

CHOLINE DERIVATIVES AND COMPOSITIONS AND USES THEREOF FOR CRYOPRESERVATION

FIELD OF THE INVENTION

[0001] The present application pertains to the field of cryopreservation and sub-zero storage of biological material. More particularly, the present application relates to choline derivatives useful as cryoprotective agents, and methods of manufacture and uses thereof in cryopreservation and sub-zero storage.

BACKGROUND

[0002] Cryopreservation remains the most common method for the long-term storage of cells and can also be used for long-term storage of tissue. However, commonly during cryopreservation a significant number of cells experience irreparable damage due to the growth of ice, ultimately resulting in decreased post-thaw recoveries or impaired function. As cell-based therapeutics continue to define new models of care, for example, in stem cell therapy, regenerative medicine and transfusion, it is becoming increasingly important to ensure the highest level of post-thaw cell viability and functionality.

[0003] The successful development and distribution of biological materials and medicinal therapeutics relies on efficient cryopreservation methods.¹⁻³ Cryopreservation employs extreme sub-zero storage temperatures (< -80 °C) to dramatically extend the shelf-life of products due to slowed biological processes. However, despite the routine use of cryopreservation in clinical and industrial applications, cryopreservation outcomes – including post-thaw viability, recovery, and functionality – remain sub-optimal. Ice formation during cryopreservation causes cells to endure significant physical and chemical stressors, which if left unmitigated, results in irreversible cellular injury that reduces the recovery and viability of cryopreserved biological materials.

[0004] Dimethyl sulfoxide (DMSO) and glycerol are the cryoprotective agents (CPA) of choice for cryopreservation protocols in both research and in clinics, and have been the key CPAs driving the development of applied cryobiology over the last 50 years.⁴⁻⁶ DMSO has an

array of physical properties that contribute to its success as a CPA; for example, DMSO has strong molecular stability, a high dielectric constant, a high affinity for water, and strong hydrogen bonding capabilities. The ability to form strong hydrogen bonding with water imparts strong antifreeze characteristics. Moreover, DMSO can readily cross biological membranes with minimal injury to cells, allowing for beneficial antifreeze and osmotic effects to occur both intracellularly and extracellularly within the cryopreserved biological sample.

[0005] The role of DMSO and glycerol as penetrative CPAs is to increase post-thaw viability; however, both DMSO and glycerol exhibit cellular toxicity and adverse biological effects that are problematic for their routine use in cryopreservation, especially for highly complex or cryo-sensitive cell types.^{5,7,8} Cells derived from T- and B-cells have proven to be even more sensitive to DMSO, with increased apoptosis, inappropriate differentiation, and growth arrest being observed.¹⁰ Furthermore, reduced functionality upon exposure to low DMSO concentrations has also been reported; a few examples of this include: increased hemolysis of erythrocytes¹⁰, inhibited disaggregation and decreased thrombin in platelets¹⁰, and reduced morphology, adhesion and differentiation in mesenchymal stem cells (MSCs).¹¹ Due to the undesirable biological effects of DMSO and glycerol in complex cell types commonly used in cell-based therapies (e.g., human induced Pluripotent Stem Cells (hiPSCs), MSCs, Hematopoietic stem and progenitor cells (HSPCs)), these CPAs are not suitable for the routine cryopreservation of emerging cell-based therapies.

[0006] In addition to the harmful biological effects observed for cell-based products, the use of DMSO and glycerol as CPAs also has inherent limitations in clinical settings. Infusion of these CPAs to patients results in harmful toxic effects, thus necessitating a time-consuming but necessary CPA removal step after thawing of the biological sample. For instance, glycerol is routinely removed from red blood cell (RBC) samples post-thaw to avoid intravascular hemolysis post-transfusion.⁵ Collectively, these limitations necessitate the design of improved non-toxic and biocompatible CPA formulations.

[0007] To mitigate the toxicity concerns with using DMSO and glycerol, efforts have been made to reduce the CPA concentration used or to combine the CPAs with other non-toxic cryoadditives, such as sugars or polymers.^{12,13} However, these protocols, despite their

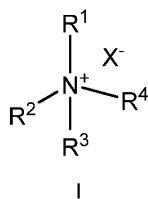
improvements in comparison with DMSO/glycerol alone, remain suboptimal. Therefore, despite the success of using DMSO and/or glycerol for the cryopreservation of a variety of cell types, there exists a need for improved cryoadditives and cryopreservation protocols that eliminate the need for DMSO and glycerol entirely.

[0008] Ice recrystallization is a significant contributor to cellular damage and death during the freezing and thawing periods of the cryopreservation process. As such, there is significant interest in designing CPAs that are effective ice recrystallization inhibitors (IRIs) to mitigate the damage inflicted by ice crystal growth and improve the overall cryopreservation outcome.¹⁴ Unfortunately, the industry standard CPAs, DMSO and glycerol, fail to address the ice recrystallization process, leading to uncontrollable ice growth and reduced post-thaw viabilities. Prior work has demonstrated that a variety of small molecules possess IRI activity and that this IRI activity can be optimized through simple chemical modifications to the molecule.^{4,5,15} Addition of these IRI active small molecules to cryomedia has successfully improved the cryopreservation outcomes (i.e., post-thaw viability, recovery, and functionality) for a variety of cell types, including complex cells such as MSCs, HSPCs, and induced Pluripotent stem cells (iPSCs).¹⁵⁻¹⁷ However, there still remains a need for an alternative to DMSO and glycerol in cryomedia.

[0009] The above information is provided for the purpose of making known information believed by the applicant to be of possible relevance to the present invention. No admission is necessarily intended, nor should be construed, that any of the preceding information constitutes prior art against the present invention.

SUMMARY OF THE INVENTION

[0010] An object of the present application is to provide choline derivatives and compositions and uses thereof for cryopreservation. In accordance with an aspect of the present application, there is provided a compound of Formula I



where

each of R^1 , R^2 and R^3 is independently H, C_1 to C_8 alkyl, C_2 to C_8 alkenyl, C_2 to C_8 alkynyl, or a C_1 to C_8 alkyl, C_2 to C_8 alkenyl, or C_2 to C_8 alkynyl that is terminally substituted with an alcohol, aryl or heteroaryl, or

one of R^1 , R^2 and R^3 is absent, H, C_1 to C_8 alkyl, C_2 to C_8 alkenyl, C_2 to C_8 alkynyl, or a C_1 to C_8 alkyl, C_2 to C_8 alkenyl, or C_2 to C_8 alkynyl that is terminally substituted with an alcohol, aryl or heteroaryl, and the remaining two of R^1 , R^2 and R^3 , together with the nitrogen to which they are attached, form a 4 to 8-membered heterocyclic ring;

R^4 is H, C_1 to C_9 alkyl, C_2 to C_9 alkenyl, C_2 to C_9 alkynyl, or a C_2 to C_9 alkyl, C_2 to C_9 alkenyl, or C_2 to C_9 alkynyl that is terminally substituted with an alcohol, $C(O)OH$, $C(O)OR_5$ or $C(O)NR_6R_7$, wherein R^5 is C_1 to C_8 alkyl, C_2 to C_8 alkenyl, or C_2 to C_8 alkynyl, each of which is optionally terminally substituted with an aryl group, alcohol group, or carboxylic acid group, and wherein each of R^6 and R^7 is independently H, C_1 to C_8 alkoxy, aryl, substituted aryl, 4-8 membered heterocyclic group, or a C_1 to C_8 alkyl, C_2 to C_8 alkenyl, or C_2 to C_8 alkynyl, each of which is optionally terminally substituted with an aryl group; and

X^- is a counterion, such as a halide ion, a phosphate, sulfate, acetate, or hexafluorophosphate ion,

with the proviso that when R^4 is $-CH_2CH_2OH$ then:

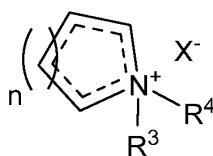
at least one of R^1 , R^2 and R^3 is not methyl,

when R^1 is methyl then R^2 and R^3 are not both H or ethyl, and

when R^1 and R^2 are both methyl then R^3 is not H or ethyl.

[0011] In accordance with some embodiments, each of R^1 , R^2 and R^3 is C_1 - C_8 alkyl (such as, ethyl, propyl, isopropyl, *n*-butyl, *sec*-butyl, *tert*-butyl or isobutyl), or C_1 - C_8 alkyl (such as, ethyl, propyl, isopropyl, *n*-butyl, *sec*-butyl, *tert*-butyl or isobutyl) terminally substituted with an aryl, or one of R^1 , R^2 and R^3 is absent H, C_1 - C_8 alkyl (such as, ethyl, propyl, isopropyl, *n*-butyl, *sec*-butyl, *tert*-butyl or isobutyl), or a C_1 - C_8 alkyl (such as, ethyl, propyl, isopropyl, *n*-butyl, *sec*-butyl, *tert*-butyl or isobutyl) terminally substituted with an aryl, and two of R^1 , R^2 and R^3 , together with the nitrogen to which they are attached, form a 4 to 8-membered heterocyclic ring. In some embodiments, at least one of R^1 , R^2 and R^3 is a C_1 - C_4 alkyl terminally substituted with an aryl, such as phenyl.

[0012] In accordance with some embodiments, the compound of Formula I has the following structure:

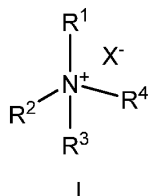


where n is an integer from 0 to 4.

[0013] In accordance with some embodiments, the counterion, X^- , is F^- , Cl^- , Br^- , I^- , a phosphate, sulfate, acetate, or hexafluorophosphate ion.

[0014] In accordance with another aspect of the present application, there is provided a composition comprising:

(a) a compound of Formula I



where

each of R^1 , R^2 and R^3 is independently H, C_1 to C_8 alkyl, C_2 to C_8 alkenyl, C_2 to C_8 alkynyl, or a C_1 to C_8 alkyl, C_2 to C_8 alkenyl, or C_2 to C_8 alkynyl that is terminally substituted with an alcohol, aryl or heteroaryl, or

one of R^1 , R^2 and R^3 is absent, H, C_1 to C_8 alkyl, C_2 to C_8 alkenyl, C_2 to C_8 alkynyl, or a C_1 to C_8 alkyl, C_2 to C_8 alkenyl, or C_2 to C_8 alkynyl that is terminally substituted with an alcohol, aryl or heteroaryl, and the remaining two of R^1 , R^2 and R^3 , together with the nitrogen to which they are attached, form a 4 to 8-membered heterocyclic ring;

R^4 is H, C_1 to C_9 alkyl, C_2 to C_9 alkenyl, C_2 to C_9 alkynyl, or a C_2 to C_9 alkyl, C_2 to C_9 alkenyl, or C_2 to C_9 alkynyl that is terminally substituted with an alcohol, $C(O)OH$, $C(O)OR^5$ or $C(O)NR^6R^7$, wherein R^5 is C_1 to C_8 alkyl, C_2 to C_8 alkenyl, or C_2 to C_8 alkynyl, each of which is optionally terminally substituted with an aryl group, alcohol group, or carboxylic acid group, and wherein each of R^6 and R^7 is independently H, C_1 to C_8 alkoxy, aryl, substituted aryl, 4-8 membered heterocyclic group, or a C_1 to C_8 alkyl, C_2 to C_8 alkenyl, or C_2 to C_8 alkynyl, each of which is optionally terminally substituted with an aryl group; and

X^- is a counterion, such as a halide ion, a phosphate, sulfate, acetate, or hexafluorophosphate ion; and

(b) a buffer, a cell culture medium or a combination thereof,

wherein the composition does not contain dimethylsulfoxide (DMSO) or glycerol, or contains < 10% by weight of DMSO and/or < 40% by weight of glycerol.

