Article

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Safety assessment of nicotinamide riboside, a form of vitamin B_3

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Abstract

Nicotinamide riboside (NR) is a naturally occurring form of vitamin B_3 present in trace amounts in some foods. Like niacin, it has been shown to be a precursor in the biosynthesis of nicotinamide adenine dinucleotide (NAD+). The safety of Niagen™, a synthetic form of NR, was determined using a bacterial reverse mutagenesis assay (Ames), an in vitro chromosome aberration assay, an in vivo micronucleus assay, and acute, 14-day and 90-day rat toxicology studies. NR was not genotoxic. There was no mortality at an oral dose of 5000 mg/kg. Based on the results of a 14-day study, a 90-day study was performed comparing NR at 300, 1000, and 3000 mg/kg/day to an equimolar dose of nicotinamide at 1260 mg/kg/day as a positive control. Results from the study show that NR had a similar toxicity profile to nicotinamide at the highest dose tested. Target organs of toxicity were liver, kidney, ovaries, and testes. The lowest observed adverse effect level for NR was 1000 mg/kg/day, and the no observed adverse effect level was 300 mg/kg/day.

Keywords

Niagen, nicotinamide riboside, NR, nicotinamide, 90-day subchronic oral toxicity, genotoxicity

Introduction

Niacin refers to nicotinamide, nicotinic acid, and derivatives that exhibit the biologic activity of nicotinamide. Nicotinamide riboside (NR) is a single chemical moiety containing nicotinamide and ribose (Figure 1) and, because it is a precursor of nicotinamide adenine dinucleotide (NAD+), is considered to be a form of vitamin B_3.[1–3] Although there is no evidence of adverse effects from naturally occurring niacin in foods, it is recognized that for nutrients, there is a daily level of intake or threshold above which the risk of adverse effects increase (defined as the tolerable upper intake level (UL).[4] A UL for NR has not been established by an authoritative or regulatory body. Therefore, to establish a UL for Niagen™, a synthetic form of NR, pivotal genotoxicity and toxicology studies were conducted.

Niacin is essential for the formation of the pyridine nucleotide coenzymes NAD+ and nicotinamide adenine dinucleotide phosphate. Both coenzymes function indispensably in oxidation–reduction reactions involved in glucose, fatty acid, ketone body, and amino acid catabolism. Although the metabolism of nicotinic acid and nicotinamide to NAD+ is tissue and cell-type dependent, in general, intracellular nicotinic acid is converted to NAD+ by the biosynthetic Preiss–Handler pathway.[5–9] In contrast, nicotinamide and NR are converted to NAD+ by way of nicotinamide mononucleotide in the salvage pathway.[10–13]

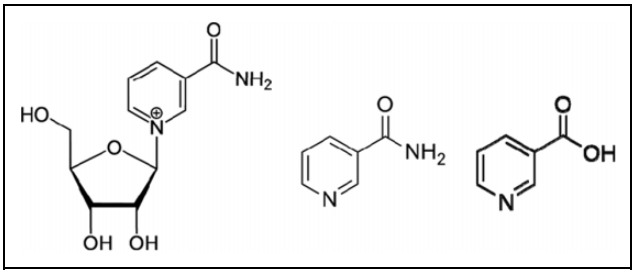

Figure 1. Structure of NR (left), nicotinamide (middle), and nicotinic acid (right). NR: nicotinamide riboside.

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Because animal toxicology studies on nicotinic acid and nicotinamide are limited and do not comply with current standardized testing protocols, such as Organization for Economic Co-operation and Development (OECD),[14–17] authoritative bodies have used the results of clinical studies in which high doses of nicotinic acid and nicotinamide have been administered to derive the ULs. The most severe forms of toxicity after nicotinic acid ingestion are hepatotoxicity and glucose intolerance and occur at doses greater than 500 mg/day. Flushing has also been reported and generally occurs at doses greater than 50 mg/day. As a result, it is considered to be the most sensitive end point of nicotinic acid effects. In contrast, after ingestion of supplemental nicotinamide, no cases of flushing or glucose intolerance have been reported and only one case of hepatitis was reported following the ingestion of greater than 3 g/day for several days.[18]

Although nicotinamide does not appear to be associated with flushing, the Institute of Medicine (IOM) established a UL of 35 mg/day for adults 19 years and older for both nicotinic acid and nicotinamide based on flushing because it is considered to be protective against potential adverse effects. The European Commission Scientific Committee on Food (SCF) established a UL of 10 mg acid/day for nicotinic acid based on flushing.[19] The SCF established a separate UL of 900 mg/day for nicotinamide based on hepatic function in diabetic subjects given nicotinamide and did not use data on flushing after nicotinic acid as the default for the UL for nicotinamide as was used by IOM.[19]

Materials and methods

Bacterial reverse mutagenicity (Ames assay)

Bacterial reverse mutation assays were performed in compliance with the OECD Principles of Good Laboratory Practices (GLPs) and Guideline No. 471.[20] Niagen (>99% NR chloride (major impurity is nicotinamide)); CAS No. 23111-00-4) was supplied by ChromaDex, Inc (Irvine, CA, USA). 2-amino anthracene, 2-nitrofluorene, sodium azide, 9-amino acridine, and 4-nitroquinoline-*N*-oxide were obtained from Sigma Aldrich Chemical Co. Inc. (St. Louis, Missouri, USA). Aroclor 1254-induced rat liver S9 homogenate was obtained from Xenometrix AG (Allschwill, Switzerland). *Salmonella typhimurium* TA98, TA100, TA1535, and TA1537 were obtained from the National Collection of Type Cultures (United Kingdom). *Escherichia coli* WP2 uvrA (pKM101) was obtained from Xenometrix.

