine! Multiple load conjugate! Molan
Ratio defined.

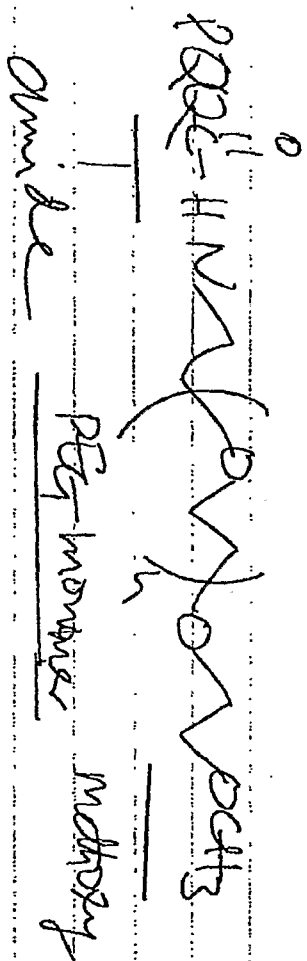
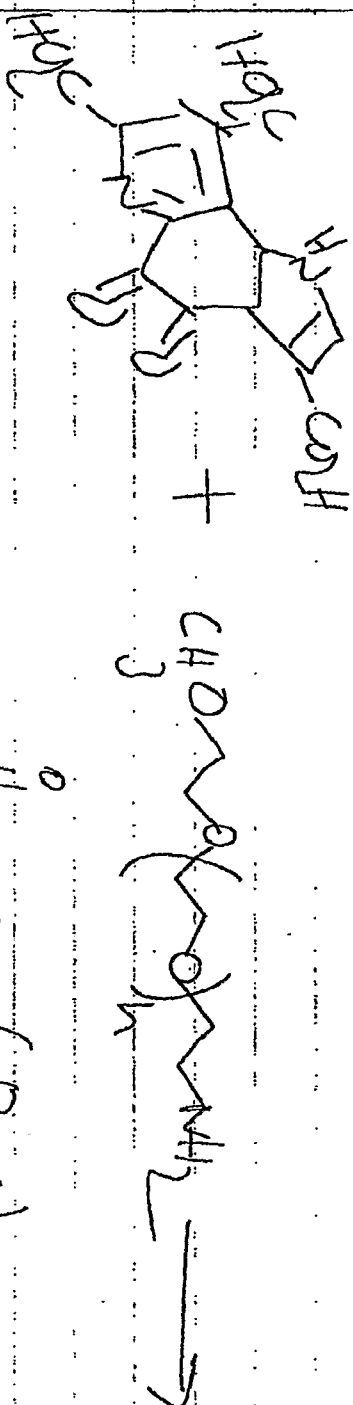


⑥ DD-Polyvinyl Carbazole
12-11-94



54a

⑦ POO-MPEG-NH₂ conjugate:

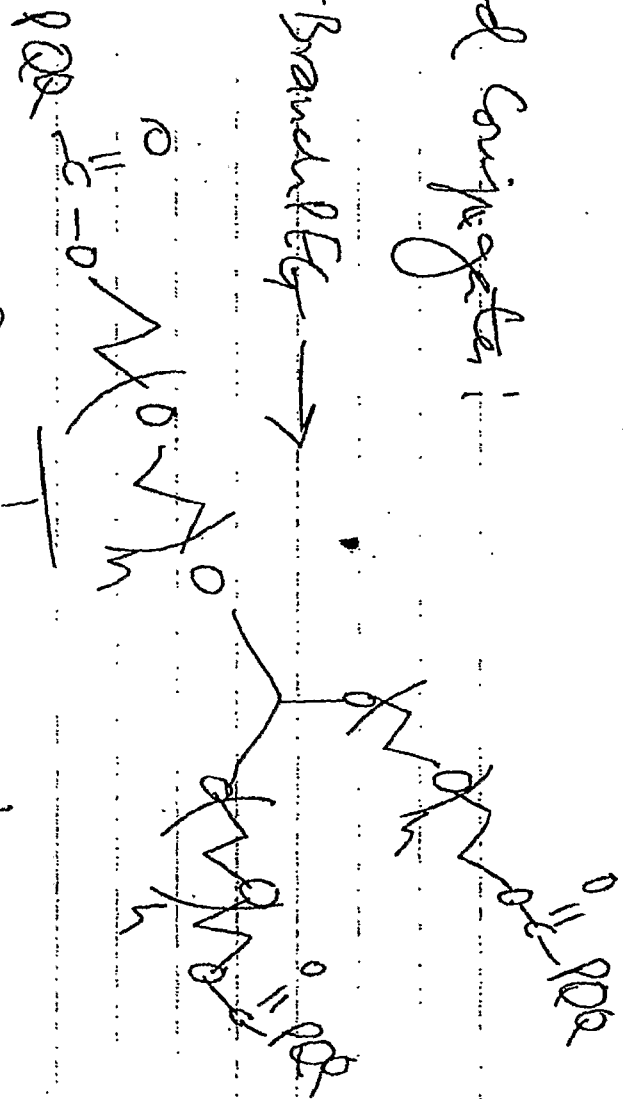


Amide PEG monomer Methyl

5412

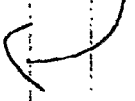
⑧ PAA-Tetrad Conjugates:

(a) PAA + 3-Branch PEG $\rightarrow$

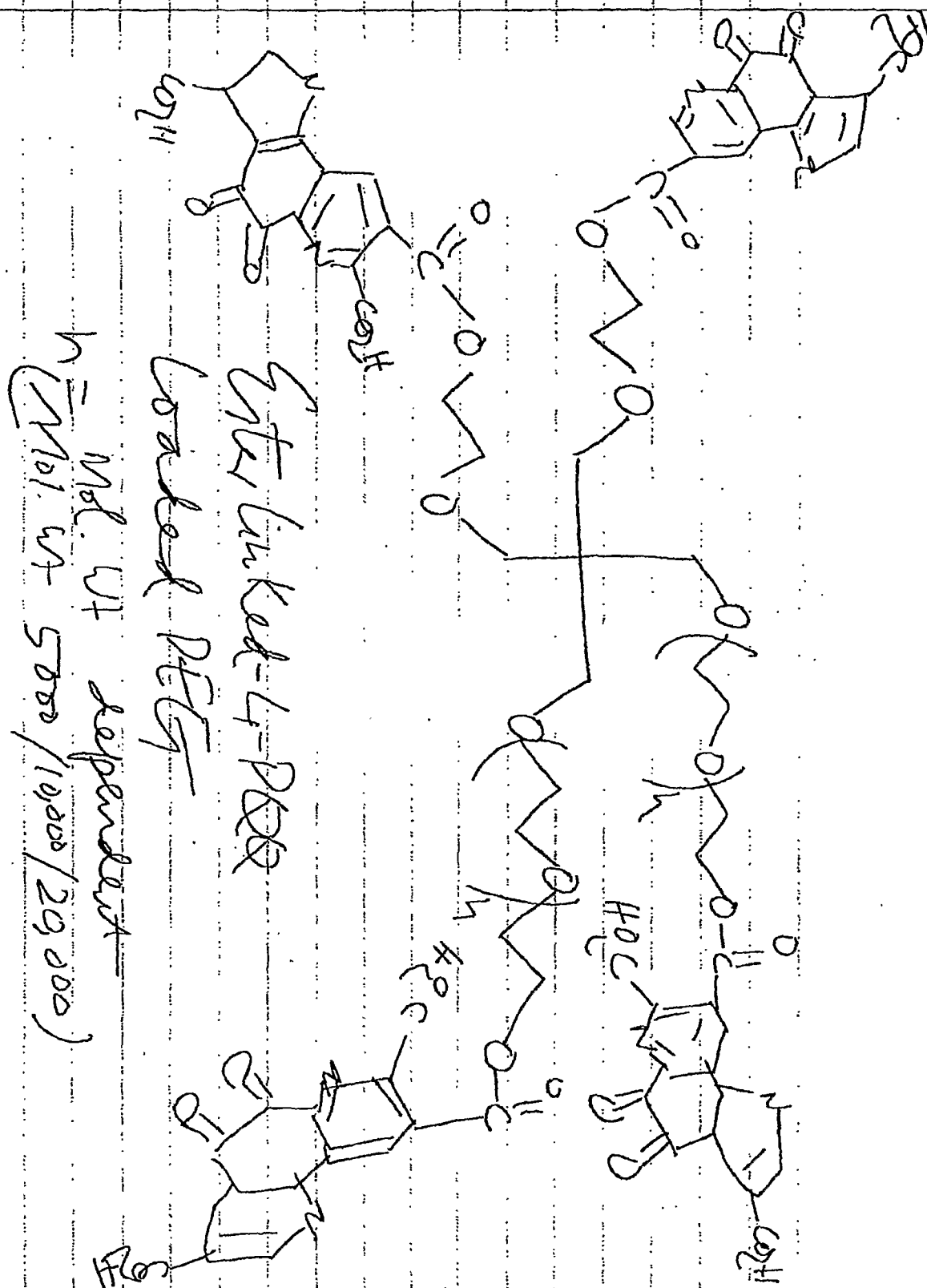


n' dependant on Mol wt.

(b) PAA + 4-Branch PEG $\rightarrow$

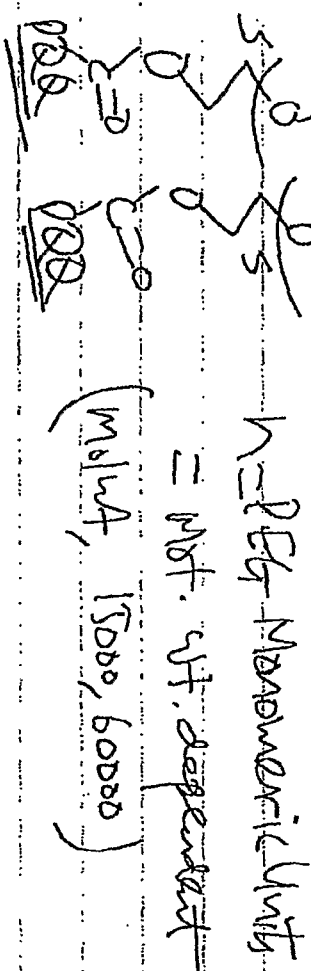
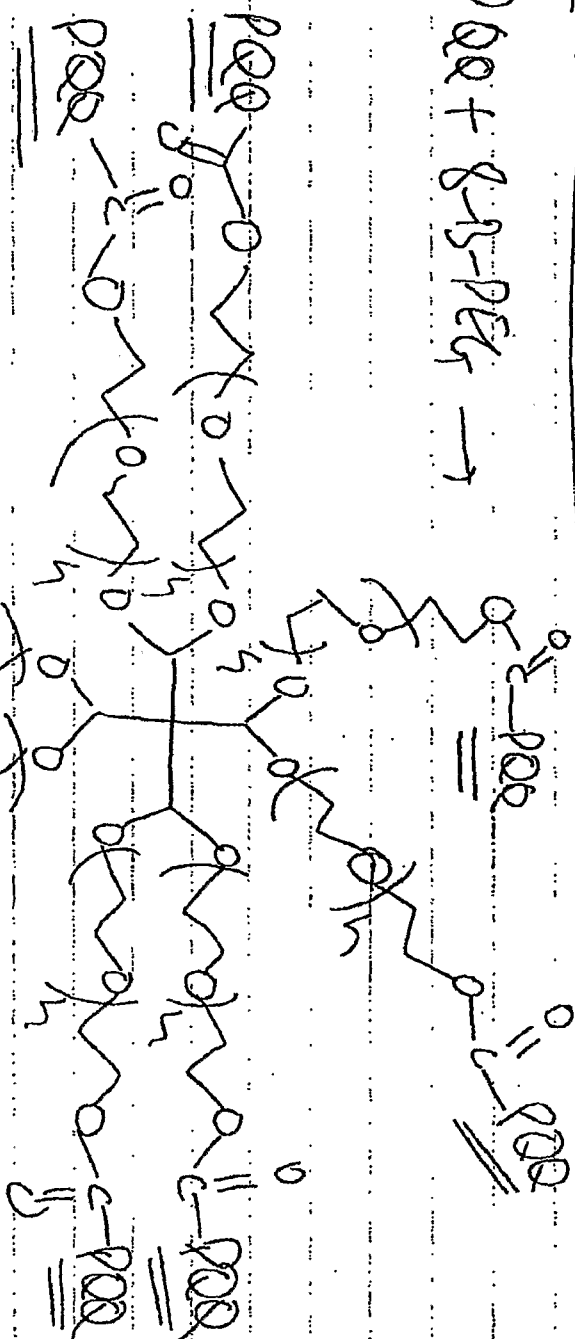
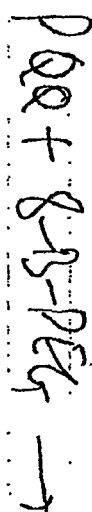


