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(54) **PROCEDE ET COMPOSITION POUR INHIBER LES
MODIFICATIONS CELLULAIRES IRRÉVERSIBLES DUES
AU STRESS**

(54) **METHOD AND COMPOSITION FOR INHIBITING CELLULAR
IRRÉVERSIBLE CHANGES DUE TO STRESS**

(57) L'invention concerne des formulations de composés naturels physiologiquement acceptables et leurs dérivés, permettant de traiter le stress cellulaire, et en particulier, l'hypoxémie. L'administration de ces formulations, comprenant essentiellement des purines, des sucres, des acides aminés et de leurs dérivés physiologiquement acceptables de ces derniers, seuls ou en association les uns avec les autres et avec d'autres composés, en particulier, des composés électroaccepteurs, permet de prolonger sensiblement la période jusqu'aux modifications cellulaires irréversibles, plus précisément, la mortalité.

(57) Formulations of naturally occurring physiologically acceptable compounds and their derivatives are provided for treatment of cellular stress, particularly hypoxia. By administering the formulations, comprising for the most part purines, sugars, amino acids and physiologically acceptable derivatives thereof, by themselves or in combination with each other and with other compounds, particularly electron acceptor compounds, the time to irreversible cellular changes, particularly mortality, can be greatly extended.

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(54) Title: METHOD AND COMPOSITION FOR INHIBITING CELLULAR IRREVERSIBLE CHANGES DUE TO STRESS (57) Abstract Formulations of naturally occurring physiologically acceptable compounds and their derivatives are provided for treatment of cellular stress, particularly hypoxia. By administering the formulations, comprising for the most part purines, sugars, amino acids and physiologically acceptable derivatives thereof, by themselves or in combination with each other and with other compounds, particularly electron acceptor compounds, the time to irreversible cellular changes, particularly mortality, can be greatly extended.		

**METHOD AND COMPOSITION FOR INHIBITING
CELLULAR IRREVERSIBLE CHANGES DUE TO STRESS**

CROSS-REFERENCE TO RELATED APPLICATIONS

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This application is a continuation-in-part of application serial no.
08/607,022, filed February 23, 1996.

BACKGROUND

10 There are many effectors of cell stress. These include infection, thermal,
cryogenic, osmotic, hyper- and hypo-gravity, transplantation, starvation, growth in
various reactors, such as bio reactors, fermentation, food preparation, etc., toxicity,
such as inhalation of various toxic gases, e.g. HCN, phosphates, thiophosphates,
etc., drug overdoses, and the like. The particular mechanism by which cells
15 respond to stress, become quiescent, and try to protect themselves from irreversible
injury is not well understood. It may involve one or more different mechanisms.

 Of particular interest is hypoxia or ischemia. Cells require energy to live and
carry out their given tasks. For many important cells in our body, oxygen is a
necessary ingredient to produce cell energy. The cell combines products from the
20 foods we eat with the oxygen we breathe to produce cell energy. This energy
powers the cell and sustains life. Without the proper amount of energy, the cell fails
to work and if the inadequate energy level is maintained for a period of time during
which the cell cannot maintain it's viable state, the cell will die. For tissues that are
carrying out many energy consuming activities, such as the brain and heart, the

absence of oxygen can result in cell death in as little as 7-15 minutes. Cells are always at work, producing energy to sustain life. When this process is disrupted, even for brief periods of time, death to a vital body part can result with death to the host. When inadequate amounts of oxygen are available to maintain normal
5 function, the cell is said to be hypoxic or experiencing hypoxia.

One may distinguish between metabolic hypoxia and bioenergetic hypoxia. These are distinguished between altered bioenergetic functions due to insufficient oxygen supply to mitochondrial cytochrome oxidase ("bioenergetic hypoxia") and altered metabolic functions due to insufficient oxygen supply to other enzymes that
10 require molecular oxygen as substrate ("metabolic hypoxia"). There is no agreement today as to the specific mechanisms associated with irreversible injury to cells during an extended oxygen starvation.

Because of the diverse conditions under which cells are stressed, both *in vitro* and *in vivo*, where one wishes to maintain their viability, there is substantial interest
15 in developing methods and compositions which will improve the outcome for the cells, in the event of oxygen starvation. There is, therefore, substantial interest in providing improved methods for reducing the probability of irreversible damage to cells during a limited period of stress, so as to allow the cells to have a higher probability of recovery upon removal of the stress condition.

20 General information concerning metabolic stress may be found in Atkinson, Cellular energy metabolism and its regulation, 1977, Academic Press, NY; Hochachka et al., Surviving hypoxia: mechanisms of control and adaptation, 1993, CRC Press, Inc., FL; and Alberts et al., Molecular biology of the cell, 1994, Garland Publishing, Inc., NY.

25 Angel K. Markov, in U.S. Patent Nos. 4,546,095, 4,703,040, 4,757,052 and 5,039,665, teaches the fructose-1,6-diphosphate, a member of the glycolytic process, is useful in the treatment of anaerobic conditions, such as hemorrhagic shock and adult respiratory distress syndrome.

30 SUMMARY OF THE INVENTION

Methods and compositions are provided for enhancing cellular resistance to irreversible changes in cellular function, including mortality, due to stress. The

stress might take any form, particularly lack of oxygen. The stress is relieved by adding compounds to the cells, which compounds are added individually or in combination, with each other and/or other compounds and include purines, purine nucleosides and derivatives thereof, sugars, electron acceptors, amino acids, and particular individual compounds, as well as reaction products thereof, in an amount sufficient to inhibit irreversible changes in cellular function, particularly leading to mortality. Combinations may be mixtures or covalently bonded combinations, particularly esters. The compositions find use in storing cells, tissues and organs, in fermentation and in the treatment of hosts under stress.

