2022-1116

United States Court of Appeals for the Federal Circuit

CHROMADEX, INC., TRUSTEES OF DARTMOUTH COLLEGE,

Plaintiffs-Appellants,

– v. –

ELYSIUM HEALTH, INC.,

Defendant-Appellee.

On Appeal from the United States District Court for the District of Delaware in No. 1:18-cv-01434-CFC-JLH, Chief Judge Colm F. Connolly

BRIEF FOR PLAINTIFFS-APPELLANTS

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FEBRUARY 2, 2022

ASSERTED CLAIMS

U.S. Patent No. 8,197,807

1. A composition comprising isolated nicotinamide riboside in combination with one or more of tryptophan, nicotinic acid, or nicotinamide, wherein said combination is in admixture with a carrier comprising a sugar, starch, cellulose, powdered tragacanth, malt, gelatin, talc, cocoa butter, suppository wax, oil, glycol, polyol, ester, agar, buffering agent, alginic acid, isotonic saline, Ringer's solution, ethyl alcohol, polyester, polycarbonate, or polyanhydride, wherein said composition is formulated for oral administration and increases NAD+ biosynthesis upon oral administration.

2. The composition of claim 1, wherein the nicotinamide riboside is isolated from a natural or synthetic source.

3. The composition of claim 1, wherein the formulation comprises a tablet, troche, capsule, elixir, suspension, syrup, wafer, chewing gum, or food.

[Appx2539]

U.S. Patent No. 8,383,086

1. [not asserted] A pharmaceutical composition comprising nicotinamide riboside in admixture with a carrier, wherein said composition is formulated for oral administration.

2. The pharmaceutical composition of claim 1, wherein the nicotinamide riboside is isolated from a natural or synthetic source.

[Appx2571]

UNITED STATES COURT OF APPEALS FOR THE FEDERAL CIRCUIT

CERTIFICATE OF INTEREST

Case Number	2022-1116
Short Case Caption	ChromaDex, Inc. v. Elysium Health, Inc.
Filing Party/Entity	ChromaDex, Inc.

Instructions: Complete each section of the form. In answering items 2 and 3, be specific as to which represented entities the answers apply; lack of specificity may result in non-compliance. **Please enter only one item per box; attach additional pages as needed and check the relevant box**. Counsel must immediately file an amended Certificate of Interest if information changes. Fed. Cir. R. 47.4(b).

I certify the following information and any attached sheets are accurate and complete to the best of my knowledge.

Date: 11/17/2021

Signature: /s/ Christopher N. Sipes

Name: Christopher N. Sipes

1. Represented Entities. Fed. Cir. R. 47.4(a)(1).	**2. Real Party in Interest.** Fed. Cir. R. 47.4(a)(2).	**3. Parent Corporations and Stockholders.** Fed. Cir. R. 47.4(a)(3).
Provide the full names of all entities represented by undersigned counsel in this case.	Provide the full names of all real parties in interest for the entities. Do not list the real parties if they are the same as the entities. ☐ None/Not Applicable	Provide the full names of all parent corporations for the entities and all publicly held companies that own 10% or more stock in the entities. ☐ None/Not Applicable
ChromaDex, Inc.	N/A	ChromaDex, Inc. is a wholly owned subsidiary of ChromaDex Corporation
Trustees of Dartmouth College	N/A	N/A

☐ Additional pages attached

4. Legal Representatives. List all law firms, partners, and associates that (a) appeared for the entities in the originating court or agency or (b) are expected to appear in this court for the entities. Do not include those who have already entered an appearance in this court. Fed. Cir. R. 47.4(a)(4).

☐ None/Not Applicable ☐ Additional pages attached

Emily Mondry (Covington & Burling LLP)	Evan Krygowski (Covington & Burling LLP) (no longer with firm)	James F. Haley, Jr. (Haley Guiliano LLP)
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Jason Reinecke (Covington & Burling LLP)	Pilar Gabrielle Kraman (Young, Conaway, Stargatt & Taylor LLP)	

5. Related Cases. Provide the case titles and numbers of any case known to be pending in this court or any other court or agency that will directly affect or be directly affected by this court's decision in the pending appeal. Do not include the originating case number(s) for this case. Fed. Cir. R. 47.4(a)(5). See also Fed. Cir. R. 47.5(b).