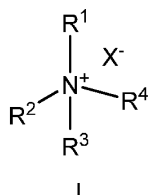
[0015] In some embodiments, the composition further comprises an ice crystal recrystallization inhibitor that does not have the structure of Formula I, and/or the composition further comprises one or more of lactobionate, glycerol, polyvinylalcohol, hydroxyethyl starch (HES), ethylene glycol (EG), propylene glycol (PG), mannitol, amino acids, urea, a reducing sugar (e.g., glucose, xylose or trehalose) and a biocompatible disaccharide or polysaccharide. The combination of the compound of Formula I and the one or more additional compound optionally forms a deep eutectic system.

[0016] In some embodiments, the composition comprises a buffer or cell culture medium that is phosphate buffered saline optionally comprising albumin, Eagle's minimum essential medium, Dulbecco's modified Eagle's medium, RPMI, fetal bovine serum, fetal calf serum, Ham's F-10, Ham's F-12, Medium 199, Hank's Buffered Salt Solution, Hank's Buffered Salt Solution and dextrose, or a combination thereof.

[0017] In some embodiments, the cryopreservation composition further comprises a biological material from a human, an animal, a plant, a fungus or a microorganism. For example, the biological material is an organ, a tissue, cells or platelets, where the cells can be or can comprise stem cells, T cells, peripheral blood mononuclear cells (PMBCs), neurons, progenitor cells, liver cells, red blood cells, immune cells, endothelial cells or cells from a cell line, preferably the cells comprise stem cells, T cells, red blood cells, progenitor cells, or liver cells.

[0018] In accordance with another aspect of the present application, there is provided a method for cryopreserving a biological material comprising:

- (a) suspending the biological material in a cryopreservation composition, wherein the cryopreservation composition comprises:
 - (i) one or more compound of Formula I



where

each of R^1 , R^2 and R^3 is independently H, C_1 to C_8 alkyl, C_2 to C_8 alkenyl, C_2 to C_8 alkynyl, or a C_1 to C_8 alkyl, C_2 to C_8 alkenyl, or C_2 to C_8 alkynyl that is terminally substituted with an alcohol, aryl or heteroaryl, or

one of R^1 , R^2 and R^3 is absent, H, C_1 to C_8 alkyl, C_2 to C_8 alkenyl, C_2 to C_8 alkynyl, or a C_1 to C_8 alkyl, C_2 to C_8 alkenyl, or C_2 to C_8 alkynyl that is terminally substituted with an alcohol, aryl or heteroaryl, and the

remaining two of R^1 , R^2 and R^3 , together with the nitrogen to which they are attached, form a 4 to 8-membered heterocyclic ring;

R^4 is H, C_1 to C_9 alkyl, C_2 to C_9 alkenyl, C_2 to C_9 alkynyl, or a C_2 to C_9 alkyl, C_2 to C_9 alkenyl, or C_2 to C_9 alkynyl that is terminally substituted with an alcohol, $C(O)OH$, $C(O)OR_5$ or $C(O)NR_6R_7$, wherein R^5 is C_1 to C_8 alkyl, C_2 to C_8 alkenyl, or C_2 to C_8 alkynyl, each of which is optionally terminally substituted with an aryl group, alcohol group, or carboxylic acid group, and wherein each of R^6 and R^7 is independently H, C_1 to C_8 alkoxy, aryl, substituted aryl, 4-8 membered heterocyclic group, or a C_1 to C_8 alkyl, C_2 to C_8 alkenyl, or C_2 to C_8 alkynyl, each of which is optionally terminally substituted with an aryl group; and

X^- is a counterion, such as a halide ion, a phosphate, sulfate, acetate, or hexafluorophosphate ion; and

(ii) a buffer, a cell culture medium or a combination thereof,

wherein the composition does not contain dimethylsulfoxide (DMSO) or glycerol, or contains <10% by weight of DMSO and/or <40% by weight of glycerol (< 40 %);

(b) cooling the suspension from step (a) to a storage temperature at or below the freezing point of the cryopreservation composition; and

(c) storing the cooled suspension at the storage temperature.

[0019] In some embodiments, the method is for cryopreserving biological material from a human, an animal, a plant, a fungus or a microorganism. For example, the biological material is an organ, a tissue, cells or platelets, where the cells can be or can comprise stem cells, T cells, peripheral blood mononuclear cells (PMBCs), neurons, progenitor cells, liver cells, red blood cells, immune cells, endothelial cells or cells from a cell line, preferably the cells comprise stem cells, T cells, red blood cells, progenitor cells, or liver cells.

[0020] In some embodiments, the step of cooling the suspension comprises performing a rate-controlled cooling of about 1°C per minute over about 16 hours.

BRIEF DESCRIPTION OF TABLES AND FIGURES

[0021] For a better understanding of the application as described herein, as well as other aspects and further features thereof, reference is made to the following description which is to be used in conjunction with the accompanying drawings, where:

[0022] Figure 1 depicts (A) ammonium-salt based compounds that have been previously found to function as IRIs but that were also found to be cytotoxic, and (B) choline chloride, which is a biocompatible alternative to previously investigated ammonium-based salts.

[0023] Figure 2 illustrates ice recrystallization inhibition activity of choline-based compounds **1-4** measured via the modified splat-cooling assay. Ice crystals images were collected in triplicate for each concentration following a 5-minute annealing period. Using the cross-sectional areas of all ice crystals within an image, the rate of ice crystal growth could be calculated and normalized (v_{norm}) to a phosphate-buffered saline (PBS) control ($n = 3$, mean $\pm$ SD) and plotted to generate a dose-response curve. From the dose-response curve, the half maximal inhibitory concentration (IC_{50}) for each IRI is extrapolated and represents a quantitative measure of IRI activity that can be directly compared across compounds (see Table 1).

[0024] Figure 3 illustrates cell viability of HepG2 cells in the presence of ChCl and modified choline-based derivatives **1-4** dissolved in supplemented Minimum Essential Media (MEM). Cells were incubated with IRIs at various concentrations for 24 hours, followed by analysis of cell viability via resazurin viability dye. Data are reported as the mean viability ($N = 2$, $n = 18$). (A) Dose-response curves were generated by plotting the % mean viability versus the concentration of IRI on a semi-logarithmic plot. Error bars indicate the standard deviation of the % mean viability for each concentration. From the dose-response curve, the 24-hour median lethal dose (LD_{50}) can be extrapolated. (B) The mean % viability for each concentration of IRI is presented for compounds **1** and **2** and error bars indicate the %SEM. Asterisks indicate statistical significance from control conditions (0 mM IRI) found using two-

way ANOVA with Šídák's multiple comparisons test (* $p < 0.05$, ** $p < 0.01$, ***, $p < 0.001$, **** $p < 0.0001$).

[0025] Figure 4 illustrates cell viability of HepG2 cells in the presence of ChCl (**1**) and modified choline-based derivatives **2**, **3**, and **8** as part of a deep eutectic system (DES) with glucose. Dose-response curves were generated by plotting the % mean viability versus the concentration of IRI within the DES on a semi-logarithmic plot.

[0026] Figure 5 schematically depicts the protocol used for Tf-1a cryopreservation studies.

[0027] Figure 6 illustrates post-thaw viability of Tf-1a cells cryopreserved with ChCl and varying concentrations of DMSO. Data presented as mean $\pm$ SEM, $n = 4$ for with $> 75,000$ events for all conditions, asterisk indicates significant difference determined using two-way ANOVA with Tukey's multiple comparison with $\geq 95\%$ confidence (* $p < 0.05$, ** $p < 0.01$).

DETAILED DESCRIPTION

[0028] Definitions

[0029] Unless defined otherwise, 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 belongs.

[0030] As used in the specification and claims, the singular forms "a", "an" and "the" include plural references unless the context clearly dictates otherwise.

[0031] The term "comprising", as used herein, will be understood to mean that the list following is non-exhaustive and may or may not include any other additional suitable items, for example one or more further feature(s), component(s) and/or ingredient(s) as appropriate.

[0032] Reference throughout this specification to "one embodiment," "an embodiment," "another embodiment," "a particular embodiment," "a related embodiment," "a certain embodiment," "an additional embodiment," or "a further embodiment" or combinations thereof means that a particular feature, structure or characteristic described in connection

with the embodiment is included in at least one embodiment. Thus, the appearances of the foregoing phrases in various places throughout this specification are not necessarily all referring to the same embodiment. Furthermore, the particular features, structures, or characteristics may be combined in any suitable manner in one or more embodiments.

[0033] The term “and/or” as used in a phrase such as “A and/or B” herein is intended to include “A and B”, “A or B”, “A”, and “B”.

[0034] The term “alkoxy”, as used herein, refers to straight or branched alkyl group bonded to an oxygen. In some embodiments, the alkoxy group includes an alkyl having 1 to about 10 carbons, or 1 to about 8 carbons, or 1 to 4 carbons. Examples include, but are not limited to, methoxy, ethoxy, propoxy, butoxy, pentoxy, hexoxy, heptoxy and octoxy. By way of example, the term “C₁-C₄-alkoxy” refers to an alkoxy having 1 to 4 carbon atoms, such as, but not limited to, methoxy, ethoxy, *n*-propoxy, 1-methylethoxy, *n*-butoxy, 1-methylpropoxy, 2-methylpropoxy and 1,1-dimethylethoxy. “Alkoxy” is intended to embrace all structural isomeric forms of an alkoxy group. For example, as used herein, propoxy encompasses both *n*-propoxy and isopropoxy, etc.

[0035] The term “alkyl”, as used herein, refers to a monovalent saturated hydrocarbon chain of 1 to about 12, or 1 to about 8, or 1 to 4 carbon atoms in length, such as, but not limited to, methyl, ethyl, propyl and butyl. The alkyl group may be a straight-chain, a branched-chain or cyclic. By way of example, the term “C₁-C₄-alkyl” as used herein refers to a saturated straight-chain or branched hydrocarbon having 1 to 4 carbon atoms. “Alkyl” is intended to embrace all structural isomeric forms of an alkyl group. For example, as used herein, propyl encompasses both *n*-propyl and isopropyl; butyl encompasses *n*-butyl, *sec*-butyl, isobutyl and *tert*-butyl.

[0036] The term “alkenyl”, as used herein, refers to a monovalent hydrocarbon chain of 2 to about 12, or 2 to about 8, or 2 to 4 carbon atoms in length that contains at least one carbon-carbon double bond. The alkenyl group may be a straight-chain, a branched-chain or cyclic. By way of example, the term “C₂-C₄-alkenyl” as used herein refers to a straight-chain or branched hydrocarbon having 2 to 4 carbon atoms and containing at least one carbon-

carbon double bond. “Alkenyl” is intended to embrace all structural isomeric forms of an alkenyl group.