10

DESCRIPTION OF THE SPECIFIC EMBODIMENTS

Treatments of stress are provided involving individual or combinations of compounds which serve to enhance mammalian cellular resistance to irreversible changes in cellular function. The stress may take any form, but a particular interest is hypoxia. The stress may be a result of a wide variety of conditions, as described above, both *in vitro* and *in vivo*. The time of onset of irreversible changes, as may be evidenced by cellular release of lactate dehydrogenase into the extra-cellular medium or formation of lactate, or cellular mortality under stress, may be greatly extended. Depending upon the particular environment in which the subject compositions are employed, the compositions may be added to an extracellular medium, administered to a host orally or parenterally, or by other convenient means.

Mammalian cells can be subjected to stress in a wide variety of environments. When growing cells, carrying out fermentation, using cells for production of various products, performing cellular modifications of substrates, and the like, cells may be subjected to stress. A particular problem is the inability to maintain a sufficient oxygen level in the medium, so that some portion of the cells will from time to time be oxygen starved. This can result in the loss of yields of a desired product, the release of cellular contents, which can interfere with the isolation and purification of product, clogging of pores of cellular reactors, and the like.

In *ex vivo* situations, where one wishes to store cells, there is great interest in maintaining the viability of the cells. This occurs in organ transplantation, cryogenic preservation of cells and tissue, and the like. The cells or organ may be maintained in a medium comprising the subject compositions during storage. Prior to
5 introducing the stress, such as freezing, one may combine the cells with the protective composition to inhibit stress during the freezing, storage, and restoration. Thus, the cells, tissue or organ may be combined with the appropriate medium containing the subject compositions and then treated in accordance with conventional conditions.

10 There are also numerous situations *in vivo*, where one is concerned with stress. The subject methods may be used for prevention before the onset of hypoxia. The situations include elective revascularization, such as surgical ischemia, transplantation, carotid endarterectomy, and surgical hypoperfusion. Specific situations involving surgical ischemia include surgery while supported by cardio-
15 pulmonary bypass, aortic surgery which includes abdominal aortic aneurysm, supra-renal or infra-renal, thoraco-abdominal aneurysm repair, aortic arch repair, particularly as in circulatory arrest. In the case of transplantation, it may involve solid organs, such as the kidney, liver, lung, heart, skin, or the like, or dispersed cells, such as bone marrow, stem cells, or hematopoietic cells.

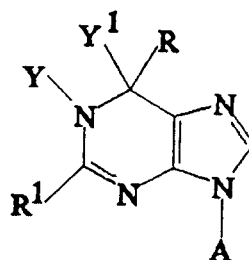
20 One may use the subject compositions to prevent injury prior to the onset of hypoxia. This may involve carotid endarterectomy, renal artery stenosis, mesenteric ischemia (bowel angina), thoracic surgery, airway surgery-apnea, peripheral revascularization, thermal-palatal grafting. Surgical hypoperfusion may involve elective hypotension with anticipated blood loss, cranial facial surgery and oncologic
25 indications. In many situations, one may use the subject compositions for rescue, which involves the use of the compositions after onset of hypoxia. This may involve non-elective revascularization, such as in the case of stroke or myocardial infarction, hemorrhagic shock, trauma, head injury, spinal cord injury or shock, severe burn and cadaveric organ harvest.

30 Other situations in which the subject compositions may find application include acute respiratory distress syndrome (ARDS), fetal distress, multisystem organ dysfunction, sepsis and sepsis syndrome, toxicities such as inhalation and

injury, poisonings, particularly involving compounds binding to metal atoms such as cyanide and carbon monoxide, extracorporeal membrane oxygenation (ECMO), and systemic wasting, as in the case of cancer and AIDS.

As indicated, individual compounds or combinations of compounds,
 5 individually or covalently linked, may be employed. In some situations an individual compound may be of greater effectiveness, while in other situations combinations of the compounds may find better application. A compound which is preferred individually for treating an indication may not be as effective when used in combination. Therefore, the preferred combinations may not select those
 10 compounds which have been found to be most effective when used individually.

Among the subject compositions which are of particular interest are physiologically acceptable purine derivatives, particularly purine ribosides which serve to extend the time for onset of irreversible changes in the cellular host, particularly changes leading to mortality. The purines have the following formula:



20 wherein:

A is a sugar or a phosphorylated sugar, particularly ribose, deoxyribose, and phosphorylated derivatives thereof, particularly 3' and/or 5'-phosphate and 5'-pyrophosphate, including derivatized phosphates, such as phosphate esters and anhydrides, particularly mixed anhydrides, where the esters may be alkyl esters of
 25 alkyl groups of from 1 to 3 carbon atoms or of sugars, e.g. ribose, deoxyribose and phosphorylated esters thereof, such as 3' and 5'-esters, and the mixed anhydrides may be anhydrides with carboxylic acids, such as pyruvate, and combinations thereof, e.g. ADP-r-r-P-r-P, where r is ribosyl and P is phosphate, where the final P may be bonded to a nucleoside;

30 R and R¹ may be nitrogen, oxygen or sulfur, where the sulfur may be substituted or unsubstituted, and the remaining valences will be satisfied by hydrogen, and where R may be taken with Y¹ to define a doubly bonded heteroatom;

Y and Y¹ are hydrogen or may be taken together to form a double bond between the atoms to which they are bonded or Y¹ may be taken together with R to form a double bond to the atom designated by R.

The core purines are guanosine, adenosine and inosine, which find use
 5 unsubstituted, as well as substituted, and include the ribosyl and 2-deoxyribosyl compounds. Various derivative compounds coming within the formula are 6-mercaptapurine riboside, adenosine diphosphate, adenosine monophosphate, S-5'-isobutyladenosine, 2,6-diaminopurine riboside, inosine monophosphate, 1-methylinosine, 2'-deoxyinosine, 2'-deoxyinosine-monophosphate, 2'-
 10 deoxyadenosine-5'-monophosphate, 2-inosine-5'-monophosphate, guanosine-monophosphate, 2'-deoxyguanosine, 2-deoxyguanosine-5'-monophosphate, S-(2-hydroxy-5-nitrobenzyl)-6-thioinosine, erythro-9-(2-hydroxy-3-nonyl) adenine and S-4-nitrobenzyl-6-thioinosine. Of particular interest are the purines indicated above, deoxy and monophosphorylated derivatives, and sulfur analogs thereof, particularly,
 15 where the sulfur is bonded to an aromatic group.