545



54T

B(c) POO-8-Branched PEG:



54v

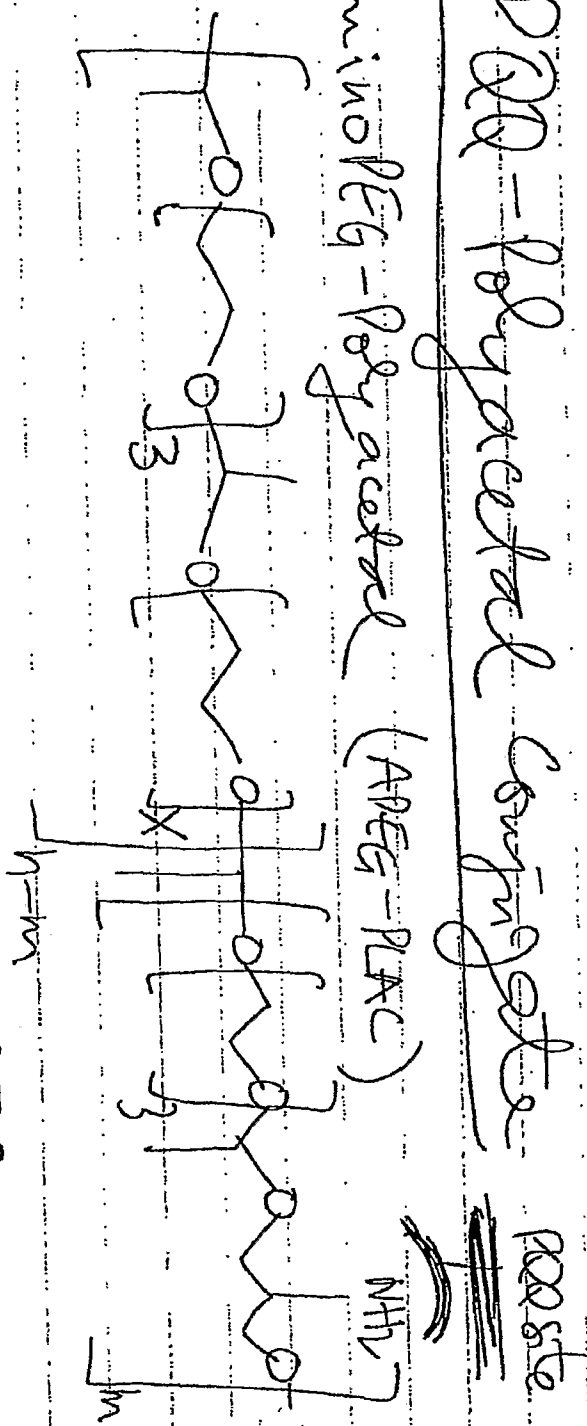
⑤ Biological Amine Conjugates:

Spermine, Spermidine based Conjug

⑩

PGA-Polyacetal Conjugate

AminoPEG-Polyacetal (APEG-PAC)



Amino-Pendant Polyacetal

5400

- pH-dependent, Biodegradable, Non-toxic (HEPES)
- comparable to $Pg(HEMA) / Pg(HDMA)$

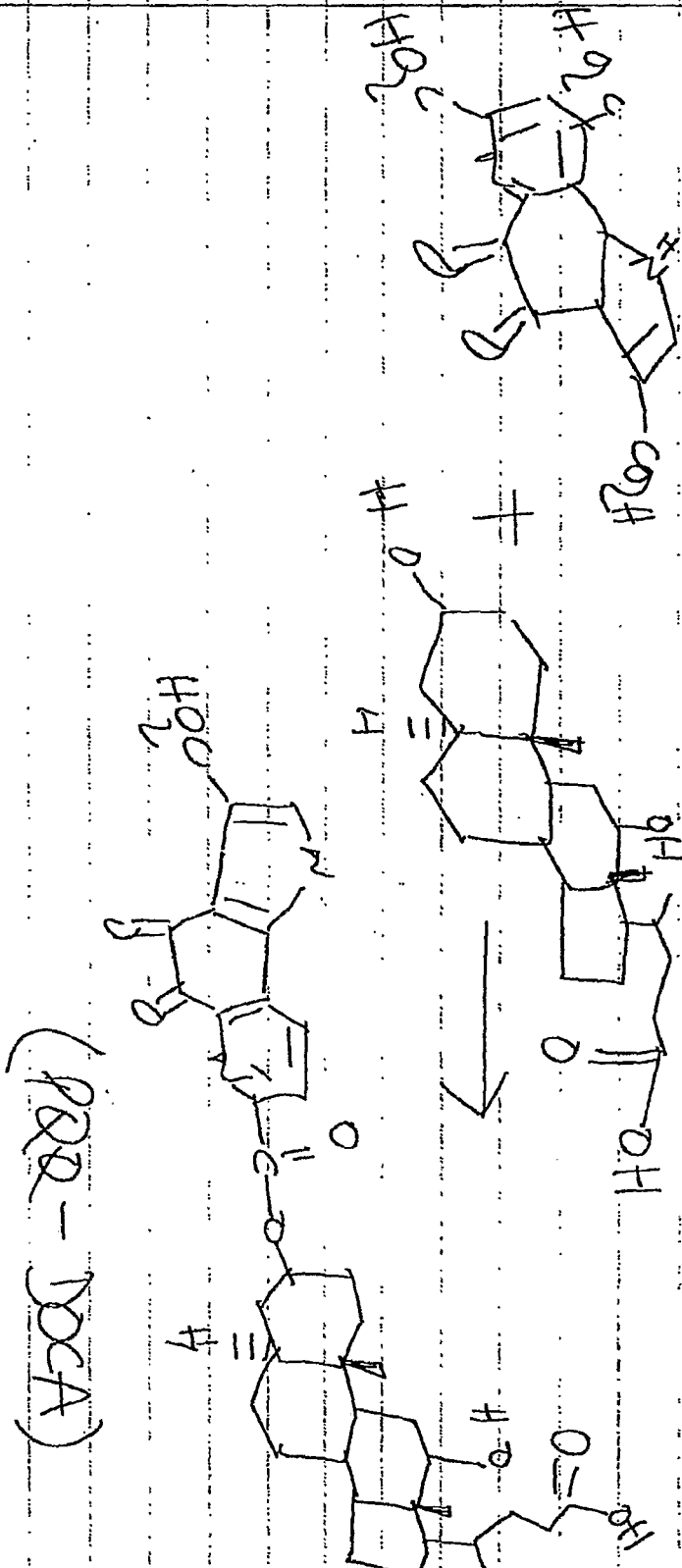
(11)

PQQ-Dendrimer conjugates:

PAMAM Based, Amino & Acid, Alcohol
ending with 4, 8, 16, 32 PQQ-moles
load.

