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(54) Title: REDUCED NICOTINAMIDERIBOSIDES FOR THE TREATMENT/PREVENTION OF LIVER DISEASE

(57) Abrégé/Abstract:
The present invention provides compounds and compositions containing reduced nicotinamide riboside for use in methods of prevention and/or treatment of liver disease and/or conditions. In one embodiment of the invention, said compounds and compositions of the invention improve liver by: maintaining or improving liver function; maintaining or improving hepatocyte function; and improving liver recovery and regeneration after injury, transplantation or surgery. In another embodiment of the invention, compounds and compositions of the invention may be used in methods to prevent and/or treat liver diseases and/or conditions such as: non-alcoholic fatty liver disease (NAFLD), alcoholic fatty liver disease, hepatic steatosis, cirrhosis and/or recovery and regeneration after liver injury, transplantation or surgery.

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(54) **Title:** REDUCED NICOTINAMIDERIBOSIDES FOR THE TREATMENT/PREVENTION OF LIVER DISEASE

(57) **Abstract:** The present invention provides compounds and compositions containing reduced nicotinamide riboside for use in methods of prevention and/or treatment of liver disease and/or conditions. In one embodiment of the invention, said compounds and compositions of the invention improve liver by: maintaining or improving liver function; maintaining or improving hepatocyte function; and improving liver recovery and regeneration after injury, transplantation or surgery. In another embodiment of the invention, compounds and compositions of the invention may be used in methods to prevent and/or treat liver diseases and/or conditions such as: non-alcoholic fatty liver disease (NAFLD), alcoholic fatty liver disease, hepatic steatosis, cirrhosis and/or recovery and regeneration after liver injury, transplantation or surgery.

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REDUCED NICOTINAMIDERIBOSIDES FOR THE TREATMENT/PREVENTION OF LIVER DISEASE

FIELD OF THE INVENTION

The present invention provides compounds and compositions containing reduced nicotinamide riboside for use in methods of prevention and/or treatment of liver disease and/or conditions. In one embodiment of the invention, said compounds and compositions of the invention improve liver by: maintaining or improving liver function; maintaining or improving hepatocyte function; and improving liver recovery and regeneration after injury, transplantation or surgery. In another embodiment of the invention, compounds and compositions of the invention may be used in methods to prevent and/or treat liver diseases and/or conditions such as: non-alcoholic fatty liver disease (NAFLD), alcoholic fatty liver disease, hepatic steatosis, cirrhosis and/or recovery and regeneration after liver injury, transplantation or surgery.

BACKGROUND TO THE INVENTION

The liver plays an important role in protein production, blood clotting, cholesterol, glucose and iron metabolism. It also produces bile which is needed to digest fats and to absorb vitamins A, D, E and K.

Liver disease is any disturbance of the liver that causes dysfunction or disease. The most common liver diseases and conditions are: non-alcoholic fatty liver disease (NAFLD), alcoholic fatty liver disease, hepatic steatosis, and/or cirrhosis. Disturbance of liver function may also occur after liver injury, transplantation or surgery. Conditions such as hepatitis A, B, C, D and E as well as infectious mononucleosis (Epstein Barr virus) and hemochromatosis may also contribute to liver dysfunction.

The ability to increase NAD+ in cells and tissues of the liver helps to maintain the activity of DNA damage-repair enzymes, preventing cell death and allowing survival and proliferation of hepatocytes to regenerate the liver.

Therefore, there is an urgent unmet need to address liver disease and/or conditions with new compounds, compositions and methods of prevention and/or treatment which influence NAD+.

SUMMARY OF THE INVENTION

The present invention provides compounds and compositions for use in methods of prevention and/or treatment of liver conditions and diseases.

In an embodiment, the composition is selected from the group consisting of: a food or beverage product, a food supplement, an oral nutritional supplement (ONS), a medical food, and combinations thereof.

In another embodiment, the present invention provides a method for increasing intracellular nicotinamide adenine dinucleotide (NAD^+) in a subject, the method comprising administering a compound or composition of the invention consisting of administering a reduced nicotinamide riboside to the subject in an amount effective to increase NAD^+ biosynthesis.

In a further embodiment, as a precursor of NAD+ biosynthesis, reduced nicotinamide riboside, can increase in NAD+ biosynthesis and provide one or more benefits to liver function.

In another embodiment, the present invention provides a unit dosage form of a composition consisting of reduced nicotinamide riboside, the unit dosage form contains an effective amount of the reduced nicotinamide riboside to increase NAD+ biosynthesis.

In one embodiment of the invention, the composition containing reduced nicotinamide riboside is provided to maintain or improve liver function in a subject.

In yet another embodiment of the invention, the composition containing reduced nicotinamide riboside is provided to enhance recovery of the liver after injury.

In a further embodiment of the invention, the composition containing reduced nicotinamide riboside is provided to enhance recovery of the liver after trauma or surgery.

In another embodiment of the invention, the composition is a nutritional composition selected from a food or beverage product, including food additives, food ingredients, functional foods, dietary supplements, medical foods, nutraceuticals, oral nutritional supplements (ONS) or food supplements.

In another embodiment of the invention, the composition containing reduced nicotinamide riboside is provided to prevent or treat non-alcoholic fatty liver disease (NAFLD), alcoholic fatty liver disease, hepatic steatosis, cirrhosis and/or End-stage liver disease (ESLD).

In another embodiment of the invention, the composition containing reduced nicotinamide riboside is provided to prevent or treat liver disease associated with hepatitis A, B, C, D and E, infectious mononucleosis (Epstein Barr virus) and/or hemochromatosis.

DETAILED DESCRIPTION OF THE INVENTION

Definitions

All percentages expressed herein are by weight of the total weight of the composition unless expressed otherwise. As used herein, “about,” “approximately” and “substantially” are understood to refer to numbers in a range of numerals, for example the range of -10% to +10% of the referenced number, preferably -5% to +5% of the referenced number, more preferably -1% to +1% of the referenced number, most preferably -0.1% to +0.1% of the referenced number.

All numerical ranges herein should be understood to include all integers, whole or fractions, within the range. Moreover, these numerical ranges should be construed as providing support for a claim directed to any number or subset of numbers in that range. For example, a disclosure of from 1 to 10 should be construed as supporting a range of from 1 to 8, from 3 to 7, from 1 to 9, from 3.6 to 4.6, from 3.5 to 9.9, and so forth.

As used in this invention and the appended claims, the singular forms “a,” “an” and “the” include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to “a component” or “the component” includes two or more components.

The words “comprise,” “comprises” and “comprising” are to be interpreted inclusively rather than exclusively. Likewise, the terms “include,” “including” and “or” should all be construed to be inclusive, unless such a construction is clearly prohibited from the context. Nevertheless, the compositions disclosed herein may lack any element that is not specifically disclosed herein. Thus, a disclosure of an embodiment using the term “comprising” includes a disclosure of embodiments “consisting essentially of” and “consisting of” the components identified. Any embodiment disclosed herein can be combined with any other embodiment disclosed herein.

Where used herein, the terms “example” and “such as,” particularly when followed by a listing of terms, are merely exemplary and illustrative and should not be deemed to be exclusive or comprehensive. As used herein, a condition “associated with” or “linked with” another condition means the conditions occur concurrently, preferably means that the conditions are caused by

the same underlying condition, and most preferably means that one of the identified conditions is caused by the other identified condition.