[0037] The term “alkynyl”, as used herein, refers to a monovalent hydrocarbon chain of 2 to about 12, or 2 to about 8, or 2 to 4 carbon atoms in length that contains at least one carbon-carbon triple bond. The alkynyl group may be a straight-chain, a branched-chain or cyclic. By way of example, the term “C₂-C₄-alkynyl” as used herein refers to a straight-chain or branched hydrocarbon having 2 to 4 carbon atoms and containing at least one carbon-carbon triple bond. “Alkynyl” is intended to embrace all structural isomeric forms of an alkynyl group.

[0038] The term “aryl”, as used herein, refers to a monocyclic or polycyclic aromatic group, such as, but not limited to, phenyl, naphthyl, thienyl and indolyl.

[0039] The term “biological material”, as used herein, refers to any substance that can be or has been removed from a plant, a fungus, a bacterial colony, a human or a non-human animal that is suitable for cryopreservation, such as, but not limited to, organs, tissues, cells, platelets, eggs, and, embryos. Alternatively, the biological material can be cultured cells, tissues or organs. Examples of “cells” include, but are not limited to, stem cells, T cells, oocytes, sperm, peripheral blood mononuclear cells (PMBCs), MSCs, iPSCs, hiPSCs, HSPCs, neurons, progenitor cells, liver cells, red blood cells, immune cells, endothelial cells, fungal cells, or cells from a cell line. In another alternative, the “cells” can be unicellular microorganisms, including, for example, bacterial or unicellular fungal (e.g., yeast) cells. The cells can be in an isolated or a purified form, or they can be in a mixture with one or more other cell types.

[0040] In some embodiments, the biological material is from a non-human animal or human source. In alternative embodiments, the biological material is from a plant source, a fungal source or a bacterial source. For example, the plant-sourced biological material can be a plant cell, tissue or organ or can be plant seed or fruit, or a part thereof.

[0041] The terms “cell medium”, “cell culture medium”, and “growth medium”, are used interchangeably herein to refer to a liquid or gel composition used to support cellular growth of cells, tissues or organs in an artificial environment. Examples of cell culture media

include, but are not limited to, Eagle's Minimum Essential Medium (MEM), Dulbecco's Modified Eagle's Medium (DMEM), Roswell Park Memorial Institute medium (RPMI), Fetal Bovine Serum (FBS), Fetal Calf Serum (FCS), Ham's F-10, Ham's F-12, Hank's buffered salt solution (HBSS), HBSS and dextrose, and Medium 199.

[0042] The terms “cryopreservation agent”, “cyroprotecting agent”, and “cyroprotectant” are used interchangeably herein to refer to a compound that assists in the cryopreservation of a biological material. Examples of cryopreservation agents include, but are not limited to, dimethyl sulfoxide (DMSO), lactobionate, glycerol, trehalose, mannitol, hydroxyethyl starch (HES), ethylene glycol (EG), propylene glycol (PG), and other biopolymers useful in cryopreservation. Examples of biopolymers that function as cryopreservation agents include, but are not limited to, polyvinyl alcohols. As used herein, the term “cryopreservation agent” is not intended to include water, RPMI, DMEM, or MEM, HBSS or dextrose alone (i.e., when not used as part of a deep eutectic system).

[0043] The terms “cryopreservation composition”, “cryopreservation formulation”, and “cryomedium” are used interchangeably herein to refer to a composition that contains one or more cryoprotective agents (CPAs) and carrier media (e.g., a buffer and/or growth medium) that is selected to provide support to cells for storage at low temperatures, which may be subzero temperatures.

[0044] The term “heterocyclic ring”, as used herein refers to four-membered to eight-membered rings that have 1 to 4 heteroatoms, such as oxygen, sulfur and/or nitrogen. These four-membered to eight-membered rings can be saturated, fully unsaturated or partially unsaturated. As used herein a “heteroaryl” is a cyclic aromatic radical having from five to ten ring atoms of which one ring atom is selected from S, O and N; zero, one or two ring atoms are additional heteroatoms independently selected from S, O and N; and the remaining ring atoms are carbon, the radical being joined to the rest of the molecule via any of the ring atoms, such as, for example, pyridyl, pyrazinyl, pyrimidinyl, pyrrolyl, pyrazolyl, imidazolyl, thiazolyl, oxazolyl, isooxazolyl, thiadiazolyl, oxadiazolyl, thiophenyl, furanyl, quinoliny, isoquinoliny, and the like. Non-limiting examples of nitrogen-containing heterocyclic rings include piperidinyl, pyrrolidinyl, pyrrolyl, pyrazolyl, pyrazolidinyl, pyridinyl, pyrimidinyl, piperazinyl, indoliny, and the like.

[0045] The term “ice recrystallization inhibitor” is used herein to refer to a type of cyroprotectant that inhibits ice recrystallization during cryopreservation. As used herein, a compound has ice recrystallization inhibitor activity if it demonstrates ice recrystallization inhibition with a half maximal inhibitory concentration (IC_{50}) of 100 mM or less. As used herein, “good” ice recrystallization inhibition is intended to refer to an IC_{50} < about 45 mM, or preferably an IC_{50} < about 35 mM.

[0046] The term “independently” is used herein when referencing alternatives for chemical substituents to indicate that each of recited substituents may be the same or different from one another.

[0047] The term “substituted” as used herein, means that any one or more hydrogens on a designated atom is replaced with a substituent, provided that the designated atom's normal valence is not exceeded, and that the substitution results in a stable compound. A substituent can be, but is not limited to, an alkyl, alkenyl, alkynyl, alkoxy, aryl or heteroaryl, as defined herein, or a halogen. It is understood that substituents and substitution patterns on the compounds of the present application can be selected by one of ordinary skill in the art to provide compounds that are chemically stable and that can be readily synthesized by techniques known in the art, as well as those methods set forth below. If a substituent is itself substituted with more than one group, it is understood that these multiple groups may be on the same atom or on different atoms, so long as a stable structure results.

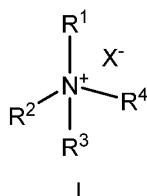
[0048] The present inventors have surprisingly found that choline salts and a class of choline-based derivatives can effectively replace DMSO and/or glycerol as cryoprotective agents (CPAs) during cryopreservation of biological material. This class of choline-based derivatives include a secondary, tertiary or quaternary ammonium group. Furthermore, the present inventors have surprisingly found that this class of choline-based derivatives exhibit ice recrystallization inhibition activity. This is the first instance of the use of ammonium salt-based CPAs that addresses the problem of ice recrystallization during cryopreservation.

[0049] The development of ice recrystallization inhibitors (IRIs) has proven to be a successful strategy to mitigate ice crystal growth and improve the cryopreservation outcome of various cell types. It has been previously reported that charged molecules

containing an ammonium counterion possess good IRI activity but suffer from cytotoxicity issues due to the toxicity of the ammonium ion.²¹ To address these toxicity concerns, the present inventors sought to optimize naturally occurring non-toxic ammonium-based metabolites for IRI activity and cryopreservation efficacy. Choline chloride (ChCl; Figure 1B) was identified by the inventors as the starting point for IRI optimization since it is biocompatible, non-toxic and has been previously used successfully in cryopreservation applications.^{18,19}

[0050] Choline-based, ammonium-containing cryopreservation agents

[0051] In accordance with one embodiment of the present application, there is provided a cryopreservation agent (CPA) having the structure of Formula I:



where

each of R¹, R² and R³ is independently H, C₁ to C₈ alkyl, C₂ to C₈ alkenyl, C₂ to C₈ alkynyl, or a C₁ to C₈ alkyl, C₂ to C₈ alkenyl, or C₂ to C₈ alkynyl that is terminally substituted with an alcohol, aryl or heteroaryl, or

one of R¹, R² and R³ is absent, H, C₁ to C₈ alkyl, C₂ to C₈ alkenyl, C₂ to C₈ alkynyl, or a C₁ to C₈ alkyl, C₂ to C₈ alkenyl, or C₂ to C₈ alkynyl that is terminally substituted with an alcohol, aryl or heteroaryl, and the remaining two of R¹, R² and R³, together with the nitrogen to which they are attached, form a 4 to 8-membered heterocyclic ring;

R⁴ is H, C₁ to C₉ alkyl, C₂ to C₉ alkenyl, C₂ to C₉ alkynyl, or a C₂ to C₉ alkyl, C₂ to C₉ alkenyl, or C₂ to C₉ alkynyl that is terminally substituted with an alcohol, C(O)OH, C(O)OR⁵ or C(O)NR⁶R⁷, wherein R⁵ is C₁ to C₈ alkyl, C₂ to C₈ alkenyl, or C₂ to C₈ alkynyl, each of which is optionally terminally substituted with an aryl group, alcohol group, or carboxylic acid group, and wherein each of R⁶ and R⁷ is independently H, C₁ to C₈ alkoxy, aryl, substituted aryl, 4-8

membered heterocyclic group, or a C₁ to C₈ alkyl, C₂ to C₈ alkenyl, or C₂ to C₈ alkynyl, each of which is optionally terminally substituted with an aryl group; and

X⁻ is a counterion, such as a halide ion, a phosphate, sulfate, acetate, or hexafluorophosphate ion.

[0052] As described above, these compounds have been found by the present inventors to function as CPAs. In particular, the CPAs of Formula I can be used to replace DMSO and/or glycerol in cryopreservation compositions, thereby avoiding the cytotoxic effects of DMSO and/or glycerol during cryopreservation.

[0053] Choline chloride (ChCl) is a non-toxic and biocompatible salt naturally found in the body, which the present inventors have found can be modified through synthetically accessible modifications to improve the IRI activity up to 10-fold over ChCl. Furthermore, these IRI active derivatives of ChCl maintain their biocompatibility at the concentrations at which they are used in cryopreservation compositions. The gap between the IRI IC₅₀ values for these compounds and the 24-hour LD₅₀ values indicates that they can be effectively implemented as IRI cryo-additives in biological systems. In this regard, the IC₅₀ values determined for the present CPAs of Formula I are significantly lower than the measured 24-hour LD₅₀ values.

[0054] Furthermore, preliminary assessment of the cryopreservation efficacy of ChCl and the *N*-ethyl modified derivative (**2**) demonstrated that these compounds have the ability to act as a CPA with a similar level of effectiveness as DMSO, such that compounds of Formula I can be used as CPAs in DMSO-free cryopreservation compositions and methods. The choline-based compounds of Formula I represent a class of CPAs having IRI activity that are amenable to large-scale preparation for use in the cryopreservation of various cell types, including cryo-sensitive and complex cell lines.

[0055] In some embodiments of the compound of Formula I, R¹, R² and R³ are not all methyl. In other embodiments, when one of R¹, R² and R³ is methyl then the other two are not both ethyl, and when two of R¹, R² and R³ are both methyl the remaining one of R¹, R² and R³ is not ethyl. In other embodiments, R¹, R², R³ and R⁴ are not all *n*-butyl. In some

embodiments, the compound of Formula I is not a choline, betaine, dimethylethanolammonium, or *N*-methylethanolammonium salt. However, the present application does relate to the use of such compounds in the cryopreservation compositions and methods, as described herein.