In combination with the purines, including purine derivatives, various physiologically acceptable electron acceptors may be used. These electron acceptors may take any one of several structures and functionalities, being electron acceptors or precursors to electron acceptors in a physiological environment, such as oxo,
 20 aromatic heterocycles, transitional metal complexes and enzymes, as well as combinations thereof. These electron acceptors include oxo compounds, both aldehydes and ketones, particularly reducing sugars (which will be described subsequently) and α - and β -ketocarboxylic acids, generally of from about 3 to 8, usually not more than 6, carbon atoms, such as pyruvate, oxaloacetate, acetoacetate,
 25 and menadione; hydroxycarboxylic acids, generally of from about 2 to 6 carbon atoms, such as hydroxypyruvate and hydroxybutyric acid, at the α or β position, other types of compounds, such as protoporphyrins, ascorbate, nicotinamide mononucleotide (oxidized or reduced), FMNH, glutathione (oxidized or reduced), superoxide dismutase, with various metal cofactors such as Mn, Mg, and Cu,
 30 deferoxamine, bathocuprine, allopurinol, N-acetyl cysteine, and the like. These compounds find particular use either in combination with the compounds indicated

above or by forming the ester of the sugar modified purine, so as to form a single compound which is readily hydrolyzable under physiologic conditions.

Various amino acids, individually or as oligopeptides, particularly dipeptides, may find application in association with the subject purines. These amino acids
5 include both natural and unnatural amino acids, and their derivatives, particularly their alkyl esters of from 1-2 carbon atoms and their amides. The amino acids will for the most part be aliphatic, but may include aromatic acids. Amino acids which may find use include glycine, N,N-dimethylglycine, glycine amide, glycine methyl ester, alanine, alanine methyl ester, serine, glutamate, glutamine, lysine, arginine,
10 sarcosine, serine, tryptophan, glycyl glycine, glycyl alanine, alanyl alanine, etc. Of particular interest are the naturally occurring amino acids of from 2 to 3 carbon atoms and their derivatives.

Other compounds of interest include various sugars and their phosphorylated derivatives, particularly sugars of from 3 to 6 carbon atoms, more usually 4 to 6
15 carbon atoms, and their oxidized derivatives, e.g. uronic acids, such as glycolic acid, 3-phosphate pyruvic acid, ribulose, xylulose, galactose, threose, 1,5-diphosphatefructose, 1,6-diphosphatefructose, 2,6-diphosphatefructose, glucose, fructose-6-phosphate, and mannose, where an individual sugar will only be used in combination with another compound of this invention when the individual compound
20 is a member of the glycolytic cycle.

Of particular interest are phosphoglycerates, where the phospho group at the 3 position is esterified with alkyl groups of from 1-2 carbon atoms and/or the 2-hydroxyl group is esterified with pyruvate.

Combinations of particular interest include a purine in conjunction with
25 either an hydroxy- or ketocarboxylic acid of from 2 to 6, usually 3 to 4, carbon atoms, and derivatives thereof, or an aliphatic amino acid of from 2 to 6 carbon atoms or dipeptide thereof, or a sugar. More particularly, the combination involving a purine, a sugar and one of the other components. The purines of particular interest in the combinations include guanosine, 7-methylinosine, 6-
30 mercaptoguanosine, 2',3'-diacetyladenosine and inosine.

Illustrative of components for such compositions are compounds such as ketone electron acceptors, e.g. pyruvate, an amino acid or derivative thereof,

particularly an amino acid of from 2-6 carbon atoms, more particularly glycine, alanine, glutamine, glutamate, where the amino acid may have C- and N-alkyl substituents of from 1 to 2 carbon atoms, particularly methyl, and reducing sugars of from 4 to 6 carbon atoms. Particularly, where the individual compounds are
5 charged, to enhance cellular absorption and transport, when possible the two compounds may be reacted to reduce the total charge, as in the case of an alcohol and an acid to produce an ester. The bonds which are formed will usually be physiologically labile, such as esters, e.g. phosphate and carboxylate, amides, anhydrides, and the like.

10 Generally, concentrations of the purine compounds as the primary compound will vary from about 0.1 μ M to 50mM, more usually from about 10 μ M to 1mM. Where combinations are used, frequently the concentration of the primary compound will be in the lower portion of the range, generally being 1mM or less. Generally, the molar ratio of the other components to the purine will be in the range of about
15 1:0.5-100, more usually 1:1-50. Since the additional materials will generally be safe at high concentrations, one may conveniently use relatively high ratios of the adjunctive materials to the purine and then reduce the concentrations to determine the optimum concentration.

Sugars and their phospho-derivatives may be used individually or in
20 combination, as a combination of sugars or with compounds other than sugars and purines, which combinations with purines have already been described. Of particular interest are xylulose and fructose, by themselves, and in combination, particularly with glucose. Other sugars and their phosphate esters, which will generally be used in combination, particularly with other sugars, more particularly
25 glucose, include erythrose 4-phosphate, ribose-1-phosphate, ribulose-1,5-bisphosphate, fructose 1,6-bisphosphate, xylulose 5-phosphate, fructose 1,6-bisphosphate, and ribulose 5-phosphate. Also of interest are sugar analogs such as phosphoenolpyruvate.

The concentration of the sugars other than glucose will generally be in the
30 range of about 0.1 to 100mM, more usually in the range of about 0.1 to 50mM, particularly in the range of about 0.5 to 10mM, while the glucose may be varied

widely depending upon the other sugar(s) and their concentration, generally varying from about 1 to 100mM, more usually from about 5 to 50mM.

Amino acids and oligopeptides which may find individual use or in combination include natural amino acids of from 2 to 6, more usually 2 to 3 carbon atoms, their amides and esters, where the total number of carbon atoms does not exceed 8, more usually 5. Of particular interest is glycine and its derivatives, such as the amide, methyl and ethyl ester, and the dipeptide glycyl-glutamine, and alanine methyl and ethyl ester, glutamate and glutamine.