The terms “food,” “food product” and “food composition” mean a product or composition that is intended for ingestion by an individual such as a human and provides at least one nutrient to the individual. A food product typically includes at least one of a protein, a lipid, a carbohydrate and optionally includes one or more vitamins and minerals. The term “beverage” or “beverage product” means a liquid product or liquid composition that is intended to be ingested orally by an individual such as a human and provides at least one nutrient to the individual.

The compositions of the present disclosure, including the many embodiments described herein, can comprise, consist of, or consist essentially of the elements disclosed herein, as well as any additional or optional ingredients, components, or elements described herein or otherwise useful in a diet.

As used herein, the term "isolated" means removed from one or more other compounds or components with which the compound may otherwise be found, for example as found in nature. For example, “isolated” preferably means that the identified compound is separated from at least a portion of the cellular material with which it is typically found in nature. In an embodiment, an isolated compound is free from any other compound.

“Prevention” includes reduction of risk, incidence and/or severity of a condition or disorder. The terms “treatment,” “treat” and “to alleviate” include both prophylactic or preventive treatment (that prevent and/or slow the development of a targeted pathologic condition or disorder) and curative, therapeutic or disease-modifying treatment, including therapeutic measures that cure, slow down, lessen symptoms of, and/or halt progression of a diagnosed pathologic condition or disorder; and treatment of patients at risk of contracting a disease or suspected to have contracted a disease, as well as patients who are ill or have been diagnosed as suffering from a disease or medical condition. The term does not necessarily imply that a subject is treated until total recovery. The terms “treatment” and “treat” also refer to the maintenance and/or promotion of health in an individual not suffering from a disease but who may be susceptible to the development of an unhealthy condition. The terms “treatment,” “treat” and “to alleviate” are also intended to include the potentiation or otherwise enhancement of one or more primary prophylactic or therapeutic measure. The terms “treatment,” “treat” and “to alleviate” are further intended to include the dietary management of a disease or condition or the dietary

management for prophylaxis or prevention a disease or condition. A treatment can be patient- or doctor-related.

The term "unit dosage form," as used herein, refers to physically discrete units suitable as unitary dosages for human and animal subjects, each unit containing a predetermined quantity of the composition disclosed herein in an amount sufficient to produce the desired effect, in association with a pharmaceutically acceptable diluent, carrier or vehicle. The specifications for the unit dosage form depend on the particular compounds employed, the effect to be achieved, and the pharmacodynamics associated with each compound in the host.

As used herein, an “effective amount” is an amount that prevents a deficiency, treats a disease or medical condition in an individual, or, more generally, reduces symptoms, manages progression of the disease, or provides a nutritional, physiological, or medical benefit to the individual. The relative terms “improve,” “increase,” “enhance,” “promote” and the like refer to the effects of the composition disclosed herein, namely a composition comprising reduced nicotinamide riboside, relative to a composition not having nicotinamide riboside but otherwise identical. As used herein, "promoting" refers to enhancing or inducing relative to the level before administration of the composition disclosed herein.

As used herein “reduced nicotinamide riboside” may also be known as protonated nicotinamide riboside, dihydronicotinamide riboside, dihydro-1-beta-D-ribofuranosyl-3-pyridinecarboxamide, or 1-(beta-D-ribofuranosyl)-dihydronicotinamide. A description of the synthesis of reduced nicotinamide riboside is given in Example 1. The location of the protonation site can give rise to different forms of “reduced nicotinamide riboside”. For example: 1,4-dihydro-1-beta-D-ribofuranosyl-3-pyridinecarboxamide; 1,2-dihydro-1-beta-D-ribofuranosyl-3-pyridinecarboxamide; and 1,6-dihydro-1-beta-D-ribofuranosyl-3-pyridinecarboxamide (Makarov and Migaud, 2019).

Liver Diseases and Conditions

Liver disease may start as inflammation and enlargement of the liver which may be initially reversible. If left untreated, it can progress to hepatic fibrosis creating a scarring of the liver which impedes blood flow through the liver. At the latter stage this is known as cirrhosis of the liver. When liver failure occurs as a result of cirrhosis this is known as End-Stage Liver Disease (ESLD). Due to its ability to increase NAD+, NRH helps to maintain the activity of DNA damage-repair enzymes, preventing cell death and allowing survival and proliferation of hepatocytes to regenerate the liver.

Non-alcoholic fatty liver disease (NAFLD)

NAFLD is a progressive complex liver disease which starts with fat accumulation in the liver without associated excessive alcohol consumption. It is strongly associated with metabolic syndrome which is the result of combination of factors of obesity, insulin resistance, and dyslipidemia. It may be exacerbated by diabetes mellitus, hyperlipidemia, insulin resistance and high blood pressure. If left untreated, NAFLD may progress to more advanced disease, such as Non-alcoholic steatohepatitis (NASH), which may evolve to fibrosis, cirrhosis, and liver cancer (HCC).

Alcoholic fatty liver disease

Normal liver function may be disrupted after excessive, chronic alcohol abuse. The liver is the primary organ required to detoxify alcohol and the hepatic tissue may be destroyed or altered resulting in fatty liver, and/or permanent scarring or cirrhosis of the liver.

Hepatic steatosis, steatohepatitis and Non-Alcoholic steatohepatitis (NASH)

NASH stands for Non-Alcoholic steatohepatitis and it represents the more severe end of the spectrum of non-alcoholic fatty liver disease. Steatohepatitis is defined as having fatty liver with inflammation. Up to 20% of adults with NASH develop cirrhosis and up to 11% may experience liver-related deaths. Many individuals develop chronic liver failure and require liver transplantation. The prevalence of NASH is 2-6% in the general population.

Cirrhosis

Cirrhosis is a condition, not a disease, that results from permanent damage or scarring of the liver. This leads to a blockage of blood flow through the liver and prevents normal metabolic and regulatory processes.

Recovery and Regeneration of the Liver after Liver Injury, Trauma from Surgery and Liver Transplantation

If a liver transplant is necessary, most patients go back to their daily activities within 6 months. People who have received a transplant need lifelong medical care, including medications to prevent their body from rejecting the new organ.

It may be appreciated that the compounds, compositions and methods of the present invention may be beneficial to prevent and/or treat the aforementioned liver diseases and conditions,

including recovery and regeneration of the liver after injury, trauma and liver transplantation, in particular, to maintain or improve liver function.

Embodiments

The present invention provides compounds and compositions containing reduced nicotinamide riboside. Another aspect of the present invention is a unit dosage form of a composition consisting of reduced nicotinamide riboside, and the unit dosage form contains the reduced nicotinamide riboside in an amount effective to increase intracellular NAD^+ in subject in need thereof.

The increase in NAD^+ biosynthesis can provide one or more benefits to the individual, for example a human (e.g., a human undergoing medical treatment), a pet or a horse (e.g., a pet or horse undergoing medical treatment), or cattle or poultry (e.g., cattle or poultry being used in agriculture) with respect to prevention or treatment of kidney disease.

For non-human mammals such as rodents, some embodiments comprise administering an amount of the composition that provides 1.0 mg to 1.0 g of the reduced nicotinamide riboside / kg of body weight of the non-human mammal, preferably 10 mg to 500 mg of the reduced nicotinamide riboside / kg of body weight of the non-human mammal, more preferably 25 mg to 400 mg of the reduced nicotinamide riboside / kg of body weight of the mammal, most preferably 50 mg to 300 mg of the reduced nicotinamide riboside / kg of body weight of the non-human mammal.

