

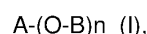
DIAGNOSIS, MONITORING, AND TREATMENT OF CONDITIONS CHARACTERIZED BY INTRACELLULAR FREE RADICALS

BACKGROUND

5 The invention features the use of coelenterazine, or a close analog, as a systemically administered real-time reporter of ROS or RNS levels in animal and human cell and organ settings. This real-time measurement can then be used to assess severity of free radical-dependent disease states, such as neurodegenerative diseases, post-organ transplant ischemia-reperfusion injury, acute radiation syndrome injury, and many others. The method can include real-time measurement of the therapeutic efficacy of PrC-210 as a free radical-scavenger capable of detoxifying both oxygen and nitrogen free radicals.

SUMMARY

15 In a first aspect, the invention features a method of measuring intracellular Reactive Oxygen Species (ROS) and Reactive Nitrogen Species (RNS) in a subject, the method including the steps of:
(i) administering to the subject a compound of formula (I):



wherein A is a radical of a luciferin, each O is an oxygen atom linking A and B, each B is, independently, a cleavable protecting group, and n is an integer from 1-4; and (ii) following step (i), extracorporeally measuring intracellular bioluminescence from the luciferin. In some embodiments, A is a radical of coelenterazine, vargulin, prolume purple, or furimazine. In particular embodiments, A is a radical of coelenterazine (e.g., enduRen or viviRen). In the compound of formula (I), each cleavable protecting group B can be selected to be cleaved by esterases or lipases.

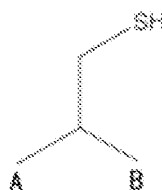
25 In some embodiments, the method further includes correlating the result of step (ii) to an intracellular ROS and RNS level in the subject.

In particular embodiments, the method further includes using the result of step (ii) to monitor the pathogenesis or severity of a disease or condition in the subject.

30 In a related aspect, the invention features a method of treating a disease or condition mediated by intracellular Reactive Oxygen Species (ROS) and Reactive Nitrogen Species (RNS) in a subject, the method including the steps of:

(a) measuring an intracellular ROS and RNS level in the subject; and

(b) on the basis of the level measured in step (a), administering to the subject an effective amount of a compound of formula (II):

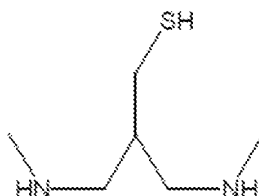


(II),

35 or a pharmaceutically acceptable acid addition salt thereof, and wherein (x) A is $-\text{CH}_2\text{NHR}'$ and B is $-\text{CH}_2\text{NHR}$, or A is $-\text{NRR}'$ and B is H; and (y) each of R and R' are, independently, selected from H, $\text{C}_1\text{-C}_6$

alkyl, and C₁-C₆ heteroalkyl, with the proviso that R and R' are not both H if B = H. Step (a) can include performing the diagnostic method of the invention for measuring the level of intracellular ROS and RNS in a subject.

In particular embodiments, the compound of formula (II) is



PrC-210,

or a pharmaceutically acceptable salt thereof.

In certain embodiments of any of the above methods, the subject has undergone a solid organ transplantation (SOT) or vascularized composite allograft transplantation (VCA) and the transplant can be monitored for acute or chronic transplant rejections and treatment success.

In some embodiments of any of the above methods, the subject suffers from a neurodegenerative disease, including but not limited to, Alzheimer's disease, Amyotrophic lateral sclerosis, Parkinson's disease or Huntington disease.

In particular embodiments of any of the above methods, the subject suffers from a neuroinflammatory disease, including but not limited to Multiple sclerosis or sterile and non-sterile encephalitis.

In certain embodiments of any of the above methods, the subject suffers from an acute brain trauma, such as Traumatic Brain injury, Spinal cord injury or stroke.

In particular embodiments of any of the above methods, the subject suffers from acute radiation syndrome, or was, is, or will be exposed to low and high energy radiation from a nuclear explosion, nuclear reactor leakage or space travel.

In some embodiments of any of the above methods, the subject suffers from neuropsychiatric disorders include, without limitation bipolar disorder (BD), schizophrenia, depression, anxiety disorders, attention deficit disorders, addictive disorders, personality disorders, autism and Asperger's disease.

In certain embodiments of any of the above methods, the subject suffers from cardiovascular diseases, including but not limited to such as arteriosclerosis, myocardial infarction, angioplasty, diseases of the heart valve, angina pectoris, peripheral circulatory disorders, vascular damage from dialysis, vascular dementia, and transient ischemic attacks.

In particular embodiments of any of the above methods, the subject suffers from myopathic diseases.

In some embodiments of any of the above methods, the subject suffers from autoimmune diseases, including but not limited to such as rheumatoid arthritis, Diabetes Type I, Morbus Crohn, colitis ulcerosa, and psoriasis,

In certain embodiments of any of the above methods, the subject suffers from diabetes Type II, obesity, and metabolic syndrome.

In some embodiments of any of the above methods, the subject suffers from aging and age-related diseases.

In certain embodiments of any of the above methods, the subject suffers from asthma, and inflammatory pulmonary conditions (e.g., chronic obstructive pulmonary disease (COPD)).

In some embodiments of any of the above methods, the subject suffers from acute injuries including but not limited to such as major injuries, major surgeries and burns.

5 In certain embodiments of any of the above methods, the subject suffers from complication of infections, including but not limited to such as post-Covid syndrome.

In some embodiments of any of the above methods, the subject suffers from a disease leading to fibrosis, such as. such as but not limited to hepatitis leading liver fibrosis, glomerulonephritis leading to kidney fibrosis and primary and secondary lung fibrosis.

10 In one particular embodiment of any of the above methods, the bioluminescence of step (ii) occurs in the absence of any luciferase enzyme.

Definitions

To facilitate the understanding of this invention, a number of terms are defined below. Terms
15 defined herein have meanings as commonly understood by a person of ordinary skill in the areas relevant to the invention. Terms such as “a”, “an,” and “the” are not intended to refer to only a singular entity, but include the general class of which a specific example may be used for illustration. The terminology herein is used to describe specific embodiments of the invention, but their usage does not limit the invention, except as outlined in the claims.

