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(71) Applicant: **ELYSIUM HEALTH, INC.** [US/US]; 434 Broadway, Second Floor, New York, New York 10013 (US).

(72) Inventors: **MARCOTULLI, Eric**; 50 Prince Street, Apt. 6A, New York, New York 10012 (US). **ALMINANA, Dan**; 50 Franklin Street, Apt. 5E, New York, New York 10013 (US). **MORRIS, Mark**; 639-B Quaker Road, Chappaqua, New York 10514 (US). **DELLINGER, Ryan**; 635 E Boxwood Lane, Azusa, California 91702 (US).

(74) Agent: **MILLS, Jeffrey K.**; Medler Ferro Woodhouse & Mills PLLC, 8201 Greensboro Drive, Suite 1060, McLean, Virginia 22102 (US).

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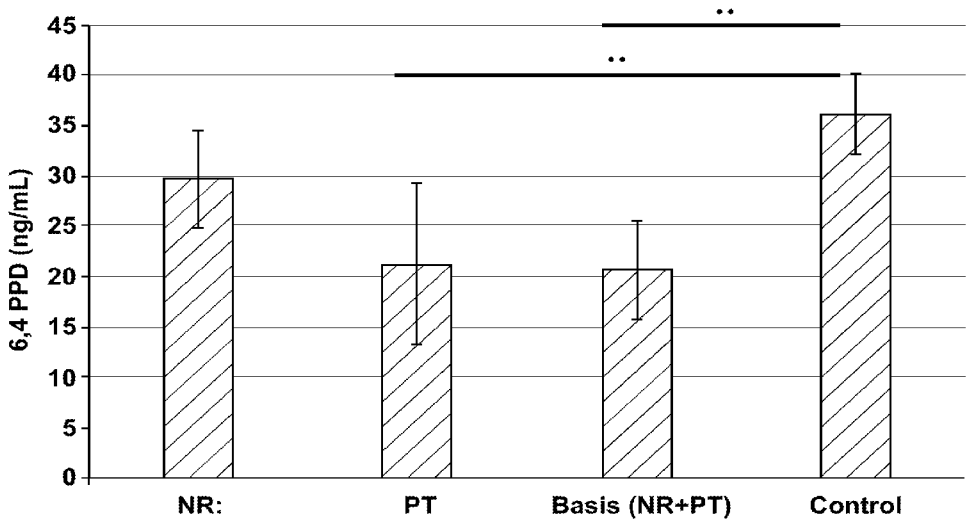

FIG. 2

(57) Abstract: The present application provides methods for treating wounds by administering a combination of nicotinamide riboside and pterostilbene. The compositions can be administered orally or as a topical formulation.

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[Continued on next page]

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METHODS FOR WOUND TREATMENT

FIELD OF THE INVENTION

[0001] The present application provides methods for treating wounds by administering a combination of nicotinamide riboside and pterostilbene. The compositions can be administered orally or as a topical formulation.

BACKGROUND OF THE INVENTION

[0002] Research in the field of wound treatment often focuses on various forms of wound dressings and methods for closing a wound using surgical or other physical methods. In addition, various topical and oral formulations have been researched to help aid and speed the healing of a wound.

[0003] The process of wound healing involves many complicated components. Immediately upon the injury insult, defense mechanisms inherent in normal body tissues are activated to restore continuity and tensile strength. Wound healing then occurs in three distinct phases.

[0004] First, is the phase of acute inflammatory response. Body fluids containing plasma proteins, fibrin, antibodies and various blood cells flow into the wound. Scab formation takes place and inflammation occurs within a few hours. Also, at this stage, neutrophils, monocytemacrophages come into play. During this acute phase, the wound is solely dependent on the closure material contained in the scab for strength.

[0005] Second, is the phase of fibroplasia. Here, via various enzymatic mechanisms, fibrin synthesis and accumulation takes place. This causes an increase in wound tensile strength and stimulation of fibroblast proliferation and growth. Fibroblasts secrete collagen, a fibrous protein as part of connective tissue. Collagen deposition begins from the fifth day and results in rapid gain in tensile strength of the wound.

[0006] The third phase is the maturational process. Tensile strength continues to increase from the cross-linking of collagen fibers. Deposition of fibrous connective tissue causes scar formation.

[0007] In all phases, compositions that can aid or speed the wound healing process are highly desired.

BRIEF SUMMARY OF THE INVENTION

[0008] The present invention fulfills these needs by providing compositions and methods for treatment of wounds.

[0009] In embodiments, provided herein is a method for treating a wound, comprising administering a composition comprising a therapeutically effective amount of nicotinamide riboside and a therapeutically effective amount of pterostilbene to a patient having the wound.

[0010] In additional embodiments, methods for treating an open wound are provided, comprising administering a composition consisting essentially of a therapeutically effective amount of nicotinamide riboside and a therapeutically effective amount of pterostilbene to a patient having the open wound.

[0011] In further embodiments, provided herein is a method for treating a closed wound, comprising administering a composition consisting essentially of a therapeutically effective amount of nicotinamide riboside and a therapeutically effective amount of pterostilbene to a patient having the closed wound.

BRIEF DESCRIPTION OF DRAWINGS

[0012] The foregoing and other features and aspects of the present technology can be better understood from the following description of embodiments and as illustrated in the accompanying drawings. The accompanying drawings, which are incorporated herein and form a part of the specification, further serve to illustrate the principles of the present technology. The components in the drawings are not necessarily to scale.

[0013] FIGs. 1A-1C show the combination of NR and PT effect on UVB-induced ear swelling at 24, 48, 72 and 96 hours.

[0014] FIG. 2 shows the effect of NR, PT and NR+PT on the formation of pyrimidine-pyrimidone (6-4) (6,4 PPD) photo-products. ** $P < 0.01$.

DETAILED DESCRIPTION OF THE INVENTION

[0015] It should be appreciated that the particular implementations shown and described herein are examples and are not intended to otherwise limit the scope of the application in any way.

[0016] The published patents, patent applications, websites, company names, and scientific literature referred to herein are hereby incorporated by reference in their entirety to the same extent as if each was specifically and individually indicated to be incorporated by reference. Any conflict between any reference cited herein and the specific teachings of this specification shall be resolved in favor of the latter. Likewise, any conflict between an art-understood definition of a word or phrase and a definition of the word or phrase as specifically taught in this specification shall be resolved in favor of the latter.

[0017] As used in this specification, the singular forms "a," "an" and "the" specifically also encompass the plural forms of the terms to which they refer, unless the content clearly dictates otherwise. The term "about" is used herein to mean approximately, in the region of, roughly, or around. When the term "about" is used in conjunction with a numerical range, it modifies that range by extending the boundaries above and below the numerical values set forth. In general, the term "about" is used herein to modify a numerical value above and below the stated value by a variance of 20%.

[0018] Technical and scientific terms used herein have the meaning commonly understood by one of skill in the art to which the present application pertains, unless otherwise defined. Reference is made herein to various methodologies and materials known to those of skill in the art.

[0019] In embodiments, provided herein are methods for treating a wound. Wounds are caused by trauma or skin breakdown. In general, wounds are considered to be acute wounds if they are new. Wounds are considered to be chronic if they are more than 3-4 weeks old.

[0020] Wounds that can be treated using the methods and compositions described herein include open wounds and closed wounds, and occur on the skin surface, or just below the skin

surface, or in mucous membranes. Wounds can also occur in internal organs due to surgery or trauma. Wounds also include ulcers, as described herein.