For humans, some embodiments comprise administering an amount of the composition that provides 1.0 mg to 10.0 g of the reduced nicotinamide riboside / kg of body weight of the human, preferably 10 mg to 5.0 g of the reduced nicotinamide riboside / kg of body weight of the human, more preferably 50 mg to 2.0 g of the reduced nicotinamide riboside / kg of body weight of the human, most preferably 100 mg to 1.0 g of the reduced nicotinamide riboside / kg of body weight of the human.

In some embodiments, at least a portion of the reduced nicotinamide riboside is isolated from natural plant sources. Additionally or alternatively, at least a portion of reduced nicotinamide riboside can be chemically synthesized. For example, according to Example 1 described below.

As used herein, a “composition consisting essentially of reduced nicotinamide riboside” contains reduced nicotinamide riboside and does not include, or is substantially free of, or completely free of, any additional compound that affects NAD+ production other than the “reduced

nicotinamide riboside". In a particular non-limiting embodiment, the composition consists of the reduced nicotinamide riboside and an excipient or one or more excipients.

In some embodiments, the composition consisting essentially of reduced nicotinamide riboside is optionally substantially free or completely free of other NAD+ precursors, such as nicotinamide riboside.

As used herein, "substantially free" means that any of the other compounds present in the composition is no greater than 1.0 wt.% relative to the amount of reduced nicotinamide riboside, preferably no greater than 0.1 wt.% relative to the amount of reduced nicotinamide riboside, more preferably no greater than 0.01 wt.% relative to the amount of reduced nicotinamide riboside, most preferably no greater than 0.001 wt.% relative to the amount of reduced nicotinamide riboside.

Another aspect of the present invention is a method for increasing intracellular NAD^+ in a mammal in need thereof, comprising administering to the mammal a composition consisting essentially of or consisting of reduced nicotinamide riboside in an amount effective to increase NAD^+ biosynthesis. The method can promote the increase of intracellular levels of NAD^+ in cells and tissues for improving cell and tissue survival and overall cell and tissue health, for example, in liver cells and tissues, especially liver cells and tissues.

Nicotinamide adenine dinucleotide (NAD+) is considered a coenzyme, and essential cofactor in cellular redox reactions to produce energy. It plays critical roles in energy metabolism, as the oxidation of NADH to NAD+ facilitates hydride-transfer, and consequently ATP generation through mitochondrial oxidative phosphorylation. It also acts as a degradation substrate for multiple enzymes (Canto et al. 2015; Imai et al. 2000; Chambon et al. 1963; Lee et al. 1991).

Mammalian organisms can synthesize NAD+ from four different sources. First, NAD+ can be obtained from tryptophan through the 10-step de novo pathway. Secondly, Nicotinic acid (NA) can also be transformed into NAD+ through the 3-step Preiss-Handler path, which converges with the de novo pathway. Thirdly, intracellular NAD+ salvage pathway from nicotinamide (NAM) constitutes the main path by which cells build NAD+, and occurs through a 2-step reaction in which NAM is first transformed into NAM-mononucleotide (NMN) via the catalytic activity of the NAM-phosphoribosyltransferase (NAMPT) and then converted to NAD+ via NMN adenylyltransferase (NMNAT) enzymes. Finally, Nicotinamide Riboside (NR) constitutes yet a fourth path to NAD+, characterized by the initial phosphorylation of NR into NMN by NR kinases (NRKs) (Breganowski et al. 2004).

Five molecules previously have been known to act as direct extracellular NAD+ precursors: tryptophan, nicotinic acid (NA), nicotinamide (NAM), nicotinic acid riboside (NaR) and nicotinamide riboside (NR). The present invention discloses a new molecule that can act as an extracellular NAD+ precursor, reduced nicotinomide riboside (NRH). The reduction of the NR molecule to NRH confers it not only a much stronger capacity to increase intracellular NAD+ levels, but also a different selectivity in terms of its cellular use.

The present invention relates to NRH, a new molecule which can act as an NAD+ precursor. This reduced form of NR, which displays an unprecedented ability to increase NAD+ and has the advantage of being more potent and faster than nicotinamide riboside (NR). NRH utilizes a different pathway than NR to synthesize NAD+, which is NRK independent. The present invention demonstrates that NRH is protected against degradation in plasma and can be detected in circulation after oral administration. These advantages of the invention support its therapeutic efficacy.

The method comprises administering an effective amount of a composition consisting essentially of reduced nicotinamide riboside or consisting of reduced nicotinamide riboside to the individual.

In each of the compositions and methods disclosed herein, the composition is preferably a food product or beverage product, including food additives, food ingredients, functional foods, dietary supplements, medical foods, nutraceuticals, oral nutritional supplements (ONS) or food supplements.

The composition can be administered at least one day per week, preferably at least two days per week, more preferably at least three or four days per week (e.g., every other day), most preferably at least five days per week, six days per week, or seven days per week. The time period of administration can be at least one week, preferably at least one month, more preferably at least two months, most preferably at least three months, for example at least four months. In some embodiments, dosing is at least daily; for example, a subject may receive one or more doses daily, in an embodiment a plurality of doses per day. In some embodiments, the administration continues for the remaining life of the individual. In other embodiments, the administration occurs until no detectable symptoms of the medical condition remain. In specific embodiments, the administration occurs until a detectable improvement of at least one symptom occurs and, in further cases, continues to remain ameliorated.

The compositions disclosed herein may be administered to the subject enterally, e.g., orally, or parenterally. Non-limiting examples of parenteral administration include intravenously, intramuscularly, intraperitoneally, subcutaneously, intraarticularly, intrasynovially, intraocularly, intrathecally, topically, and inhalation. As such, non-limiting examples of the form of the composition include natural foods, processed foods, natural juices, concentrates and extracts, injectable solutions, microcapsules, nano-capsules, liposomes, plasters, inhalation forms, nose sprays, nosedrops, eyedrops, sublingual tablets, and sustained-release preparations.

The compositions disclosed herein can use any of a variety of formulations for therapeutic administration. More particularly, pharmaceutical compositions can comprise appropriate pharmaceutically acceptable carriers or diluents and may be formulated into preparations in solid, semi-solid, liquid or gaseous forms, such as tablets, capsules, powders, granules, ointments, solutions, suppositories, injections, inhalants, gels, microspheres, and aerosols. As such, administration of the composition can be achieved in various ways, including oral, buccal, rectal, parenteral, intraperitoneal, intradermal, transdermal, and intratracheal administration. The active agent may be systemic after administration or may be localized by the use of regional administration, intramural administration, or use of an implant that acts to retain the active dose at the site of implantation.

In pharmaceutical dosage forms, the compounds may be administered as their pharmaceutically acceptable salts. They may also be used in appropriate association with other pharmaceutically active compounds. The following methods and excipients are merely exemplary and are in no way limiting.

For oral preparations, the compounds can be used alone or in combination with appropriate additives to make tablets, powders, granules or capsules, for example, with conventional additives, such as lactose, mannitol, corn starch or potato starch; with binders, such as crystalline cellulose, cellulose functional derivatives, acacia, corn starch or gelatins; with disintegrators, such as corn starch, potato starch or sodium carboxymethylcellulose; with lubricants, such as talc or magnesium stearate; and if desired, with diluents, buffering agents, moistening agents, preservatives and flavoring agents.

The compounds can be formulated into preparations for injections by dissolving, suspending or emulsifying them in an aqueous or non-aqueous solvent, such as vegetable or other similar oils, synthetic aliphatic acid glycerides, esters of higher aliphatic acids or propylene glycol; and if

desired, with conventional, additives such as solubilizers, isotonic agents, suspending agents, emulsifying agents, stabilizers and preservatives.