20 As used herein, the term “about” refers to a value that is within 10% above or below the value being described.

As used herein, any values provided in a range of values include both the upper and lower bounds, and any values contained within the upper and lower bounds.

As used herein, the term “pharmaceutically acceptable salt” represents those salts of the
25 compounds described that are, within the scope of sound medical judgment, suitable for use in contact with the tissues of humans and animals without undue toxicity, irritation, allergic response and the like and are commensurate with a reasonable benefit/risk ratio. Pharmaceutically acceptable salts are well known in the art. For example, pharmaceutically acceptable salts are described in: Berge et al., *J. Pharmaceutical Sciences* 66:1-19, 1977 and in *Handbook of Pharmaceutical Salts: Properties, Selection, and Use*, (Eds. P.H. Stahl and C.G. Wermuth), Wiley-VCH, 2008. These salts may be acid addition salts
30 involving inorganic or organic acids. The salts can be prepared in situ during the final isolation and purification of the compounds described herein or separately by reacting the free base group with a suitable acid.

As used herein, the term “effective amount” refers to an amount sufficient to effect beneficial or
35 desired results, such as diagnostic results of determining disease activity, and, as such, determining the “therapeutically effective amount” depends upon the context in which it is being applied. For example, in the context of administering a luciferin prodrug administered to provide real-time reporting of intracellular ROS or RNS levels in a subject. The method can include real-time measurement of the therapeutically effective amount of aminothiols (e.g., PrC-210) required to detoxify both oxygen and nitrogen free radicals.
40 Thus, an effective amount of an aminothiol compound is, for example, an amount sufficient to ameliorate the symptoms or progression of diseases characterized by intracellular ROS and RNS species. The

methods of the invention can include systemic (e.g., intravenous) or local administration (e.g., topical or local injection) of aminothiols compound, as needed depending upon the nature of the condition being treated.

As used herein, and as well understood in the art, 'to diagnose' and 'to monitor' a disease activity and "to treat" a condition or "treatment" of various diseases and disorders is an approach for obtaining beneficial or desired results, such as clinical results. Beneficial or desired results can include, but are not limited to, alleviation of one or more symptoms or conditions; diminishment of extent of disease, disorder, or condition; stabilizing (i.e., not worsening) state of disease, disorder, or condition; delay or slowing the progress of the disease, disorder, or condition; amelioration or palliation of the disease, disorder, or condition; and remission (whether partial or total), whether detectable or undetectable. "Palliating" a disease, disorder, or condition means that the extent and/or undesirable clinical manifestations of the disease, disorder, or condition are lessened and/or time course of the progression is slowed or lengthened, as compared to the extent or time course in the absence of treatment.

The term "subject," as used herein, can be a human, non-human primate, or other mammal, such as but not limited to dog, cat, horse, cow, pig, goat, monkey, rat, mouse, and sheep. In preferred embodiments, the subject is a human.

As used herein, the term "pharmaceutical composition" refers to an active compound, formulated together with one or more pharmaceutically acceptable excipients. In some embodiments, a compound of the invention is present in unit dose amount appropriate for administration in a therapeutic regimen that shows a statistically significant probability of achieving a predetermined therapeutic effect when administered to a relevant population. In certain embodiments, pharmaceutical compositions may be specially formulated for administration in solid or liquid form, including those adapted for the following: oral administration, for example, drenches (aqueous or non-aqueous solutions or suspensions), tablets, or capsules; and parenteral administration, for example, by subcutaneous, intramuscular, or intravenous injection.

The term "pharmaceutically acceptable excipient," as used herein, refers to any inactive ingredient (for example, a vehicle capable of suspending or dissolving the active compound) that is biocompatible and suitable for administration to a subject. Typical excipients include, for example: antiadherents, antioxidants, binders, coatings, compression aids, disintegrants, dyes, emollients, emulsifiers, diluents, film formers or coatings, flavors, fragrances, glidants, lubricants, preservatives, printing inks, sorbents, suspending or dispersing agents, sweeteners, or waters of hydration. Excipients include, but are not limited to: butylated optionally substituted hydroxytoluene (e.g., BHT), calcium carbonate, calcium phosphate dibasic, calcium stearate, croscarmellose, crosslinked polyvinyl pyrrolidone, citric acid, crospovidone, cysteine, ethylcellulose, gelatin, optionally substituted hydroxypropyl cellulose, optionally substituted hydroxypropyl methylcellulose, lactose, magnesium stearate, maltitol, mannitol, methionine, methylcellulose, methyl paraben, microcrystalline cellulose, polyethylene glycol, polyvinyl pyrrolidone, povidone, pregelatinized starch, propyl paraben, retinyl palmitate, shellac, silicon dioxide, sodium carboxymethyl cellulose, sodium citrate, sodium starch glycolate, sorbitol, starch, stearic acid, sucrose, talc, titanium dioxide, vitamin A, vitamin E,

vitamin C, and xylitol. Those of ordinary skill in the art are familiar with a variety of agents and materials useful as excipients.

The term “alkyl,” as used herein, refers to a branched or straight-chain monovalent saturated aliphatic radical containing only C and H when unsubstituted. The monovalency of an alkyl group does not include the optional substituents on the alkyl group. For example, if an alkyl group is attached to a compound, monovalency of the alkyl group refers to its attachment to the compound and does not include any additional substituents that may be present on the alkyl group. In some embodiments, the alkyl group may contain, e.g., 1-6, 1-4, or 1-2 carbon atoms (e.g., C₁-C₆, C₁-C₄, or C₁-C₂). Examples include, but are not limited to, methyl, ethyl, isobutyl, sec-butyl, tert-butyl, 2-methylpropyl, and 2,2-dimethylpropyl.

The term “C₁-C₆ heteroalkyl,” as used herein, refers to a branched or straight-chain monovalent saturated aliphatic radical containing C, H, and from one or three N atoms.