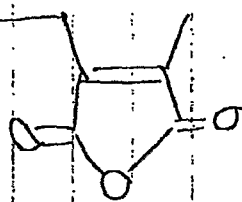
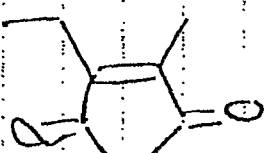
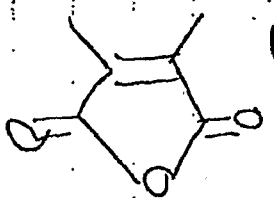
54X

⑫ PQQ - Deoxy Cholic Acid (DCA) conjugate!

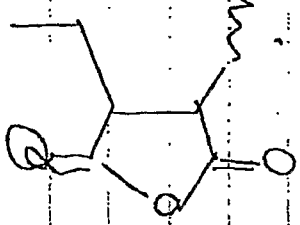
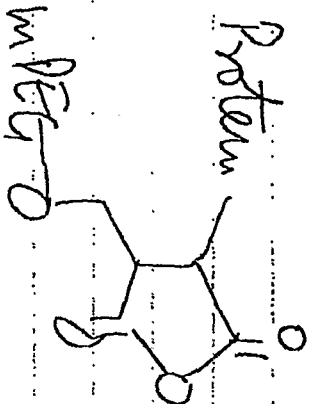
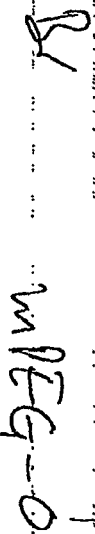


549

⑬ Polymer-Protein-POO- conjugate!



Protein/Peptide →



(Protein = Albumin, Avidin etc.)

Henry Lane

Permade

Arginine-rich

Bymer

220

Computer

5

2000

Wt.

15. (f, g)

[illegible]
$$\log(x) = \log - \log$$

My - ex - am - ple - Ar - tist

Ag 2

27

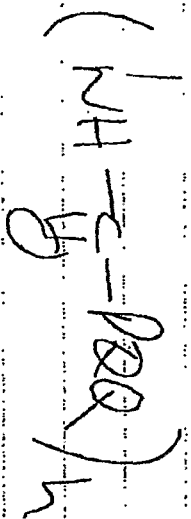
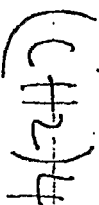
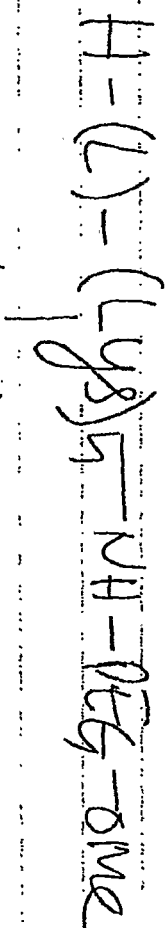
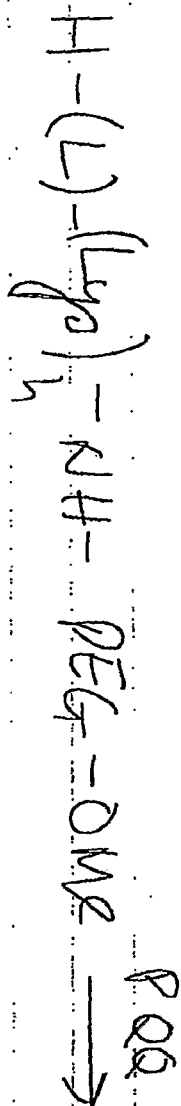
Argentina

100 mg.

$$L_f = L_{\text{force}}$$

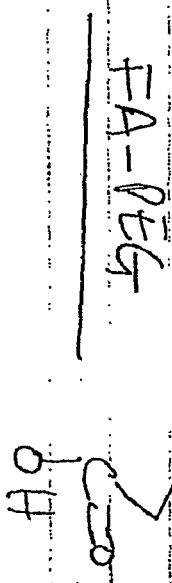
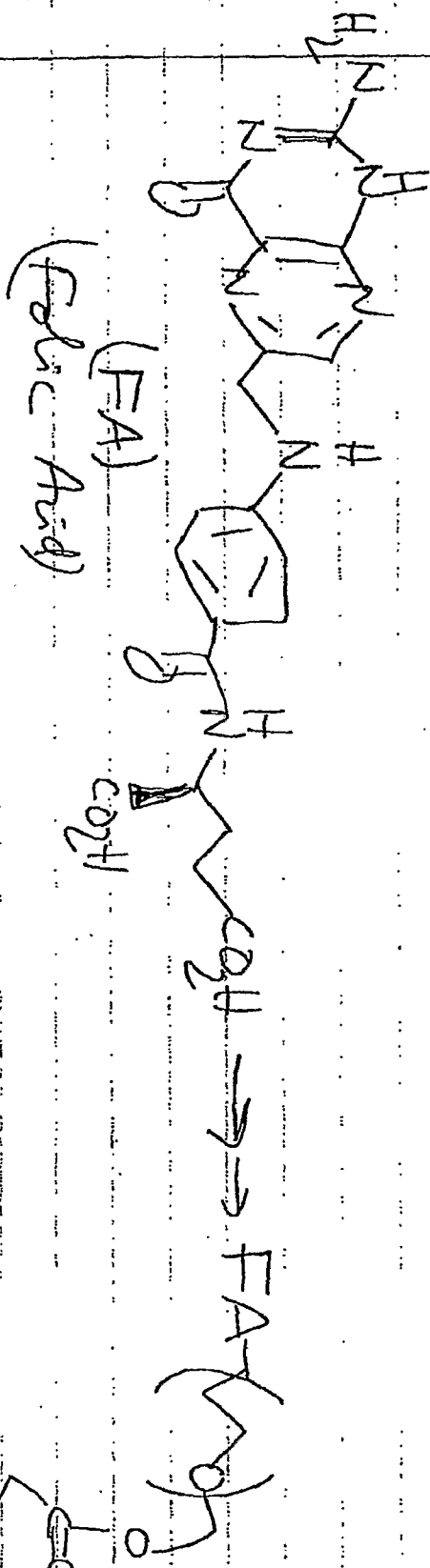
544A