The compounds can be utilized in an aerosol formulation to be administered by inhalation. For example, the compounds can be formulated into pressurized acceptable propellants such as dichlorodifluoromethane, propane, nitrogen and the like.

Furthermore, the compounds can be made into suppositories by mixing with a variety of bases such as emulsifying bases or water-soluble bases. The compounds can be administered rectally by a suppository. The suppository can include a vehicle such as cocoa butter, carbowaxes and polyethylene glycols, which melt at body temperature, yet are solidified at room temperature.

Unit dosage forms for oral or rectal administration such as syrups, elixirs, and suspensions may be provided wherein each dosage unit, for example, teaspoonful, tablespoonful, tablet or suppository, contains a predetermined amount of the composition. Similarly, unit dosage forms for injection or intravenous administration may comprise the compounds in a composition as a solution in sterile water, normal saline or another pharmaceutically acceptable carrier, wherein each dosage unit, for example, mL or L, contains a predetermined amount of the composition containing one or more of the compounds.

Compositions intended for a non-human animal include food compositions to supply the necessary dietary requirements for an animal, animal treats (e.g., biscuits), and/or dietary supplements. The compositions may be a dry composition (e.g., kibble), semi-moist composition, wet composition, or any mixture thereof. In one embodiment, the composition is a dietary supplement such as a gravy, drinking water, beverage, yogurt, powder, granule, paste, suspension, chew, morsel, treat, snack, pellet, pill, capsule, tablet, or any other suitable delivery form. The dietary supplement can comprise a high concentration of the UFA and NORC, and B vitamins and antioxidants. This permits the supplement to be administered to the animal in small amounts, or in the alternative, can be diluted before administration to an animal. The dietary supplement may require admixing, or can be admixed with water or other diluent prior to administration to the animal.

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DESCRIPTION OF FIGURES

Figure 1. Chemical structure of nicotinamide riboside in its oxidized (NR) and reduced (NRH) forms

1: 1-b-D-ribofuranosyl-3-pyridinecarboxamide salt

2: 1,4-dihydro-1-b-D-ribofuranosyl-3-pyridinecarboxamide

3: 1,2-dihydro-1-b-D-ribofuranosyl-3-pyridinecarboxamide

4: 1,6-dihydro-1-b-D-ribofuranosyl-3-pyridinecarboxamide

X^-: anion (e.g. triflate)

Figure 2. Dose-response experiments revealed that NRH could significantly increase NAD+ better than NR

Starting at levels at a concentration of 10 μM, NRH achieved similar increases in intracellular NAD+ levels to those reached with NR at 50-fold higher concentrations. NRH achieved maximal effects on NAD+ synthesis around the millimolar range, managing to increase intracellular NAD+ levels by more than 10-fold.

Figure 3. NHR acts rapidly after 5 minutes from treatment.
NRH actions were also extremely fast, as significant increases in NAD+ levels were observed within 5 minutes after NRH treatment. Peak levels of NAD+ were achieved between 45 minutes and 1 h after treatment.

Figure 4. NRH leads to NAD+ biosynthesis through an adenosine kinase dependent path.
AML12 cells were treated with an adenosine kinase inhibitor (5-IT; 10 mM) for 1 hour prior to NRH treatment at the doses indicated. Then, 1 hour later, acidic extracts were obtained to measure NAD^+ levels. All values in the figure are expressed as mean +/- SEM of 3 independent experiments. * indicates statistical difference at $p< 0.05$ vs. the respective vehicle treated group.

Figure 5. NRH is an orally active NAD+ precursor in mice.
8 week-old C57Bl/6NTac mice were orally gavaged with either saline (as vehicle), NR (500 mg/kg) or NRH (500 mg/kg). After 1 hour, liver, skeletal muscle and kidney NAD^+ levels were evaluated. All results are expressed as mean +/-SEM of n=5 mice per group. * indicates statistical difference at $p<0.05$ vs. vs. saline-treated mice. # indicates statistical difference at $p<0.05$ vs. NR treated mice.

Figure 6. NRH is found intact in mice tissues after administration.
8 week-old C57Bl/6NTac mice were orally gavaged with either saline (as vehicle), and NRH (250 mg/kg). After 2 hours, liver, skeletal muscle and kidney NRH levels were evaluated. All results are expressed as mean +/-SEM of n=4 mice per group, as areas under the signal by LC-MS analysis, corrected by total protein amount of tissue.

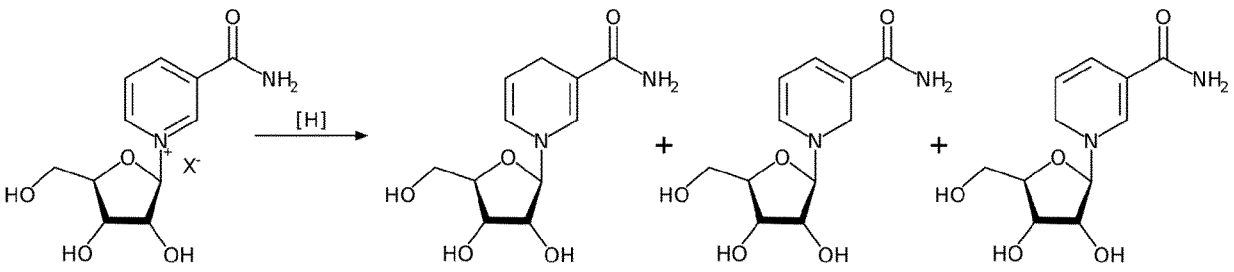
Figure 7: NRH increases hepatic mitochondrial NAD+

While NR, NRH and NAM all increase intrahepatic NAD+, only NRH increases mitochondrial NAD+ levels. All results are mean +/- SEM of n=4 animals per group. * indicates statistical difference at $p<0.05$ vs. control/vehicle group.

EXAMPLES

Example 1: Synthesis of the reduced form of nicotinamide riboside (NRH)

Reduced nicotinamide riboside (NRH) was obtained from NR (1) by reduction of pyridinium salts (for example, triflate) to dihydropyridines (1,2-, 1,4-, and 1,6-dihydropyridines) as shown below



1: 1-b-D-ribofuranosyl-3-pyridinecarboxamide salt

2: 1,4-dihydro-1-beta-D-ribofuranosyl-3-pyridinecarboxamide

3: 1,2-dihydro-1-beta-D-ribofuranosyl-3-pyridinecarboxamide

4: 1,6-dihydro-1-beta-D-ribofuranosyl-3-pyridinecarboxamide

X^-: anion (e.g. triflate)

Sodium borohydride ($NaBH_4$) and sodium dithionite ($Na_2S_2O_4$) were used as reducing agents for *N*-substituted pyridinium derivatives. Regioselectivity of reducing agents differ, leading to either only one dihydropyridine or a mixture of all 3 isomers in different proportions (2,3,4).

Dithionate reduction of pyridinium salts, carrying electron withdrawing substituents in positions 3 and 5, yielded almost exclusively 1,4-dihydropyridine products. The reduction was made in mild conditions (e.g. in aqueous sodium bicarbonate or potassium phosphate dibasic medium), due to instability of the reduced products in acidic media. To perform the reduction, hydroxyl groups in the ribofuranose moiety were protected with either benzyl or acetyl substituents. Deprotection was then be done by sodium hydroxide in methanol under ball mill conditions, after reduction.