[0021] "Open wounds" are wounds where the skin surface is broken or penetrated. Open wounds include, for example:

- Abrasions - An abrasion occurs when skin rubs or scrapes against a rough or hard surface. Road rash is an example of an abrasion. Generally not a lot of bleeding.

- Lacerations or incisions - A laceration or incision is a deep cut or tearing of the skin. Accidents with knives, tools, and machinery are frequent causes of lacerations. In the case of deep lacerations, bleeding can be rapid and extensive. Generally a laceration is with jagged edges, an incision with clean edges, and includes surgical incisions.

- Punctures - A puncture is a small hole caused by a long, pointed object, such as a nail or needle. Sometimes, a bullet can cause a puncture wound. Punctures may not bleed much, but these wounds can be deep enough to damage internal organs.

- Avulsions - An avulsion is a partial or complete tearing away of skin and the tissue beneath. Avulsions usually occur during violent accidents, such as body-crushing accidents, explosions, and gunshots. They bleed heavily and rapidly.

- Burns – includes thermal, chemical and electrical burns. As used herein "burns" explicitly excludes burns caused by exposure to sun (i.e., sun burns). Thus, "wounds" and "open wounds" also explicitly exclude sun burns.

- Bites and stings.

[0022] "Closed wounds" are wounds where the skin surface is not broken or penetrated, and in general the wound occurs just below the skin surface. Closed wounds include, for example:

- Contusions: blunt trauma causing pressure damage to the skin and / or underlying tissues (includes bruises).

- Blisters: fluid filled pockets under the skin.

- Seroma: a fluid filled area that develops under the skin or body tissue (commonly occur after blunt trauma or surgery).
- Hematoma: a blood filled area that develops under the skin or body tissue (occur due to internal blood vessel damage to an artery or vein).
- Crush injuries: can be caused by extreme forces, or lesser forces over a long period of time.

[0023] "Ulcers" - Ulcers are lesions that wear down the skin or mucous membrane that can have various causes depending on their location. Ulcers are a gradual disturbance of tissues by an internal cause that originate from an impaired immune system or nervous system. Cells require blood, oxygen, and nutrients and anything that reduces the supply of these requirements can lead to ulcer formation. The most common types of internally originating skin ulcers are diabetic foot ulcers, venous leg ulcers, and pressure ulcers.

- Skin ulcer types:
    - Pressure ulcer: injury that causes breakdown of the skin and often the underlying tissue as well. Pressure ulcers can range in severity from discolored skin areas to large open wounds that expose the underlying bone or muscle.
    - Diabetic Foot Ulcer (DFU): a major complication of diabetes that occurs when neuropathic (nerve) and vascular (blood vessel) complications of the disease cause altered or complete loss of feeling in the foot and/or leg. Pressure from shoes, cuts or any injury to the foot may go unnoticed causing a DFU.
    - Venous ulcer (VLU): an open sore that develops when the skin is broken and air or bacteria gets into the underlying tissues. VLUs are caused by venous disease; a disease of the veins of the leg.
    - Ulcerative dermatitis: an ulcer due to a dermatological condition
    - Genital ulcer: painful, non-sexually acquired genital ulceration.

[0024] The methods described herein suitably "treat" a wound, which means that the methods and compositions result in the closure or healing of a wound.

[0025] Suitably, methods for treating a wound are provided that comprise administering a composition comprising a therapeutically effective amount of nicotinamide riboside and a therapeutically effective amount of pterostilbene to a patient having the wound.

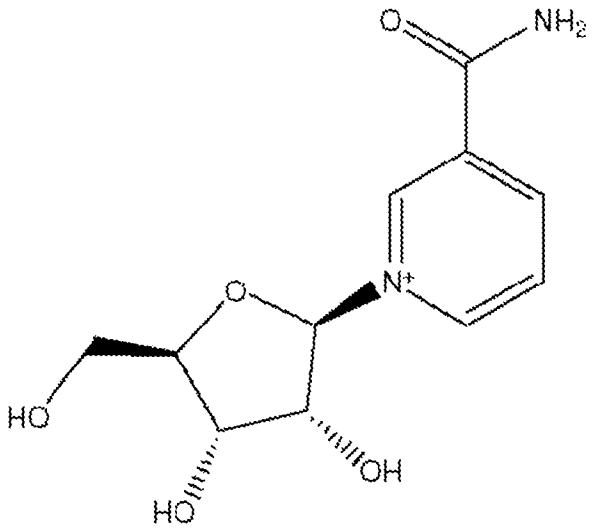
[0026] As described herein, the methods suitably include administering orally the composition to the patient having the wound. Oral administration can include providing a capsule, tablet, lozenge, syrup, liquid, etc., to a patient via the mouth. Additional methods and forms of oral administration are described throughout.

[0027] In further embodiments, the methods include administering topically the composition to the patient having the wound. Topical administration includes the application of a liquid, cream, foam, gel, salve, or other topical form as described herein, directly to the surface of the skin of the patient having the wound. Suitably, the topical application is made directly to the wound itself, as well as surrounding tissue to aid in healing of the wound.

[0028] Compositions for Use in Treatment

[0029] In exemplary embodiments, the nicotinamide riboside and pterostilbene for use in the methods described herein are contained within the same formulation, suitably an oral formulation, including a liquid, drops, a spray, a solution, a gel, a powder, a suspension, or in a solid dosage form such as a lozenge, a capsule, a tablet, a pill, a gel-cap, a buccal or sub-lingual strip, etc.

[0030] As discussed herein, in embodiments, the compositions contain nicotinamide riboside, a precursor of coenzyme NAD^+, which is involved in metabolic processes such as energy production, DNA repair, cellular detoxification, the inflammatory response, and protein folding. The chemical structure of nicotinamide riboside is provided below.

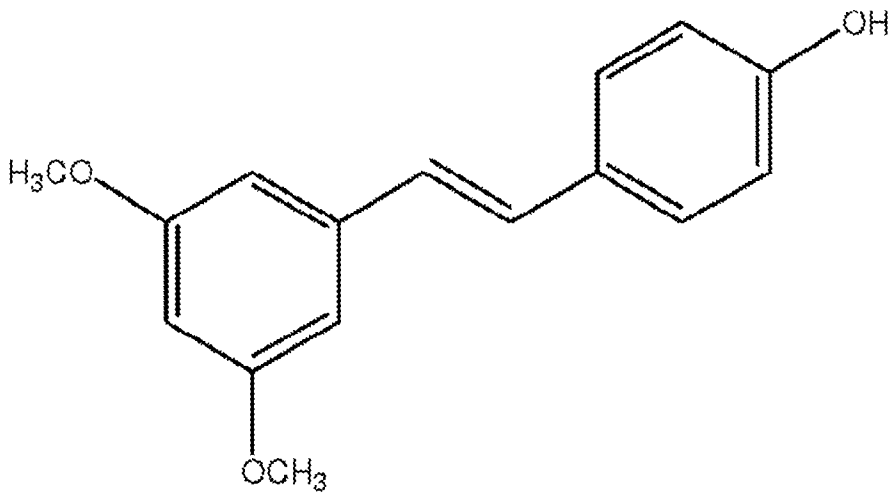


[0031] Nicotinamide riboside has four asymmetric centers and that any optical isomer, as separated, pure or partially purified optical isomers and any mixtures thereof including racemic mixtures can be used. The enantiomeric form can be in enantiomeric excess, e.g., essentially in a pure form. Accordingly, some embodiments relate to nicotinamide riboside having an enantiomeric excess of at least 60%, at least 70%, at least 80%, at least 85%, at least 90%, at least 96%, at least 98%, and ranges therebetween.