The mutagenicity of Niagen was determined using the plate incorporation and preincubation methods. In the plate incorporation method, 50, 159, 501, 1582, and 5000 μg Niagen was mixed with selective top agar, the tester strains *S. typhimurium* TA98, TA100, TA1535, and TA1537 or *E. coli* WP2 *uvr*A pKM101, histidine and biotin or tryptophan (depending on the type stain used), with and without a metabolic activation system (S9 mix). The mixture was overlaid onto solidified Vogel–Bonner minimal E basal agar,[21] and the plates were examined for the presence of a background lawn and precipitate, and the number of revertant colonies were counted manually. In a confirmatory assay, the tester strains were preincubated with 99, 265, 699, 1869, or 5000 μg Niagen in the presence or absence of the S9 mix. The plates were examined for the presence of a background lawn and precipitate, and the number of revertant colonies were counted manually.

All experiments were performed in triplicate with vehicle and strain-specific positive controls. In the presence of the S9 mix, the positive controls for all strains were 2-aminoanthracene. In the absence of the S9 mix, the positive controls for strains TA98, TA100 and TA1535, TA1537, and WP2 *uvr*A pKM101 were 2-nitrofluorene, sodium azide, 9-aminoacridine, and 4-nitroquinoline-*N*-oxide, respectively. Niagen was considered cytotoxic if there was a 50% reduction in the mean number of revertants per plate compared to the mean vehicle control and/or at least a moderate reduction in the background lawn. Niagen was considered mutagenic if there was a concentration-related increase in the number of revertants per plate in at least one tester strain over a minimum of two increasing concentrations of Niagen. For the strains TA98, TA100, and WP2 *uvr*A pKM101, the result was considered positive if the mean number of revertants was equal to or greater than two times the number of revertants obtained with the negative control. For the strains TA1535 and TA1537, the result was considered positive if the mean number of revertants was equal to or greater than three times the number of revertants obtained with the negative control.

In vitro chromosomal aberration assay

In vitro chromosomal aberration assays were performed in compliance with the OECD Principles of GLP and Guideline No. 473. Niagen (>99% NR

chloride) was supplied by ChromaDex, Inc. Cyclophosphamide monohydrate and ethyl methanesulphonate were obtained from Sigma Aldrich Chemical Co., Inc. Aroclor 1254-induced rat liver S9 homogenate was obtained from Xenometrix AG. Human peripheral blood lymphocytes (PBLs) were obtained from whole blood harvested from a healthy donor who was approximately 35 years old, had no history of smoking or alcoholism, and had not received medication for 1 month prior to the blood draw. The whole blood was cultured at 37C and 5% carbon dioxide in complete medium (Roswell Park Memorial Institute (RPMI) 1640, 10% fetal bovine serum (FBS), amphotericin, penicillin, streptomycin) supplemented with heparin and phytohemagglutinin (PHA) for 3 days per OCED Guideline 473 and International Conference on Harmonisation of Technical Requirements for Pharmaceuticals for Human Use-harmonized guidances on genotoxicity testing of pharmaceuticals.

To determine the clastogenic activity of Niagen, the PHA-stimulated whole blood cultures were centrifuged, and the resulting PBLs were resuspended in complete medium containing, either vehicle (water), 1.25, 2.5, or 5 mg/mL of Niagen, or the appropriate positive control (cyclophosphamide monohydrate and ethyl methanesulphonate), and then supplemented with or without the metabolic activation system (S9 mix). The mixtures were then incubated at 37°C and 5% carbon dioxide. At 3 hr a subset of cultures were harvested, washed, and reincubated in complete medium for an addition 19 h. Three h before harvesting, colchicine was added to the cultures to a final concentration of 2 μg/mL. The cells were harvested by centrifugation, resuspended in 0.56% prewarmed potassium chloride, and incubated at room temperature for 25 to 30 min. The cell suspension was centrifuged, and the cellular pellet was resuspended and fixed with cold fixative (acetic acid: methanol (1:3)). After the final fixative incubation, the cell suspension was dropped onto clean cold slides, stained with 5% Giemsa, and scored for the presence of metaphase cells and the presence of aberrations. To determine the mitotic index, which was used as an indicator of cytotoxicity, a minimum of 1000 cells were scored for each group and the total number of metaphases was divided by the number of cells counted. The quotient was then multiplied by 100. To determine the types of aberrations (chromatid gaps, chromosomal gaps, chromosomal breaks, chromatid breaks, deletions, and fragments), a minimum of 300 metaphases containing 46 $\pm$ 2 centromere regions were counted and the number of cells containing one or more different types of aberrations were recorded. The data were then subjected to a one-tailed Fisher exact test. Niagen was considered cytotoxic if there was a 45 $\pm$ 5% reduction in the mitotic index compared to the vehicle control. Niagen was considered mutagenic if there was a concentration-related and statistically significant increase ($p < 0.05$) in the number of chromosome aberrations.

In vivo micronucleus assay

The in vivo micronucleus assay performed in compliance with the OECD principles of GLP and Guideline No. 474, and the recommendations of the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC) and Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCESA), Government of India. Sprague–Dawley rats were obtained from Harlan Laboratories (The Netherlands). Teklab global rodent diet was obtained from Harlan Laboratories. Niagen (>99% NR chloride) was supplied by ChromaDex, Inc. Cyclophosphamide was obtained from Sigma Aldrich Chemical Co., Inc.

All rats were housed at three rats per sex per cage, acclimatized for at least 5 days prior to treatment, and provided feed and water ad libitum throughout the study. Prior to dosing, the rats were randomized by body weight to six groups ($n = 6$/sex/group). At dosing, a single dose of vehicle (water), 500, 1000, and 2000 mg/kg of Niagen or 40 mg/kg cyclophosphamide was administered by gavage at a rate of 10 mL/kg body weight. Twenty-four hours after dosing the vehicle, 500 mg/kg Niagen-, 1000 mg/kg Niagen-, 2000 mg/kg Niagen-, and 40 mg/kg cyclophosphamide-treated groups were euthanized. Forty-eight hours after dosing, an additional 2000 mg/kg Niagen-treated group was also euthanized. The animals were observed for mortality at 1 and 2 h and then twice daily after dosing for 2 days. Clinical signs were monitored 1 and 2 h after dosing and then once daily for 2 days.