Example 2: Measurement of NRH and other NAD+ related metabolites in biological samples

Levels of NRH and other NAD-related metabolites in biological samples were obtained by using a cold liquid-liquid extraction using a mixture of methanol:water:chloroform in 5:3:5 (v/v), from

which the polar phase was recovered for hydrophilic interaction ultra-high performance liquid chromatography mass spectrometry (UHPLC-MS) analysis. The UHPLC consisted of a binary pump, a cooled autosampler, and a column oven (DIONEX Ultimate 3000 UHPLC+ Focused, Thermo Scientific), connected to a triple quadrupole spectrometer (TSQ Vantage, Thermo Scientific) equipped with a heated electrospray ionisation (H-ESI) source. Of each sample, 2 µL were injected into the analytical column (2.1 mm x 150 mm, 5 µm pore size, 200 Å HILICON iHILIC®-Fusion(P)), guarded by a pre-column (2.1 mm x 20 mm, 200 Å HILICON iHILIC®-Fusion(P) Guard Kit) operating at 35 °C. The mobile phase (10 mM ammonium acetate at pH 9, A, and acetonitrile, B) was pumped at 0.25 mL/min flow rate over a linear gradient of decreasing organic solvent (0.5-16 min, 90-25% B), followed by re-equilibration for a total run time of 30 min. The MS operated in positive mode at 3500 V with multiple reaction monitoring (MRM). The software Xcalibur v4.1.31.9 (Thermo Scientific) was used for instrument control, data acquisition and processing. Retention time and mass detection was confirmed by authentic standards.

Structure elucidation of the used NRH for biological studies was confirmed by nuclear magnetic resonance (NMR).

Example 3: NRH is a potent NAD+ precursor

AML12 hepatocytes were treated with NRH, and it was observed that the ability of NRH to increase intracellular NAD+ was superior to that of NR.

Dose-response experiments revealed that NRH could significantly increase NAD+ levels at a concentration of 10 µM (Figure 2). Even at such relatively low dose, NRH achieved similar increases in intracellular NAD+ levels to those reached with NR at 50-fold higher concentrations. NRH achieved maximal effects on NAD+ synthesis around the millimolar range, managing to increase intracellular NAD+ levels by more than 10-fold.

NRH actions were also extremely fast (Figure 3), as significant increases in NAD+ levels were observed within 5 minutes after NRH treatment. Peak levels of NAD+ were achieved between 45 minutes and 1 h after treatment, as also occurred with NR.

The ability of NRH to potently increase NAD+ was tested as well in other cell type models. NRH treatment highly elevated NAD+ levels in C2C12 myotubes, INS1-cells and 3T3 fibroblasts, supporting the notion that NRH metabolism is widely conserved among different cell types.

Example 4: Pathway of NRH-induced NAD+ synthesis

A path in which NRH would be converted to NMNH, then to NADH and this would be finally oxidized to NAD+. Accordingly, NRH and NMNH could be detected intracellularly 5 minutes after NRH, but not NR, treatment. Interestingly, NRH treatment also led to an increase in intracellular NR and NMN, greater than that triggered by NR itself, opening the possibility that NRH could synthesize NAD+ by being oxidized to NR, using then the canonical NRK/NMNAT path.

In order to understand the exact path by which NRH synthesizes NAD+, we initially evaluated whether NRH, could be transported into the cell by equilibrative nucleoside transporters (ENTs). Confirming this possibility, NRH largely lost its capacity as an extracellular NAD+ precursor in the presence of an agent blocking ENT-mediated transport, such as S-(4-nitrobenzyl)-6-thioinosine (NBTI). Nevertheless, a substantial action of NRH remained even after ENT blockage, suggesting that NRH might be able to enter the cell through additional transporters.

The action of NRH was also NAMPT-independent, based on experiments using FK866, a NAMPT inhibitor. If NRH led to NAD+ synthesis via the formation of NMNH, this hypothetical path would require the phosphorylation of NRH into NMNH. Given the essential and rate-limiting role of NRK1 in NR phosphorylation, we wondered whether the ability of NRH to boost NAD+ levels was NRK1 dependent. To answer this question, we evaluated NRH action in primary hepatocytes from either control or NRK1 knockout (NRK1KO) mice. While after 1 hour of treatment NR failed to increase NAD+ levels in NRK1KO derived primary hepatocytes, NRH action was not affected by NRK1 deficiency. These results indicate that NRH action is NRK1 independent. Further, they rule out the possibility that NRH-induced NAD+ transport is driven by NRH oxidation into NR.

Considering the molecular structure of NRH, we reasoned that an alternative nucleoside kinase could be responsible for the phosphorylation of NRH. Confirming this expectation, the adenosine kinase (AK) inhibitor 5-iodotubercidin (5-IT) fully ablated the action of NRH. The role of AK in NRH-mediated NAD+ synthesis was confirmed using a second, structurally different, AK inhibitor, ABT-702. Metabolomic analyses further confirmed that upon inhibition of AK, the generation of NMNH, NADH and NAD+ was fully blunted, even if NRH was effectively entering the cell. Interestingly, 5-IT treatment also prevented the formation of NR and NMN after NRH treatment.

This indicates that the occurrence of NR after NRH treatment cannot be attributed simply to direct NRH intracellular oxidation to NR. As a whole, these experiments depict adenosine

kinase as the enzymatic activity catalyzing the conversion of NRH into NMNH, initiating this way the transformation into NAD+.

As a follow-up step, NMNAT enzymes could catalyze the transition from NMNH to NADH. Accordingly, the use of gallotannin as a NMNAT inhibitor largely compromised NAD+ synthesis after NRH treatment. Yet, part of the NRH action remained after gallotannin treatment when NRH was used at maximal doses. However, NRH action was totally blocked by gallotannin at submaximal doses, suggesting that the remaining effect at 0.5 mM could be attributed to incomplete inhibition of NMNAT activity by gallotannin. Altogether, these results indicate that adenosine kinase and NMNATs vertebrate the path by which NRH leads to NAD+ synthesis via NADH.

Example 5: NRH is detectable in circulation after IP injection

NR degradation to NAM has been proposed as a limitation for its pharmacological efficacy. To evaluate whether NRH was also susceptible to degradation to NAM, we spiked NRH or NR in isolated mouse plasma. After 2 h of incubation, NR levels decayed in plasma, in parallel to an increase in NAM. In contrast, NAM was not generated from NRH, as its levels remained stable during the 2 h test. We also tested the stability of NRH in other matrixes. Given our previous experiments in cultured cells, we verified that NRH did not degrade to NAM in FBS supplemented media, as occurs with NR. Finally, we also certified NRH stability in water (pH=7, at room temperature) for 48 h.

The above results prompted us to test whether NRH could act as an effective NAD+ precursor in vivo. For this, we first intraperitoneally (IP) injected mice with either NR or NRH (500 mg/kg). After 1 h, both compounds increased NAD+ levels in liver (Figure 5), muscle and kidney. As expected, NAM levels were highly increased in circulation upon NR administration, while only a very mild increase was observed with NRH. Importantly, NRH was detectable in circulation after IP injection.

To our surprise, NR was detectable in circulation after NRH treatment at much higher levels than those detected after NR injection itself. Given that NRH incubation in isolated plasma did not lead to NR production, the appearance of NR might be consequent to intracellular production and release to circulation. Similarly, the residual appearance of NAM after NRH treatment might be explained by the degradation of released NR or by the release of intracellular NAM as a product of NAD+ degradation, as NRH did not significantly alter NAM levels when incubated in isolated plasma.