For the most part, the subject compounds are physiologically safe up to relatively high concentrations. The particular compositions may be optimized for any combination in association with the indication for which it is used, the manner of administration, and the like. The determination of the optimum formulation can be performed in accordance with conventional ways and will vary whether the compositions are being used in culture or *ex vivo* or *in vivo*, as will be described below. The subject compositions are used as therapeutics to treat a condition involving stress, where the condition may be chronic or acute, usually acute, particularly involving specific episodes and not part of a dietary regimen, where the subject compositions are taken as part of a diet or in conjunction with a diet, to provide specific dietary supplements to augment a dietary deficiency. Thus, the individual components are not provided to enhance caloric content, substitute for a vitamin, amino acid or nucleotide deficiency, nor taken daily as a dietary supplement, such as vitamins and minerals. Rather, the subject compositions are taken in response to an anticipated or occurring stress situation, particularly hypoxia, resulting from a cellular stress due to an external event, generally resulting in a reduction in the energy charge-adenylate and total adenine nucleotide content of the cells. Therefore, the subject compositions will be formulated to provide the active components in a form which can act on the cells to relieve the stress, providing for anaerobic metabolism to at least enhance the energy charge-adenylate and total adenine nucleotide content of the cells, as compared to the levels which would occur under the stress conditions.

Formulations of the subject compositions may be prepared as concentrates for dilution prior to use. The amount of a subject compound which will be administered

will be dependant upon the safety level of the compound, the context in which it is used, the manner of administration, whether localized or systemic, whether oral or parenteral, the particular indication, the frequency of administration, whether as a bolus, continuous, e.g. intravenous, or the like, or other conventional
5 considerations.

For *in vitro* or *ex vivo* administration, the compounds may be provided in the medium, as a single bolus, by repetitive addition, by continual infusion, or the like. During the course of the treatment, the concentration of the subject compositions may be monitored to insure that the desired level is maintained. For *in vivo*
10 administration, where the administration is oral, the subject compositions may be provided as tablets, capsules, powders, solutions, dispersions, or the like. For rectal administration, the subject compositions may be provided as suppositories, as solutions for enemas, or other convenient application. Otherwise, the subject compositions may be administered intravascularly, arterially or venous,
15 subcutaneously, intraperitoneally, intraorgan, intramuscularly, or the like.

For oral administration, the subject compositions may be compounded with other physiologically acceptable materials which can be ingested, such as in foods, e.g. food bars, beverages, powders, food additives, candies and the like

To determine the optimum concentration for any application, conventional
20 techniques may be employed. Thus, for *in vitro* and *ex vivo* use, a variety of concentrations may be used and various assays employed to determine the degree of dysfunction of the cells when exposed to stress, particularly hypoxia. One technique which may be employed for mammalian cells, is the extra- and intracellular distribution of lactate dehydrogenase over time. The longer the period that the
25 intracellular lactate dehydrogenase is maintained as against the proportion in the extracellular medium, the better the cells are protected from irreversible changes leading to mortality. Conveniently, the lactate dehydrogenase assay described in the experimental section may be employed. Alternatively or in conjunction with the lactate dehydrogenase assay, one may also look for the formation of lactate. The
30 greater the formation of lactate, the greater the preservation of the cells.

For preservation of cells, the subject compositions may be added for a short time, usually at least about 0.5 hour and then removed, maintained for long periods

of time with the cells, including weeks or months or longer, may be replenished periodically, or the like. Since the subject compounds are for the most part safe, without significant side effects, the subject compounds may be maintained for the entire stress period or for an extended period in anticipation of stress.

- 5 In a number of situations there may be oxygen deprivation or other stress due to physical activity, such as exercise, or sports activities, or surgery, or the like. The subject compositions may be formulated as foods, either solid or liquid, such as food bars, cereals, cooked foods, beverages, or the like. Usually, the amount of the active ingredient will be at least 0.1g per serving, and may be 0.5g per serving or
- 10 more, generally no exceeding about 5g, more usually not exceeding about 2g per serving. The subject compositions may combined with particular foods to enhance their effectiveness, such as high protein foods, foods which enhance transport across the gut, such as gums, foods which provide for compatible energy sources, and the like.
- 15 The following examples are offered by way of illustration and not by way of limitation.

EXPERIMENTAL

Generalized Protocol for Assaying Protection

- 20 I. Protocol for harvesting and assaying WIF B cells for LDH release with various compounds.
- A. Plating cells onto coverslips:
1. Autoclave glass coverslips 18 mm diam. (VWR). Insert 9
 sterile coverslips into a petri dish and arrange in 3 rows of 3.
- 25 Adhere the coverslips to the petri dish by flaming tweezers and melting plastic over coverslips in 4 or more places around each coverslip.
2. From a culture flask (T75), check cell density. Cells should not be confluent, they should appear in clumps or in log phase
- 30 growth.

3. Aspirate off media. Add 25 ml PBS to bottom of flask and do not disturb adherent cells. Tilt flask to gently wash adherent cells and aspirate.
- 5 4. Add 2 ml of prepared trypsin solution to bottom and gently overlay solution to adherent cells. Let stand for 3 minutes. Tap flask fairly vigorously to dislodge adherent cells from surface. Add 8 ml of WIF B media with serum over any remaining adherent cells and mix gently to get cell suspension.
- 10 a. For WIF B media, serum, and trypsin solution components and protocols, see below.
5. From suspension, determine cell density. Each petri dish is 60cm² and the desired density is 20,000 cells/cm² for 1.2 million cells per dish. Make volume up to 15 ml of total media to add to each petri dish.
- 15 6. The next day transfer coverslips to 6 well plates with 5 ml of WIF B media in each well. Change media every other day until the 7th day. Change media every day until cells are used. Cells will be used between 9 and 11 days in culture starting from the day cells were plated onto coverslips. 10
- 20 days in culture is optimum.
- B. Assaying WIF B cells for LDH release.
1. On the day of the assay, remove media from wells and wash cells with 1 x 5 ml each well Balanced Salt solution (below). pH 7.4 total mOsm = 288: Balanced Salt solution contains:
- 25 1.3 mM CaCl₂, 1.2 mM MgCl₂, 5 mM KCl, 25 mM Hepes, 1 mM KH₂PO₄, 115 mM NaCl.
2. Dilute each compound with respective Balanced Salt solution containing respective amounts of NaCl to make up for osmolality (refer below for calculation sample). Make serial
- 30 dilutions in isotonic Balanced Salt solution above. For testing compounds 1 mM or less, dilute in 115 mM HB (above) or

make 250 mM (500x) solution in DMSO and insert 10 μ l in each well and mix.