Immediately after euthanization, bone marrow was harvested from the femurs of each animal. The centrifuged cell suspension was smeared on two slides, which were then air-dried, fixed in methanol, and stained using a May-Gruenwald and Giemsa solution. The test item was considered toxic if the polychromatic erythrocyte/total erythrocyte ratio was less than that in vehicle control group. Niagen would be considered mutagenic if at least one of the treatment groups

exhibited a statistically significant ($p < 0.05$) increase in the frequency of micronucleated immature erythrocytes when compared with the concurrent vehicle control.

Acute toxicity study

An acute toxicity study was performed in male and female Sprague–Dawley rats in compliance with the OECD Principles of GLP, the Guidance for Industry, Single Dose Acute Toxicity Testing for Pharmaceuticals from the United States Food and Drug Administration, and the recommendations of AAALAC and CPCESA. The rats were obtained from Harlan Laboratories. Nutrilab Rodent Pellet feed was obtained from Provimi Animal Nutrition (Bangalor, India). Niagen (>99% NR chloride) was supplied by ChromaDex, Inc.

All rats were housed as two to three rats per sex per cage, acclimatized for at least 5 days prior to treatment, and, except for the overnight fast prior to dosing, provided feed and water ad libitum throughout the study. Prior to dosing, the rats were randomized by body weight to two groups ($n = 5$/sex/group). At dosing, a single dose of vehicle (water) or 5000 mg/kg of Niagen was administered by gavage at a rate of 10 mL/kg body weight. Over the course of the following 14 days, all rats were monitored for morbidity, mortality, and visible clinical signs. Detailed clinical examinations were conducted on days 1, 8, and 15 and included evaluations of the skin, fur, eyes, mucous membranes, occurrence of secretions and excretions and autonomic activity, changes in gait, posture, response to handling, and presence of clonic or tonic movements, stereotypes, or bizarre behaviors. Body weights were recorded prior to treatment on day 1 and then on days 8 and 15. Food consumption for each cage was measured on days 8 and 15, and food consumption per rat was calculated by dividing the total food consumption during the interval per cage by the number of rats multiplied by the number of days. On day 15, all animals were euthanized and examined for gross pathological changes. Analyses were conducted using two-tailed tests for a minimum significance level of 5%, comparing the Niagen and vehicle-treated group for each sex. All quantitative variables, including body weight, body weight gain, and food consumption, were subjected to Student's *t*-test. Males and females were considered separately for each analyses, and a p value of <0.05 was considered statistically significant.

14-Day repeat dose study

A 14-day repeat dose study was conducted in male and female Sprague–Dawley rats in compliance with the OECD Guideline 407, but for 14 days instead of 28 days, and the recommendations of the AAALAC and CPCESA. The rats were obtained from Harlan Laboratories. Nutrilab Rodent Pellet feed was obtained from Provimi Animal Nutrition. Niagen (>99% NR chloride) was supplied by ChromaDex, Inc.

All rats were housed as two to three rats per sex per cage, acclimatized for at least 5 days prior to treatment, and provided feed and water ad libitum throughout the study. Prior to dosing, the rats were randomized to five groups ($n = 5$/sex/group) according to body weight. During the 14-day treatment period, each group was gavaged daily with either vehicle (water) or 750, 1500, 2500, or 5000 mg/kg/day of Niagen at a rate of 10 mL/kg body weight. Dose formulation analyses showed that Niagen was completely soluble in water and the dose formulations were homogeneous and contained the targeted concentrations of NR. Stability analyses showed that when Niagen was dissolved in water, NR was stable up to 6 h at room temperature and 7 days at 2–8°C.

During the 14-day treatment period, all rats were monitored for morbidity, mortality, and visible clinical signs. Detailed clinical examinations were conducted on days 1, 8, and 15 and included evaluations of the skin, fur, eyes, mucous membranes, occurrence of secretions and excretions and autonomic activity, changes in gait, posture, response to handling, and presence of clonic or tonic movements, stereotypes, or bizarre behaviors. Body weights were recorded prior to treatment on day 1 and then on days 8 and 15. Food consumption for each cage was measured on days 8 and 15 and food consumption per rat was calculated by dividing the total food consumption during the interval per cage by the number of rats multiplied by the number of days. On day 15, all animals were euthanized and examined for gross pathological changes.

All analyses were conducted using two-tailed tests for a minimum significance level of 5%, comparing the Niagen and vehicle-treated group for each sex. All quantitative variables, including body weight, body weight gain, and food consumption, were tested for normality and homogeneity of variances within the group before performing a one-factor analysis of variance (ANOVA) by treatment. When the data were

found to be nonoptimal, the data were log transformed prior to performing the ANOVA. Comparisons of the differences of the means between the Niagen- and vehicle-treated groups were performed using Dunnett's post hoc test. When normality/homogeneity was significant, even after transformation, the data were subjected to the Kruskal–Wallis test, followed by Dunn's post hoc test. Males and females were considered separately for each analyses, and a p value of <0.05 was considered statistically significant.

Subchronic toxicity study

A 90-day oral subchronic toxicity study in male and female Sprague–Dawley rats was conducted in compliance with the OECD Principles of GLP, the OECD Guideline 408 for testing of chemicals, and the recommendations of AAALAC and CPCESA. The rats were obtained from Harlan Laboratories. Nutrilab Rodent Pellet feed was obtained from Provimi Animal Nutrition. Niagen (>99% NR chloride) was supplied by ChromaDex, Inc. Nicotinamide ($\geq$ 99.5%; CAS No. 98-92-0) was obtained from Sigma Aldrich.