[0032] Nicotinamide riboside is a quaternary salt and forms an ionic bond with a counteranion. Examples of counteranions include the anions of suitable organic acid such as formic, acetic, trichloroacetic, trifluoroacetic, propionic, benzoic, cinnamic, citric, fumaric, glycolic, itaconic, lactic, methanesulfonic, maleic, malic, malonic, mandelic, oxalic, picric, pyruvic, salicylic, succinic, methane sulfonic, ethanesulfonic, tartaric, ascorbic, pamoic, bismethylene salicylic, ethanedisulfonic, gluconic, citraconic, aspartic, stearic, palmitic, EDTA, glycolic, p-aminobenzoic, glutamic, benzenesulfonic, p-toluenesulfonic acids, theophylline acetic acids, as well as the 8-halotheophyllines, for example 8- bromotheophylline and the like. Further examples of pharmaceutical acceptable inorganic or organic acid counteranions include the pharmaceutically acceptable salts listed in J. Pharm. Sci.66, 2 (1977)). In certain other embodiments, the active agent is a derivative, salt, solvate, or prodrug of nicotinamide riboside. In some embodiments, the ribose in nicotinamide riboside is β-D-ribose. In certain embodiments,

nicotinamide riboside may be substituted or combined with nicotinamide mononucleotide, niacinamide, nicotinamide, nicotinic acid, and/or niacin.

[0033] Suitably, the compositions also include pterostilbene. Pterostilbene is a polyphenol based derivative of resveratrol and, like the NAD^+ precursor, promotes metabolic health. The chemical structure of pterostilbene is provided below:



[0034] In some embodiments, a derivative, salt, solvate, or prodrug of pterostilbene can be used in the compositions described herein. In certain embodiments, pterostilbene may be substituted and/or combined with epsilon-viniferin and/or resveratrol.

[0035] As described herein, the compositions for use in the treatment methods are suitably formulated for oral delivery, i.e., in an oral formulation. Oral solid dosage forms are described generally in Remington's Pharmaceutical Sciences, 18thEd.1990 (Mack Publishing Co. Easton Pa.18042) at Chapter 89. Solid dosage forms include tablets, capsules, pills, troches or lozenges, cachets, pellets, powders, or granules or incorporation of the material into particulate preparations of polymeric compounds such as polylactic acid, polyglycolic acid, etc., or into liposomes. Such compositions may influence the physical state, stability, rate of *in vivo* release, and rate of *in vivo* clearance of the disclosed. See, e.g., Remington's Pharmaceutical Sciences, 18thEd. (1990, Mack Publishing Co., Easton, Pa. 18042) pages 1435-1712. The compositions may be prepared in liquid form, or may be in dried powder (e.g., lyophilized) form. Liposomal or proteinoid encapsulation may be used to formulate the compositions. Liposomal encapsulation

may be used and the liposomes may be derivatized with various polymers (e.g., U.S. Patent No.5,013,556). See also, Marshall, K. In: Modern Pharmaceutics Edited by G. S. Banker and C. T. Rhodes Chapter 10, 1979. The formulation may include a peptide (or chemically modified forms thereof) and inert ingredients which protect compounds in the stomach environment, and release of the biologically active material in the intestine.

[0036] Nicotinamide riboside, niacinamide, nicotinamide, nicotinic acid, pterostilbene, nicotinamide mononucleotide, niacin, epsilon-viniferin, resveratrol or derivatives thereof may be chemically modified so that oral delivery of the compound is efficacious. Contemplated chemical modification is the attachment of at least one moiety to the component molecule itself, where the moiety permits uptake into the blood stream from the stomach or intestine, or uptake directly into the intestinal mucosa. Also contemplated is the increase in overall stability of the component or components and increase in circulation time in the body. Certain embodiments may be pharmaceutical compositions. Certain embodiments may be nutritional supplements.

[0037] Certain embodiments provide liquid dosage forms for oral administration, including pharmaceutically acceptable emulsions, solutions, suspensions, and syrups, which may contain other components including inert diluents, adjuvants such as wetting agents, emulsifying and suspending agents, and sweetening, and flavoring agents. The methods can involve the use of a composition which is administered as a liquid with an active agent (i.e., nicotinamide riboside and/or pterostilbene) dissolved (e.g., solution) or dispersed (e.g., suspension) in the composition. The solution or suspension may be prepared using one or more pharmaceutically acceptable excipients. Suitable excipients include, but are not limited to, surfactants, humectants, plasticizers, crystallization inhibitors, wetting agents, bulk filling agents, solubilizers, bioavailability enhancers, pH adjusting agents, flavorants and combinations

[0038] Controlled release oral formulations may be provided. Controlled release may include, but is not limited to, delayed release and pH-dependent release. In certain embodiments, the nicotinamide riboside and pterostilbene, or derivatives thereof can be incorporated into microcapsules, microparticulates, nanoparticulates, etc. through use of coatings to affect release of the active principle. In certain embodiments, nicotinamide riboside and pterostilbene, or derivatives thereof can be incorporated into an inert matrix which permits release by either

diffusion or leaching mechanisms, e.g., gums. Slowly degenerating matrices may also be incorporated into the formulation.

[0039] Modified release oral formulations may be provided. Modified release may allow for specific release profiles.

[0040] Extended release oral formulations may be provided. Extended release may allow for release of active ingredient over a desired time period.

[0041] Additional discussions for varying release formulations and related terms may be found in Lesczek Krowczynski, Extended-Release Dosage Forms, 1987 (CRC Press, Inc.).

[0042] In certain aspects, the form of a controlled, modified or extended release oral formulation is a tablet, capsule, or microbeads for oral administration. In other aspects, controlled, modified or extended release formulations comprising suitable and effective treatment amounts of the desired components may be pills, powders, granules, sterile parenteral solutions or suspensions, oral solutions or suspensions, oil-water emulsions as well as implants and microencapsulated delivery systems.

[0043] Other formulations may provide controlled, modified or extended release profiles. Compositions of the present invention may comprise conventional pharmaceutical binders, excipients and additives, which may act to control, modify or extend release when used in sufficient quantities. Coating agents, e.g., plasticizers, may be used to enhance the controlled, modified or extended release features of the compositions of the invention.

[0044] For oral formulations, the location of release may be the stomach, the small intestine (the duodenum, the jejunum, or the ileum), or the large intestine. The release can avoid the deleterious effects of the stomach environment, either by protection of the agent (or derivative) or by release of the agent (or derivative) beyond the stomach environment, such as in the intestine. To ensure full gastric resistance a coating temporally impermeable to at least pH 5.0 is useful. Examples of the more common inert ingredients that are used as enteric coatings are cellulose acetate trimellitate (CAT), hydroxypropylmethylcellulose phthalate (HPMCP), polyvinyl acetate phthalate (PVAP), poly(methacrylic acid-co-ethyl acrylate) 1:1, cellulose acetate phthalate (CAP), poly(methacylic acid-co-methyl methacrylate) 1:1, poly(methacylic

acid-co-methyl methacrylate) 1:2, and natural shellac resin. These coatings may be used as mixed films.

[0045] In exemplary embodiments, the methods described herein utilize the oral administration of soft capsules containing nicotinamide riboside and pterostilbene or their equivalents. The soft capsule can be prepared using techniques well known in the art. For example, soft capsules are typically produced using a rotary die encapsulation process. Active agent formulations are fed into the encapsulation machine by gravity. In an embodiment, the formulation comprises pharmaceutical excipients such as olive oil, gelatin, glycerin, purified water, beeswax yellow, sunflower lecithin, silicon dioxide, titanium dioxide, F. D. & C Blue 1 and F. D. & C Red 4, microcrystalline cellulose, hypromellose, vegetable magnesium stearate, and/or silica.