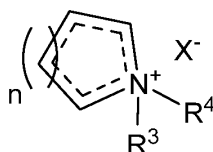
[0056] In some embodiments, selection of the R^1 , R^2 and R^3 groups in the compound of Formula I is made to increase hydrophobicity about the nitrogen atom in comparison to ChCl.

[0057] When R^1 , R^2 and/or R^3 is an alkyl group it is a branched or straight alkyl group having from 1 to 8 carbon atoms, or from 1 to 4 carbon atoms. In some embodiments, one or more of R^1 , R^2 and R^3 is methyl, ethyl, propyl, isopropyl, *n*-butyl, *sec*-butyl, *tert*-butyl, or isobutyl.

[0058] In some embodiments, one or more of R^1 , R^2 and R^3 is a C_1 - C_8 alkyl terminally substituted with an aryl or a heteroaryl, or a C_1 - C_4 alkyl terminally substituted with an aryl or a heteroaryl. For example, the aryl group can be phenyl. In another example, the heteroaryl group is pyrrolyl, pyrrolidinyl, pyridinyl or pyrimidinyl. In one embodiment, one or more of R^1 , R^2 and R^3 is benzyl, or only one of R^1 , R^2 and R^3 is benzyl.

[0059] In an alternative embodiment, one of R^1 , R^2 and R^3 is absent, H, C_1 - C_9 alkyl (e.g., a C_1 - C_4 alkyl), or a C_1 - C_8 alkyl terminally substituted with an aryl or heteroaryl (e.g., C_1 - C_4 alkyl terminally substituted with an aryl, such as phenyl, or heteroaryl, such as pyrrolyl, pyrrolidinyl, pyridinyl or pyrimidinyl), and the other two of R^1 , R^2 and R^3 , together with the nitrogen to which they are attached, form a 4 to 8-membered heterocyclic ring.

[0060] In one embodiment, the compound of Formula I has the structure:



wherein n is an integer from 0 to 4, and R^3 , R^4 and X^- are as defined herein, including in the specific embodiments; and wherein the dashed line indicates the optional presence of one or more double bonds in the heterocyclic ring.

[0061] As would be appreciated by the skilled person, in the embodiments in which only one or two of R^1 , R^2 and R^3 are not H, the nitrogen will remain positively charged at physiological pH, and the remaining one or two of R^1 , R^2 and R^3 are H.

[0062] In some embodiments, the ethanol chain of choline is modified to alter its hydrophilicity or to introduce another terminal functional group. In some embodiments, R^4 is H. In other embodiments, R^4 is an aliphatic chain that is a C_1 to C_9 alkyl, C_2 to C_9 alkenyl, C_2 to C_9 alkynyl, C_1 to C_5 alkyl, C_2 to C_5 alkenyl, or C_2 to C_5 alkynyl.

[0063] In other embodiments, R^4 is an aliphatic chain that is a C_1 to C_9 alkyl, C_2 to C_9 alkenyl, C_2 to C_9 alkynyl, C_1 to C_5 alkyl, C_1 to C_5 alkenyl, or C_1 to C_5 alkynyl, in which the aliphatic chain is terminally substituted with an alcohol, carboxylic acid, carboxylate, amide, or ester. The terminal ester group is $C(O)OR^5$, wherein R^5 is C_1 to C_8 alkyl, C_1 to C_8 alkenyl, or C_1 to C_8 alkynyl, each of which is, optionally, terminally substituted with an aryl, alcohol, or carboxylic acid/carboxylate. The terminal amide group is $C(O)NR^6R^7$, wherein each of R^6 and R^7 is independently H, C_1 to C_8 alkoxy, aryl, substituted aryl, 4-8 membered heterocyclic group, or a C_1 to C_8 alkyl, C_2 to C_8 alkenyl, or C_2 to C_8 alkynyl, each of which is optionally terminally substituted with an aryl group.

[0064] The counterion X^- in the compound of Formula I is a biologically compatible ion or is present at a concentration that is biocompatible. In particular, the counterion is selected so that it does not adversely affect cryopreservation based on its cytotoxicity and/or the concentration at which it becomes cytotoxic. The counterion is selected such that it is not cytotoxic at the concentrations used during cryopreservation. In some embodiments, the counterion is one that has been used in regulatory approved pharmaceuticals and/or is generally recognized as safe (GRAS). Non-limiting examples of suitable counterions include halide, a phosphate, sulfate, acetate, or hexafluorophosphate.

[0065] Compositions for Preserving Biological Material

[0066] Also provided herein are compositions for preserving biological material comprising one or more ammonium compounds of Formula I, optionally in combination with one or more cryoprotecting agents (e.g., other IRIs), as defined herein. The cryopreservation compositions of the present application are suitable for use in cryopreservation, in which

the compositions comprising the biological material are frozen, or for subzero preservation without freezing. In both instances, the presence of one or more choline-derivative compounds of the present application aids in cryopreservation, for example by inhibiting ice recrystallization, during cooling and during warming following cryopreservation. The preservation compositions of the present application can also be used in non-cryopreservation storage methods, such as, for example, hypothermic/vitrification storage methods and normothermic storage methods.

[0067] In a particular embodiment, the at least one compound of Formula I is present in the composition at a concentration of less than about 500 mM, preferably less than about 400 mM, more preferably less than about 300 mM, even more preferably less than about 200 mM, still more preferably less than about 100 mM, and most preferably less than about 50 mM. In another embodiment, the at least one compound of Formula I is present in the composition at a concentration of about 0.5 mM to (and including) about 500 mM, or about 40 mM to (and including) about 400 mM, or about 100 mM to about 250 mM.

[0068] In a particular embodiment, compositions for cryopreserving a biological material are provided herein, wherein the composition additionally comprises at least one, at least two, or at least three, cryopreservation agents selected from the group consisting of lactobionate, glycerol, polyvinylalcohol, hydroxyethyl starch (HES), ethylene glycol (EG), propylene glycol (PG), polyvinyl alcohol, other biopolymers useful in cryopreservation, mannitol, amino acids, urea, reducing sugars and biocompatible disaccharides or polysaccharides. In some embodiments, the reducing sugar is glucose, xylose, trehalose, or a combination thereof. In some embodiments, the at least one additional cryopreservation agent, when present, is in the composition at a concentration of at least about 0.1% wt%, about 0.1 wt% to (and including) about 30 wt%, preferably about 0.1 wt% to (and including) about 20 wt%, preferably about 5 wt% to (and including) about 30 wt%, and preferably about 5 wt% to (and including) about 20 wt%. In embodiments in which the additional cryopreservation agent(s) form part of a deep eutectic system (as described below), the the additional cryopreservation agent(s) may be present at an amount of great that 10 wt%, greater than 20 wt% or greater than 30 wt%. Alternatively, or in addition, the composition additionally comprises at least one, at least two, or at least three additional IRIs, such as those described in international PCT Publication No., WO 2013/175299.

[0069] In some embodiments, the cryopreservation composition includes a deep eutectic system (DES) comprising one or more CPA of Formula I. DES's can be attractive in cryopreservation compositions due to their sustainable properties such as high biodegradability, cost-effectiveness, and low toxicity. These systems can be formed by natural primary metabolites, such as sugars, amino acids, or organic acids. The components are mixed at a particular molar ratio, presenting a high melting point depression, often called the "eutectic point", compared to that of the individual constituents, therefore becoming liquid at room temperature or near room temperature. In one embodiment, the cryopreservation composition contains a DES comprising a CPA of Formula I in combination with one or more reducing sugar (e.g., glucose, xylose, trehalose). Incorporation of the compound of Formula I in a DES can reduce the CPA's cytotoxicity, which is already low (as shown in the Examples), thereby allowing the use of higher concentrations of the CPA.

[0070] The cryopreservation compositions of the present application do not require the presence of DMSO or glycerol to provide cryoprotection of a biological material during cryostorage. Accordingly, in some embodiments the cryopreservation composition does not contain DMSO or glycerol or contains less DMSO and/or glycerol than typically used during cryopreservation. In other embodiments, the cryopreservation composition comprises less than 10 wt% DMSO, or less than 2 wt% of DMSO. Alternatively, or in addition, the cryopreservation composition comprises less than 40 wt% of glycerol.

[0071] In a particular embodiment, compositions for cryopreserving a biological material are provided herein which further comprise a biological material. The biological material can be, for example, organs, tissues, cells, platelets, eggs, and embryos. Alternatively, the biological material can be cultured cells, tissues or organs. Examples of "cells" include, but are not limited to, stem cells, T cells, oocytes, sperm, peripheral blood mononuclear cells (PMBCs), neurons, progenitor cells, liver cells, red blood cells, immune cells, endothelial cells or cells from a cell line. The cells can be in an isolated or a purified form, or they can be in a mixture with one or more other cell types.

[0072] In some embodiments, the biological material is from a non-human animal or human source. For example, the biological material from a non-human animal or human source can be a cell, tissue or organ. In alternative embodiments, the biological material is from a plant

source. For example, the plant-sourced biological material can be a plant cell, tissue or organ or can be plant seed or fruit, or a part thereof. In other embodiments, the biological material is from a fungal source. In yet other embodiments, the biological material comprises microorganisms of one or more types or species.

[0073] In a particular embodiment, compositions for cryopreserving a biological material are provided herein further comprising a buffer, cell culture or growth medium. Examples of suitable cell culture media include, but are not limited to, Eagle's Minimum Essential Medium (MEM), Dulbecco's Modified Eagle's Medium (DMEM), Roswell Park Memorial Institute medium (RPMI), Fetal Bovine Serum (FBS), Fetal Calf Serum (FCS), Ham's F-10, Ham's F-12, Hank's buffered salt solution (HBSS), HBSS and dextrose, Medium 199 and combinations thereof. In some embodiments the cryopreservation compositions comprise a buffer to maintain the pH at about physiological pH during cryopreservation.

[0074] In another embodiment, the compositions for cryopreservation can contain both biological material and cell medium as provided above.

[0075] Kits for Preserving Biological Material

[0076] Kits for cryopreserving a biological material are provided herein comprising at least one compound of Formula I or composition for cryopreserving a biological material as described herein. In some embodiments, the kit comprises at least one compound of Formula I and one or more further cryopreservation agents (e.g., an IRI). In this embodiment, the at least one compound of Formula I and one or more further cryopreservation agents (e.g., an IRI) can be in the same composition or in separate compositions. Additionally, they can be co-packaged for common presentation or packaged individually. The kit can additionally include instructions for cryopreservation of various types of biological material. The kits provided herein can further comprise a cell culture or growth medium, which are optionally provided in a separate container. The kits provided herein can further contain one or more containers suitable for cryostorage of the biological material in the cryopreservation composition.

[0077] Methods for Cryopreserving Biological Material

[0078] Methods for cryopreserving a biological material are provided herein by suspending the biological material in a solution of at least one ammonium compound of Formula I to form a suspension and freezing the suspension or cooling the suspension to a sub-zero storage temperature.

[0079] In some embodiments, the method comprises adding a biological material to a solution comprising at least one ammonium compound of Formula I, and then cryopreserving the biological material in cryogenic vials or other suitable container. The vials can be frozen under rate controlled freezing conditions, such as freezing at 1°C per minute over 16 hours. The vials can be stored using standard cryopreservation techniques, and then they can be thawed when required by removing the vials from the cold storage, and thawing using standard protocols. Examples of standard cryopreservation techniques include freezing in liquid nitrogen to about -196 °C and freezing in dry ice to about -80 °C. Examples of standard thawing protocols include, but are not limited to, ambient thaw or rapid thaw in a water bath at a temperature that is at or between room temperature and 37 °C. Alternatively, a controlled, slow thaw protocol can be used.