All rats were housed as two to three rats per sex per cage, acclimatized for at least 5 days prior to treatment, and, except for the overnight fast prior to euthanasia on day 91, provided feed and water ad libitum throughout the study. Prior to dosing, the rats were randomized by body weight to 5 groups ($n = 10$/sex/group). During the 90-day treatment period, each group was gavaged daily with either vehicle (water), 300, 1000, 3000 mg/kg of Niagen or 1260 mg/kg/day of nicotinamide, which is equivalent to 3000 mg/kg/day of Niagen on a molar basis. Dose formulation analyses showed that both Niagen and nicotinamide were completely soluble in water and the dose formulations contained the targeted concentrations of NR or nicotinamide. Stability analyses showed that when Niagen and nicotinamide were dissolved in water, both NR and nicotinamide were stable up to 6 h at room temperature and 7 days at 2–8°C. The formulations for the study were stored at 2–8°C and used within 7 days. The parameters evaluated during the study were twice daily checks for mortality, daily evaluations for clinical signs, weekly detailed clinical examinations, and weekly body weight and food consumption measurements. Ophthalmological examinations were performed prior to treatment and prior to killing. On day 91, after urine was collected from all animals after an overnight fast, the animals were anesthetized, and blood was collected from the sublingual vein for hematology, coagulation, and clinical chemistry evaluations. Then the animals were euthanized by exsanguination under deep anesthesia and subjected to necropsy and gross pathological examination. Hematological parameters included differential leukocyte count, reticulocytes, leukocytes, erythrocytes, eosinophils, neutrophils, lymphocytes platelets, basophils, monocytes, and large unstained cell counts, hemoglobin, hematocrit, mean corpuscular volume, mean cell hemoglobin, mean hemoglobin concentration, prothrombin time, and activated partial thromboplastin time. Plasma clinical chemistry parameters included total protein, albumin, bile acids, aspartate aminotransferase (AST), alanine aminotransferase (ALT), gamma-glutamyl transpeptidase, globulin, alkaline phosphatase (ALP), total cholesterol (TC), triglycerides, glucose, blood urea nitrogen (BUN), creatinine, inorganic phosphorous, calcium, magnesium, sodium, potassium, and chloride levels. The organs that were collected, weighed, and preserved included the adrenals, aorta, bone marrow smear, brain including medulla/pons, cerebellum and cerebrum, cecum, colon, duodenum, epididymides, esophagus, eyes with optic nerve, biceps femoris muscles, femur bone with joint gross lesions, heart, ileum with Peyer's patches, jejunum, kidneys, liver, lungs with main bronchi and bronchioles, mandibular lymph nodes, mesenteric lymph nodes, mammary gland, ovaries, oviducts, pancreas, pituitary, prostate, seminal vesicles and coagulating glands, rectum, salivary glands (mandibular, parotid and sublingual), sciatic nerve, skin (inguinal region), spinal cord at three levels—cervical, mid-thoracic and lumbar—spleen, sternum with marrow, stomach, testes, thymus, thyroid and parathyroid, tongue, trachea, urinary bladder, uterus with cervix, and vagina. The preserved tissues were processed and embedded in paraffin, sectioned, and stained with hematoxylin and eosin. Histopathology was performed on all preserved organs of the vehicle control, positive control nicotinamide, and Niagen high dose groups. Liver, kidneys, thyroids, testes, epididymides (male), ovaries (female), and adrenals were examined at lower dose Niagen groups as treatment-related microscopic lesions were found in high-dose group. Urine was analyzed for volume, osmotic pressure, specific gravity, pH, and concentrations of glucose, bilirubin, ketone, blood, protein, urobilinogen, nitrate, and the presence of leukocytes. All analyses were conducted using two-tailed tests for a minimum significance level of 5%, comparing the Niagen and vehicle-treated

group for each sex. All quantitative variables, including body weight, body weight gain, and food consumption, were tested for normality and homogeneity of variances within the group before performing a one-factor ANOVA by treatment. When the data were found to be nonoptimal, the data were log transformed prior to performing the ANOVA. Comparisons of the means differences between the Niagen- and vehicle-treated groups was performed using Dunnett's post hoc test. When normality/homogeneity was significant, even after transformation, the data were subjected to the Kruskal–Wallis test followed by Dunn's post hoc test. Males and females were considered separately for each analyses, and a p value of <0.05 was considered statistically significant.

Results

Bacterial reverse mutagenicity (Ames assay)

Niagen was not cytotoxic at any of the doses used in this study (data not shown) and, compared to the vehicle control, did not increase the number of revertant colonies in any of the frameshift or base-pair tester strains either when incubated in the presence or absence of the S9 mix or using the plate incorporation or preincubation methods (Supplemental Table 1). In contrast, all positive controls (2-aminoanthracene 2-nitrofluorene, 9-aminoacridine, sodium azide, 4-nitroquinoline-*N*-oxide) significantly increased the number of revertant colonies ($p < 0.05$), demonstrating both the sensitivity and validity of the assay. Therefore, Niagen was not mutagenic under the conditions used in the studies.

In vitro chromosomal aberration assay

Niagen was not cytotoxic to ex vivo human peripheral blood lymphocytes at any of the concentrations used in the study as determined by the mitotic index (data not shown) and, compared to the vehicle control, did not increase the number of aberrant metaphases when incubated with or without S9 mix for 3 h (Supplemental Table 2). Moreover, the types of aberrations (chromatid gaps, chromosomal gaps, chromosomal breaks, and chromatid breaks) detected in the vehicle- and Niagen-treated cells were similar. In contrast, the positive controls, cyclophosphamide and ethyl methansulphonate, significantly increased the number of aberrant metaphases ($p < 0.05$), characterized as chromatid gaps, chromosomal gaps, chromosomal breaks, chromatid breaks, deletions, and fragments, thus confirming the sensitivity and validity of the assay. Similar results were also found when the lymphocytes were incubated with increasing amounts of Niagen for 22 h in the absence of the S9 mix (data not shown). Niagen was therefore not clastogenic under the conditions used in the study.

In vivo micronucleus assay

No mortalities or clinical signs of toxicity were observed in any of the rats receiving Niagen. In addition, bone marrow analyses showed that compared to the negative control, the administration of 500, 1000, and 2000 mg/kg of Niagen did not result in cytotoxicity or increase the percentage of polychromatic erythrocytes (Supplemental Table 3). In contrast, the positive control cyclophosphamide induced a statistically significant ($p < 0.05$) increase in the percentage of polychromatic erythrocytes at 24 h, demonstrating both the sensitivity and validity of the assay. Therefore, Niagen was not genotoxic under the conditions used in this study.