(15) PEG-Polypeptide-Polypeptide

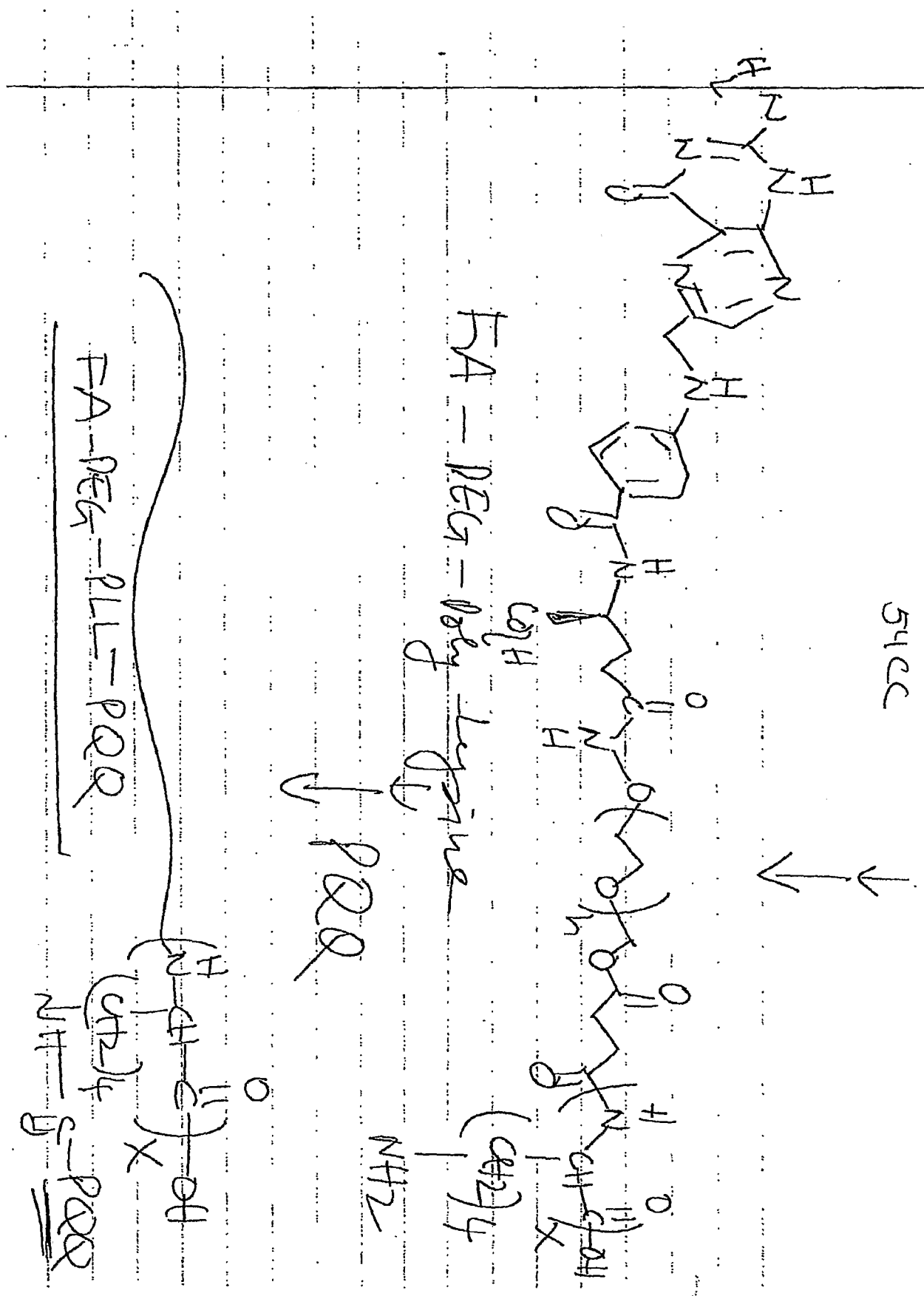


54BB

⑥ Folic Acid-PEG-PQQ Conjugate



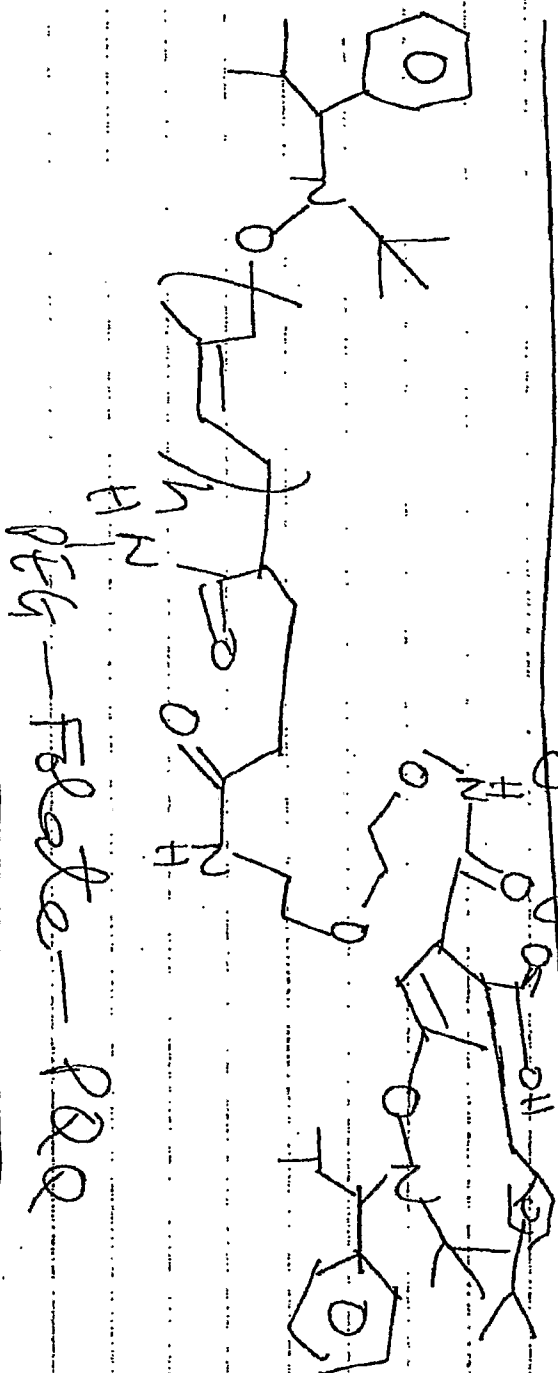
PEG-Lysine
 (PLL)