Example 6: NRH is detectable after oral administration as an orally bioavailable NAD+ precursor that overcomes direct degradation in plasma

Oral administration of NRH led to very similar results to those observed after IP administration. First, NRH had a more potent effect on hepatic NAD+ levels than NR. NRH was detectable in plasma 1 h after oral administration. In contrast, NR levels were undetectable at 1 h after NR administration. As expected, NR treatment led to large increases in circulating NAM, which where ~4-fold higher than those observed after NRH treatment. Quantification measurements revealed that after oral gavage, NRH concentration in plasma reached 11.16 ± 1.74 micromolar, which is enough to effectively drive NAD+ synthesis. These results illustrate that NRH is a potent orally bioavailable NAD+ precursor that overcomes direct degradation to NAM in plasma.

Example 7: NRH is found intact in liver, kidney and muscle after oral administration

NRH is not only found in circulation but it was also found intact, in high levels, in mice liver, kidney and muscle 2 hours after gavage (Figure 6). This indicates that oral administration of NRH allows for efficient biodistribution in target tissues.

Example 8: NRH increases hepatic mitochondrial NAD+

10 week old male mice were fasted for 2 hours prior to intraperitoneal injection (IP) with saline as control/vehicle, nicotinamide riboside (NR; 500 mg/kg) or equimolar amounts of dihydronicotinamide riboside (NRH) or nicotinamide (NAM). Then, 1 hour later, mice were euthanized and a piece of liver was snap frozen while another piece was used for mitochondrial isolation following a published procedure (Frezza et al., 2007; Nature Protocols). The piece of snap frozen liver was weighted and submitted to acidic extraction to evaluate NAD+ levels through enzymatic methods. For isolated mitochondria, protein was quantified and 200 micrograms of protein were used for acidic extraction and NAD+ measurement through enzymatic methods. Figure 7 shows that while NR, NRH and NAM all increase intrahepatic NAD+, only NRH increases mitochondrial NAD+ levels. All results are mean +/- SEM of n=4 animals per group. * indicates statistical difference at $p<0.05$ vs. control/vehicle group.

CLAIMS

1. Reduced nicotinamide riboside for use in a method of increasing intracellular NAD+ in a subject comprising delivering to the subject in need an effective unit dose form of reduced nicotinamide to prevent and/or treat liver diseases or conditions.

2. Reduced nicotinamide riboside for use according to claim 1 wherein said reduced nicotinamide riboside is selected from:

(i) 1,4-dihydro-1-beta-D-ribofuranosyl-3-pyridinecarboxamide;

(ii) 1,2-dihydro-1-beta-D-ribofuranosyl-3-pyridinecarboxamide or

(iii) 1,6-dihydro-1-beta-D-ribofuranosyl-3-pyridinecarboxamide.

3. Reduced nicotinamide riboside for use according to claims 1 or 2 wherein the reduced nicotinamide riboside is 1,4-dihydro-1-beta-D-ribofuranosyl-3-pyridinecarboxamide.

4. Composition comprising reduced nicotinamide riboside according to any one of claims 1-3 wherein said composition is for use to prevent and/or treat liver diseases or conditions.

5. Composition according to claim 4 wherein said composition consists essentially of reduced nicotinamide riboside without other NAD+ precursors for use to prevent and/or treat liver diseases or conditions.

6. Composition according to any one of claims 4 to 5 containing reduced nicotinamide riboside for use to maintain or improve liver function in a subject.

7. Composition according to any one of claims 4 to 6 containing reduced nicotinamide riboside for use to prevent and/or treat liver diseases and conditions selected from the group consisting of: non-alcoholic fatty liver disease (NAFLD), alcoholic fatty liver disease, hepatic steatosis, cirrhosis and/or recovery and regeneration after liver injury, transplantation or surgery.

8. Composition according to any one of claims 4 to 6 containing reduced nicotinamide riboside for use to prevent and/or treat liver diseases and conditions associated with hepatitis A,B,C,D and E, infectious mononucleosis (Epstein Barr virus) and/or hemochromatosis.

9. Composition according to any one of claims 4 to 6 containing reduced nicotinamide riboside for use to enhance regeneration and recovery of liver tissue after injury, trauma or liver transplantation surgery.

10. Composition according to any one of claims 4 to 9 wherein said composition is a nutritional composition selected from a: food or beverage product, including food additives, food ingredients, functional foods, dietary supplements, medical foods, nutraceuticals, oral nutritional supplements (ONS) or food supplements.

11. Reduced nicotinamide riboside according to any one of claims 1 to 3 or a composition according to any one of claims 4 to 10 containing nicotinamide riboside for use to prevent and/or treat a liver disease and/or condition wherein the liver disease and/or condition is selected from the group consisting of: non-alcoholic fatty liver disease (NAFLD), alcoholic fatty liver disease, hepatic steatosis, cirrhosis and/or recovery and regeneration after liver injury, transplantation or surgery.

12. Reduced nicotinamide riboside according to any one of claims 1 to 3 or a composition according to any one of claims 4 to 10 containing for use to prevent and/or treat a liver disease and/or condition wherein the liver disease and/or condition is associated with hepatitis A,B,C,D and E, infectious mononucleosis (Epstein Barr virus) and/or hemochromatosis.

13. Method for increasing intracellular NADH in a subject mammal, comprising delivering to the mammal in need of such treatment an effective amount of reduced nicotinamide riboside according to any one of claims 1 to 12 in an effective unit dose form for use to prevent and/or treat liver diseases or conditions.

14. Method according to claim 13 wherein the liver disease or condition is selected from the group of: non-alcoholic fatty liver disease (NAFLD), alcoholic fatty liver disease, hepatic steatosis, cirrhosis and/or recovery and regeneration after liver injury, transplantation or surgery.

15. Method according to claim 13 or 14 wherein the liver disease and/or condition is associated with hepatitis A,B,C,D and E, infectious mononucleosis (Epstein Barr virus) and/or hemochromatosis.

16. Method according to any one of claims 13 to 15 for use to prevent and/or treat liver disease or conditions in a subject in need comprising the steps of:

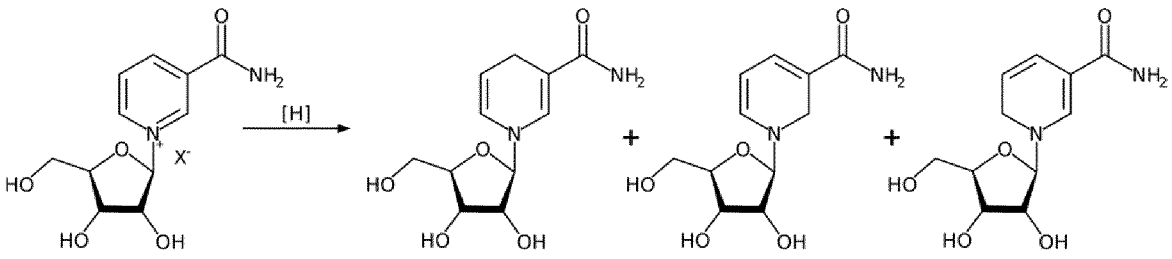
i) providing the subject a composition consisting essentially of reduced nicotinamide riboside and

ii) administering the composition to said subject.

17. Method according to claim 16 wherein the subject is selected from the group consisting of: human, dog, cat, cow, horse, pig, or sheep.