[0046] A capsule shell can comprise one or more plasticizers such as glycerin, sorbitol, sorbitans, maltitol, glycerol, polyethylene glycol, polyalcohols with 3 to 6 carbon atoms, citric acid, citric acid esters, triethyl citrate and combinations thereof. In an embodiment, the plasticizer is glycerin.

[0047] In addition to the plasticizer(s), the capsule shell can include other suitable shell additives such as opacifiers, colorants, humectants, preservatives, flavorings, and buffering salts and acids.

[0048] Opacifiers are used to opacify the capsule shell when the encapsulated active agents are light sensitive. Suitable opacifiers include, but not limited to, titanium dioxide, zinc oxide, calcium carbonate and combinations thereof. In an embodiment, the opacifier is titanium dioxide.

[0049] Colorants can be used to for marketing and product identification and/or differentiation purposes. Suitable colorants include synthetic and natural dyes and combinations thereof.

[0050] Humectants can be used to suppress the water activity of the softgel. Suitable humectants include glycerin and sorbitol, which are often components of the plasticizer composition. Due to the low water activity of dried, properly stored softgels, the greatest risk

from microorganisms comes from molds and yeasts. For this reason, preservatives can be incorporated into the capsule shell. Suitable preservatives include alkyl esters of p-hydroxy benzoic acid such as methyl, ethyl, propyl, butyl and heptyl (collectively known as "parabens") or combinations thereof.

[0051] One composition for use in the methods of treatment described herein is referred to as "BASIS®" and includes nicotinamide ribose (250 mg) and pterostilbene (50 mg) as the active ingredients. This can be in a capsule formed of microcrystalline cellulose, hypromellose, vegetable magnesium stearate, olive oil, gelatin, glycerin, purified water, beeswax yellow, sunflower lecithin, silicon dioxide, titanium dioxide, F. D. & C Blue 1 and F. D. & C Red 4, or vegetarian hard capsules made solely of plant materials. Any embodiment may include microcrystalline cellulose, hypromellose, vegetable magnesium stearate, and/or silica. Other pharmaceutical excipients that can be included in the disclosed formulations, include acetyl-L-carnitine, N-acetyl cysteine, α-lipoic acid, biotin, vitamin B6, vitamin B12, folic acid, resveratrol, vinpocetine, chromium picolinate, vitamin D3, naringin, quercetin, and creatine.

[0052] In additional embodiments, the nicotinamide riboside and pterostilbene for use in the methods described herein are contained within the same formulation, suitably a topical formulation. As used herein "topical formulation" refers to any form of a composition or formulation that can be applied to the surface of the skin, including the scalp, face, eyebrows, eyelashes, back, neck, trunk, legs, etc. Topical formulations include liquids, foams, powders, emulsions, sprays, creams, lotions, serums, salves, balms, etc. Examples of components that can be included in topical formulations in addition to the nicotinamide riboside and pterostilbene include various moisturizers, humectants, odor modifiers, buffers, pigments, preservatives, Vitamins such as A, C and E, emulsifiers, dispersing agents, wetting agents, odor-modifying agents, gelling agents, stabilizers, propellants, antimicrobial agents, sunscreens, enzymes and the like.

[0053] The methods described herein, whether administered orally or topically, comprise administering a therapeutically effective amount of nicotinamide riboside and a therapeutically effective amount of pterostilbene to a patient having the wound. As used herein "therapeutically effective" refers to the amount of nicotinamide riboside and/or pterostilbene needed to produce

close or heal a wound. In certain embodiments, nicotinamide mononucleotide, niacinamide, nicotinamide, nicotinic acid and/or niacin may be substituted for nicotinamide riboside. In certain embodiments, a combination of nicotinamide riboside, nicotinamide mononucleotide, and/or niacin may be used. In certain embodiments epsilon-viniferin and/or resveratrol may be substituted for pterostilbene. In certain embodiments, a combination of pterostilbene, epsilon-viniferin, and/or resveratrol maybe used.

[0054] In exemplary embodiments, nicotinamide riboside or its equivalents, alone or in combination with pterostilbene or its equivalents, is administered to a patient (either via a single composition, or in multiple, separate compositions) in an amount of between about 50 mg and about 1500 mg, between about 100 mg and about 1500 mg, between about 100 mg and about 1000 mg, between about 125 mg and about 900 mg, between about 150 mg and about 850 mg, between about 200 mg to 700 mg, between about 200 mg to about 500 mg, between about 1000 mg and about 1500 mg, or about 100 mg, about 150 mg, about 200 mg, about 250 mg, about 300 mg, about 350 mg, about 400 mg, about 450 mg, about 500 mg, about 550 mg, about 600 mg, about 650 mg or about 700 mg nicotinamide. Suitably, these amounts are administered to a patient on a daily basis in the form of a single composition or multiple compositions. The administration of nicotinamide riboside can be orally or topically, as described herein.

[0055] In exemplary embodiments, pterostilbene or its equivalents, alone or in combination with nicotinamide riboside or its equivalents, is administered to a patient (either via a single composition or in multiple, separate compositions) in an amount between about 25 mg and about 1000 mg, between about 100 mg and about 1000 mg, between about 25 mg and about 500 mg per day, between about 25 mg and about 200 mg, between about 25 mg and about 250 mg, between about 30 mg and about 225 mg, between about 40 mg and about 200 mg, between about 45 mg and about 250 mg, or about 25 mg, about 30 mg, about 40 mg, about 50 mg, about 60 mg, about 70 mg, about 80 mg, about 90 mg, about 100 mg, about 110 mg, about 120 mg, about 130 mg, about 140 mg, or about 150 mg pterostilbene. Suitably, these amounts are administered to a patient on a daily basis in the form of a single composition or multiple compositions. The administration of pterostilbene can be orally or topically, as described herein.

[0056] In exemplary embodiments, the compounds, compositions or pharmaceutical compositions are prepared as oral formulations containing nicotinamide riboside and pterostilbene in the same composition. In further embodiments, the compounds, compositions or pharmaceutical compositions are prepared as topical formulations containing nicotinamide riboside and pterostilbene in the same composition.

[0057] In additional exemplary embodiments, the compounds, compositions or pharmaceutical compositions are prepared as oral formulations containing nicotinamide riboside and pterostilbene in different compositions, including different oral delivery types or formulations. In further embodiments, the compounds, compositions or pharmaceutical compositions are prepared as topical formulations containing nicotinamide riboside and pterostilbene in different compositions, including different topical delivery types or formulations.

[0058] Suitably, the methods of treatment include administering a composition comprising a combination of nicotinamide riboside at about 200 mg to about 700 mg and pterostilbene at about 25 mg to about 200 mg to the patient, including a composition comprising amount 500 mg of nicotinamide riboside and about 100 mg pterostilbene. In embodiments, the methods of treatment include administering a composition including a combination of nicotinamide riboside at about 250 mg and pterostilbene at about 50 mg to the patient. In additional embodiments, the methods of treatment include administering two or more doses of such as composition, such that a total of about 500 mg nicotinamide riboside and about 100 mg pterostilbene is administered to the patient on a daily basis. The administration of the composition can be orally or topically, as described herein.