☐ None/Not Applicable ☐ Additional pages attached

ChromaDex, Inc. v. Elysium Health, Inc., No. 8:16-cv-2277 (C.D. Cal.)	ChromaDex, Inc. v. Thorne Research, Inc., No. 1:21-cv-4241 (S.D.N.Y.)	
Thorne Research, Inc. v. Trustees of Dartmouth College, No. IPR2021-00268		
Thorne Research, Inc. v. Trustees of Dartmouth College, No. IPR2021-00491		

6. Organizational Victims and Bankruptcy Cases. Provide any information required under Fed. R. App. P. 26.1(b) (organizational victims in criminal cases) and 26.1(c) (bankruptcy case debtors and trustees). Fed. Cir. R. 47.4(a)(6).

☑ None/Not Applicable ☐ Additional pages attached

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I. INTRODUCTORY STATEMENT

This appeal in a patent case raises three issues. Although discrete, they have this in common: the district court made significant errors in each, and those errors all are subject to *de novo* review by this Court.

First, the district court invalidated on summary judgment all asserted claims for lack of patent-eligible subject matter under 35 U.S.C. § 101. The court erroneously ruled under *Alice* Step One that the claims were directed to a patent-ineligible product of nature and did so by failing to consider the claims as a whole, failing to consider compelling evidence of inherency, and failing to follow binding precedent from this Court. The court likewise erred under *Alice* Step Two by failing to recognize the clear inventive step that included recognizing the potential and harnessing the power of isolating and thereby concentrating the natural substance and incorporating it into a pharmaceutical composition for oral administration.

Second, the district court held on a motion to dismiss that Plaintiff-Appellant ChromaDex, Inc. and its affiliates lacked standing to sue, notwithstanding their status as exclusive licensees under an express grant of exclusionary rights. Among other errors, the district court concluded that the mere presence of two corporate affiliates covered by the same license (commonly-owned affiliates ChromaDex and Healthspan), each with a conditional right to sublicense, deprived both entities of

standing. But this Court's precedents hold that parties can share exclusionary rights, and in a suit brought by such entities against an accused infringer, the exclusive licensees possess standing so long as the defendant has no ability to obtain a license from any other entity, as was the case here. In addition, the district court's conclusion that Healthspan could have single-handedly granted Elysium a license was itself erroneous, when it could not do so without Dartmouth's consent, and where the commercial reality—dismissed or ignored by the district court—made any possibility of licensing Elysium nonexistent.

Third, the district court's claim construction decision erroneously construed the key claim term, "nicotinamide riboside." While the claim construction did not figure into the court's ruling on § 101, in the event this Court reverses that ruling and remands, a correction of that claim construction error would materially advance and simplify further proceedings in this case. This Court should thus exercise its discretion to review and correct that error. The district court's construction (which materially broadened the subject term beyond its ordinary meaning) was not only circular; it variously characterized a sentence in the specification as explicit lexicography, implicit lexicography, and a "disclaimer"—when it was none of them.

II. STATEMENT OF RELATED CASES

No other appeal from the same proceeding has previously come before this or any other appellate court. One *inter partes* review ("IPR") involving one of the patents at issue in this appeal, U.S. Patent No. 8,383,086 ("the '086 Patent), has come before this Court in *Elysium Health, Inc. v. Trustees of Dartmouth College*, No. 19-1630, 796 F. App'x 745 (Fed. Cir. 2020). The Court summarily affirmed the Patent Trial and Appeal Board's ("the Board") final written decision, which held claim 2 patentable. Claim 2 is the only claim of the '086 Patent at issue in this proceeding. The Board's decision also held claims 1 and 3–5, which are not at issue, unpatentable.

Counsel is also aware of the following pending cases that are related to the patents in suit: *ChromaDex, Inc. v. Elysium Health, Inc.*, No. 8:16-cv-2277-CJC-DFM (C.D. Cal.); *Thorne Research, Inc. v. Trustees of Dartmouth College*, No. IPR2021-00268 (P.T.A.B.); *Thorne Research, Inc. v. Trustees of Dartmouth College*, No. IPR2021-00491 (P.T.A.B.); *ChromaDex, Inc. v. Thorne Research, Inc.*, No. 1:21-cv-4241-ER (S.D.N.Y.).

III. JURISDICTIONAL STATEMENT

The district court had subject matter jurisdiction over this patent infringement action pursuant to 28 U.S.C. § 1338(a). This Court has jurisdiction over this appeal pursuant to 28 U.S.C. § 1295(a)(1). This appeal is from a final

judgment entered on October 6, 2021 (Appx11406-11407). A Notice of Appeal was timely filed on November 2, 2021 (Appx11413-11415).