- 5

3. Incubate compounds (5ml each well) with cells for half an hour at 37°C. After incubation, swirl gently the six well plates and take 50 μ l of sample from each well and insert into 96 well microtiter plate. This is the time 0 (background) point. Add 5 μ l 100mM DNP or 5 μ l of 100 μ M, antimycin A in DMSO to each well (final concentration 100 μ M, 100nM, respectively). Use 2 pipets to mix gently. Take 50 μ l at each

10

time point and insert into 96 well microtiter. Keep microtiter plate at 4°C.
4. At the end of the time course, lyse the remaining cells on the coverslip by adding 10X lysine buffer to remaining media and aspirate vigorously with P1000.

15

a. 10X lysing buffer contains: 10% triton-X in 115mM NaCl HB buffer. This lysate will include amount LDH remaining in the cells + in the media which = total LDH activity.
- 20

5. Remove 50 μ l at each time point. At the end of each time course, assay for LDH activity using Boehringer Mannheim Kit (notebook 005 pg. 26) except decrease assay volume to 100 μ l.
- 25

6. Using Sigma Chicken Liver LDH #L6504 as standard, dilute down standards in 115mM NaCl HB to = 400, 200, 100, 50, 25, 12.5, 0 U/l. Assay 50 μ l of each as above.
- C. Calculations:

 1. LDH media OD-bkg: raw OD values - 115mM NaCl HB background.
 2. LDH (U/l): LDH activity calculated based on standard curve extrapolation. Note that U/l are not based on final

30

concentration of LDH but rather the inserted U/l of standards

above. The true final U/l in the cuvette would be the values divided by 2 (50 μ l of 100 μ l assay).

- 5
3. Dilution factor: 50 μ l taken per assay will decrease total volume of sample in each well. For example, the dilution factor of zero time point will be 100 (50 μ l of 5ml). The second time point 50 μ l will be taken from 4.95ml or 99 dilution factor, etc.
- 10
4. Adjusted LDH (U/l): LDH (U/l) multiplied by dilution factor.
5. LDH-time 0: This is time 0 LDH or initial background release if any subtracted from values during time course to observe DNP or Antimycin A dependent LDH release during the time course. Time 0 LDH values were also subtracted from LDH value from lysate.
- 15
6. % LDH release: LDH-time 0 values divided by LDH in Lysate or total LDH activity.
- 20
7. % LDH of Time 0: This is to monitor the amount of release at time 0 due to toxicity of compound or possibly other factors. Compounds exhibiting high time 0 background will be tested to see if the compound interferes with the assay or it will be concluded that compound may induce LDH release.

D. Calculations: for lactate:

1. Lactate OD-bkg: raw OD values - 115mM NaCl HB background.
2. Lactate μ M: lactate calculation extrapolated from the lactate standard curve. Note that unlike the LDH calculation, the lactate concentration stated is the concentration within the cuvette.
3. Dilution factor: above except will be multiplied by 0.01 for below calculation.
4. Total lactate nmol: lactate μ M multiplied by 4.8 (since 50 μ l sample in 240 μ l assay) = μ M in actual sample. This value is multiplied by 5ml = total nmol lactate in 5ml. The total nmol lactate is then multiplied by the dilution factor above.

15 Cells used in assays and their preparation for the assays

Cells are maintained in Cassio modified HAM F-12 medium supplemented with 33 mM sodium bicarbonate, 50 μ g/ml streptomycin, 200 Unit/ml penicillin G, 0.5 μ g/ml amphotericin B, 10 mM sodium hypoxanthine, 40 μ M aminopterin, and 1.6 mM thymidine (HAT), and 5% fetal bovine serum. The WIF-B cells are derived from a fusion of a HGPRTase⁻ rat hepatoma cell (FAO) and a normal human lung fibroblast (WI-38). The fibroblasts confer the ability of the fused cells to grow in the presence of HAT. The cells are kept at 37°C in a humidified incubator with 7% CO₂ atmosphere. Cells are grown on plastic tissue culture flask or on glass coverslips. The medium was replaced every other day to day 7, then changed daily thereafter.

Subculturing was performed by dislodging cells from the flask by treatment with a solution containing 0.05% trypsin, 0.5mM EDTA, 137mM NaCl, 5.4mM KCl, and 0.058% NaHCO₃. Following harvesting, WIF-B cells were seeded onto plastic flasks or glass coverslips at a density of 1-2 x 10⁴ cells/cm². Cells used for carrying the cell line were grown to a density between 1-1.5 x 10⁵/cm² in about 7 days. For assays, cells were grown to higher densities and used 9-11 days post seeding.

Various compounds were tested as set forth in the following table. To the extent feasible, the groups of compounds were classified according to a potential mechanism by which the compounds may be active.

5

Table

Class	Action	Example
Class-1	decrease redox stress	purine-ribonucleoside
Class-2	receptor	purine-ribonucleotide
Class-3	receptor	ribulose bis 1,5-PO ₄
10 Class-4	ATP supplement	fructose
Class-5	redox-sink	pyruvate, acetoacetate
Class-6	endogenous protect	Vit-E, glutathione
Class-7	-	amino acids, glycine, alanine
Class-8	-	-
15 Class-9	-	-
Class-10	misc.	nucleotide transport inhibitors, apoptosis inhibitors

In the following table, Table 1, a number of compounds were tested in the LDH assay and their activity compared to the activity of guanosine in reducing the proportion of extracellular LDH as compared to total LDH after contact of the cells with an inhibitor. Based on their chemical structure and/or mode of action, the various compounds were collected together in classes for comparison.