As used herein, the term “luciferin” refers to a class of light-emitting biological pigments found in organisms capable of bioluminescence as well as synthetic analogues or functionally equivalent chemicals, which can be oxidized to produce oxyluciferin and energy in the form of light. D-luciferin, or 2-(6-hydroxybenzothiazol-2-yl)-2-thiazoline-4-carboxylic acid, was first isolated from the firefly *Photinus pyralis*. Various chemically distinct forms of luciferin have been discovered and studied from various different organisms, mainly from the ocean, for example fish and squid, however, many have been identified in land dwelling organisms, for example, worms, beetles and various other insects (Day et al. (2004) *Luminescence* 19:8-20). As used herein, luciferin also includes derivatives or analogues of luciferin. In addition to entirely synthetic luciferin, such as cyclic alkylaminoluciferin (CycLucl), there are at least five general types of biologically evolved luciferin, which are each chemically different and catalyzed by chemically and structurally different luciferases that employ a wide range of different cofactors. First, is firefly luciferin, the substrate of firefly luciferase, which requires ATP for catalysis (EC 1.13.12.7). Second, is bacterial luciferin, also found in some squid and fish, which consists of a long chain aldehyde and a reduced riboflavin phosphate. Bacterial luciferase is FMNH-dependent. Third, is dinoflagellate luciferin, a tetrapyrrolic chlorophyll derivative found in dinoflagellates (marine plankton), the organisms responsible for night-time ocean phosphorescence. Dinoflagellate luciferase catalyses the oxidation of dinoflagellate luciferin and consists of three identical and catalytically active domains. Fourth, is the imidazolopyrazine vargulin, which is found in certain ostracods and deep-sea fish, for example, *Porichthys*. Last, is coelenterazine (an imidazolopyrazine), the light-emitter of the protein aequorin, found in radiolarians, ctenophores, cnidarians, squid, copepods, chaetognaths, fish and shrimp.

As used herein, the term “coelenterazine” refers to a class of luciferins that function as a substrate which occurs in cnidarians, copepods, chaetognaths, ctenophores, decapod shrimps, mysid shrimps, radiolarians and some fish taxa (Greer and Szalay (2002) *Luminescence* 17:43-74). For *Renilla* luciferase for example, coelenterazine analogues/derivatives are available that result in light emission between 418 and 547 nm (Loening et al. (2007) *Nature Methods* 4:641-643). A coelenterazine analogue/derivative (400A, DeepBlueC) has been described emitting light at 400 nm with *Renilla* luciferase (WO 01/46691, incorporated herein by reference). Other examples of coelenterazine luciferins are enduRen, prolume purple, prolume purple II, prolume purple III, viviRen and furimazine. Other examples of coelenterazine analogues/derivatives include, but are not limited to, compounds disclosed in

U.S. Pat. No. 9,624,425 and U.S. Pat. Pub. No. 20140302539, each of which is incorporated herein by reference.

Other features and advantages of the invention will be apparent from the following Detailed Description, the drawings, and the claims.

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Brief Description of the Drawings

Figure 1 depicts the chemical reaction of photon release from coelenterazine.

Figure 2 depicts chemical structure comparisons between coelenterazine, enduRen and vivRen.

Figure 3 shows a comparison of mouse body distribution between injected coelenterazine and
10 VivRen. Coelenterazine mainly induces photon signals within the vessels when injected intravenously, and not in specific organ sites. VivRen results in a well distributed full-body release of photons.

Figure 4 (Panels a and b) show treatment of U373 MG human astrocytoma cells in tissue culture with phorbol myristate (PMA). PMA addition to culture medium induces production of superoxide (measured by its reaction with coelenterazine), and in panel b, we saw a PrC-210 dose-dependent
15 capture and suppression (to background) of the generated superoxide. Panel c shows a PMA dose-dependent production of superoxide in a prior-irradiated mouse (measured by its reaction with VivRen), and data showing that an intraperitoneal injection of PrC-210 (0.5 MTD, 252 mg/kg bw), prior to PMA administration to the mouse, caused a significant reduction in the detectable level of mouse superoxide.

Figure 5 shows a comparison of mouse body distribution between intravenous vs subcutaneous
20 injection of vivRen in mice.

Figure 6 shows imaged ROS levels which follow organ and time specific distribution patterns after irradiation with 8.68 Gy.

Figure 7 (A) shows free-radical levels in mouse after irradiation with 8.68 Gy; Free-radical level is suppressed to background by systemically administered PrC-210. (B,C) Caspase 3/7 marker levels in
25 mouse plasma after irradiation with 8.68 Gy, this biomarker is elevated with no PrC-210 treatment and it is suppressed to background by PrC-210, i.e., (B) with 0.5 MTD PrC-210 given 30min before or (C) 0.3 MTD PrC-210 24 hours after irradiation with 8.68 Gy.

Figure 8 shows ROS (A), MDA (B), and Caspase 1 (C) levels in mouse brain after irradiation with 8.68 Gy; both, ROS and MDA levels are suppressed to background by systemically administered PrC-
30 210.

Figure 9 shows ROS, Caspase 8 and Caspase 3/7 marker levels in mouse brain after irradiation with 8.68 Gy. These biomarkers are elevated with no treatment and they are suppressed to background by PrC-210. Caspase levels 'mirror' ROS levels.

Figure 10 shows (A) quantitative measurement of mouse brain ROS level (measured by its
35 reaction with IV VivRen) in three SOD1^{G93A} mutant mice and a wildtype control. (B) Body weights over time of the same three SOD1^{G93A} mice. Weight loss was progressive along with onset of ALS motor symptoms and onset of hind-limb paralysis in correlation with ROS levels elevations in (A). Administration of systemic PrC-210 (0.1 MTD, IP) was associated with an immediate plateau in body weight loss.

Figure 11 shows the whole-body free-radical levels after irradiation with different radiation doses
40 either without PrC-210, or A) with different doses of PrC-210 given intravenously 30 minutes before

radiation and free-radical imaging 30 minutes after radiation, or B) different dose of PrC-210 given intravenously immediately before free-radical imaging 30 minutes after radiation.