540D

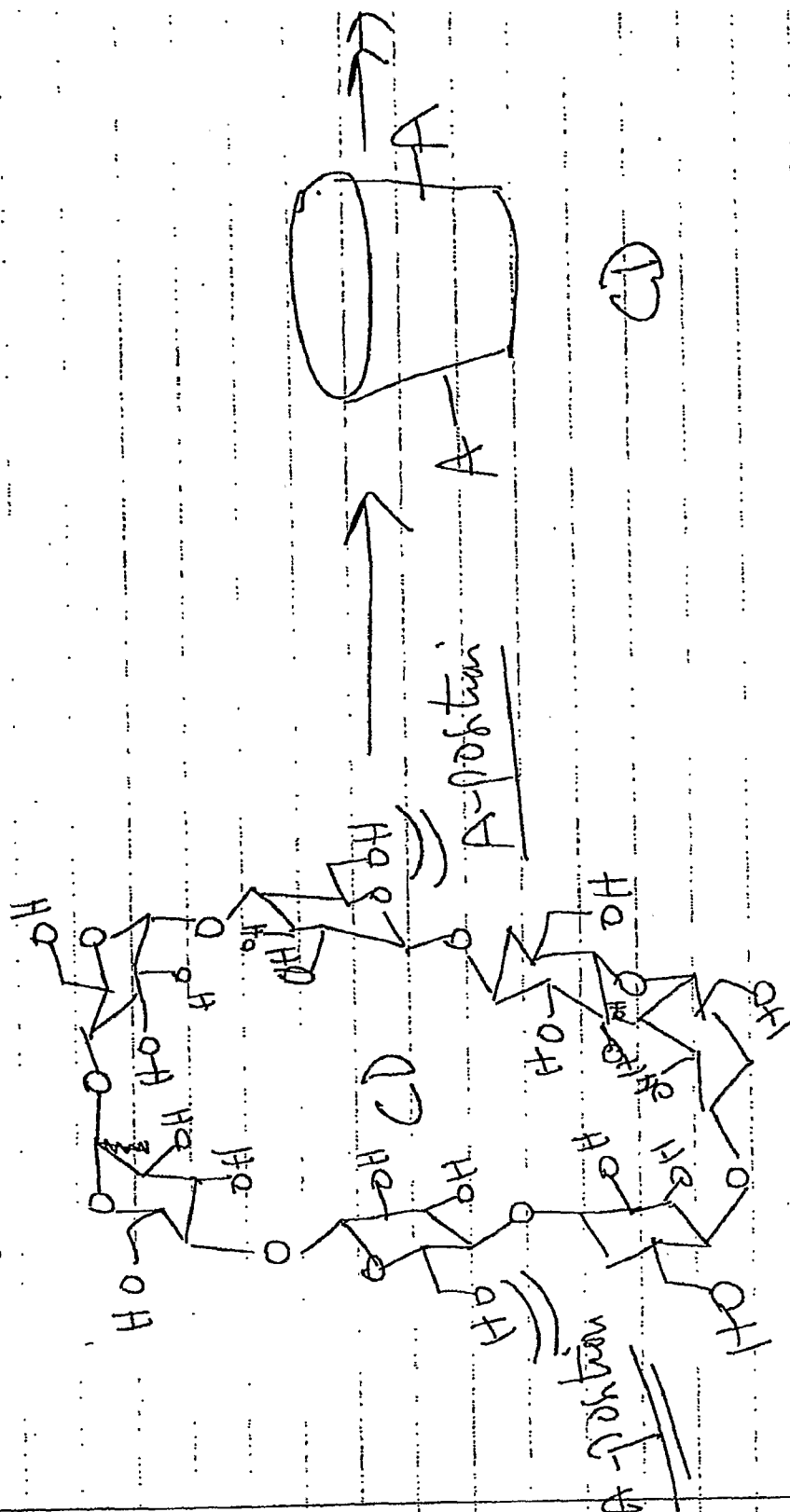
(17)