[0059] In certain embodiments a composition may be administered in a dosage regimen over days, weeks, or months. Dosages may be multiple times per day or singular doses per day. Each dosage when dosages are administered over multiple days, weeks, or months may not be equal amounts. Dosage amounts during a dosage regimen may vary according to the amounts and ranges disclosed herein. Suitably, the compositions described herein are administered on a daily basis for a period of at least 4 weeks, suitably for a period of at least 1 month, or for a period of at least 2 months, at least 3 months, at least 4 months, at least 5 months, at least 6 months, etc.

The compositions described herein can also be administered to a patient for 1 or more years, including for the lifetime of a patient. Dosage can be provided orally or topically, as described herein.

[0060] In further embodiments, methods are provided herein for treating an open wound, comprising administering a composition consisting essentially of a therapeutically effective amount of nicotinamide riboside and a therapeutically effective amount of pterostilbene to a patient having the open wound. In still further embodiments, methods are provided herein for treating an closed wound, comprising administering a composition consisting essentially of a therapeutically effective amount of nicotinamide riboside and a therapeutically effective amount of pterostilbene to a patient having the open wound.

[0061] In compositions and formulations that "consist essentially" of the recited ingredients, such compositions and formulations contain the recited components and those that do not materially affect the basic and novel characteristics of the claimed formulations. Components that do not materially affect the basic and novel characteristics of the claimed formulations are those that do not limit the ability of the nicotinamide riboside and pterostilbene to heal the wound. Suitably, compositions and formulations that consist essentially of the recited ingredients specifically exclude other active agents for treatment of the wound.

[0062] In oral compositions and formulations that "consist essentially" of the recited ingredients, such oral compositions and formulations contain the recited components and those that do not materially affect the basic and novel characteristics of the claimed formulations. Components that do not materially affect the basic and novel characteristics of the claimed oral formulations are those that do not limit the ability of the nicotinamide riboside and pterostilbene to heal the wound, and include various oral excipients. Suitably, oral compositions and formulations that consist essentially of the recited ingredients specifically exclude other active agents for treatment of the wound.

[0063] In topical compositions and formulations that "consist essentially" of the recited ingredients, such topical compositions and formulations contain the recited components and those that do not materially affect the basic and novel characteristics of the claimed formulations. Components that do not materially affect the basic and novel characteristics of the

claimed topical formulations are those that do not limit the ability of the nicotinamide riboside and pterostilbene to heal the wound, and include various topical excipients. Suitably, topical compositions and formulations that consist essentially of the recited ingredients specifically exclude other active agents for treatment of the wound.

[0064] In embodiments, the methods of treatment described herein can be utilized in combination with well-known wound dressings and bandages. While other compounds can be provided in addition the nicotinamide riboside and pterostilbene formulations described herein, suitably no other compounds are provided to the patient to aid in the wound healing.

[0065] In exemplary embodiments, the methods described herein result in a healing rate of the wound that is at least 10% faster than without the administration of the compositions and formulations described herein. As used herein a "healing rate" refers to the time needed for wound closure or the time needed for the skin to return to an unwounded state. Healing rate can be measured using any well-known method including for example, measurements of wound area, wound perimeter, or mutually perpendicular diameters (largest diameter of the wound, and diameter taken at right angle to largest diameter), with respect to time. In exemplary embodiments, healing rate refers to the advance of the wound margin towards the wound center, measured with respect to time (i.e., per day), which allows for comparison of wounds with different initial areas. *See, e.g.*, Cukjati *et al.*, "A reliable method of determining wound healing rate," *Medical and Biological Engineering & Computing 39*:263-271 (2001), the disclosure of which is incorporated by reference herein in its entirety. The healing rate of a wound (suitably measured in days until the wound is healed) with the administration of the compositions and formulations described herein (i.e., treated) is compared to the healing rate of a wound without the administration (i.e., control), and the difference in rate (i.e., length of time in days) is compared to the control rate to determine the increase in healing rate. The compositions and methods described herein can also be compared to other compounds or wound dressings acting as controls to measure an increase in healing rate of the present compounds in comparison to other methods and formulations.

$$\%\ Increase\ in\ healing\ rate = \frac{[(healing\ rate\ of\ control) - (healing\ rate\ of\ treated)]}{healing\ rate\ of\ control} * 100$$

[0066] In further embodiments, the methods described herein (i.e., treated) result in a healing rate of the wound that is at least 15% faster than without the administration of the compositions and formulations described herein (i.e., control), suitably at least 20% faster, at least 25% faster, at least 30% faster, at least 35% faster, at least 40% faster, at least 45% faster, at least 50% faster, at least 55% faster, at least 60% faster, at least 65% faster, at least 70% faster, or between 10%-50% faster, between 10%-40% faster, between 10%-30% faster, between 10%-20% faster, or 10%, 20%, 30%, 40%, or 50% faster.

[0067] In further embodiments, the administration described herein results in an increase in an immune response of the patient as a result of the combination of nicotinamide riboside and pterostilbene. This immune response can happen more quickly, or provide more neutrophils, and monocytemacrophages to the wound site.

[0068] Embodiment 1 is a method for treating a wound, comprising administering a composition comprising a therapeutically effective amount of nicotinamide riboside and a therapeutically effective amount of pterostilbene to a patient having the wound.

[0069] Embodiment 2 includes the method of embodiment 1, comprising administering orally the composition to the patient having the wound.

[0070] Embodiment 3 includes the method of embodiment 1, comprising administering topically the composition to the patient having the wound.

[0071] Embodiment 4 includes the method of any of embodiments 1-3, wherein the composition comprises a combination of nicotinamide riboside at about 200 mg to about 700 mg and pterostilbene at about 25 mg to about 200 mg.

[0072] Embodiment 5 includes the method of any of embodiments 1-4, wherein the composition comprises a combination of nicotinamide riboside at about 250 mg and pterostilbene at about 50 mg.

[0073] Embodiment 6 includes the method of any of embodiments 1-5, wherein the wound is an open wound, a closed wound, or an ulcer.

[0074] Embodiment 7 includes the method of embodiment 6, wherein the open wound is selected from the group consisting of a laceration, an incision, a puncture, an avulsion or an abrasion.

[0075] Embodiment 8 includes the method of embodiment 6, wherein the closed wound is selected from the group consisting of a contusion, a blister, a seroma and a hematoma.

[0076] Embodiment 9 is a method for treating an open wound, comprising administering a composition consisting essentially of a therapeutically effective amount of nicotinamide riboside and a therapeutically effective amount of pterostilbene to a patient having the open wound.

[0077] Embodiment 10 includes the method of embodiment 9, comprising administering orally the composition to the patient having the wound.

[0078] Embodiment 11 includes the method of embodiment 9, comprising administering topically the composition to the patient having the wound.

[0079] Embodiment 12 includes the method of any of embodiments 9-11, wherein the composition consists essentially of a combination of nicotinamide riboside at about 200 mg to about 700 mg and pterostilbene at about 25 mg to about 200 mg.

[0080] Embodiment 13 includes the method of any of embodiments 9-12, wherein the composition consists essentially of a combination of nicotinamide riboside at about 250 mg and pterostilbene at about 50 mg.

[0081] Embodiment 14 includes the method of any of embodiments 9-13, wherein the open wound is selected from the group consisting of a laceration, an incision, a puncture, an avulsion or an abrasion.

[0082] Embodiment 15 is a method for treating a closed wound, comprising administering a composition consisting essentially of a therapeutically effective amount of nicotinamide riboside and a therapeutically effective amount of pterostilbene to a patient having the closed wound.

[0083] Embodiment 16 includes the method of embodiment 15, comprising administering orally the composition to the patient having the wound.

[0084] Embodiment 17 includes the method of embodiment 15, comprising administering topically the composition to the patient having the wound.