Acute toxicity study

No mortalities, clinical signs, or gross pathological lesions were observed in males or females at the single tested dose of 5000 mg/kg of Niagen. There were also No statistically significant differences in body weight in both sexes compared to the vehicle-treated males and females throughout the study. Cumulative body weight gain (days 1–15) was significantly lower in female rats as compared to vehicle-treated group. Because the change in body weight at day 15 was minimal (−3%), this was considered as treatment related but non-adverse. There were no statistically significant changes in food consumption in either sex. Niagen administration resulted in no mortality at a dose of 5000 mg/kg in the acute toxicity study.

14-Day toxicology study

A minimal test-article related reduction in mean body weight compared to the vehicle-treated group was observed in male rats at 2500 mg/kg/day (7–8% reduction) and 5000 mg/kg/day (8–9% reduction) on days 8, 11, 14, and 15. A decrease in overall (days 1–14) feed consumption was also observed at 5000 mg/kg/day (8%) in male rats. No test item-related changes were observed in body weight and feed consumption in female rats. No gross pathological lesions were observed in male and female rats. Based on the

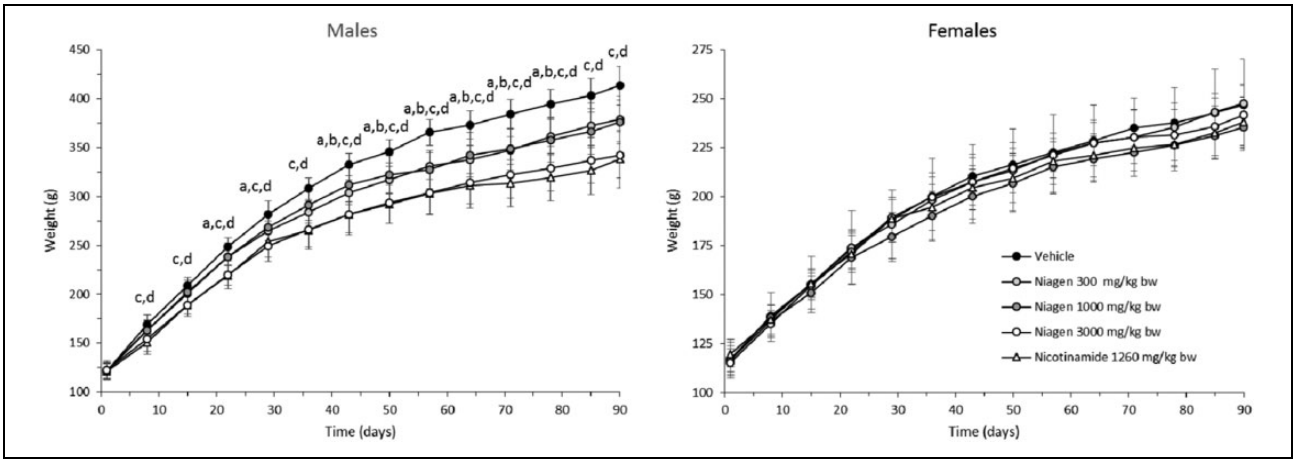

Figure 2. Body weights of male and female rats treated with vehicle control, NR or nicotinamide for 90-days. "a", "b", "c", and "d" denote significant ($p < 0.05$) differences between rats treated with 300, 1000, and 3000 mg/kg/day of Niagen, or 1260 mg/kg/day of nicotinamide, respectively, and rats treated with the vehicle control. NR: nicotinamide riboside.

combination of both a body weight reduction of approximately 10% and the feed intake reduction 5000 mg/kg/day was considered to be too high for the 90-day study. Based on the body weight reduction of 7–8% at 2500 mg/kg/day in the 14-day study, the doses of 300, 1000, and 3000 mg/kg/day were chosen for the 90-day subchronic toxicity study in rats.

90-Day subchronic toxicity study

Mortality, body weight, and feed consumption. No treatment-related mortality or clinical signs were observed at any dose level in this study. Compared to vehicle-treated controls, a significant ($p < 0.05$) treatment-related decrease in body weight (17% reduction) was noted in male rats at the high dose (3000 mg/kg/day) of Niagen; a similar decrease in body weight was observed at an equimolar dose (1260 mg/kg/day) of nicotinamide (Figure 2). Significant ($p < 0.05$) 8–9% reductions in body weight (<10%) were also observed at the 300 and 1000 mg/kg/day dose of Niagen in male rats. This decrease was <10% and therefore not considered to be adverse. No statistically significant differences in body weights were seen in Niagen- or nicotinamide-treated females. In male rats, decreases in feed consumption were noted at 3000 mg/kg/day of Niagen (9–14%) and 1260 mg/kg/day of nicotinamide (9–17%) throughout the treatment period. Decreases in feed consumption also occurred at 300 mg/kg/day on days 57–64 and at 1000 mg/kg/day on days 50–57. In female rats, decreases in feed consumption occurred at days 1–8 in the nicotinamide-treated group and at days 15–22 in the 3000 mg/kg/day Niagen group.

Hematological, clinical chemistry, and urinalysis tests. Similar treatment-related changes in hematology parameters were observed at the high dose Niagen- and nicotinamide-treated groups (Table 1). Statistically significant ($p < 0.05$) treatment-related increases in white blood cells (WBCs) and neutrophils occurred in both males and females. Statistically significant ($p < 0.05$) increases in monocytes were also noted in females treated with 3000 mg/kg/day of Niagen and 1260 mg/kg/day of nicotinamide. At 1000 mg/kg/day of Niagen, significant increases in WBCs and neutrophils were observed in males and females, respectively. There were no significant changes in hematological parameters at male or female rats treated with 300 mg/kg/day of Niagen. Importantly, the significant effects were not associated with any inflammatory changes in any of the organs examined. All other changes in hematology parameters, including those determined to be statistically significant, were considered to be due to normal biological variation, and not due to the administration of the test item.