DETAILED DESCRIPTION

- 5 This invention features the use of luciferin prodrugs administered to provide a real-time reporter of ROS and RNS levels in animal and human cell and organ settings. This real-time measurement can then be used to assess activity and severity of free radical-dependent disease states, such as neurodegenerative diseases, post-organ transplant ischemia-reperfusion injury, acute radiation syndrome injury, and many others. The method can include real-time measurement of the therapeutic efficacy of
- 10 PrC-210 as a free radical-scavenger capable of detoxifying both oxygen and nitrogen free radicals.

Luciferins and Luciferin Prodrugs

- The methods of the invention feature the use of the bioluminescent luciferin coelenterazine (Shimomura O, Johnson, FH, "Chemical nature of bioluminescence systems in coelenterates,"
- 15 Proceedings of the National Academy of Sciences of the United States of America; April, 1975), which emits one photon when oxidized by a free radical molecule (Figure 1). The emitted photons can be quantitatively measured using a bioluminescence monitor, e.g., Lago X, Spectral Instruments, (<https://spectralinvivo.com/imaging-systems/>). The light-emitting coelenterazine oxidation reaction doesn't require the presence of any additional catalytic enzyme. Therefore, coelenterazine can react with, and
- 20 then release a photon, anywhere and any time it comes in close contact with a free-radical species. Coelenterazine is not dependent on an enzyme, such as luciferase, to be oxidized and then release a photon. This enzyme independent reaction makes the luciferin derivative, such as coelenterazine, a stand-alone diagnostic for the detection of free-radicals in situ.

- Coelenterazine is a class of luciferins that function as a substrate which occurs in cnidarians, copepods, chaetognaths, ctenophores, decapod shrimps, mysid shrimps, radiolarians and some fish taxa (Greer and Szalay (2002) Luminescence 17:43-74). For Renilla luciferase for example, coelenterazine analogues/derivatives are available that result in light emission between 418 and 547 nm (Loening et al. (2007) Nature Methods 4:641-643). A coelenterazine analogue/derivative (400A, DeepBlueC) has been described emitting light at 400 nm with Renilla luciferase (WO 01/46691, incorporated herein by
- 25 reference). Other examples of coelenterazine luciferins are enduRen, prolume purple, prolume purple II, prolume purple III, viviRen and furimazine. Other examples of coelenterazine analogues/derivatives useful in the methods of the invention include, but are not limited to, compounds disclosed in U.S. Pat. No. 9,624,425 and U.S. Pat. Pub. No. 20140302539, each of which is incorporated herein by reference. There are other luciferin compounds, such as firefly luciferin, vargulin, etc, which could be substituted for
- 30 coelenterazine. This invention uses coelenterazine because this compound emits blue light and thereby has a higher tissue penetration. This is important, as this invention is used to measure emitted photons from tissue sites in living organisms. Tissues and organs such as brain, kidneys, heart, intestines are located millimeters below the body surface. The emitted blue light, due to its shorter wavelength, more efficiently penetrates body tissues, and is thus detectable at the body surface. For example, vargulin also
- 40 emits blue light, and therefore could substitute for coelenterazine. Further, the addition or reduction of benzyl rings could increase or decrease the rigidity or change the polarity of the compound, and thus,

could change to emission to even shorter (ultraviolet 380-10nm) or longer (near infrared 500 – 2500 nm) wavelengths, depending on the detection need.

Administration of Luciferin Prodrugs

5 The animal and human dose of the coelenterazine reporter of this invention depends on factors including the route of administration, the disease to be diagnosed or monitored and treated, and subject's physical characteristics, e.g., age, weight, and general health, the target organ and the target species, such as humans, mice or pig. Typically, the administered amount of a coelenterazine reporter molecule disclosed herein (e.g. coelenterazine), contained within a single dose, may be an amount that allows one
10 to differentiate free-radical concentrations above the free-radical background level. The administered dose will be adapted by the clinician based on animal and human studies and in accordance with conventional factors such as the extent of the disease, the method and location of measuring free-radicals, such as via full-body photometry, endoscopy, intra-organ sensors, or transcutaneous or topical measurement, and different physiologic parameters of the subject, and is also dependent on the safety
15 margin of the compound, which could be 100 times the currently used compound concentration. For example, the LD50 of orally applied viviRen in rats is 14,500 mg/kg, which is 100 times the dose we used in our experiments. As we have shown in Figure 4 for Phorbol myristate (PMA) treated U373 cells and irradiated ICR mice, a standard curve will need to be established to demonstrate the free-radical background and the therapeutic window in a species. In this model, PMA induces the production of
20 superoxide radicals which are free-radical species with a very short half-life. Phorbol myristate (PMA)-induced production of superoxide radical in human U373 astrocytoma cells in tissue culture (Figure 2, panel A), and PrC-210 concentration-dependent suppression (capture) of the generated superoxide (B). U373 cells were grown in DMEM media containing 10% FBS; PMA was added to media in 10% ethanol:90% saline. Thirty seconds later, coelenterazine was added to media, and coelenterazine photon
25 emission was then continuously monitored in an IVIS imaging chamber. PrC-210 was added to media 10 min prior to PMA addition. In panel C, PMA in a 20% ethanol:80% saline solution was injected IV into the tail veins of ICR mice. Ten minutes later, ViviRen was likewise injected into the mouse tail vein. Levels of superoxide-ViviRen conjugate in mice were monitored by quantifying photon emission in an IVIS chamber. As shown, IP injection of a 0.5 MTD dose of PrC-210 (252 mg/kg) just prior to IV PMA
30 administration to mice conferred a significant reduction in measurable superoxide-viviRen product.