[0085] Embodiment 18 includes the method of any of embodiments 15-17, wherein the composition consists essentially of a combination of nicotinamide riboside at about 200 mg to about 700 mg and pterostilbene at about 25 mg to about 200 mg.

[0086] Embodiment 19 includes the method of any of embodiments 15-17, wherein the composition consists essentially of a combination of nicotinamide riboside at about 250 mg and pterostilbene at about 50 mg.

[0087] Embodiment 20 includes the method of any of embodiments 15-19, wherein the closed wound is selected from the group consisting of a contusion, a blister, a seroma and a hematoma.

[0088] Embodiment 21 includes the method of any of embodiments 1-20, wherein the administration results in a healing rate of the wound that is at least 20% faster than without the administration.

[0089] Embodiment 22 includes the method of embodiment 21, wherein the healing rate of the wound is at least 40% faster than without the administration.

Examples

Example 1: Prevention of Ear-induced swelling using nicotinamide riboside and pterostilbene

[0090] In these experiments, the ability of a combination of nicotinamide riboside and pterostilbene to inhibit ultraviolet B radiation (UVB)-induced inflammation (ear swelling), was investigated. This provides an experimental measure of wound healing in response to treatment with nicotinamide riboside and pterostilbene.

[0091] These experiments used 0.04% pterostilbene (PT) and 0.2% nicotinamide riboside (NR). Mice were divided into 4 groups (n=5 per group):

[0092] Group 1: Standard diet (SD)

[0093] Group 2: SD supplemented with 0.04% PT

[0094] Group 3: SD, plus water containing 0.2% NR

[0095] Group 4: SD supplemented with 0.04% PT, plus water containing 0.2% NR

[0096] Mice were fed their respective diets for four weeks prior to UVB exposure. The thickness of the ear was measured prior to UVB exposure (using a spring-loaded micrometer), and then the mice were placed in individual compartments and their ears exposed once to 3,500 J/m^2 UVB. Ear thickness was subsequently measured at 24-, 48- and 96-hours post-exposure. A separate cohort of mice were euthanized 24-hours post-exposure in order to harvest back skin for evaluation of 6,4 pyrimidine-pyrimidone (6,4 PPD) photo-product formation.

[0097] The combination of NR and PT was found to act synergistically to reduce UVB-induced ear swelling and protect against UVB-induced DNA damage.

[0098] 0.04% PT and 0.2% NR were administered individually and in combination in order to explore whether the ingredients act synergistically to reduce UVB-induced ear swelling. A significant reduction ($P<0.01$) in ear swelling was observed in mice treated with the combination of NR+PT (BASIS) relative to control mice (FIGs. 1A-1D). This reduction in ear swelling was not observed in mice fed NR or PT alone.

[0099] A subset of animals was harvested 24-hours post-UVB exposure, in order to quantify levels of 6,4 PPD photo-products in skin harvested form the back. 6,4 PPD is a marker of DNA damage. In mice treated with either PT or NR+PT (BASIS), a significant reduction ($P<0.01$ for both PT and NR+PT-treated animals) was observed (FIG. 2), indicating a reduction in DNA damage.

Example 2: In vitro Scratch Test to Measure the Effect of Nicotinamide Riboside and Pterostilbene on Cell Migration.

[00100] In these experiments, a *in vitro* scratch test is utilized to examine the effect of nicotinamide riboside (NR) and pterostilbene (PT), separately and in combination, to determine the effect on cell migration as a measure of wound healing.

[00101] The scratch test is carried out as described in Liang *et al.*, "*In vitro* scratch assay: a convenient and inexpensive method for analysis of cell migration *in vitro*," *Nature Protocols 2*:329-333 (2007) (the disclosure of which is incorporated by reference herein in its entirety, particularly for the methods of carrying out the scratch test). The test involves creating a "scratch" in a cell monolayer, capturing images of the cells at the beginning and at regular intervals during cell migration to close the scratch, and comparing the images to quantify the migration rate of the cells. Compared to other methods, the *in vitro* scratch assay is suitable for studies on the effects of cell–matrix and cell–cell interactions on cell migration, and mimics cell migration during wound healing *in vivo,* and are compatible with imaging of live cells during migration. Exemplary cells that can be used in the assays include fibroblasts, epithelial cells and endothelial cells.

[00102] Following proper coating of cell culture dishes and passage of the cells in culture, a scratch is generated in a cell monolayer. In embodiments, a green fluorescent protein can be transfected into the cells to follow a desired gene or protein during the healing process, as described in Liang *et al.*

[00103] Nicotinamide riboside (NR) and pterostilbene (PT), separately and in combination, are then added to different cell culture plates, along with appropriate controls examining vehicle only (e.g., buffer, etc.). The amounts of NR and PT are scaled appropriately to maintain a desired ratio of the two compounds to examine their effects, and can be increased or decreased by varying amounts (e.g. 0.1-100 times), to examine various ranges.

[00104] Cells are observed immediately following the "scratch", and then every hour for 2-24 hours, as desired. Observation can be made using fluorescence or optical microscopy. Measurements are made of the rate of cell migration as they migrate and grow to close the scratch.

[00105] The methods are expected to demonstrate an increased closure rate of the scratch with the combination of Nicotinamide riboside (NR) and pterostilbene (PT), compared to either compound separately and to controls with only buffer or media utilized.

Example 3: Increase in wound healing rate with nicotinamide riboside and pterostilbene in mouse wound healing model

[00106] In these experiments, the ability of a combination of nicotinamide riboside and pterostilbene to increase the healing rate of wounds in mice is examined. This provides an experimental measure of wound healing in response to treatment with nicotinamide riboside and pterostilbene, and how the healing rate is increased relative to controls.

[00107] These experiments use 0.04% pterostilbene (PT) and 0.2% nicotinamide riboside (NR). Mice are divided into 4 groups (n=5 per group):

[00108] Group 1: Standard diet (SD)

[00109] Group 2: SD supplemented with 0.04% PT

[00110] Group 3: SD, plus water containing 0.2% NR

[00111] Group 4: SD supplemented with 0.04% PT, plus water containing 0.2% NR

[00112] Mice are fed their respective diets for four weeks prior to injury.

[00113] A mouse excisional wound is created in accordance with Wang *et al.*, "The mouse excisional wound splinting model, including applications for stem cell transplantation," *Nature Protocols 8*:302-309 (2013) (the disclosure of which is incorporated by reference herein in its entirety, particularly for the methods described therein related to generation of the wound in the mouse model). The mouse excisional wound splinting model utilizes a splinting ring tightly adhered to the skin around the wound, preventing local skin contraction. The wound therefore heals through granulation and re-epithelialization, a process similar to that occurring in humans. The model, takes 2–4 weeks to carry out. The wound is created using a sterile biopsy punch to create two symmetrical full-thickness excisional wounds.

[00114] Wound area, perimeter and distance of wound margin relative the wound center are measured daily for a period of 15-30 days during the healing process to determine the effect of PT, NR and the combination of PT and NR.

[00115] 0.04% PT and 0.2% NR are administered individually and in combination in order to explore whether the ingredients act synergistically to increase the wound healing rate. The combination of 0.04% PT and 0.2% NR is expected to statistically increase the wound healing rate relative to controls.

Example 4: Increase in wound healing rate with nicotinamide riboside and pterostilbene in human wound healing models

[00116] In these experiments, wound healing models in human patients are utilized to examine the effect of Nicotinamide riboside (NR) and pterostilbene (PT), both alone and in combination, on the rate of wound healing. Exemplary methods include those detailed in Wilhelm *et al.*, "Models of wound healing: an emphasis on clinical studies," *Skin Research and Technology 23*:3-12 (2017) (the disclosure of which is incorporated by reference herein in its entirety, including the methods described therein regarding models of wound healing).