Niagen produced statistically significant ($p < 0.05$) increases at 3000 mg/kg/day in ALT, ALP, GGT, triglycerides and bile acids. ALT and triglycerides were also significantly increased at 1000 mg/kg/day of Niagen in females (Table 2). Comparable effects on clinical chemistries were seen in the nicotinamide-treated group. A minimal but statistically significant decrease in sodium in females and chloride in males and females

Table 1. Summary of significant changes-hematological parameters day 91.[a]

	Doses (mg/kg/day)				
Parameters	Vehicle 0	Niagen 300	Niagen 1000	Niagen 3000	Nicotinamide 1260[b]
Males					
White Blood Cells	8.47 ± 1.97	9.16 ± 1.54	11.11 ± 1.77[c]	13.51 ± 2.64[c]	14.11 ± 1.66[c]
Total neutrophils	2.12 ± 0.7	2.30 ± 1.67	2.86 ± 1.20	5.76 ± 1.47[c]	6.02 ± 0.7[c]
Total monocytes	0.28 ± 0.09	0.33 ± 0.30	0.40 ± 0.19	0.42 ± 0.09	0.45 ± 0.12
Females					
White blood cells	6.25 ± 1.20	6.23 ± 1.84	7.40 ± 1.01	10.38 ± 1.51[c]	9.99 ± 2.64[c]
Total neutrophils	0.93 ± 0.31	1.11 ± 0.57	1.63 ± 0.29[c]	3.36 ± 1.19[c]	4.13 ± 1.56[c]
Total monocytes	0.16 ± 0.07	0.15 ± 0.05	0.15 ± 0.04	0.32 ± 0.08[c]	0.33 ± 0.21[c]

[a]Values represented as mean ± SD.
[b]Equimolar ratio to Niagen 3000 mg/kg/day.
[c]Significantly higher than the vehicle control group at $p < 0.05$.

Table 2. Summary of significant changes-clinical chemistries day 91.[a]

	Doses (mg/kg/day)				
Parameters	Vehicle 0	Niagen 300	Niagen 1000	Niagen 3000	Nicotinamide 1260[b]
Males					
ALT (U/L)	85.23 ± 26.11	75.94 ± 12.20	106.17 ± 45.34	159.35 ± 27.56[c]	152.11 ± 25.50[c]
AST (U/L)	122.18 ± 27.34	116.10 ± 13.94	138.23 ± 49.03	132.93 ± 23.63	139.47 ± 26.99
ALP (U/L)	99.85 ± 22.69	120.94 ± 16.68	110.96 ± 18.67	131.63 ± 19.73[c]	139.48 ± 24.88[c]
GGT (U/L)	3.09 ± 0.90	4.13 ± 1.22	3.47 ± 1.20	3.82 ± 1.22	3.81 ± 1.16
Trig (mg/dL)	49.84 ± 18.96	59.30 ± 29.48	69.65 ± 29.91	128.34 ± 47.88[c]	98.57 ± 34.72[c]
Sodium (mmol/L)	139.15 ± 3.37	141.03 ± 6.05	137.99 ± 3.52	137.1 ± 4.7	135.88 ± 1.9
Chloride (mmol/L)	101.55 ± 2.66	102.13 ± 4.41	99.21 ± 1.3	97.34 ± 3.24[c]	97.52 ± 1.25[c]
Females					
ALT (U/L)	56.09 ± 15.54	56.34 ± 10.47	76.50 ± 20.42[c]	121.81 ± 22.35[c]	125.22 ± 30.29[c]
AST (U/L)	101.02 ± 18.67	106.1 ± 18.52	126.69 ± 35.51	126.2 ± 33.09	126.31 ± 16.24[c]
ALP (U/L)	60.79 ± 5.16	69.66 ± 11.23	85.96 ± 22.06	105.10 ± 26.99[c]	114.41 ± 30.91[c]
GGT (U/L)	3.15 ± 1.17	3.9 ± 0.92	3.83 ± 1.42	5.01 ± 1.36[c]	5.46 ± 1.75[c]
Triglycerides (mg/dL)	28.38 ± 6.9	28.24 ± 7.92	46.69 ± 12.97[c]	61.85 ± 23.31[c]	87.00 ± 38.48[c]
Sodium (mmol/L)	137.71 ± 1.28	137.86 ± 1.25	136.68 ± 1.46[c]	134.42 ± 1.22[c]	135.83 ± 1.17[c]
Chloride (mmol/L)	102.12 ± 0.96	101.43 ± 1.13	100.69 ± 1.52	98.12 ± 1.63[c]	98.93 ± 1.33[c]

ALT: alanine aminotransferase, AST: aspartate aminotransferase, ALP: alkaline phosphatase; GGT: gamma-glutamyl transpeptidase.
[a]Values represented as mean ± SD.
[b]Equimolar ratio to Niagen 3000 mg/kg/day.
[c]Significantly higher than the vehicle control group at $p < 0.05$.

was noted at 3000 mg/kg/day of Niagen. Similar results were seen in the nicotinamide-treated group. A significant reduction ($p < 0.05$) in sodium was also observed in females treated with 1000 mg/kg/day. All other changes in clinical chemistry parameters were considered incidental as the magnitude of the changes were minimal and within normal biological variation.

Urinalysis showed increased urine volume in both nicotinamide and high-dose Niagen-treated males (data not shown). This effect was considered a treatment-related effect, which may have been correlated with microscopic changes in the adrenal glands. Urine pH was decreased in males (7.6 ± 0.64 in controls vs. 6.11 ± 0.16 in high dose) and females (7.2 ± 0.53 in controls vs. 6.3 ± 6.3 ± 0.35 in high dose) at 3000 mg/kg/day of Niagen, but not in the males and females treated with nicotinamide. The slightly acidic pH may have been due to the excretion of test item and an acidic metabolite.