Measuring Intracellular ROS/RNS Levels

 The invention further features methods to measure organ and "disease relevant" free-radical concentrations. As such, it may at times be necessary to assure that only intracellular free-radicals are
35 measured. To assure this, the luciferin reporter molecule, such as coelenterazine, needs to be linked to a cleavable blocking group, which blocks any oxidation, and associated photon emission, as long as the blocking group is attached to the luciferin. These blocking groups need to be chosen so that they do not inhibit uptake of the luciferin reporter into cells, or penetration of the blood-brain-barrier. Good examples of these combination luciferins ("blocked luciferins") are viviRen and enduRen (Otto-Duessel M, Khankaldyyan V, Gonzalez-Gomez I, Jensen MC, Laug WE, Rosol M., "In Vivo Testing of Renilla Luciferase Substrate Analogs in an Orthotopic Murine Model of Human Glioblastoma," *Molecular*
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Imaging, 5(2), 2006), which are analogs of coelenterazine (Figure 2). The linked blocking groups are cleaved by cellular lipases and esterases once the compound has entered the cell. For the cleavage of the blocking group any endogenously present enzyme of all cells can be used as cleavage mechanism. This cleavage then activates coelenterazine, or the respective analog, so it can be oxidized to release a photon. Coelenterazine without a blocking group structure will be mostly oxidized either at the point of administration or intravascular, before it is able to reach the target organ (see coelenterazine example in Figure 3). It has been shown in this invention, that the intracellular release leads to an even distribution of the luciferin throughout the body (see *viviRen* in Figure 3).

The photon signal emitted by the luciferin substrate, such as from coelenterazine or its analog, is measured in a Bioluminescent Machine, such as the IVIS machine from Perkin Elmer. Other methods to measure photons are, for example, Charged-coupled devices (CCD) or Complementary Metal-Oxide-Semiconductor (CMOS) devices. As the invention is not relying on a catalytic enzyme, such as luciferase, the amount of measured luminescent signal is significantly lower in comparison to the amounts measured from mutant cells, which express such catalytic enzymes, and therefore cannot be measured by using the standard measurement protocol. To receive enough photon signal for a differentiating analysis, the exposure time often requires a longer acquisition time. The acquisition time could be reduced, if: i) the photon sensitivity of the measuring Bioluminescent Machine is increased, ii) the amount of bioluminescent compound is increased, and/or iii) the wavelength is optimized to the specific organ or measuring modality.

Potential imaging applications include:

Photon detection outside of, or within, the body is done by cameras that encompass the ability to translate the captured photons into electrons, such as digital cameras, including DSLRs, mirrorless cameras, and smartphone cameras, which encompass either CCD (Charge-coupled device) or CMOS (Complementary Metal-Oxide-Semiconductor) sensors to capture images. Both CCD and CMOS sensors are used to detect light and convert it into electrical signals, which are then processed into images.

Cutaneous measurement: wearable device encompassing a CCD (Charge-coupled device) camera or smart phone cameras CMOS (complementary Metal-oxide semiconductor) sensor, such as to image through thin dermal layers (e.g. wrist or finger) to determine overall inflammatory state (e.g. monitoring for Alzheimer's, Multiple sclerosis (MS), rheumatoid arthritis, lupus, or vasculitis).

Sub-cutaneous measurement: analogous to those used for continuous glucose monitoring devices, such as optical sensors or transcutaneous fiber optics attached to a CCD camera. This could be used to evaluate the overall inflammatory state in autoimmune diseases, such as Multiple sclerosis (MS), rheumatoid arthritis, the state of arteriosclerosis, aging and/or neurodegeneration, such as in Alzheimer's disease, ALS or Parkinson's disease.

Intra-muscular measurement: intra-muscular imaging needles are known in the art (see, e.g., Sanchez, Gabriel N., et al., *Neuron* 88.6 (2015): 1109-1120) to evaluate muscular degeneration (potential decrease of signal) or inflammation (increase of signal) (e.g. ALS, MS).

Intra-organ measurement can be made using, e.g., an intra-organ placed fiberoptic connected to a CCD camera to measure for example an inflammatory disease activity in the brain, liver, kidney, pancreas etc.

Hollow organ imaging, such as imaging of digestive tract, respiratory system, nasal cavity, kidneys, can be performed with CCD scopes for imaging the digestive tract (routine or intraoperative Laparoscopic imaging for e.g. tumor or inflammatory bowel disease detection); the respiratory system (bronchoalveolar imaging for e.g. inflammation); the nasal cavity, including the possibility for deep brain
5 imaging e.g. pituitary gland, corpus callosum from the nasal cavity for e.g. Alzheimer's, ALS); the imaging of the kidneys (Ureteroscopy for e.g. renal disease), or arthroscopy to determine the state of arthritis.

Arterial imaging for imaging arteries for determining the state of arteriosclerosis, or of the heart to determine the state of myocarditis.

10 Amino thiols

The amino thiols useful in the treatment methods of the invention can be synthesized, e.g., as described in U.S. Patent No. 7,314,959.

Amino thiol Dosing

15 The administered dose of the compound of the disclosure depends on factors including the route of administration, the disease to be treated, and physical characteristics, e.g., age, weight, and general health, of the subject. Typically, the amount of a compound disclosed herein (e.g., PrC-210) contained within a single dose or multiple doses over a longer period of time may be an amount that effectively treats the disease without inducing significant toxicity. The dosage may be adapted by the clinician in
20 accordance with conventional factors such as the extent of the disease and different parameters of the subject. Typically, a pharmaceutical composition of the disclosure can be administered in an amount from about 0.001 mg up to about 500 mg/kg/day of an amino thiol, such as PrC-210.

For every species an age, skin pigmentation and weight dependent 'Normal' Free-Radical Range can be established, which serves as comparator to determine deviation of Free-Radicals from the normal
25 Range (see, e.g., Curtis et al., Temporal variations of skin pigmentation in C57BL/6 mice affect optical bioluminescence quantitation. Mol Imaging Biol. 2011;13(6):1114-1123.). Studies in animals and humans can be used to establish the amount and dosing regimen for PrC-210 to keep Free-Radicals within this Normal Range per species and disease.