[0080] In a particular embodiment, methods for cryopreserving a biological material are provided herein by suspending biological material in a solution of at least one ammonium compound of Formula I to form a suspension, which is contained in a vial or other suitable container, wherein the vial is frozen directly in a storage unit without a rate controlled freezing protocol.

[0081] Methods for Inhibiting Ice Recrystallization in Biological Material

[0082] Also provided herein is a method for inhibiting ice recrystallization during cryopreservation or sub-zero storage of a biological material, which method comprises suspending a biological material in a solution of at least one ammonium compound of Formula I reported herein to form a suspension and cryopreserving the resultant suspension or cooling the resultant suspension to a sub-zero storage temperature.

[0083] To gain a better understanding of the invention described herein, the following examples are set forth. It should be understood that these examples are for illustrative purposes only. Therefore, they should not limit the scope of this invention in any way.

EXAMPLES

[0084] EXAMPLE 1: Modification of Choline Chloride (ChCl) to Improve IRI activity

[0085] Expanding upon previous work demonstrating that ammonium (NH_4^+) is beneficial for IRI activity, the IRI activity of ChCl was determined using a modified splat-cooling assay. Surprisingly, ChCl (**1**) does not exhibit meaningful IRI activity ($\text{IC}_{50} = 235 \text{ mM}$, 95% CI [140 – 344]), and therefore, the present inventors sought to improve the IRI activity of the compound through simple chemical modifications.

[0086] Methodology

[0087] Briefly, the modified splat-cooling assay used in the present study represents IRI activity as a dose-response curve of the normalized ice crystal growth rate (y-axis) versus the concentration of IRI (x-axis). The experimental details of the assay used in this study have been previously published and demonstrated to be appropriate for providing accurate ice recrystallization activity data.²²

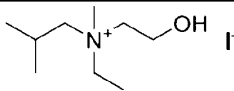
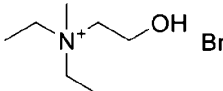
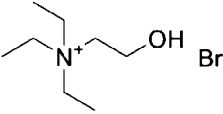
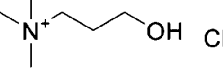
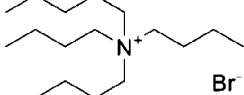
[0088] Solutions of the choline-derivatives were initially prepared with a range of concentrations solubilized in phosphate-buffered saline (PBS). The upper plateau of the curve was defined at 100 by the control sample, where the control was PBS, which is non-IRI active. The IC_{50} value and Hill slope for each compound were then determined by nonlinear regression using a 4-parameter sigmoidal dose-response fitting equation. The lower bound was defined with the assay development in terms of what is considered rate = 0.

[0089] The rate of ice crystal growth was normalized to the non-IRI active PBS control. From the dose-response curve, a half-maximal inhibitory concentration (IC_{50}) was extrapolated for each, where smaller IC_{50} values indicate smaller ice crystals and more effective inhibition.²²

[0090] Results

[0091] All *N*-alkyl modifications tested exhibited improvements to IRI activity in comparison to the parent ChCl molecule, with some modifications surprisingly increasing the IRI activity up to 10-fold (Figure 2, Table 1).

#	Compound	IC ₅₀ (mM)	LD ₅₀ (mM)
1		234	83
Choline acetate		80	
2		23	41
3		22	21
4		18	58
5		197	N/A
6		263	N/A
7		No measurable IRI activity	N/A
8		43	46
9		14	41
10		46	42

#	Compound	IC ₅₀ (mM)	LD ₅₀ (mM)
11	 I ⁻	82	N/A
12	 Br ⁻	67	N/A
13	 Br ⁻	45	N/A
14	 Cl ⁻	56	N/A
15	 Br ⁻	2	--

[0092] Substitution of one *N*-methyl branch on ChCl for another *N*-alkyl chain (i.e., *N*-ethyl, *N*-isopropyl, *N*-butyl, etc.) resulted in at least a 5-fold improvement in IRI activity, with *N*-ethyl (**2**, IC₅₀ = 23 mM) and *N*-isopropyl derivatives (**3**, **4**, IC₅₀ = 18 mM, 22 mM) exhibiting the highest IRI activities. Substitution of two *N*-methyl branches on ChCl for another *N*-alkyl chain showed similar improvements to the IRI activity in comparison to ChCl, with IC₅₀ values in the range of 14 to 83 mM. Notably, substitution of two *N*-methyl branches for two *N*-butyl branches yielded an improved IC₅₀ of 14 mM. Finally, derivatives of ChCl with modifications to all three *N*-methyl branches were generated, with IC₅₀ values within the range of 10 to 46 mM. Interestingly, these highly hydrophobic trisubstituted derivatives possessed less IRI activity than tetrabutylammonium bromide (**15**), which was found to possess an IRI activity of 2 mM but is a known cytotoxic molecule.

[0093] All the above-described modifications to the *N*-alkyl position(s) resulted in a notable increase in IRI activity over ChCl, demonstrating that increased hydrophobicity is important for IRI activity.

[0094] To alter the hydrophilicity of the molecule, derivatives of ChCl with modifications to the ethanol chain (5-6) were synthesized and studied. These molecules were found to possess essentially equivalent IRI activity to the parent ChCl molecule (IC_{50} = 197 and 263 mM, for 5 and 6, respectively, versus 235 mM for ChCl). These findings suggest that the presence of the hydroxyl group and the length of the ethanol chain has little to no impact on the inhibition of ice crystal growth. Collectively, the IRI activity of ChCl was improved using synthetically accessible modifications that are amenable to large-scale synthesis.

[0095] EXAMPLE 2: Biocompatibility of choline-derivatives in HepG2 Cells

[0096] An important parameter for the development of new CPA formulations is the biocompatibility and cytotoxicity of the cryo-additive. While ChCl is a nontoxic molecule, it was necessary to determine the cytotoxicity, if any, of the novel modified choline-based molecules.

[0097] Methodology

[0098] The human hepatocellular carcinoma cell line (HepG2) is a widely used adherent cell line that exhibits high similarities to normal endogenous liver cells.²⁴ As such, HepG2 cells are commonly used to assess the cytotoxicity of small molecules and were selected as representative of cytotoxicity of other cell types.

[0099] The viability of HepG2 cells in the presence or absence of IRIs was determined after a 24-hour incubation period using resazurin as a measure of the metabolic activity of the cells following a previously published and validated method.²⁵ This assay afforded a preliminary median lethal dose (LD_{50}) measurement that goes beyond the typical exposure timepoint of CPAs during cryopreservation (typically only a few minutes versus 24 hours). In addition, this assay provides the ability to compare concentration-dependence of HepG2 viability for each IRI molecule. Collectively, this cytotoxicity data provides an indication of which IRIs are biocompatible and, therefore, useful in cellular applications with minimal toxicity or adverse biological effects.

[00100] Results

[00101] Only ChCl-based IRIs displaying moderate to strong IRI activity ($IC_{50} < 30$ mM) were assessed for cytotoxicity (Figure 3, Table 1). As a frame of reference, ChCl, which is considered non-toxic and biocompatible, exhibited a 24 hour LD_{50} of 83 mM (Figure 3, Table 1). While the cytotoxic effect was increased for the modified choline-based derivatives in comparison to ChCl, most derivatives showed LD_{50} values greater than their IC_{50} values, indicating that these molecules have the potential to be used as IRIs in cell-based systems. The *N*-ethyl choline-based derivative **2** displayed 50% cell viability at a concentration of 40 mM, while its IC_{50} was approximately half that at 23 mM. Similarly, HepG2 cells treated with the *N*-isopropyl choline bromide derivative **4** exhibited 50% viability at a concentration of 58 mM, whereas the IC_{50} value was found to be 18 mM.

[00102] The *N*-ethyl derivative **2** was studied further due to its relatively strong IRI activity (10-fold improvement to the parent ChCl with an IC_{50} of 23 mM) and weak cytotoxicity (24-hour LD_{50} of 40 mM). To further explore the biocompatibility of IRI **2**, the present inventors compared the concentration-dependence of the 24-hour cytotoxicity data of **2** to ChCl, which is biocompatible (Figure 3B). The *N*-ethyl derivative **2** displayed no significant differences in % viability at low concentrations of 0 – 25 mM, indicating that at its IC_{50} value of 23 mM, the molecule is non-toxic and biocompatible. At higher concentrations of 50 and 75 mM, significant differences were observed between ChCl and **2**, indicating that at these higher concentrations some cytotoxicity may be observed. However, since these % mean viabilities were measured at a 24-hour timepoint, the toxicity observed in this experiment is much higher than what would be experienced during use in cryopreservation since cells will be only minimally exposed to the CPA. Overall, this result is extremely promising for the CPA-potential of IRI **2** since it appears to exhibit a similar toxicity profile to that of ChCl (which is non-toxic) at IRI-relevant concentrations.

[00103] The *N*-alkyl modifications have minimal impact on the cytotoxicity of the IRI molecule in comparison to ChCl. These results further demonstrate that these choline-based derivatives have sufficiently high biocompatibility to provide meaningful IRI activity without or with only minimal toxic effects. Considering the wide IC_{50} to LD_{50} gap observed at this

extreme 24-hour timepoint, these modified choline-based derivatives can function as effective IRIs in biological systems.

[00104] The choline derivatives were used to prepare DES with glucose at a 2:1 molar ratio over a range of concentrations. As illustrated in Figure 4, the cell viability was improved when employing the choline derivatives as part of a DES with glucose; the cytotoxicity of the choline derivatives was mitigated by formation of a DES.

[00105] EXAMPLE 3: Use of Choline-based Derivative Ammonium Compounds in DMSO-free Cryopreservation Compositions

[00106] Recent studies have shown that ChCl (in complex with the cryoprotective, 1,2-propanediol) and betaine (a zwitterionic ammonium compound), have the ability to improve the cryopreservation outcome and have demonstrated success as a CPA.^{19,26} Further, betaine, used with electroporation or as part of a deep eutectic system has been shown to modify the thermal properties of water in a similar fashion to DMSO and has been proposed as a potential DMSO replacement.^{27,28}

[00107] Building on this previous work and the present cytotoxicity results in HepG2s, the present inventors sought to assess the ability of ChCl and the present choline-based derivative compounds (exemplified by the *N*-ethyl modified IRI **2**) to act as a CPA in a typical cryopreservation protocol, as illustrated in Figure 5

[00108] A cryopreservation freeze-thaw experiment was performed using ChCl or the *N*-ethyl modified IRI **2** and Tf-1a cells. The Tf-1a cell line is a model cell line for human primitive myeloid progenitor cells (PMCs), which is useful for demonstrating the CPA potential of these IRIs in a complex and cryopreservation-sensitive cell line. The present inventors assessed the ability of ChCl and **2** to improve post-thaw viability of Tf-1a cells in the presence of various concentrations of DMSO (0, 2, and 10% DMSO).