20

Table 1

25

compound	activity (% of guanosine)	maximum effective concentration
Class-1		
guanosine	100	500μM
Adenosine	85	500μM
30 Inosine	90	500μM
6-mercaptopurine riboside	90	500μM

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	N6-cyclopentyladenosine	20	500µM
	2'-deoxyadenosine	70	500µM
	Xanthosine	10	500µM
	Benzoyl adenosine	10	500µM
5	1-methylinosine	20	500µM
	5'-deoxy 5' methylthioadenosine	10	500µM
	6-dimethylallylamino purine riboside	10	500µM
	5' amino 5'-deoxyadenosine	10	500µM
	Adenosine periodate	10	500µM
10	Adenosine 5' monosulfate	40	500µM
	7-methylinosine	40	500µM
	alpha-adenosine	10	500µM
	6-Cl purine riboside	30	500µM
	5'-Cl-5'-deoxyadenosine	5	500µM
15	2'-deoxyinosine	30	500µM
	2'-deoxyguanosine	40	500µM
	2,6 diaminopurine 2'-deoxy riboside	70	500µM
	5'-deoxyadenosine	30	500µM
	5-Iodotubercidin	50	500µM
20	6-mercaptopurine	5	500µM
	6-mercaptopguanosine	70	500µM
	2-amino 6-Cl-purine riboside	40	500µM
	N2-methylguanosine	50	500µM
	5'-S-isobutyladenosine	50	500µM
25	Adenosine 5' succinate	10	500µM
	2'3' DiO acetyl adenosine	40	500µM
	6-hydroxyguanidine purine riboside	2	500µM
	2-Phenylaminoadenosine	70	500µM
	5-Iodoadenosine	5	500µM
30	2'-O-methyladenosine	10	500µM
	S-(2hydroxy 5-nitrobenzyl) 6-thioinosine *	150	100µM
Class-2			
	ADP	80	500µM
35	ATP	75	500µM
	AMP	85	500µM

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	3' AMP	60	20mM
	IMP	85	500μM
	Cordycepin	60	20μM
	2'deoxy-IMP	70	500μM
5	2'deoxy-AMP	70	500μM
	N2,2'0-dibutylguanosine3'5'cyclic monophosphate	4	500μM
	N2,2'0-dibutyladenosine3'5'cyclic monophosphate	2	500μM
	NAD	50	500μM
	2'deoxy-GMP	70	500μM
10			
	Class-3		
	3'5' adenosine diphosphate (3'5'PAP)	60	20mM
	Ribulose 1,5 Diphosphate	65	20mM
	Glucose 1,6 Diphosphate	5	20mM
15	Fructose 2,6 Diphosphate	30	20mM
	2'5' adenosine diphosphate (2'5'PAP)	10	20mM
	Adenosine 5'diphosphoribose (ADP-ribose)	50	800μM
	Class-4		
20	Fructose	50	30mM
	3-phosphoglycerate	5	10mM
	Glucose	20	30mM
	Fructose 1,6 Diphosphate	20	30mM
	Fructose-6-phosphate	10	30mM
25	Mannose	20	30mM
	Class-5		
	Pyruvate	30	20mM
	Phenylalanine	30	30mM
30	alpha-Ketoglutarate	5	20mM
	malate	5	20mM
	Acetoacetate	10	20mM
	Class-6		
35	Arginine	60	30mM
	Glutamate	20	30mM

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	Glutathione (oxidized)	20	20mM
	N,N-dimethylglycine	10	20mM
	Glycine amide	110	20mM
	Glycine methyl ester	120	20mM
5	Alanine methyl ester	120	2mM
Class-7			
	alanine	100	5mM
	Glycine	100	5mM
10	Serine	10	30mM
	Glutamate	20	30mM
	Tryptophan	60	30mM
	Glutamine	60	30mM
	Lysine	5	1mM
15	Sarcosine	100	5mM
Class-10			
	Aurintricarboxylic acid	10	10μM
	Taurine	20	30mM
20	cytosine	30	500μM
	Thymidine	30	500μM
	Theophylline	4	500μM
	Orotate	10	500μM
	Cycloserine	40	20mM
25	Methylene Blue	60	40μM
	S-4 Nitrobenzyl 6-thioinosine (NBTI)	60	500μM
	erythro-9-(2-hydroxy-3-nonyl)adenine EHNA	95	500μM
	Mycophenolic Acid	50	500μM
30	Activity of each compound is expressed as % of the lead compound guanosine (guanosine at 100%) at the compound's maximal effective concentration		

* = not based on maximum effective concentration

In addition, following the above procedures, combinations of compounds were employed.

Table 2

5	Time, hour	% LDH Release			
	Nothing	Guanosine, 500 μ M	Guanosine, 500 μ M; Pyruvate, 1mM	Guanosine, 500 μ M; Pyruvate, 1mM; Glycine, 1mM	
	2	50	0	0	0
	4	70	10	5	0
	7		25	25	5
10	9	90	60	60	10

In the next study, protection as a result of adding guanosine at 500 μ M was determined by performing the LDH release analysis where the guanosine was added at different times after 100 μ M antimycin A was added, followed by determination of the LDH release over the next two hours. The following table indicates the results.

Table 3

20	Time delay, hour	% LDH Release	
		Nothing	Guanosine, 500 μ M
	0	65	4
	0.5	89	8
	1	91	24
25	1.5	93	46
	2	103	75

It is evident from the above results that administering the guanosine to the cells as long as 1.5 hours after the stress has been initiated still provides a significant degree of protection.

The following procedures were used to develop the subsequent data.