30 Pharmaceutical Compositions

A pharmaceutical composition of the invention contains one or more of the compounds disclosed herein (e.g., one or more of the compounds of formula (I), formula (IA), and Table 1) as the therapeutic compound. In addition to a therapeutically effective amount of the compound, the pharmaceutical compositions also contain a pharmaceutically acceptable excipient, which can be formulated by methods
35 known to those skilled in the art. In some embodiments, the pharmaceutical compositions for treating diseases contain one or more of the compounds disclosed herein (e.g., one or more of the compounds of formula (I), formula (IA), and Table 1) may be formulated and/or administered with or without other therapeutics for a particular condition. Examples of such therapeutics (second therapeutic agents) are described herein.

The compounds disclosed herein (e.g., the compounds of formula (I), formula (IA), and Table 1) may be used in the form of free base, or in the form of salts, and as solvates. All forms are within the scope of the disclosure.

Exemplary routes of administration of the pharmaceutical compositions (or the compounds of the composition) include oral, sublingual, buccal, transdermal, intradermal, intramuscular, parenteral, intravenous, intra-arterial, intracranial, subcutaneous, intraorbital, intraventricular, intraspinal, intraperitoneal, intranasal, inhalation, and topical administration.

Methods of Treatment

In certain embodiments, the invention includes correlating the intracellular bioluminescence to an intracellular ROS and RNS level in the subject. The measured level can be used to monitor the pathogenesis or severity of a disease or condition in the subject. The measured level can also be used to determine an effective amount of an aminothiols (a compound of formula (II), such as PrC-210) needed to treat the ROS and RNS in the subject.

The methods can be useful for treating, monitoring, and assessing a subject who has undergone a solid organ transplantation (SOT) or vascularized composite allograft transplantation (VCA), wherein the transplant can be monitored for acute or chronic transplant rejections and treatment success.

The methods can be useful for treating, monitoring, and assessing a subject who suffers from a neurodegenerative disease, including but not limited to, Alzheimer's disease, Amyotrophic lateral sclerosis, Parkinson's disease or Huntington disease.

The methods can be useful for treating, monitoring, and assessing a subject who suffers from neuropsychiatric disorders include, without limitation bipolar disorder (BD), schizophrenia, depression, anxiety disorders, attention deficit disorders, addictive disorders, personality disorders, autism and Asperger's disease.

The methods can be useful for treating, monitoring, and assessing a subject who suffers from cardiovascular diseases, including but not limited to such as arteriosclerosis, myocardial infarction, angioplasty, diseases of the heart valve, angina pectoris, peripheral circulatory disorders, vascular damage from dialysis, vascular dementia, and transient ischemic attacks.

The methods can be useful for treating, monitoring, and assessing a subject who suffers from a neuroinflammatory disease, including but not limited to Multiple sclerosis or sterile and non-sterile encephalitis.

The methods can be useful for treating, monitoring, and assessing a subject who suffers from an acute brain trauma, such as Traumatic Brain injury, Spinal cord injury or stroke.

The methods can be useful for treating, monitoring, and assessing a subject who suffers from myopathic diseases.

The methods can be useful for treating, monitoring, and assessing a subject who suffers from autoimmune diseases, including but not limited to such as rheumatoid arthritis, Diabetes Type I, Morbus Crohn, colitis ulcerosa, and psoriasis,

The methods can be useful for treating, monitoring, and assessing a subject who suffers from diabetes Type II, obesity, and metabolic syndrome.

The methods can be useful for treating, monitoring, and assessing a subject who suffers from aging and age-related diseases.

The methods can be useful for treating, monitoring, and assessing a subject who suffers from asthma, and inflammatory pulmonary conditions (e.g., chronic obstructive pulmonary disease (COPD)).

5 The methods can be useful for treating, monitoring, and assessing a subject who suffers from acute injuries including but not limited to such as major injuries, major surgeries and burns.

The methods can be useful for treating, monitoring, and assessing a subject who suffers from from complication of infections, including but not limited to such as post-Covid syndrome.

10 The methods can be useful for treating, monitoring, and assessing a subject who suffers from a disease leading to fibrosis, such as. such as but not limited to hepatitis leading liver fibrosis, glomerulonephritis leading to kidney fibrosis and primary and secondary lung fibrosis.

The methods can be useful for treating, monitoring, and assessing a subject who suffers from acute radiation syndrome, or was, is, or will be exposed to low and high energy radiation from a nuclear explosion, nuclear reactor leakage or space travel.

15 The following examples are meant to illustrate the invention. They are not meant to limit the invention in any way.

EXAMPLES

Example 1. Continuous Monitoring of Free-Radical Levels In Vivo.

20 ICR mice received 8.68 Gy whole-body radiation at Time "0." At the times indicated (2 hr to 196 hr following irradiation) beneath the images (Figure 6), individual mice were anesthetized with 3% isoflurane, and a bolus of viviRen was injected IV into the mouse tail vein. Total mouse body ROS-ViviRen produced photons were then continuously monitored and quantified in an IVIS chamber. It can be seen in the image panels, that the free-radical levels follow an organ-specific pattern over time.

25 Interestingly, this pattern reflects the time-dependent development of organ specific post-radiation toxicities reported by the CDC (see, e.g., <https://www.cdc.gov/nceh/radiation/emergencies/arsphysicianfactsheet.htm>).

Example 2. Monitoring Free-Radical Levels Following Irradiation.

30 ICR mice received 8.68 Gy whole-body radiation (Figure 7) at Time "0." At the times indicated on the X axis, individual mice were anesthetized with 3% isoflurane, and a bolus of ViviRen was injected IV into the mouse tail vein. Mouse whole-body ROS-ViviRen produced photons were then continuously monitored and quantified in an IVIS chamber. Treatment of mice with PrC-210, either i) 0.5 MTD (252 mg/kg) 30 min before irradiation, or ii) 0.3 MTD (151 mg/kg) 24 hr after irradiation, conferred profound

35 suppressions of ROS signal intensities in the body of the intact mice. Interestingly, caspase 3/7 in plasma mirrors the free-radical concentrations seen in the IVIS chamber during the first 48 hours post radiation; the markers remain fully suppressed to background for 6 days after PrC-210 treatment. Free-radical levels follow the same pattern, except for a peak after six days, which based on the organ patterns in Figure 6, appears to be a sign of initial multi-organ-failure, with liver and lung being involved.