[00109] Methodology

[00110] As per the recommended cryopreservation protocol for Tf-1a cells, the Tf-1a cells were suspended in complete growth medium either in the presence or absence of IRI and supplemented with either 0, 2, 5, or 10% DMSO. 1 mL cryovials containing the

cryopreservation suspensions were first cooled to -80°C in a rate-controlled Mr. Frosty™ container for at least 12 hours, followed by transfer to storage in liquid nitrogen vapours for a minimum additional 12 hours. Cryovials were thawed using rapid thawing methods in a 37°C water bath, followed by analysis of post-thaw viability by flow cytometry.

[00111] The post-thaw viabilities were obtained through propidium iodide (PI) staining and flow cytometry. In some instances, Calcium AM staining was used in tandem with PI to provide a live:dead staining comparison.

[00112] Results

[00113] The post-thaw viabilities obtained are shown in Figure 6. Both ChCl (**1**) and the *N*-ethyl modified derivative (**2**) were effective as CPAs for the cryopreservation of Tf-1a cells. When 0% DMSO and either **1** or **2** are present, a significant improvement to post-thaw viability was observed in comparison to 0% DMSO alone ($p < 0.01$ and $p < 0.001$, respectively). Moreover, when the cryomedium was supplemented with only **1** or **2** (0% DMSO present), there were no significant differences between the IRI-treated cell populations and the industry standard 5 and 10% DMSO-treated cell populations, thereby indicating that ChCl and the modified *N*-ethyl choline derivative can both function as CPAs in DMSO-free cryopreservation formulations.

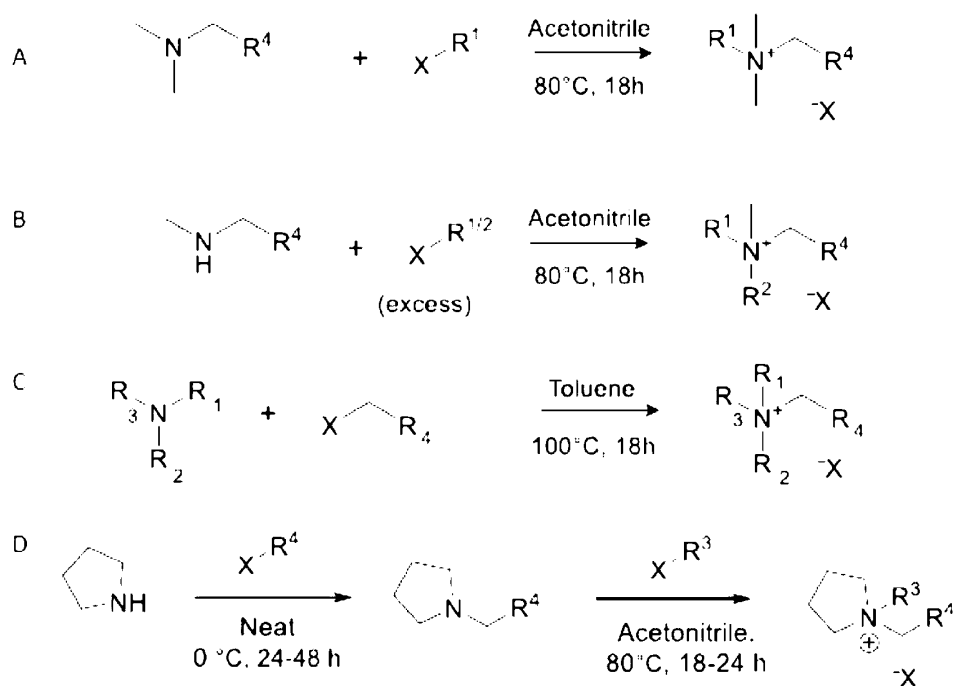
[00114] Due to the toxicity of DMSO and DMSO's inability to effectively cryopreserve emerging cell therapies, this finding is extremely relevant to the current issues facing the field of cryopreservation. The ability of the present choline-based CPAs to effectively achieve comparable post-thaw viability to 5% and 10% DMSO for complex cell types, such as Tf-1a, means that the compounds of the present application provide a biocompatible and non-toxic alternative for use in DMSO-free CPA formulations.

[00115] The post-thaw viability within each IRI treatment was also assessed with varying DMSO concentrations to determine the potential benefits of using DMSO and choline-based IRIs in tandem. The post-thaw viability of Tf-1a cells treated with **1** decreased with increasing DMSO concentrations, which is indicative of a non-beneficial interaction between ChCl and DMSO. Interestingly, the post-thaw viability of cells treated with 2% DMSO and **2** was significantly worse than using **2** and either 0% or 10% DMSO.

[00116] Collectively, these findings illustrate that choline-based CPAs perform better without the addition of DMSO, or with the addition of less than 2% DMSO, and, without wishing to be bound by theory, there is a likely an interaction between the ammonium salt and DMSO that hinders the efficacy of the CPA formulations when both compounds are present.

[00117] EXAMPLE 4: Synthesis of Choline-based Derivative Ammonium Compounds

[00118] Choline-based derivative ammonium compounds of the present application have been synthesized using different synthetic protocols, as summarized in Scheme 1 below:



[00119] General Methodology

[00120] General Protocol for Reaction A:

[00121] A flame dried round-bottom flask was fit with a rubber septum and purged under argon. Anhydrous acetonitrile (1.5 M) was added by syringe, followed by the desired

tertiary amine starting material such *N,N*-dimethylethanolamine, *N,N*-dimethylglycine (7.104 mmol, 1 eq) and the desired alkyl halide (8.524 mmol, 1.2 eq). The mixture was refluxed at 80°C with stirring overnight. The resulting solid was separated from the reaction mixture using suction filtration, washed with ethyl acetate, and the solvent was evaporated. In some cases, a recrystallization with toluene was necessary to obtain the final product (65 – 94 % yield).

[00122] General Protocol for Reaction B:

[00123] A flame dried round-bottom flask was fit with a rubber septum and purged under argon. Anhydrous acetonitrile (1.5 M) was added by syringe, followed by the desired tertiary amine starting material such *N,N*-dimethylethanolamine, *N,N*-dimethylglycine (7.104 mmol, 1 eq) and the desired alkyl halide in excess (28.416 mmol, 4 eq). The mixture was refluxed at 80°C with stirring overnight. The resulting solid was separated from the reaction mixture using suction filtration, washed with ethyl acetate, and the solvent was evaporated. In some cases, a recrystallization with toluene was necessary to obtain the final product as a faint yellow/orange or white solid (0 – 73 % yield).

[00124] General Protocol for Reaction C:

[00125] A flame dried round-bottom flask was fit with a rubber septum and purged under argon. Anhydrous toluene (3.2 M) was added by syringe, followed by the desired substituted tertiary amine (12.625 mmol, 1 eq) and corresponding the halogenated alkanol, alkanoic acid, or alkyl alkanoate starting material (15.15 mmol, 1.2 eq). The mixture was refluxed at 110°C with stirring overnight. The resulting solid was separated from the reaction mixture using suction filtration, washed with ethyl acetate, and the solvent was evaporated. In some cases, a recrystallization with toluene was necessary to obtain the final product as a faint yellow/orange or white solid (0 – 92 % yield).

[00126] General Protocol for Reaction D:

[00127] A flame dried round-bottom flask was fit with a rubber septum, purged under argon, and cooled to 0°C. The desired heterocycle (8.12 mmol, 1 eq) and halogenated alkanol, alkanoic acid, or alkyl alkanoate starting material (8.12 mmol, 1 eq) was added to

the flask, and the flask was allowed to stir until solidification (approximately 10-15 minutes). The resulting viscous oil was purified via recrystallization using acetonitrile or an acetonitrile-methanol mixture (approximately 10:1) and filtered to obtain the final product as a pure oil (yield approximately 72%).

[00128] References

1. Mazur, P. Cryobiology: The Freezing of Biological Systems: The Responses of Living Cells to Ice Formation Are of Theoretical Interest and Practical Concern. *Science* **1970**, 168 (3934), 939–949. <https://doi.org/10.1126/science.168.3934.939>.
2. Baust, J. G.; Gao, D.; Baust, J. M. Cryopreservation: An Emerging Paradigm Change. *Organogenesis* **2009**, 5 (3), 90–96. <https://doi.org/10.4161/org.5.3.10021>.
3. Meneghel, J.; Kilbride, P.; Morris, G. J. Cryopreservation as a Key Element in the Successful Delivery of Cell-Based Therapies—A Review. *Front. Med.* **2020**, 7, 592242. <https://doi.org/10.3389/fmed.2020.592242>.
4. Capicciotti, C. J.; Mancini, R. S.; Turner, T. R.; Koyama, T.; Alteen, M. G.; Doshi, M.; Inada, T.; Acker, J. P.; Ben, R. N. O -Aryl-Glycoside Ice Recrystallization Inhibitors as Novel Cryoprotectants: A Structure–Function Study. *ACS Omega* **2016**, 1 (4), 656–662. <https://doi.org/10.1021/acsomega.6b00163>.
5. Capicciotti, C. J.; Kurach, J. D. R.; Turner, T. R.; Mancini, R. S.; Acker, J. P.; Ben, R. N. Small Molecule Ice Recrystallization Inhibitors Enable Freezing of Human Red Blood Cells with Reduced Glycerol Concentrations. *Sci. Rep.* **2015**, 5 (1), 9692. <https://doi.org/10.1038/srep09692>.
6. Meryman, H. T. Cryopreservation of Living Cells: Principles and Practice. *Transfusion (Paris)* **2007**, 47 (5), 935–945. <https://doi.org/10.1111/j.1537-2995.2007.01212.x>.
7. Hess, J. R. Red Cell Freezing and Its Impact on the Supply Chain. *Transfus. Med.* **2004**, 14 (1), 1–8. <https://doi.org/10.1111/j.0958-7578.2004.00472.x>.
8. Mitrus, I.; Smagur, A.; Fidyk, W.; Czech, M.; Prokop, M.; Chwieduk, A.; Glowala-Kosinska, M.; Czerw, T.; Sobczyk-Kruszelnicka, M.; Mendrek, W.; Michalak, K.; Sadus-Wojciechowska, M.; Najda, J.; Holowiecki, J.; Giebel, S. Reduction of DMSO Concentration in Cryopreservation Mixture from 10% to 7.5% and 5% Has No Impact on Engraftment after Autologous Peripheral Blood Stem Cell Transplantation: Results