A. Cell Culture Conditions for Screening

Cells are cultured directly (without cover slip) in tissue culture wells of various sizes. Typically, sterile microplates of opaque or clear plastic of 6, 12, 24,
5 48 or 96 wells are used.

B. Method of Injury

To inflict chemical hypoxia, 100nM antimycin A dissolved in Balanced Salt Solution (BSS) is added. To perform anoxia, the cells in BSS are placed in an
10 hypoxic chamber where air is replaced with nitrogen. Test molecules of interest are added at the start of the injury. All solutions containing test molecules are checked prior to use in terms of pH and osmolarity. When test molecules are present at concentrations higher than 2mM, the amount of NaCl in the BSS is adjusted to ensure iso-omolarity. cells are incubated at 37°C under all injury conditions.
15

C. Cellular Adenine Nucleotide Measurements

To examine cellular energy charge, cells are treated as described above with hypoxia in the presence or absence of cytoprotective molecules. Aliquots of incubation solution, which contains the released cytosolic components are withdrawn
20 as described above. After specified time intervals, treatment is stopped by rapidly removing the incubation solution, rinsing the monolayers with ice-cold BSS, and lysing the cells in ice-cold water (0.9ml). Perchloric acid (0.1ml 4N) is added to the cell lysates to precipitate the macromolecules. The acid soluble small molecules are recovered in the supernatant after removal of the insoluble materials by
25 centrifugation and neutralized by extraction with tri-N-octylamine:1,1,2-trichlorotrifluoroethane (20μl, 4x). The protein content in the acid precipitates is determined by Bradford Assay.

Cellular nucleotides are analyzed by ion-pairing high pressure liquid chromatography with a C₈-reverse phase column (Mac-Mod 15cm x 4.6mM). The
30 system is equilibrated at a flow rate of 0.7ml/min with buffer A (50mM K₃PO₄, pH 6 containing 5mM tetrabutylammonium phosphate). Elution is achieved with a gradient of acetonitrile in buffer A up to 30% (1%/min). Under these conditions,

all the nucleotides as well as the adenine base and adenosine are resolved to baseline separation. The elution profiles are monitored by optical density at 260nm and the amounts of material are determined by integrating the area under each peak and comparing the values with a known amount of authentic standards. The nucleotide
 5 levels are represented as nmol/mg cell protein. Total adenine content (TANC) and adenylate energy charge (EC-A) are calculated as follows:

$$\text{TANC} = \sum(\text{ATP} + \text{ADP} + \text{AMP}), \text{ where the unit of the values is nmol/mg cytosolic protein;}$$

$$\text{EC-A} = ([\text{ATP}] + 0.5[\text{ADP}]/([\text{ATP}] + [\text{ADP}] + [\text{AMP}])).$$

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D. Assaying WIF-B Cells for Plasma Membrane Damage

Cell injury is monitored by plasma membrane integrity. This is measured by the leakage of lactate dehydrogenase (LDH), a strictly cytosolic enzyme, from the cytosol into the extracellular space or by measuring the binding of a dye (SYTOX,
 15 green nucleic acid stain, S-7020, Boehringer Mannheim) to nucleic acids resulting in fluorescence at 524nm. Cells are grown in opaque tissue culture plates and injured in the presence or absence of test molecules as described above. The dye (1 μ M final concentration) is added at the start of the experiment and remains to the end. At designated time points during the course of the experiments, cells are excited at
 20 504nm and the fluorescence intensity measured at 524nm. To determine % nucleic binding, the same calculations are performed as for LDH.

The compositions found to be effective to inhibiting membrane injury as indicated by LDH release and/or SYTOX binding, and enhancing EC-A and TANC as compared to a control are as follows:

25

7-Me-inosine 250 μ M	xylulose 2mM	
guanosine 250 μ M	xylulose 2mM	
guanosine 250 μ M	xylulose 2mM	α -ketobutyric acid 10mM
guanosine 250 μ M	xylulose 2mM	β -hydroxybutyric acid 10mM
30 guanosine 250 μ M	fructose 10mM	

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5	guanosine 250 μ M	fructose 10mM	β -hydroxybutyric acid 2.5 mM
	6-HS-guanosine 250 μ M	xylulose 2mM	α -ketobutyric acid 10mM
	2',3'-diacetyladenosine 250 μ M	xylulose 2mM	
	2',3'-diacetyladenosine 250 μ M	xylulose 2mM	β -hydroxybutyric acid 10mM
	2',3'-diacetyladenosine 250 μ M	xylulose 2mM	α -ketobutyric acid 10mM
10	inosine 500 μ M		pyruvate 1mM
	guanosine 500 μ M		pyruvate 0.1mM alanine 1mM
	guanosine 500 μ M		glycine 1mM
	guanosine 500 μ M		pyruvate 10mM glycine 1mM
	guanosine 500 μ M		β -hydroxybutyric acid 1mM
15	guanosine 500 μ M		α -ketoglutaric/asp 10mM
	guanosine 500 μ M		α -ketobutyric acid 10mM
	guanosine 500 μ M		β -hydroxypyruvic acid 1mM
	guanosine 500 μ M		glutamine 10mM
	guanosine 500 μ M		glutamate 10mM
20	guanosine 500 μ M	fructose 30mM	pyruvate 10 mM
	guanosine 500 μ M	mannose 3mM	
	guanosine 500 μ M	glucose 2 mM	
	guanosine 500 μ M	galactose 3mM	
	guanosine 500 μ M	ribulose 3mM	
	guanosine 500 μ M	threose 3mM	
	guanosine 500 μ M	xylulose 5mM	

The above data demonstrate that by employing the subject compounds with cells under stress, either by themselves or in conjunction with other compounds, including compounds which have been known or not known to provide for protection against stress, the time to irreversible changes leading to cellular mortality can be greatly extended. The results demonstrate that there is substantial improvement available, by including the subject compounds which are generally regarded as safe and can be used in relatively high concentrations, by themselves, or in combination with other substances. These compositions can provide extensive opportunities in a variety of situations involving *in vitro*, *ex vivo* and *in vivo* situations. Thus, cells and organs may be stably maintained for extended periods of time, even under such stresses as cryogenic preservation and reoxygenation upon thawing. The subject compositions may also be used *in vivo*, by administering systemically or locally in situations where hypoxia may be anticipated or has occurred.