Example 3. Monitoring Reductions in Free-Radical Levels Following Irradiation and Dosing with PrC-210.

To visualize the post-radiation ROS response, ICR mice received 8.68 Gy whole-body radiation (Figure 8) at Time "0." At the times indicated on the X axis, individual mice were anesthetized with 3% isoflurane, and a bolus of ViviRen was injected IV into the mouse tail vein. Mouse brain ROS-ViviRen produced photons were then continuously monitored and quantified in an IVIS chamber. Treatment of mice with PrC-210, either i) 0.5 MTD (252 mg/kg) 30 min before irradiation, or ii) 0.3 MTD (151 mg/kg) 24 hr after irradiation, conferred profound suppressions of ROS signal intensities in the intact mice.

To measure the impact of radiation-induced ROS on mouse brains, ICR mice received 8.68 Gy whole-body radiation (Fig. 7), they were euthanized at the indicated hours following irradiation, and brains were removed and homogenized in Tris buffer (pH 7.4). Homogenates were analyzed fresh or stored at -80°C prior to analysis. Levels of malondialdehyde (MDA, ROS-oxidized lipids) and Caspase 1 (inflammasome-induced through free-radicals) in brain homogenates were measured as described previously in our work (Verhoven, B.M., Fahl, W.E. et al, Transplantation Direct 6:8 e578-586, 2020). MDA and Caspase 1 levels were normalized to 100 ug protein in brain homogenates. Mice either received a saline (control), PrC-210 (0.5 MTD, 252 mg/kg bw) IP injection 30 min before irradiation or PrC-210 (0.3 MTD, 151 mg/kg) 24 hr after irradiation. Three replicate MDA and Caspase 1 analyses were done. P value for comparison of PrC-210 treated vs control mice is indicated. Brain ROS levels with and without PrC-210 treatment correlate nearly perfectly with MDA and Caspase 1 levels over the entire timespan of the experiment. Malondialdehyde (MDA) as a lipid peroxidation marker (Stefan Gawel, Maria Wardas, Elzbieta Niedworok, Piotr Wardas Wiad Lek, 2004;57(9-10):453-5.) and Caspase 1 in secondary response to free-radical increase (Fabio Martinon, Signaling by ROS drives inflammasome activation, European Journal of Immunology Volume40, Issue3, March 2010, Pages 616-61) increases are both the result of increased free-radical levels and oxidative stress.

Example 4. Caspase Levels Track Free-Radical Levels Following Irradiation and PrC-210 Therapy.

As in Example 3, ICR mice received 8.68 Gy whole-body radiation (Figure 8) at Time "0." At the times indicated on the X axis, individual mice were anesthetized with 3% isoflurane, and a bolus of ViviRen was injected IV into the mouse tail vein. Mouse brain ROS-ViviRen produced photons were then continuously monitored and quantified in an IVIS chamber. Treatment of mice with PrC-210, either i) 0.5 MTD (252 mg/kg) 30 min before irradiation, or ii) 0.3 MTD (151 mg/kg) 24 hr after irradiation, conferred profound suppressions of ROS signal intensities in the intact mice. Different from Example 3, this time Caspase 8 and Caspase 3/7 were analyzed in the brain samples taken at the different times indicated on the X axis. There were two main findings from this experiment: i) Caspase 8 and Caspase 3/7 activations mirrored the ROS levels with a slight delay, which makes sense, as the apoptotic pathways first need to get activated in response to the free-radical induced oxidative stress, and ii) free-radical levels were suppressed in the same manner following the same suppression pattern from PrC-210 treatment as the Caspase 8 and Caspase 3/7 activity over the time of the experiment.

Example 5. Continuous Monitoring of Free-Radical Levels in Brain Cells In Vivo.

This experiment was designed to illuminate any relationship between brain free-radical levels and the onset and progression of neurodegenerative diseases, such as Amyotrophic Lateral Sclerosis. In Figure 10, Panel A the quantitative measurement of mouse brain ROS level (measured by its reaction with IV ViviRen) in three SOD1^{G93A} mutant mice and a wildtype control can be seen. Figure 10, Panel B shows the body weights over time of the same three Panel A SOD1^{G93A} mice. Weight loss was progressive and was going into exponential decline along with onset of ALS motor symptoms.

There were two main findings from this experiment: i) The timing of motor symptom onset in the three mice was directly correlated with the increased levels of free-radicals in the three mice. ii) The administration of systemic PrC-210 (0.1 MTD, IP) was associated with an immediate plateau in body weight and the weight loss was stopped.

Example 6. Measuring the Relationship between Radiation Dose and Free-radical Levels, and Free-radical Scavenging with PrC-210.

This experiment was designed to illuminate (i) the relationship between different administered radiation doses to mice and the free-radical levels that result in mouse organs as a result of the radiation, and (ii) the ability of PrC-210 to suppress the radiation-induced mouse organ free-radical levels through its scavenging capability.

Figure 11 A and B show a linear relationship between radiation doses and free-radical levels measured with viviRen 30 minutes after radiation.

Figure 11 A shows that PrC-210 injected intravenously 30 min before radiation reduces free-radical levels in a PrC-210 dose-dependent manner.

Both Figures A and B show that increasing PrC-210 doses are able to suppress free-radicals to background with increasing radiation doses, which means that it should be possible to define a PrC-210 dose, for any given radiation dose, at which radiation induced free-radicals are suppressed to background, whether the with the PrC-210 is given before or after radiation.

Figure B shows that PrC-210 reduces radiation induced free-radicals in a dose-dependent manner.

Both Figure 11 panels show a linear relationship between administered radiation dose and organ free-radical production measured with viviRen. The panels also show a dose-dependent relationship between radiation induced free-radical levels and their suppression by PrC-210 doses. These data can be used i) to measure the cell/organ biological impact of a given radiation dose by measuring cell/organ free-radical levels with viviRen, and ii) to measure the free-radical scavenging efficacy of PrC-210 on free-radical level suppression, independent on whether PrC-210 is given before or after radiation.