Folic Acid-PEG-Polyacrylate-PQQ

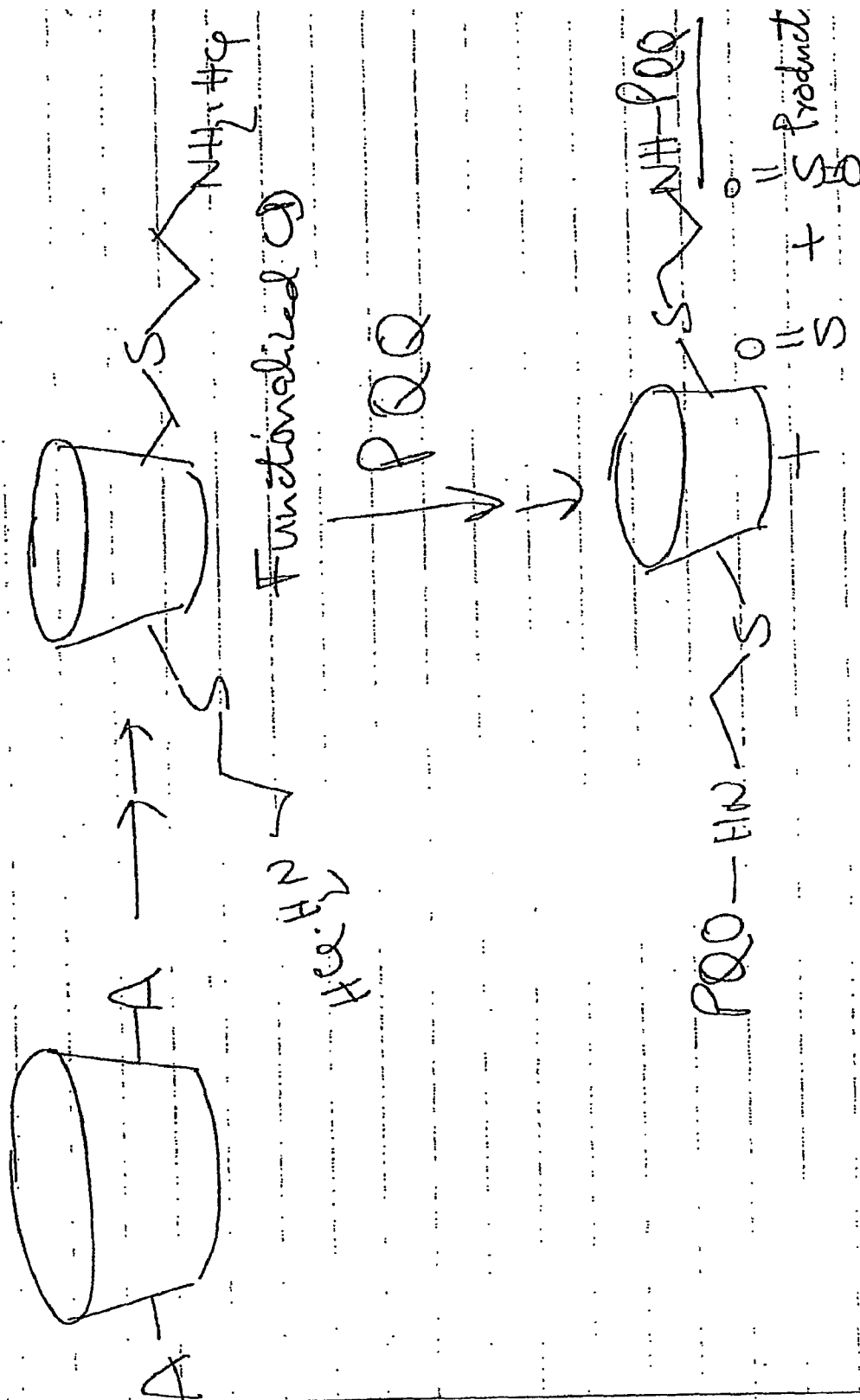
PEG-Folate-PQQ

33125

(18) Cyclodextrin- β -D-Galactose conjugate:

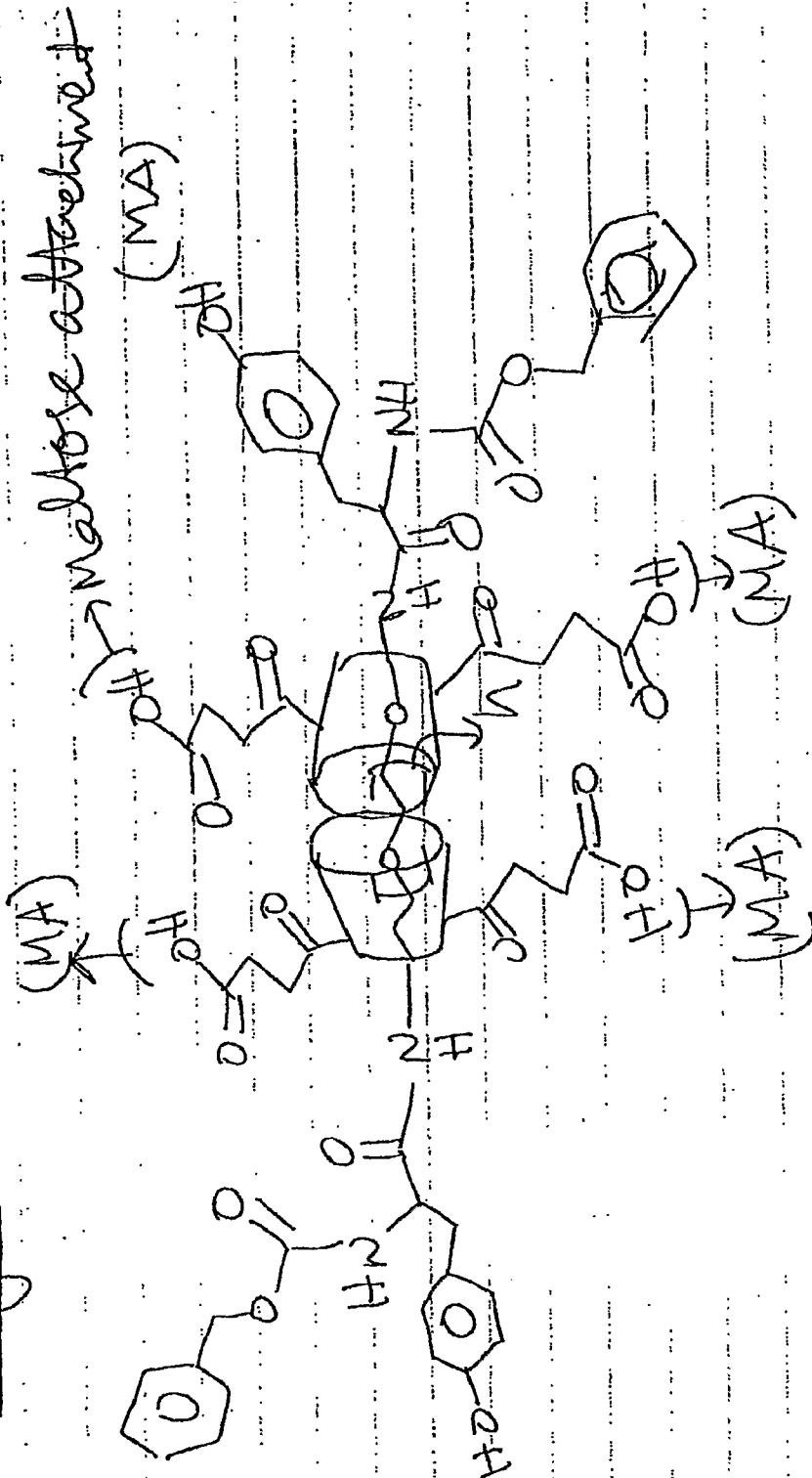


54 FF

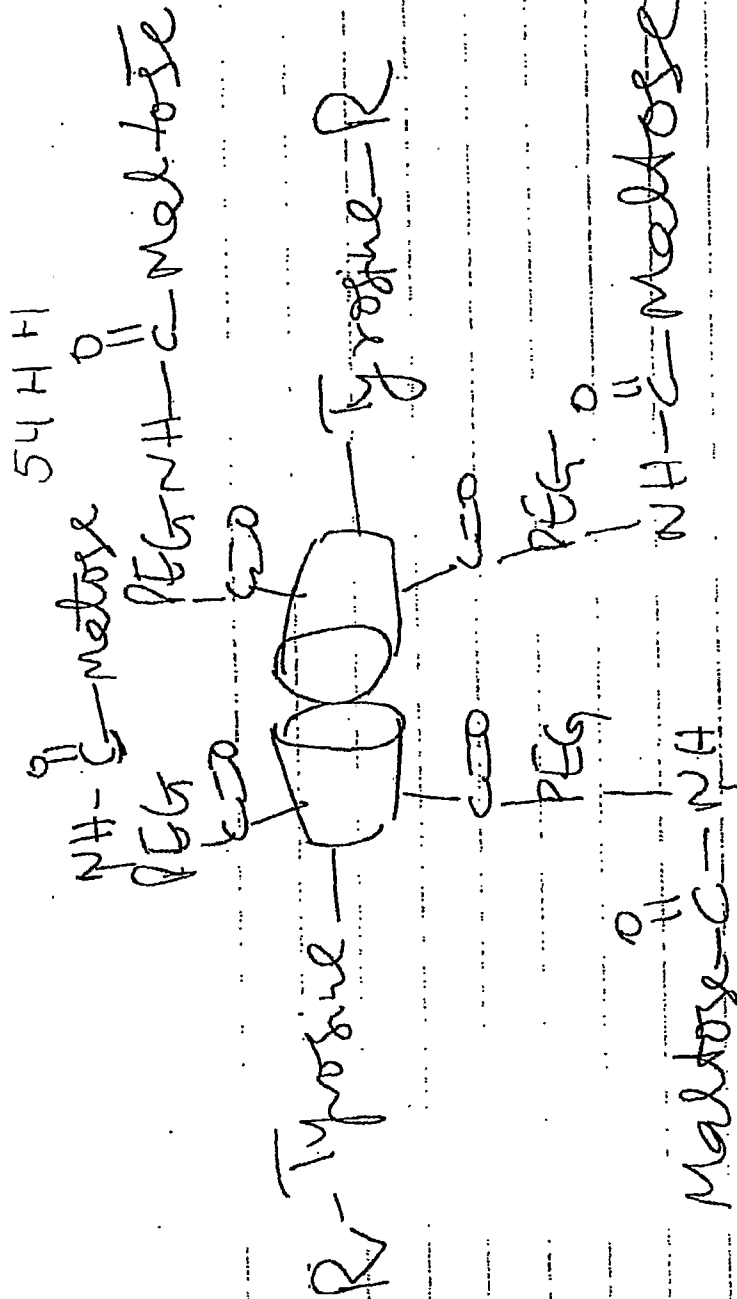


5466

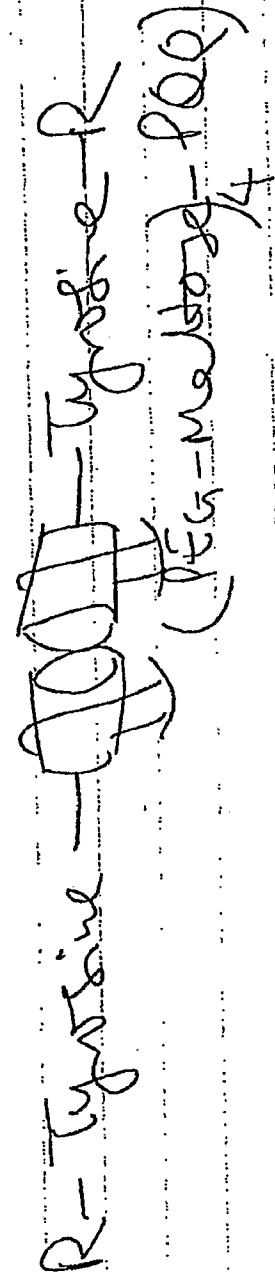
(19) polyoxetane-PEG-maltose-tyrosine-PQ1-



MA via -NH₂ functionalization



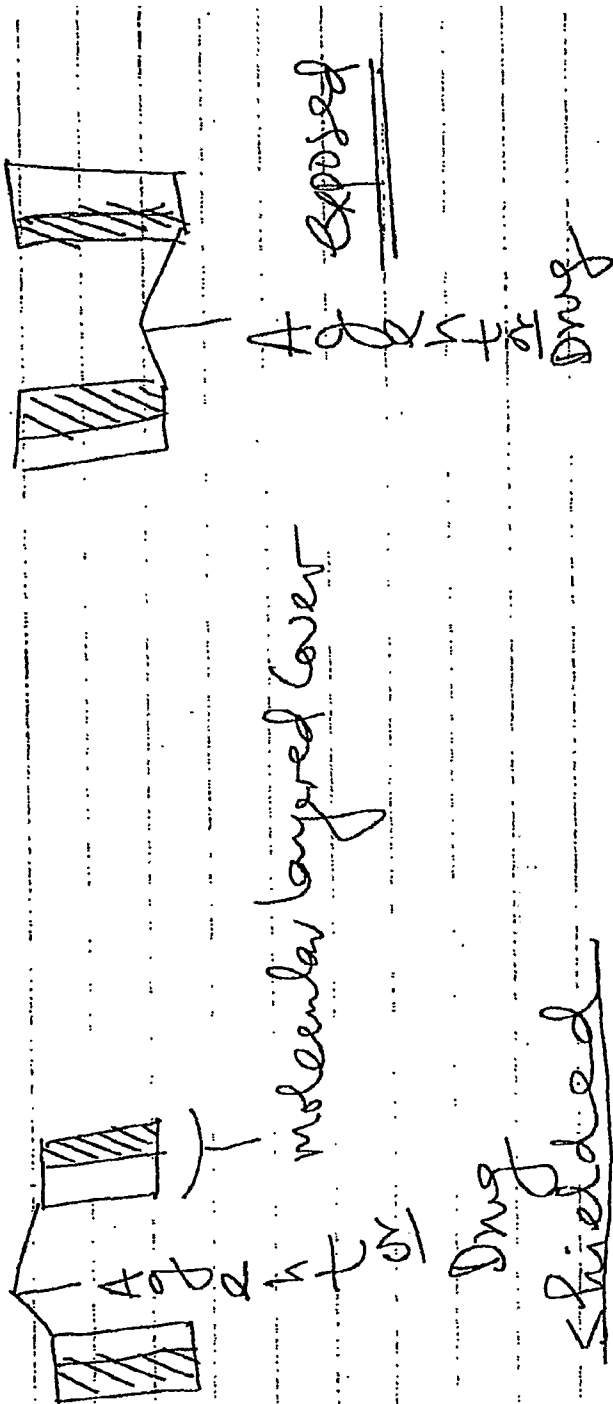
↓ PQQ (Molar Ratio)



54 II

(20) Molecular Umbrella Based Configuration

- Detachable by Triol linkage
- In shielded and exposed forms



5435

Sulfated Seccharide