All publications and patent applications mentioned in this specification are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

The invention now being fully described, it will be apparent to one of ordinary skill in the art that many changes and modifications can be made thereto without departing from the spirit or scope of the appended claims.

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WHAT IS CLAIMED IS:

1. A method for delaying the time that mammalian cells under stress undergo irreversible change related to said stress, said method comprising:
adding to a medium comprising said cells in an amount sufficient to delay the time for said irreversible change, a formulation comprising at least one agent consisting of a physiologically acceptable purine derivative other than adenosine or adenosine triphosphate, their deoxy derivatives or a sugar or sugar derivative, with the proviso that when said agent is a member of the glycolytic cycle or finds use in a diet or dietary supplement, said formulation comprises at least two of said agents or at least one agent and an electron acceptor compound other than said sugar or an amino acid of from 2 to 6 carbon atoms or a dipeptide comprising said amino acid.
2. A method according to Claim 1, wherein said stress is hypoxia.
3. A method according to Claim 2, wherein said purine derivative is guanosine, inosine or the deoxy derivatives thereof.
4. A method according to Claim 1, wherein said formulation comprises a purine or purine derivative and at least one of an electron acceptor and an amino acid.
5. A method according to Claim 4, wherein said electron acceptor is a reducing sugar.
6. A method of inhibiting the effects of hypoxia in a cell or tissue culture, said method comprising:
adding to said culture in an amount sufficient to delay the time for cells in said cell culture to undergo irreversible changes related to said hypoxia a formulation comprising at least one agent which consists of an N⁹-ribosylated purine or phosphate derivative thereof other than adenosine or adenosine triphosphate, their deoxy derivatives or a reducing sugar

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or derivative thereof, with the proviso that when said agent is a member of the glycolytic cycle or finds use in a diet or dietary supplement, said formulation comprises at least two of said agents or at least one agent and at least one of an electron acceptor compound other than said reducing sugar or an amino acid of from 2 to 6 carbon atoms or a dipeptide comprising said amino acid.

7. A method according to Claim 6, wherein said N⁹-ribosylated purine is guanosine, inosine or 6-mercaptapurine riboside or the deoxy derivatives thereof and said sugar is fructose or xylulose.

8. A method of inhibiting the effects of hypoxia in a mammalian host suffering from hypoxia, said method comprising:

adding to said mammalian host in an amount sufficient to delay the time for cells in said mammalian host to undergo irreversible changes related to said hypoxia a formulation comprising at least one agent which consists of an N⁹-ribosylated purine or phosphate derivative thereof other than adenosine or adenosine triphosphate, their deoxy derivatives or a reducing sugar or derivative thereof, with the proviso that when said agent is a member of the glycolytic cycle or finds use in a diet or dietary supplement, said formulation comprises at least two of said agents or at least one agent and at least one of an electron acceptor compound other than said reducing sugar or an amino acid of from 2 to 6 carbon atoms or a dipeptide comprising said amino acid.

9. A method according to Claim 8, wherein said N⁹-ribosylated purine is guanosine, inosine or 6-mercaptapurine riboside or the deoxy derivatives thereof and said sugar is fructose or xylulose.

10. A composition comprising a concentrate capable of forming a pharmacologically acceptable solution of a N⁹-ribosylated purine, derivative thereof or deoxy derivative thereof, other than adenosine, in combination with at least one of an electron acceptor, a

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reducing sugar or derivative thereof and an amino acid, wherein said solution comprises said purine at a concentration in the range of about 0.1 μ M to 5mM and said electron acceptor, reducing sugar or phosphate ester derivative thereof and/or amino acid are in a molar ratio of purine to other component of 1:1-100.

11. A composition according to Claim 10, wherein said electron acceptor is an α - or β -ketocarboxylic acid, said reducing sugar is fructose or xylulose and said amino acid is a naturally occurring amino acid of from 2 to 3 carbon atoms.
12. A composition according to Claim 11, wherein said purine derivative is guanosine, said α -ketocarboxylic acid is pyruvate, said sugar is xylulose and said amino acid is glycine.
13. A cellular composition comprising dispersed cells, tissue or viable organ and in an amount sufficient to delay the time for occurrence of an irreversible change in said cells, tissue or viable organ leading to mortality, a composition comprising a N⁹-ribosylated purine, derivative thereof or deoxy derivative thereof, other than adenosine, in combination with at least one of an electron acceptor, a reducing sugar or derivative thereof and an amino acid, wherein said solution comprises said purine at a concentration in the range of about 0.1 μ M to 5mM and said electron acceptor, reducing sugar or phosphate ester derivative thereof and/or amino acid are in a molar ratio to said purine 1-100:1.
14. A cellular composition according to Claim 13, wherein said N⁹-ribosylated purine is guanosine and further comprising at least one of an electron acceptor a reducing sugar or phosphate ester thereof in a molar ratio of 1-100:1 to said purine.
15. In a method for transplanting cells or an organ, where said cells or organ are maintained for a period of time between isolation and implantation, the improvement which comprises:

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storing said cells or organ during said period of time in a composition comprising at least one of an N⁹-ribosylated purine other than adenosine or a reducing sugar, with the proviso that said reducing sugar is other than glucose in the absence of said N⁹-ribosylated purine.

16. A method according to Claim 15, wherein said composition further comprises an α - or β - hydroxy- or ketocarboxylic acid.

17. A food composition comprising as an additive an N⁹-ribosylated purine or phospho ester derivative and at least one of an electron acceptor, a sugar other than glucose and an amino acid, in an amount sufficient for delaying the period of time that mammalian cells under stress undergo irreversible change related to said stress.

18. A food composition according to Claim 17, comprising an N⁹-ribosylated purine.

19. A food composition according to Claim 17, wherein said food composition is a food bar or a beverage.