[00117] For example, the following models can be utilized to examine the effects of NR and PT, alone and in combination, of

1. Tape stripping – involving removal of the stratum corneum with adhesive tape. Generally leaving the epidermal compartment in tact. Healing can be measured by examining the change in transepidermal water loss as a function of time, as a measure of the change in skin permeability. Re-epithelization can also be measured as a determination of the wound healing rate.

2. Abrasive wound model – generally involving the infliction of standardized, superficial abrasions by repeatedly abrading the skin with a surgical brush until the first signs of uniform glistening and punctuate bleeding are observed. Wound healing can then be observed, generally without scarring.

3. Laser wound model – can be initiated using CO_2 or Er:YAG lasers to create wounds of various depths, suitably with a surface area of a few square centimeters. In general, part of the epidermis is removed.

4. Microdermabrasion – generally involving the use of a spray of microcrystals or a diamond abrasive surface to remove part of the epidermis.

5. Biopsy – utilizes a fill thickness wound, including the removal of epidermis and dermis, often with a punch or scalpel/

[00118] Following the generation of one or more of these wound types, a patient or patient group is provided with oral or topical Notinamide riboside (NR), and pterostilbene (PT), or both NR and PT, in a suitable oral or topical formulation. Amounts of NR and PT are described herein, as are suitably formulation forms. Control groups utilizing only oral or topical carriers or excipients are also utilized.

[00119] In embodiments, nicotinamide riboside is administered (orally or topically) at about 250 mg to one patient group; pterostilbene at about 50 mg to one patient group; and another patient group receives a combination of NR at 250 mg and PT at 50 mg, for example orally as BASIS. The wound is followed for a period of 7 days to 4 weeks or more, with wound measurements made on a weekly basis or twice per week, observing the closure or healing of the wound to calculate a wound healing rate.

[00120] It is expected that the combination of NR and PT results in an increased wound healing rate compared to either compound alone, or in comparison to controls without active agents, suitably with an increase in healing rate that is suitably at least 20% faster.

[00121] It is to be understood that while certain embodiments have been illustrated and described herein, the claims are not to be limited to the specific forms or arrangement of parts described and shown. In the specification, there have been disclosed illustrative embodiments and, although specific terms are employed, they are used in a generic and descriptive sense only and not for purposes of limitation. Modifications and variations of the embodiments are possible in light of the above teachings. It is therefore to be understood that the embodiments may be practiced otherwise than as specifically described.

[00122] While various embodiments have been described above, it should be understood that they have been presented only as illustrations and examples of the present technology, and not by way of limitation. It will be apparent to persons skilled in the relevant art that various changes in form and detail can be made therein without departing from the spirit and scope of the present technology. Thus, the breadth and scope of the present technology should not be limited by any of the above-described embodiments, but should be defined only in accordance with the appended claims and their equivalents. It will also be understood that each feature of each embodiment discussed herein, and of each reference cited herein, can be used in combination with the features of any other embodiment. All patents and publications discussed herein are incorporated by reference herein in their entirety.

CLAIMS

What is claimed is:

1. A method for treating a wound, comprising administering a composition comprising a therapeutically effective amount of nicotinamide riboside and a therapeutically effective amount of pterostilbene to a patient having the wound.

2. The method of claim 1, comprising administering orally the composition to the patient having the wound.

3. The method of claim 1, comprising administering topically the composition to the patient having the wound.

4. The method of any one of claims 1-3, wherein the composition comprises a combination of nicotinamide riboside at about 200 mg to about 700 mg and pterostilbene at about 25 mg to about 200 mg.

5. The method of any one of claims 1-4, wherein the composition comprises a combination of nicotinamide riboside at about 250 mg and pterostilbene at about 50 mg.

6. The method of any one of claims 1-5, wherein the wound is an open wound, a closed wound, or an ulcer.

7. The method of claim 6, wherein the open wound is selected from the group consisting of a laceration, an incision, a puncture, an avulsion or an abrasion.

8. The method of claim 6, wherein the closed wound is selected from the group consisting of a contusion, a blister, a seroma and a hematoma.

9. A method for treating an open wound, comprising administering a composition consisting essentially of a therapeutically effective amount of nicotinamide riboside and a therapeutically effective amount of pterostilbene to a patient having the open wound.

10. The method of claim 9, comprising administering orally the composition to the patient having the wound.

11. The method of claim 9, comprising administering topically the composition to the patient having the wound.

12. The method of any one of claims 9-11, wherein the composition consists essentially of a combination of nicotinamide riboside at about 200 mg to about 700 mg and pterostilbene at about 25 mg to about 200 mg.

13. The method of any one of claims 9-12, wherein the composition consists essentially of a combination of nicotinamide riboside at about 250 mg and pterostilbene at about 50 mg.

14. The method of any one of claims 9-13, wherein the open wound is selected from the group consisting of a laceration, an incision, a puncture, an avulsion or an abrasion.

15. A method for treating a closed wound, comprising administering a composition consisting essentially of a therapeutically effective amount of nicotinamide riboside and a therapeutically effective amount of pterostilbene to a patient having the closed wound.

16. The method of claim 15, comprising administering orally the composition to the patient having the wound.

17. The method of claim 15, comprising administering topically the composition to the patient having the wound.

18. The method of any one of claims 15-17, wherein the composition consists essentially of a combination of nicotinamide riboside at about 200 mg to about 700 mg and pterostilbene at about 25 mg to about 200 mg.

19. The method of any one of claims 15-17, wherein the composition consists essentially of a combination of nicotinamide riboside at about 250 mg and pterostilbene at about 50 mg.

20. The method of any one of claims 15-19, wherein the closed wound is selected from the group consisting of a contusion, a blister, a seroma and a hematoma.

21. The method of any one of claims 1-20, wherein the administration results in a healing rate of the wound that is at least 20% faster than without the administration.

22. The method of claim 21, wherein the healing rate of the wound is at least 40% faster than without the administration.