18. Method according to claim 17 wherein the subject is a human.

Figure 1



1 2 3 4

Figure 2

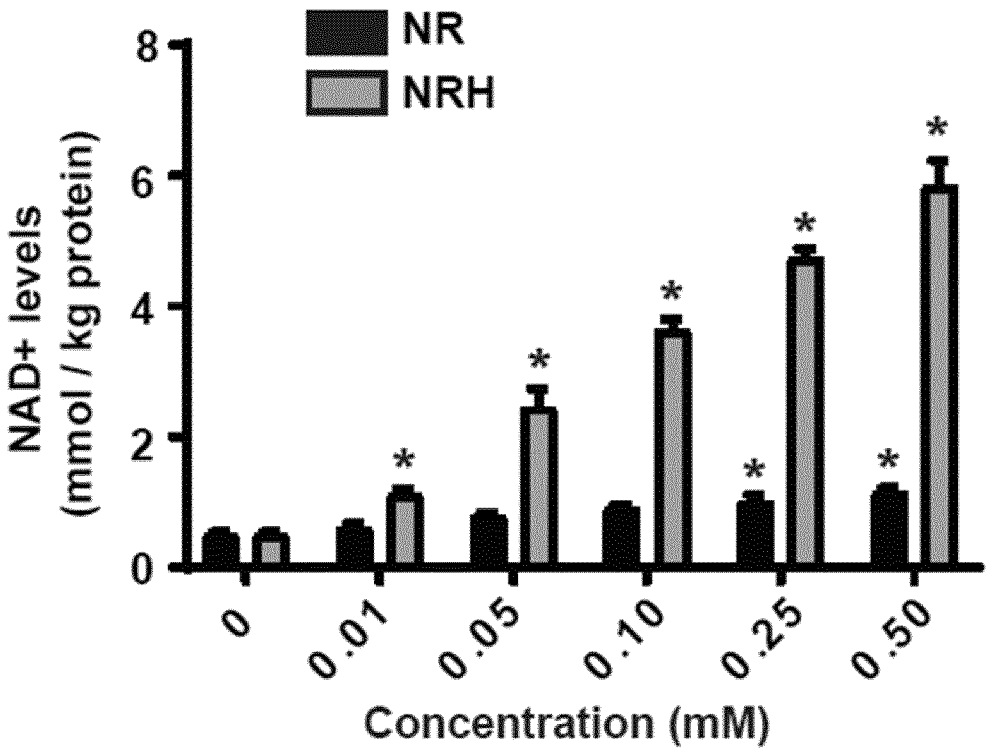

NR
NRH
NAD+ levels
(mmol / kg protein)
8
6
4
2
0
*
0
0.01
0.05
0.10
0.25
0.50
Concentration (mM)

Figure 3

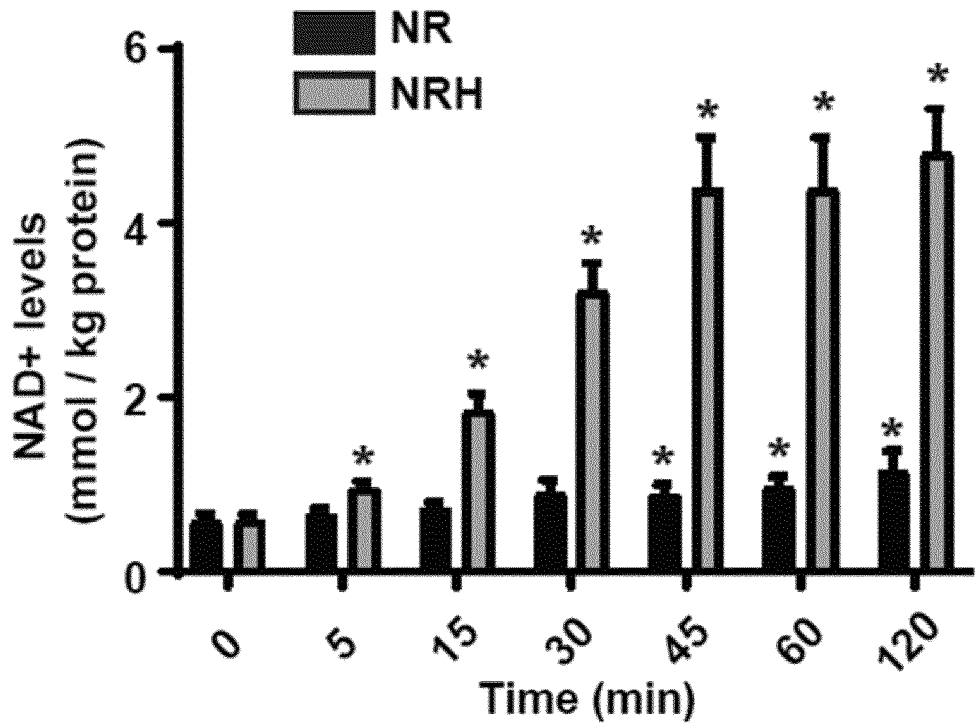

NR
NRH
NAD+ levels
(mmol / kg protein)
0
2
4
6
*
0
5
15
30
45
60
120
Time (min)