Table 3. Summary of significant changes-organ weight ratio relative to body weight at day 91.[a]

	Dose (mg/kg/day)				
Parameters	Vehicle 0	Niagen 300	Niagen 1000	Niagen 3000	Nicotinamide 1260[b]
Males					
Terminal body weight	395.7 ± 18.36	363.56 ± 23.22	354.23 ± 21.85[c]	317.21 ± 25.8[c]	312.87 ± 26.74[c]
Organ/body weight					
Liver	2.958 ± 0.143	3.013 ± 0.163	3.200 ± 0.18[c]	3.600 ± 0.272[c]	3.703 ± 0.116[c]
Kidneys	0.715 ± 0.047	0.701 ± 0.033	0.777 ± 0.02[c]	0.876 ± 0.063[c]	0.833 ± 0.086[c]
Brain	0.507 ± 0.027	0.526 ± 0.038	0.550 ± 0.024[c]	0.572 ± 0.042	0.583 ± 0.043[c]
Heart	0.368 ± 0.019	0.356 ± 0.018	0.373 ± 0.015	0.384 ± 0.02	0.387 ± 0.015
Thymus	0.068 ± 0.011	0.076 ± 0.015	0.086 ± 0.014[c]	0.07 ± 0.009	0.068 ± 0.008
Adrenals	0.012 ± 0.001	0.013 ± 0.001	0.014 ± 0.001[c]	0.014 ± 0.001[c]	0.015 ± 0.001[c]
Females					
Terminal Body Wt.	232.29 ± 8.1	234.43 ± 23.28	219.51 ± 9.92	216.19 ± 14.75	215.61 ± 11.16[c]
Organ/body weight					
Liver	2.902 ± 0.191	3.003 ± 0.327	3.295 ± 0.181[c]	4.046 ± 0.174[c]	4.465 ± 0.239[c]
Kidneys	0.676 ± 0.053	0.645 ± 0.06	0.678 ± 0.058	0.822 ± 0.044[c]	0.766 ± 0.019[c]
Brain	0.78 ± 0.034	0.788 ± 0.055	0.801 ± 0.047	0.798 ± 0.041	0.781 ± 0.031
Heart	0.392 ± 0.016	0.393 ± 0.015	0.398 ± 0.022	0.433 ± 0.032[c]	0.424 ± 0.029[c]
Thymus	0.09 ± 0.011	0.097 ± 0.024	0.093 ± 0.013	0.087 ± 0.014	0.084 ± 0.017
Adrenals	0.027 ± 0.003	0.028 ± 0.003	0.027 ± 0.002	0.029 ± 0.004	0.027 ± 0.003
Ovaries	0.036 ± 0.005	0.037 ± 0.005	0.036 ± 0.006	0.045 ± 0.008 [c]	0.049 ± 0.007[c]

[a]Values represented as mean ± SD.
[b]Equimolar ratio to Niagen 3000 mg/kg/day.
[c]Significantly higher/lower than the vehicle control group at $p < 0.05$.

Gross pathology. Bilateral small-size testes observed in 3000 mg/kg/day Niagen- and nicotinamide-treated males was considered to be treatment related and associated with degeneration/atrophy of the seminiferous tubules.

One incidence of auxiliary region subcutaneous nodule microscopically associated with adenocarcinoma of mammary gland was observed in one Niagen high-dose male. This neoplastic change was considered an incidental tumor of spontaneous origin as it is reported to occur naturally in young Sprague–Dawley male rats.[22]

All other gross pathologic findings were considered incidental and not related to test item as they were randomly distributed in different groups and were not dose-dependent.

Organ weights. At 3000 mg/kg/day, there were statistically significant reductions in absolute organ weights of brain, spleen, testes, epididymides, prostate, thyroid/parathyroid, pituitary and heart in males; brain and pituitary absolute organ weights were reduced and liver and kidney absolute organ weights were increased in females. In the nicotinamide-treated group, there were statistically significant reductions in absolute organ weights of brain, spleen, testes, epididymis, prostate, thyroid/parathyroid, pituitary, thymus and heart in males; brain and pituitary absolute organ weights were reduced and liver and ovary weights were increased in females. At 1000 mg/kg/day, there were statistically significant reductions in absolute organ weights of thyroid/parathyroid, pituitary and heart in males; no effects on absolute organ weights were seen in females. At 300 mg/kg/day, there were statistically significant reductions in absolute organ weights of brain and heart in males; no effects on absolute organ weights were seen in females.

Relative organ weight changes in the 3000 mg/kg/day Niagen-treated rats were similar to those of animals ingesting an equimolar dose of nicotinamide (Table 3). Treatment-related organ weight changes were observed in liver, kidneys, testes, epididymides and ovaries in 3000 mg/kg/day Niagen- and nicotinamide-treated groups. At 1000 mg/kg/day of Niagen, increases in liver and kidney weights were statistically significant. All other relative to body weight organ weight changes, which reached

statistical significance were likely secondary to decrease in terminal body weight and/or random biological variation and not considered treatment related.

Relative to brain weight, at 3000 mg/kg/day, there were statistically significant reductions in heart, epididymides, prostate and thyroid/parathyroid in males; liver, heart, ovaries and kidney were increased in females. In the nicotinamide-treated group, there were statistically significant reductions in weights of spleen, epididymides, testes and heart in males; liver weight was increased. At 1000 mg/kg/day there were no changes in organ weights relative to brain weight in males and a statistically significant increase in liver weight in females. No changes in organ weights relative to brain weights were seen in either males or females treated with 300 mg/kg/day of Niagen.

Histopathological findings. Treatment-related histopathological changes were observed in liver, thyroid, kidneys, testes, epididymides, ovaries, and adrenals in both the 3000 mg/kg/day Niagen-treated and nicotinamide-treated groups. All other microscopic findings were considered incidental and not related to test item, as they were randomly distributed among groups. Importantly, the treatment-related histopathological changes noted in Niagen at the high dose were similar to the findings observed in the equimolar nicotinamide group.

In the livers of 3000 mg/kg/day Niagen-treated and nicotinamide-treated males and females, centrilobular hepatocellular hypertrophy was reported. This was characterized by enlarged hepatocytes containing granular eosinophilic cytoplasm, follicular cell hypertrophy, characterized by enlarged follicular epithelium, which contained pale eosinophilic cytoplasm and small clear vacuoles, and hepatocyte single cell necrosis, which was considered a treatment-related adverse change. In the kidneys, chronic progressive nephropathy characterized by presence of foci or areas of basophilic tubules, with or without simple tubular hyperplasia, hyaline casts, atrophic tubules, dilated tubule, focal glomerular sclerosis/atrophy and mononuclear cell infiltration was seen.