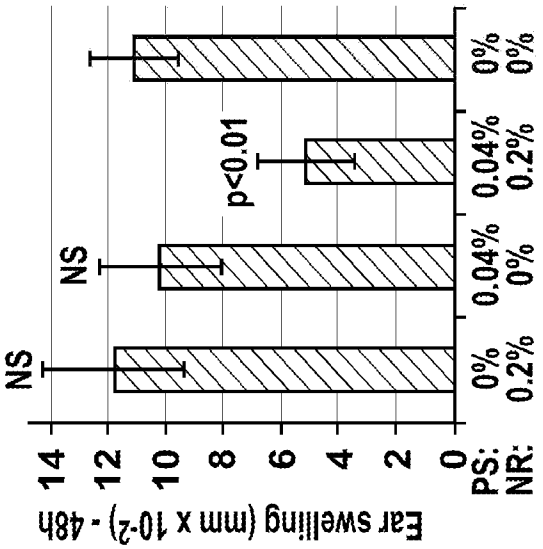


FIG. 1B

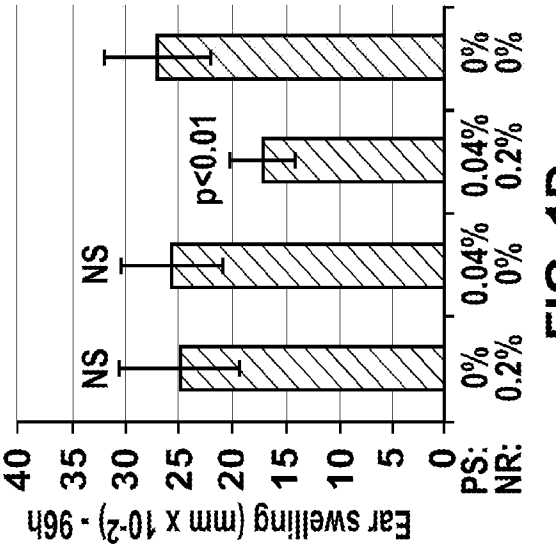


FIG. 1D

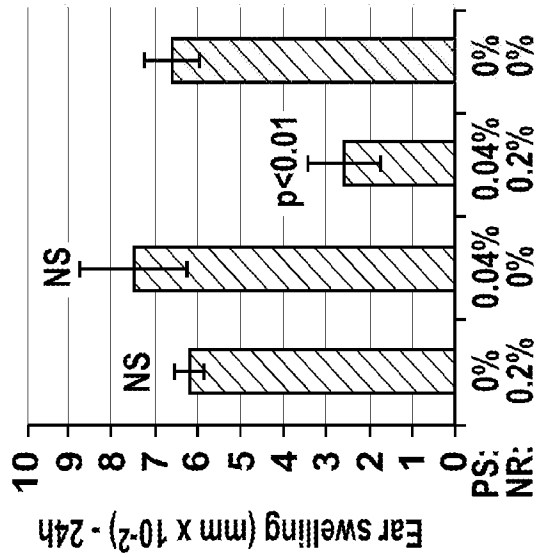


FIG. 1A

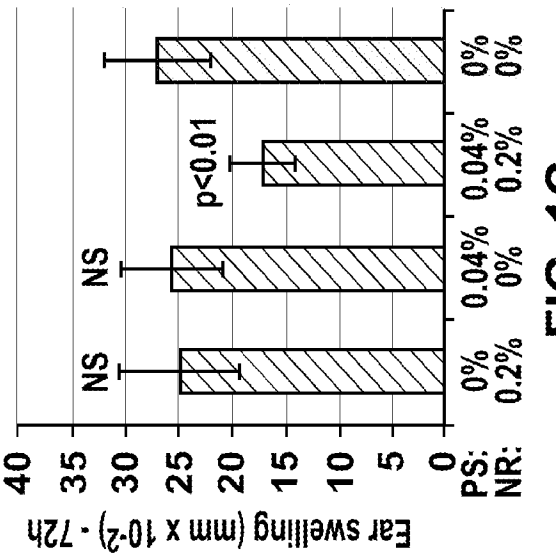


FIG. 1C

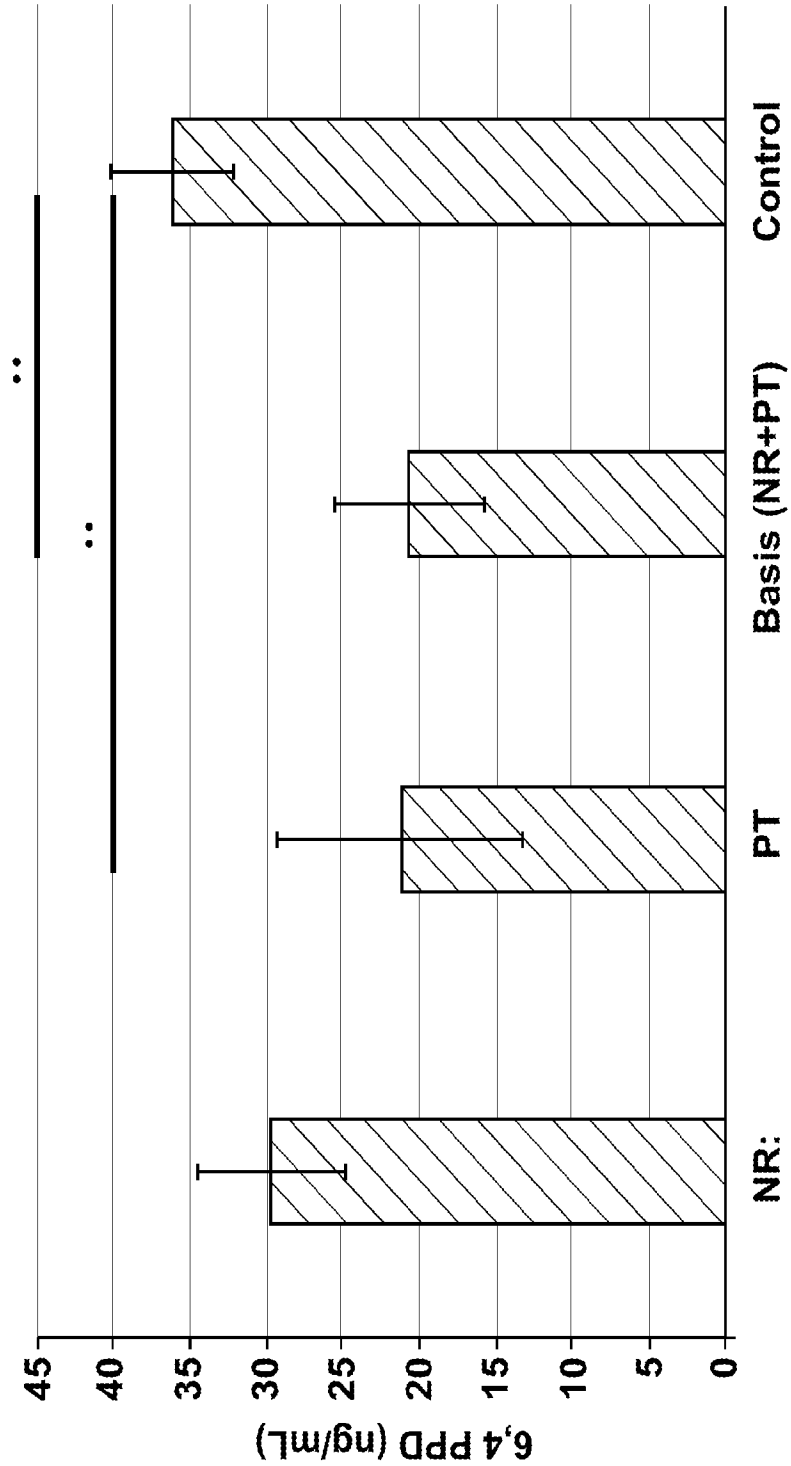


FIG. 2

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2020/022596

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - A61K 31/706; A61K 31/09; A61P 17/02; C07H 19/048 (2020.01)
CPC - A61K 31/706; A61K 31/09; A61P 17/02; C07H 19/048 (2020.02)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
See Search History document

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
USPC - 514/43 (keyword delimited)

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
See Search History document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	WO 2018/236814 A2 (GANAPATI et al) 27 December 2018 (27.12.2018) entire document	1-3, 9-11, 15-17 --- 4, 12, 18, 19
Y	US 2018/0353497 A1 (ELYSIUM HEALTH INC) 13 December 2018 (13.12.2018) entire document	4, 12, 18, 19
A	US 2016/0272668 A1 (CHROMADEX INC et al) 22 September 2016 (22.09.2016) entire document	1-4, 9-12, 15-19

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
24 April 2020	11 MAY 2020
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, VA 22313-1450 Facsimile No. 571-273-8300	Authorized officer Blaine R. Copenheaver PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2020/022596

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 5-8, 13, 14, 20-22
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.