Figure 4

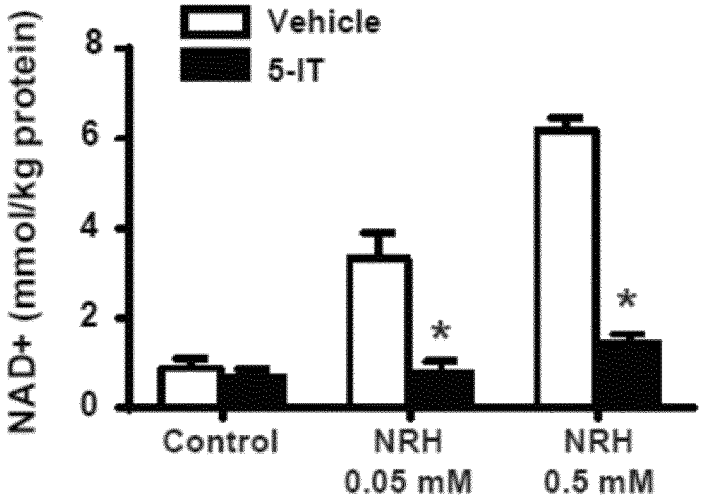

Vehicle
5-IT
NAD+ (mmol/kg protein)
8
6
4
2
0
*
*
Control
NRH
0.05 mM
NRH
0.5 mM

Figure 5

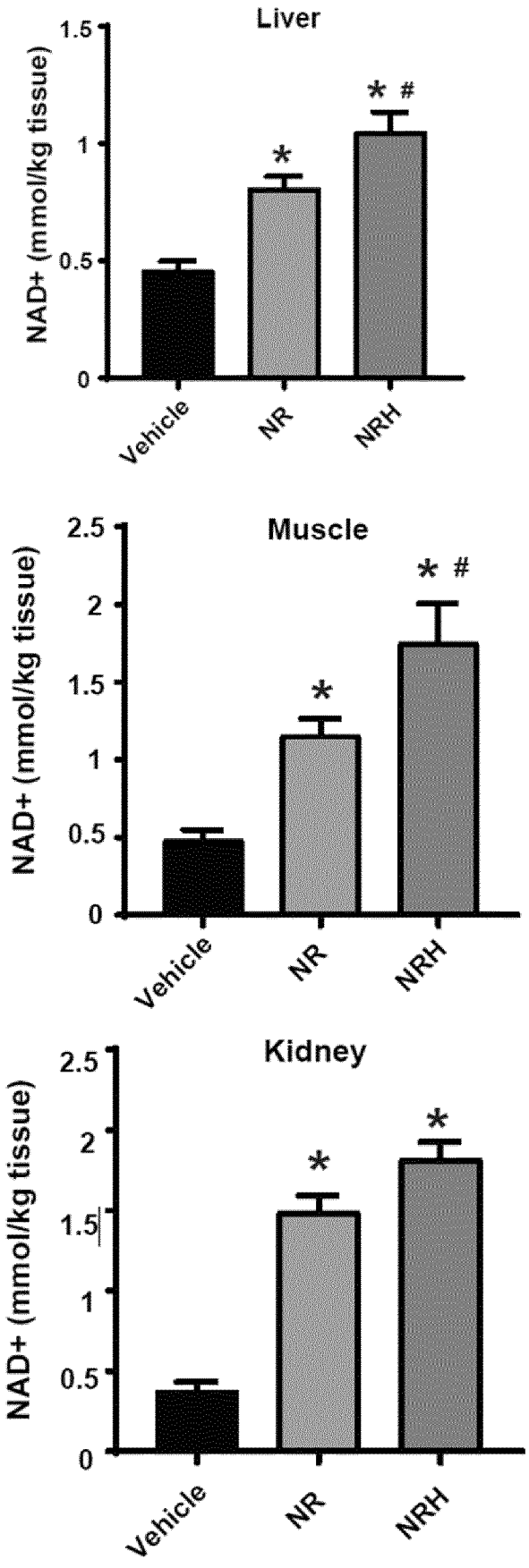

Liver
NAD+ (mmol/kg tissue)
1.5
1
0.5
0
* #
*
Vehicle
NR
NRH
Muscle
NAD+ (mmol/kg tissue)
2.5
2
1.5
1
0.5
0
* #
*
Vehicle
NR
NRH
Kidney
NAD+ (mmol/kg tissue)
2.5
2
1.5
1
0.5
0
*
*
Vehicle
NR
NRH

Figure 6

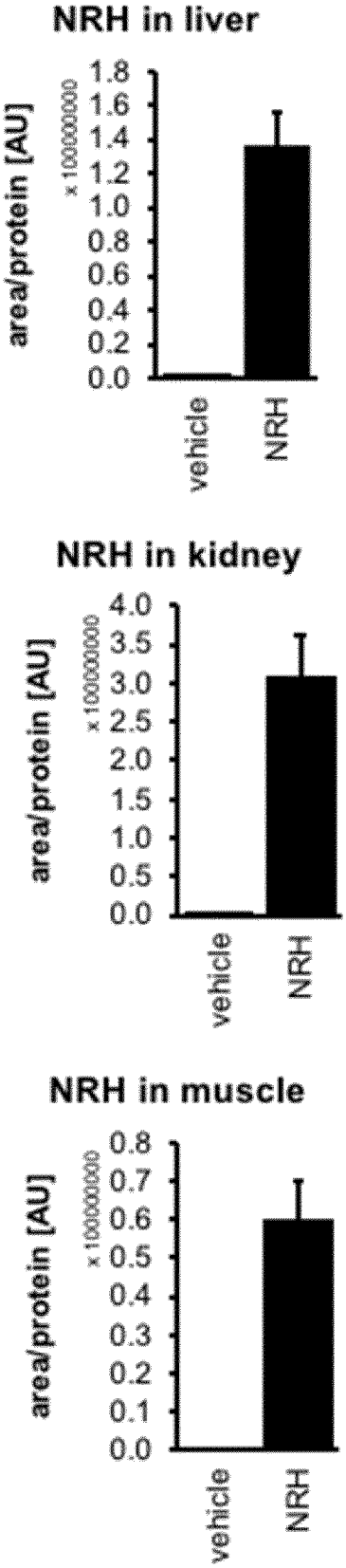
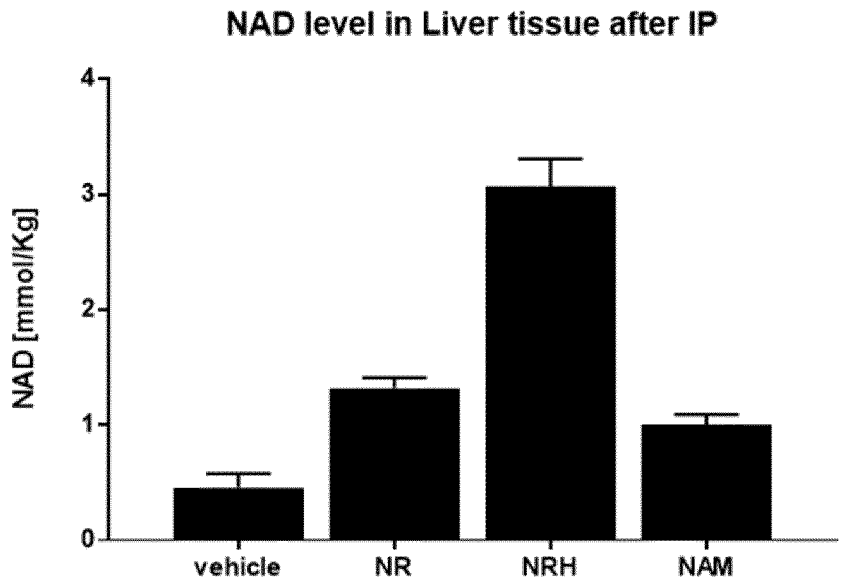
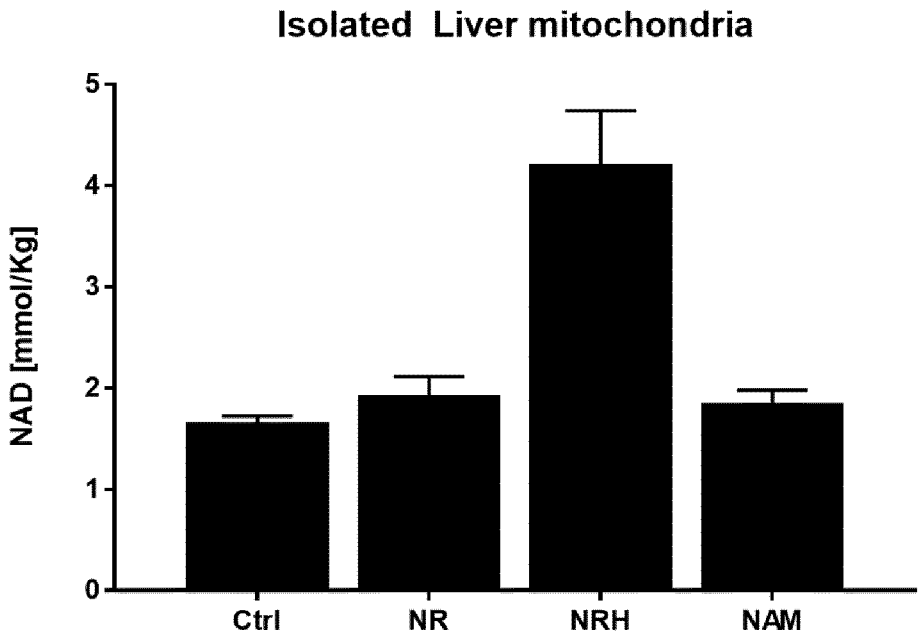

NRH in liver
area/protein [AU]
x 1000000000
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
vehicle
NRH
NRH in kidney
area/protein [AU]
x 1000000000
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
vehicle
NRH
NRH in muscle
area/protein [AU]
x 1000000000
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
vehicle
NRH

Figure 7


NAD level in Liver tissue after IP
NAD [mmol/Kg]
0
1
2
3
4
vehicle
NR
NRH
NAM


Isolated Liver mitochondria
NAD [mmol/Kg]
0
1
2
3
4
5
Ctrl
NR
NRH
NAM