In male rats, both 3000 mg/kg/day Niagen-treated and nicotinamide-treated rats exhibited degeneration/atrophy of seminiferous tubules characterized by the presence of some tubules containing degenerating germ cells. Some tubules were also depleted of all germ cells and lined only by Sertoli cells and others were partially depleted of germ cells. Degenerating tubules contained multinucleated germ cells, spermatid head retention, Sertoli cell cytoplasmic vacuolation and disorganization of germ cells. Reduced sperm and cell debris in epididymal lumen in nicotinamide and Niagen high-dose males were considered treatment-related effects.

In female rats of the 3000 mg/kg/day Niagen-treated and nicotinamide-treated groups, hypertrophy of corpora lutea was seen. The affected ovaries contained large-sized corpora lutea and lightly eosinophilic cytoplasm of the enlarged luteal cells.

In male and female rats of the 3000 mg/kg/day Niagen-treated and nicotinamide-treated groups, hypertrophy of the zona glomerulosa of the adrenal cortex was considered a treatment-related non-adverse change. Hypertrophy of zona glomerulosa was characterized by increased thickness of zona glomerulosa layer and cytoplasm of hypertophic cells was lightly eosinophilic.

Discussion

The safety of a synthetic form of NR was evaluated in an Ames assay, in vitro chromosome aberration assay, in vivo micronucleus assay and acute, 14-day, and 90-day rat toxicology studies to derive a UL.

The determination of a UL for any vitamin is based on well-established principles of risk assessment and relies on data concerning adverse health effects from excessive nutrient intakes in epidemiologic studies, clinical trials, and experimental studies. Several factors associated with these various data sources influence the derivation of a UL. Among the most important of these factors are the intake at which adverse effects occur (the lowest observed adverse effect level(LOAEL)) and the maximum level of intake, which is always less than the LOAEL, at which no adverse health effects are observed (the no observed adverse effect level (NOAEL)).[4]

There are no animal toxicology studies on nicotinic acid and nicotinamide that comply with current standardized testing protocols from which to derive a UL. Therefore, authoritative bodies have used the results of clinical studies in which high doses of nicotinic acid and nicotinamide have been administered. A UL of 35 mg/day for adults was established for both nicotinic acid and nicotinamide by the IOM based on the flushing in humans as the critical adverse effect.[4] However, nicotinamide does not induce flushing when either given as an intravenous injection or orally at high-doses to patients with diabetes,[19] and, therefore, it is currently thought that the flushing effects are

related to the presence of the carboxyl group on the pyridine nucleus of nicotinic acid (Figure 1).[19] NR also lacks this carboxyl group and thus may not produce the flushing response that is associated with high nicotinic acid intakes (Figure 1). Furthermore, in vitro studies have shown that NR does not induce the GPR109A-mediated calcium flux, which is believed to be required for nicotinic acid-induced flushing.[23,24] It is therefore appropriate to derive the ULs for nicotinamide and NR based on end points other than flushing.

Consistent with this, the European Commission and UK Expert Group on Vitamins and Minerals derived independent ULs for nicotinamide and nicotinic acid. The SCF[19] set the UL for nicotinamide of 900 mg/day based on long-term studies in patients with Type 1 diabetes mellitus, at dosages of 2–3 g of nicotinamide/day, whereas the UK Expert Group on Vitamins and Minerals set the UL at 500 mg/day[25] based on human studies where large doses (up to 3000 mg/day for periods of up to 3 years) appeared to be well tolerated.

NR is not genotoxic. In addition, NR is thought to exhibit the same toxicity profile as nicotinamide, because evidence from a single dose pharmacokinetic study in humans suggests that it is metabolized in a manner similar to nicotinamide (unpublished results). Because there are no publicly available 90-day studies on either nicotinamide or NR, a toxicology study was completed where 300, 1000, or 3000 mg/kg body weight/day of NR, or 1260 mg/kg body weight/day of nicotinamide, which is equivalent to 3000 mg/kg/day dose of NR on a molar basis, was administered to rats over the course of 90 days. Adverse effects at 3000 mg/kg body weight/day of NR included treatment-related adverse effects in liver, kidneys, testes, epididymides and ovaries. These effects included increases in clinical chemistry parameters related to hepatocyte damage (ALT, ALP, and GGT) and a corresponding increase in liver weight, centrilobular hepatocellular hypertrophy, and single cell necrosis. In addition, thyroid follicular cell hypertrophy and increased kidney weight with exacerbation of chronic progressive nephropathy were observed. Statistically significant but minor reductions in sodium and chloride were seen and microscopically associated with hypertrophy of zona glomerulosa in adrenals at 3000 mg/kg. Importantly, these effects also occurred in the nicotinamide groups with a similar magnitude.

NR administration at 1000 mg/kg/day dose level resulted in treatment-related organ weight changes in liver and kidney and increases in neutrophils, ALT and triglycerides, which were statistically significant in female rats only. Although these changes were considered adverse, based on their dose-dependent responsiveness, the increases in ALT and triglycerides occurred only in one gender and were below the twofold increase that is typically used as the cutoff for a biologically significant effect in the absence of histological results.[26] The kidney weight increases at this dose also occurred in the absence of corresponding histopathology. Therefore, the liver and kidney effects at 1000 mg/kg/day were considered to be treatment related, but mild and potentially adaptive in nature due to prolonged exposure to this form of niacin. There were no treatment-related adverse effects noted at 300 mg/kg/day, although there was a slight decrease (8%) in overall body weight (day 90) at 300 mg/kg/day, which was considered adaptive. The NOAEL and LOAEL for NR were determined to 300 and 1000 mg/kg body weight/day, respectively.

A UL for human exposure to NR is derived by application of a 100-fold safety factor to the NOAEL determined from this 90-day study; the UL for NR is 3 mg/kg/day or 180 mg/day, assuming a body weight of 60 kg. Importantly, because the UL for NR falls below the ULs for nicotinamide and the adverse effects associated with ingestion of NR were similar to those found with equimolar amounts of nicotinamide, this level of intake provides an adequate margin of safety to protect consumers from the adverse effects associated with NR ingestion.

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Authors note

All authors contributed equally to this manuscript.

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Supplemental material

The online [appendices/data supplements/etc] are available at http://het.sagepub.com/supplemental.

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