- of a Prospective, Randomized Study. *Bone Marrow Transplant*. **2018**, 53 (3), 274–280. <https://doi.org/10.1038/s41409-017-0056-6>.
9. Timm, M.; Saaby, L.; Moesby, L.; Hansen, E. W. Considerations Regarding Use of Solvents in in Vitro Cell Based Assays. *Cytotechnology* **2013**, 65 (5), 887–894. <https://doi.org/10.1007/s10616-012-9530-6>.
 10. Yi, X.; Liu, M.; Luo, Q.; Zhuo, H.; Cao, H.; Wang, J.; Han, Y. Toxic Effects of Dimethyl Sulfoxide on Red Blood Cells, Platelets, and Vascular Endothelial Cells *in Vitro*. *FEBS Open Bio* **2017**, 7 (4), 485–494. <https://doi.org/10.1002/2211-5463.12193>.
 11. Pal, R.; Mamidi, M. K.; Das, A. K.; Bhonde, R. Diverse Effects of Dimethyl Sulfoxide (DMSO) on the Differentiation Potential of Human Embryonic Stem Cells. *Arch. Toxicol.* **2012**, 86 (4), 651–661. <https://doi.org/10.1007/s00204-011-0782-2>.
 12. Motta, J. P. R.; Paraguassú-Braga, F. H.; Bouzas, L. F.; Porto, L. C. Evaluation of Intracellular and Extracellular Trehalose as a Cryoprotectant of Stem Cells Obtained from Umbilical Cord Blood. *Cryobiology* **2014**, 68 (3), 343–348. <https://doi.org/10.1016/j.cryobiol.2014.04.007>.
 13. Rodrigues, J. P.; Paraguassú-Braga, F. H.; Carvalho, L.; Abdelhay, E.; Bouzas, L. F.; Porto, L. C. Evaluation of Trehalose and Sucrose as Cryoprotectants for Hematopoietic Stem Cells of Umbilical Cord Blood. *Cryobiology* **2008**, 56 (2), 144–151. <https://doi.org/10.1016/j.cryobiol.2008.01.003>.
 14. Balcerzak, A. K.; Capicciotti, C. J.; Briard, J. G.; Ben, R. N. Designing Ice Recrystallization Inhibitors: From Antifreeze (Glyco)Proteins to Small Molecules. *RSC Adv* **2014**, 4 (80), 42682–42696. <https://doi.org/10.1039/C4RA06893A>.
 15. Jahan, S.; Adam, M. K.; Manesia, J. K.; Doxtator, E.; Ben, R. N.; Pineault, N. Inhibition of Ice Recrystallization during Cryopreservation of Cord Blood Grafts Improves Platelet Engraftment. *Transfusion (Paris)* **2020**, 60 (4), 769–778. <https://doi.org/10.1111/trf.15759>.
 16. Khan, S.; Poisson, J.; Davila, L.; Ben, R. N.; Courtman, D. W. Small-Molecule Ice Recrystallization Inhibitors Improve Post-Thaw Recovery of Mesenchymal Stromal Cells. *Cytotherapy* **2019**, 21 (5), S76. <https://doi.org/10.1016/j.jcyt.2019.03.477>.
 17. Briard, J. G.; Jahan, S.; Chandran, P.; Allan, D.; Pineault, N.; Ben, R. N. Small-Molecule Ice Recrystallization Inhibitors Improve the Post-Thaw Function of Hematopoietic

- Stem and Progenitor Cells. *ACS Omega* **2016**, 1 (5), 1010–1018.
<https://doi.org/10.1021/acsomega.6b00178>.
18. Talevi, R.; Barbato, V.; Mollo, V.; Fiorentino, I.; De Stefano, C.; Guarino, F. M.; Gualtieri, R. Replacement of Sodium with Choline in Slow-Cooling Media Improves Human Ovarian Tissue Cryopreservation. *Reprod. Biomed. Online* **2013**, 27 (4), 381–389. <https://doi.org/10.1016/j.rbmo.2013.07.003>.
19. Svedentsov, E. P.; Zaitseva, O. O.; Tumanova, T. V.; Solomina, O. N.; Khudyakov, A. N. Use of Choline Chloride for Leukocyte Cryopreservation (–40°C). *Bull. Exp. Biol. Med.* **2009**, 148 (3), 461–463. <https://doi.org/10.1007/s10517-010-0736-3>.
20. Stachecki, J. J.; Cohen, J.; Willadsen, S. M. Cryopreservation of Unfertilized Mouse Oocytes: The Effect of Replacing Sodium with Choline in the Freezing Medium. *Cryobiology* **1998**, 37 (4), 346–354. <https://doi.org/10.1006/cryo.1998.2130>.
21. Ampaw, A. A.; Newell, K.; Ben, R. N. Investigating the Solubility and Activity of a Novel Class of Ice Recrystallization Inhibitors. *Processes* **2021**, 9 (10), 1781. <https://doi.org/10.3390/pr9101781>.
22. Abraham, S.; Keillor, K.; Capicciotti, C. J.; Perley-Robertson, G. E.; Keillor, J. W.; Ben, R. N. Quantitative Analysis of the Efficacy and Potency of Novel Small Molecule Ice Recrystallization Inhibitors. *Cryst. Growth Des.* **2015**, 15 (10), 5034–5039. <https://doi.org/10.1021/acs.cgd.5b00995>.
23. Balcerzak, A. K.; Febbraro, M.; Ben, R. N. The Importance of Hydrophobic Moieties in Ice Recrystallization Inhibitors. *RSC Adv.* **2013**, 3 (10), 3232. <https://doi.org/10.1039/c3ra23220d>.
24. Gerets, H. H. J.; Tilmant, K.; Gerin, B.; Chanteux, H.; Depelchin, B. O.; Dhalluin, S.; Atienzar, F. A. Characterization of Primary Human Hepatocytes, HepG2 Cells, and HepaRG Cells at the mRNA Level and CYP Activity in Response to Inducers and Their Predictivity for the Detection of Human Hepatotoxins. *Cell Biol. Toxicol.* **2012**, 28 (2), 69–87. <https://doi.org/10.1007/s10565-011-9208-4>.
25. Anoopkumar-Dukie, S.; Carey, J. B.; Conere, T.; O'Sullivan, E.; Van Pelt, F. N.; Allshire, A. Resazurin Assay of Radiation Response in Cultured Cells. *Br. J. Radiol.* **2005**, 78 (934), 945–947. <https://doi.org/10.1259/bjr/54004230>.

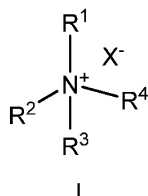
26. Yang, J.; Cai, N.; Zhai, H.; Zhang, J.; Zhu, Y.; Zhang, L. Natural Zwitterionic Betaine Enables Cells to Survive Ultrarapid Cryopreservation. *Sci. Rep.* **2016**, *6* (1), 37458. <https://doi.org/10.1038/srep37458>.
27. Gao, L.; Zhou, Q.; Zhang, Y.; Sun, S.; Lv, L.; Ma, P.; Yang, J.; Liu, M.; Zhang, L.; Wang, X.; Zhan, L. Dimethyl Sulfoxide-Free Cryopreservation of Human Umbilical Cord Mesenchymal Stem Cells Based on Zwitterionic Betaine and Electroporation. *Int. J. Mol. Sci.* **2021**, *22* (14), 7445. <https://doi.org/10.3390/ijms22147445>.
28. Jesus, A. R.; Meneses, L.; Duarte, A. R. C.; Paiva, A. Natural Deep Eutectic Systems, an Emerging Class of Cryoprotectant Agents. *Cryobiology* **2021**, *101*, 95–104. <https://doi.org/10.1016/j.cryobiol.2021.05.002>

[00129] All publications, patents and patent applications mentioned in this Specification are indicative of the level of skill of those skilled in the art to which this invention pertains and are herein incorporated by reference to the same extent as if each individual publication, patent, or patent applications was specifically and individually indicated to be incorporated by reference.

[00130] The invention being thus described, it will be obvious that the same may be varied in many ways. Such variations are not to be regarded as a departure from the spirit and scope of the invention, and all such modifications as would be obvious to one skilled in the art are intended to be included within the scope of the following claims.

We Claim:

1. A compound of Formula I



where

each of R^1 , R^2 and R^3 is independently H, C_1 to C_8 alkyl, C_2 to C_8 alkenyl, C_2 to C_8 alkynyl, or a C_1 to C_8 alkyl, C_2 to C_8 alkenyl, or C_2 to C_8 alkynyl that is terminally substituted with an alcohol, aryl or heteroaryl, or

one of R^1 , R^2 and R^3 is absent, H, C_1 to C_8 alkyl, C_2 to C_8 alkenyl, C_2 to C_8 alkynyl, or a C_1 to C_8 alkyl, C_2 to C_8 alkenyl, or C_2 to C_8 alkynyl that is terminally substituted with an alcohol, aryl or heteroaryl, and the remaining two of R^1 , R^2 and R^3 , together with the nitrogen to which they are attached, form a 4 to 8-membered heterocyclic ring;

R^4 is H, C_1 to C_9 alkyl, C_2 to C_9 alkenyl, C_2 to C_9 alkynyl, or a C_2 to C_9 alkyl, C_2 to C_9 alkenyl, or C_2 to C_9 alkynyl that is terminally substituted with an alcohol, $C(O)OH$, $C(O)OR_5$ or $C(O)NR_6R_7$, wherein R^5 is C_1 to C_8 alkyl, C_2 to C_8 alkenyl, or C_2 to C_8 alkynyl, each of which is optionally terminally substituted with an aryl group, alcohol group, or carboxylic acid group, and wherein each of R^6 and R^7 is independently H, C_1 to C_8 alkoxy, aryl, substituted aryl, 4-8 membered heterocyclic group, or a C_1 to C_8 alkyl, C_2 to C_8 alkenyl, or C_2 to C_8 alkynyl, each of which is optionally terminally substituted with an aryl group; and

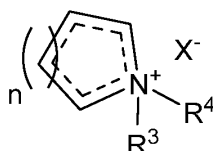
X^- is a counterion, such as a halide ion, a phosphate, sulfate, acetate, or hexafluorophosphate ion,

with the proviso that when R^4 is $-CH_2CH_2OH$ then:

at least one of R^1 , R^2 and R^3 is not methyl,

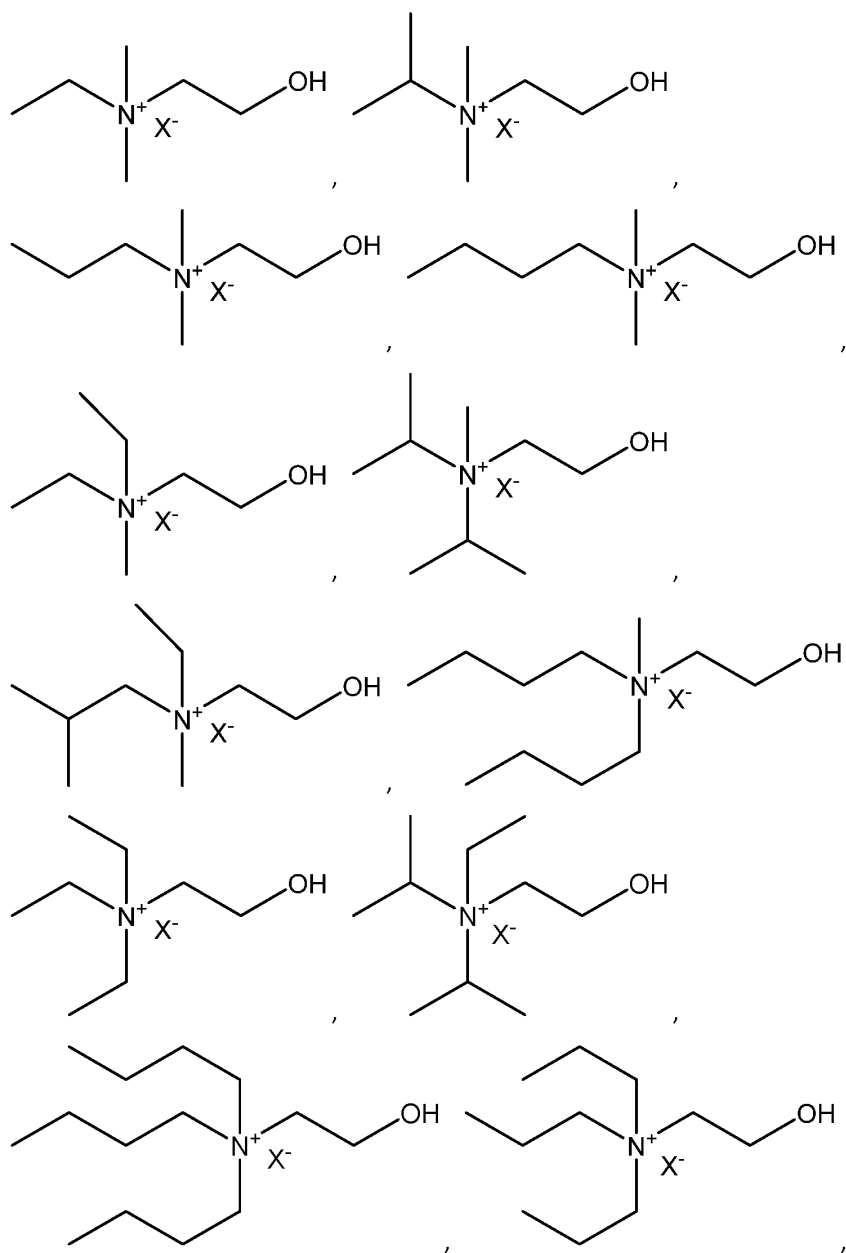
when R^1 is methyl then R^2 and R^3 are not both H or ethyl, and
 when R^1 and R^2 are both methyl then R^3 is not H or ethyl.