Other Embodiments

Various modifications and variations of the described compositions, methods, and uses of the invention will be apparent to those skilled in the art without departing from the scope and spirit of the invention. Although the invention has been described in connection with specific embodiments, it should be understood that the invention as claimed should not be unduly limited to such specific embodiments.

Indeed, various modifications of the described modes for carrying out the invention that are obvious to those skilled in the art are intended to be within the scope of the invention.

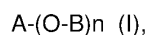
Other embodiments are in the claims.

What is claimed is:

CLAIMS

1. A method of measuring intracellular Reactive Oxygen Species (ROS) and Reactive Nitrogen Species (RNS) in a subject, the method comprising the steps of:

(i) administering to the subject a compound of formula (I):



wherein A is a radical of a luciferin, each O is an oxygen atom linking A and B, each B is, independently, a cleavable protecting group, and n is an integer from 1-4; and

(ii) following step (i), extracorporeally measuring intracellular bioluminescence from the luciferin.

2. The method of claim 1, wherein A is a radical of coelenterazine, vargulin, prolume purple, or furimazine.

3. The method of claim 2, wherein A is a radical of coelenterazine.

4. The method of claim 3, wherein the radical of coelenterazine is enduRen or viviRen.

5. The method of any one of claims 1-4, wherein each cleavable protecting group B is selected to be cleaved by esterases or lipases.

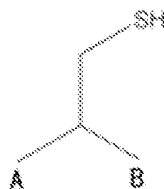
6. The method of any one of claims 1-5, further comprising correlating the result of step (ii) to an intracellular ROS and RNS level in the subject.

7. The method of any one of claims 1-5, further comprising using the result of step (ii) to monitor the pathogenesis or severity of a disease or condition in the subject.

8. A method of treating a disease or condition mediated by intracellular Reactive Oxygen Species (ROS) and Reactive Nitrogen Species (RNS) in a subject, the method comprising the steps of:

(a) measuring an intracellular ROS and RNS level in the subject; and

(b) on the basis of the level measured in step (a), administering to the subject an effective amount of a compound of formula (II):



(II),

or a pharmaceutically acceptable acid addition salt thereof, and wherein

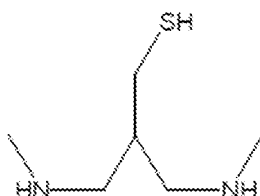
(x) A is $-\text{CH}_2\text{NHR}'$ and B is $-\text{CH}_2\text{NHR}$, or $A = -\text{NRR}'$ and $B = \text{H}$; and

(y) each of R and R' are, independently, selected from H, $\text{C}_1\text{-C}_6$ alkyl, and $\text{C}_1\text{-C}_6$ heteroalkyl,

with the proviso that R and R' are not both H if B = H.

9. The method of claim 8, wherein step (a) comprises performing the method of any one of claims 1-7.

10. The method of claim 8, wherein the compound of formula (II) is



PrC-210,

or a pharmaceutically acceptable salt thereof.

11. The method of any one of claims 1 to 10, wherein the subject has undergone a solid organ transplantation (SOT) or vascularized composite allograft transplantation (VCA) and the transplant can be monitored for acute or chronic transplant rejections and treatment success.

12. The method of any one of claims 1 to 10, wherein the subject suffers from a neurodegenerative disease, including but not limited to, Alzheimer's disease, Amyotrophic lateral sclerosis, Parkinson's disease or Huntington disease.

13. The method of any one of claims 1 to 10, wherein the subject suffers from a neuroinflammatory disease, including but not limited to Multiple sclerosis or sterile and non-sterile encephalitis.

14. The method of any one of claims 1 to 10, wherein the subject suffers from an acute brain trauma, such as Traumatic Brain injury, Spinal cord injury or stroke.

15. The method of any one of claims 1 to 10, wherein the subject suffers from acute radiation syndrome, or was, is, or will be exposed to low and high energy radiation from a nuclear explosion, nuclear reactor leakage or space travel.

16. The method of any one of claims 1 to 10, wherein the subject suffers from neuropsychiatric disorders include, without limitation bipolar disorder (BD), schizophrenia, depression, anxiety disorders, attention deficit disorders, addictive disorders, personality disorders, autism and Asperger's disease.

17. The method of any one of claims 1 to 10, wherein the subject suffers from cardiovascular diseases, including but not limited to such as arteriosclerosis, myocardial infarction, angioplasty, diseases of the heart valve, angina pectoris, peripheral circulatory disorders, vascular damage from dialysis, vascular dementia, and transient ischemic attacks.

18. The method of any one of claims 1 to 10, wherein the subject suffers from myopathic diseases.

19. The method of any one of claims 1 to 10, wherein the subject suffers from autoimmune diseases, including but not limited to such as rheumatoid arthritis, Diabetes Type I, Morbus Crohn, colitis ulcerosa, and psoriasis.

20. The method of any one of claims 1 to 10, wherein the subject suffers from diabetes Type II, obesity, and metabolic syndrome.

21. The method of any one of claims 1 to 10, wherein the subject suffers from aging and age-related diseases.

22. The method of any one of claims 1 to 10, wherein the subject suffers from asthma, and inflammatory pulmonary conditions (e.g., chronic obstructive pulmonary disease (COPD)).

23. The method of any one of claims 1 to 10, wherein the subject suffers from acute injuries including but not limited to such as major injuries, major surgeries and burns.

24. The method of any one of claims 1 to 10, wherein the subject suffers from complication of infections, including but not limited to such as post-Covid syndrome.

25. The method of any one of claims 1 to 10, wherein the subject suffers from a disease leading to fibrosis, such as. such as but not limited to hepatitis leading liver fibrosis, glomerulonephritis leading to kidney fibrosis and primary and secondary lung fibrosis.

26. The method of any one of claims 1-10, wherein the bioluminescence of step (ii) occurs in the absence of any luciferase enzyme.