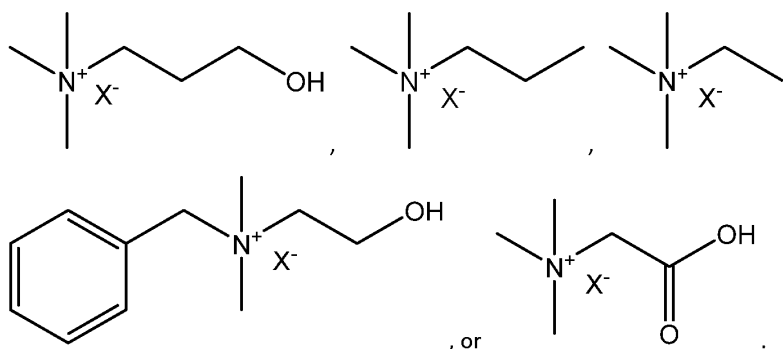
2. The compound according to claim 1, wherein:
 each of R^1 , R^2 and R^3 is C_1 - C_8 alkyl, or C_1 - C_8 alkyl terminally substituted with an aryl,
 or
 one of R^1 , R^2 and R^3 is absent H, C_1 - C_8 alkyl, or a C_1 - C_8 alkyl terminally substituted
 with an aryl, and two of R^1 , R^2 and R^3 , together with the nitrogen to which
 they are attached, form a 4 to 8-membered heterocyclic ring.
3. The compound according to claim 1 or 2, wherein each of R^1 , R^2 and R^3 is
 independently methyl, ethyl, propyl, isopropyl, *n*-butyl, *sec*-butyl, *tert*-butyl, isobutyl,
 or a C_1 - C_4 alkyl terminally substituted with an aryl.
4. The compound according to any one of claims 1 to 3, wherein at least one of R^1 , R^2
 and R^3 is ethyl, propyl, isopropyl, *n*-butyl, *sec*-butyl, *tert*-butyl or isobutyl.
5. The compound according to any one of claims 1 to 4, wherein at least one of R^1 , R^2
 and R^3 is a C_1 - C_4 alkyl terminally substituted with an aryl, such as phenyl.
6. The compound according to claim 1 or 2, having the following structure



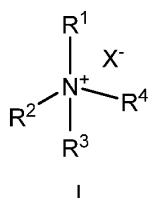
where n is an integer from 0 to 4.

7. The compound according to any one of claims 1 to 6, wherein X^- is F^- , Cl^- , Br^- , I^- , a
 phosphate, sulfate, acetate, or hexafluorophosphate ion.
8. The compound according to claim 1, which is





9. A composition comprising:
 (a) a compound of Formula I



where

each of R^1 , R^2 and R^3 is independently H, C_1 to C_8 alkyl, C_2 to C_8 alkenyl, C_2 to C_8 alkynyl, or a C_1 to C_8 alkyl, C_2 to C_8 alkenyl, or C_2 to C_8 alkynyl that is terminally substituted with an alcohol, aryl or heteroaryl, or

one of R^1 , R^2 and R^3 is absent, H, C_1 to C_8 alkyl, C_2 to C_8 alkenyl, C_2 to C_8 alkynyl, or a C_1 to C_8 alkyl, C_2 to C_8 alkenyl, or C_2 to C_8 alkynyl that is terminally substituted with an alcohol, aryl or heteroaryl, and the remaining two of R^1 , R^2 and R^3 , together with the nitrogen to which they are attached, form a 4 to 8-membered heterocyclic ring;

R^4 is H, C_1 to C_9 alkyl, C_2 to C_9 alkenyl, C_2 to C_9 alkynyl, or a C_2 to C_9 alkyl, C_2 to C_9 alkenyl, or C_2 to C_9 alkynyl that is terminally substituted with an alcohol, $C(O)OH$, $C(O)OR_5$ or $C(O)NR_6R_7$, wherein R^5 is C_1 to C_8 alkyl, C_2 to C_8 alkenyl, or C_2 to C_8 alkynyl, each of which is optionally terminally substituted with an aryl group, alcohol group, or carboxylic acid

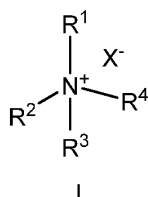
group, and wherein each of R^6 and R^7 is independently H, C_1 to C_8 alkoxy, aryl, substituted aryl, 4-8 membered heterocyclic group, or a C_1 to C_8 alkyl, C_2 to C_8 alkenyl, or C_2 to C_8 alkynyl, each of which is optionally terminally substituted with an aryl group; and

X^- is a counterion, such as a halide ion, a phosphate, sulfate, acetate, or hexafluorophosphate ion; and

(b) a buffer, a cell culture medium or a combination thereof, wherein the composition does not contain dimethylsulfoxide (DMSO) or glycerol, or contains < 10% by weight of DMSO and/or < 40% by weight of glycerol.

10. The composition of claim 9, wherein the compound is as defined in any one of claims 1 to 8.
11. The composition of claim 9 or 10, wherein the composition further comprises an ice crystal recrystallization inhibitor that does not have the structure of Formula I.
12. The composition of any one of claims 9 to 11, wherein the composition further comprises one or more of lactobionate, glycerol, polyvinylalcohol, hydroxyethyl starch (HES), ethylene glycol (EG), propylene glycol (PG), mannitol, amino acids, urea, a reducing sugar and a biocompatible disaccharide or polysaccharide.
13. The composition of claim 12, wherein the reducing sugar is glucose, xylose or trehalose.
14. The composition of any one of claims 9 to 13, wherein the buffer or cell culture medium is phosphate buffered saline optionally comprising albumin, Eagle's minimum essential medium, Dulbecco's modified Eagle's medium, RPMI, fetal bovine serum, fetal calf serum, Ham's F-10, Ham's F-12, Medium 199, Hank's Buffered Salt Solution, Hank's Buffered Salt Solution and dextrose, or a combination thereof.

15. The cryopreservation composition of any one of claims 9 to 14, further comprising a biological material from a human, an animal, a plant, a fungus or a microorganism.
16. The cryopreservation composition of claim 15, wherein the biological material is an organ, a tissue, cells or platelets.
17. The cryopreservation composition of claim 16, wherein the cells comprise stem cells, T cells, peripheral blood mononuclear cells (PMBCs), neurons, progenitor cells, liver cells, red blood cells, immune cells, endothelial cells or cells from a cell line, preferably the cells comprise stem cells, T cells, red blood cells, progenitor cells, or liver cells.
18. A method for cryopreserving a biological material comprising:
 - (a) suspending the biological material in a cryopreservation composition, wherein the cryopreservation composition comprises:
 - (i) one or more compound of Formula I



where

each of R^1 , R^2 and R^3 is independently H, C_1 to C_8 alkyl, C_2 to C_8 alkenyl, C_2 to C_8 alkynyl, or a C_1 to C_8 alkyl, C_2 to C_8 alkenyl, or C_2 to C_8 alkynyl that is terminally substituted with an alcohol, aryl or heteroaryl, or

one of R^1 , R^2 and R^3 is absent, H, C_1 to C_8 alkyl, C_2 to C_8 alkenyl, C_2 to C_8 alkynyl, or a C_1 to C_8 alkyl, C_2 to C_8 alkenyl, or C_2 to C_8 alkynyl that is terminally substituted with an alcohol, aryl or heteroaryl, and the remaining two of R^1 , R^2 and R^3 , together with the nitrogen to which they are attached, form a 4 to 8-membered heterocyclic ring;

R^4 is H, C_1 to C_9 alkyl, C_2 to C_9 alkenyl, C_2 to C_9 alkynyl, or a C_2 to C_9 alkyl, C_2 to C_9 alkenyl, or C_2 to C_9 alkynyl that is terminally substituted with an alcohol, $C(O)OH$, $C(O)OR_5$ or $C(O)NR_6R_7$, wherein R^5 is C_1 to C_8 alkyl, C_2 to C_8 alkenyl, or C_2 to C_8 alkynyl, each of which is optionally terminally substituted with an aryl group, alcohol group, or carboxylic acid group, and wherein each of R^6 and R^7 is independently H, C_1 to C_8 alkoxy, aryl, substituted aryl, 4-8 membered heterocyclic group, or a C_1 to C_8 alkyl, C_2 to C_8 alkenyl, or C_2 to C_8 alkynyl, each of which is optionally terminally substituted with an aryl group; and

X^- is a counterion, such as a halide ion, a phosphate, sulfate, acetate, or hexafluorophosphate ion; and

(ii) a buffer, a cell culture medium or a combination thereof,

wherein the composition does not contain dimethylsulfoxide (DMSO) or glycerol, or contains <10% by weight of DMSO and/or <40% by weight of glycerol (< 40 %);

(b) cooling the suspension from step (a) to a storage temperature at or below the freezing point of the cryopreservation composition; and

(c) storing the cooled suspension at the storage temperature.

19. The method according to claim 18, wherein the compound of Formula I is as defined in any one of claims 1 to 8.
20. The method according to claim 18 or 19, wherein the biological material is from a human, an animal, a plant, a fungus or a microorganism.
21. The method of claim 20, wherein the biological material is an organ, a tissue, cells or platelets.
22. The method of claim 21, wherein the cells comprise stem cells, T cells, peripheral blood mononuclear cells (PMBCs), neurons, progenitor cells, liver cells, red blood cells, immune cells, endothelial cells or cells from a cell line, preferably the cells comprise stem cells, T cells, red blood cells, progenitor cells, or liver cells.

23. The method of any one of claims 18 to 22, wherein cooling the suspension comprises performing a rate-controlled cooling of about 1°C per minute over about 16 hours.