IV. STATEMENT OF THE ISSUES

On *de novo* review:

1. Did the district court err in holding all asserted claims patent-ineligible as directed to a natural product on summary judgment, after effectively distilling the claims down to a single element—isolated nicotinamide riboside ("NR")—even though *isolated* NR is not itself a natural substance, and then disregarding several other claim elements as well as the inherent properties of the claimed combination?

2. Did the district court err in finding that exclusive licensees ChromaDex and Healthspan lacked standing to sue for infringement occurring after March 12, 2017, even though they shared an express grant of exclusive rights, Elysium could not obtain a license from any other entity, and the licensees themselves did not have unrestricted rights to sublicense Elysium under the asserted patents and would not do so in any event?

3. Did the district court err in circularly construing the term "nicotinamide riboside" to include not only "nicotinamide riboside," but also esters and other derivatives thereof, where there was no explicit or implicit lexicography,

no disclaimer, and there existed unrebutted extrinsic evidence that militated against the district court's construction?

V. STATEMENT OF THE CASE

A. The Parties

Plaintiff ChromaDex has been, and continues to be, the industry leader in the science, research, and development of isolated nicotinamide riboside ("NR"), a unique form of vitamin B3 for use in oral dietary supplements. ChromaDex is a global nutraceutical company that sells, among other products, dietary supplements. These include products sold under the brand TRU NIAGEN®, a pharmaceutical composition of NR which embodies the inventions of U.S. Patent Nos. 8,197,807 (Appx2510-2540) and 8,383,086 (Appx2542-2573) (the '807 and '086 Patents) here at issue.

Plaintiff Dartmouth is a nonprofit educational research institution, and is the assignee of the foregoing patents, which name as inventor Dr. Charles M. Brenner, at one time a professor of genetics and biochemistry at the Dartmouth Medical School.

Defendant Elysium sells, among other products, a dietary supplement BASIS® through the Internet to customers nationwide. Elysium initially purchased NIAGEN® NR (the active ingredient in ChromaDex's TRU NIAGEN®) from ChromaDex for incorporation into Elysium's BASIS® product, eventually utilizing isolated NR from alternative sources.

B. Dartmouth's License To ChromaDex

In 2012 Dartmouth granted ChromaDex and its Subsidiaries an exclusive, royalty-bearing license under the '807 and '086 Patents "to make, have made, use, and/or sell" products and processes covered by the patents ("Original Agreement"). (Appx1498-1508 §§ 1.02, 2.01[1].) The license was for products in "the Field," which included dietary supplements. The territorial scope was worldwide. (Appx1498-1499 §§ 1.03, 1.04, 2.01.) Because the license to ChromaDex and its Subsidiaries was "exclusive," Dartmouth was bound not to license any other entity. (Appx1499 § 2.01.) Consistent with this grant, Dartmouth gave ChromaDex exclusionary rights, including the ability to enforce the patents against third-party infringers in patent infringement lawsuits. (Appx1505 § 8.01.)

The agreement also gave ChromaDex the right to grant sublicenses to third parties, but only with the consent of Dartmouth, which would not be unreasonably withheld. (Appx1499 § 2.02.) Dartmouth gave its consent in the license itself for a sublicense to Opko Health, Inc. (*Id.*) It did not provide consent for a sublicense to any other third party. (*Id.*)

On March 12, 2017, ChromaDex's parent company, ChromaDex Corporation, acquired Healthspan Research LLC ("Healthspan"). (Appx1590

[1] Section 1.01 defined "Dartmouth Patent Rights" to include U.S. Patent No. 8,197,807 and "continuations…thereof." (Appx1498 § 1.01.) The '086 Patent, which issued after the execution of the agreement, is a continuation of the '807 Patent. (Appx124.)

¶¶ 3-4; Appx3.) Thereafter, the ChromaDex organization, through Healthspan, sold the TRU NIAGEN® dietary supplement, which incorporated NIAGEN® NR, directly to consumers. (Appx1590 ¶¶ 3-4.) Healthspan's sales of TRU NIAGEN® competed directly with Elysium's sales of BASIS®. (Appx1590 ¶ 4; Appx1465-1466 ¶¶ 9, 13.)

Although Healthspan was not a subsidiary of ChromaDex, but rather an affiliated "sister" company commonly owned by ChromaDex Corporation (Appx1590 ¶ 3), ChromaDex treated Healthspan, *de facto*, as its subsidiary for purposes of the license agreement and paid royalties to Dartmouth on Healthspan's sales of TRU NIAGEN®. (Appx1523-1524.) The parties made this course of dealing explicit in a September 2019 Restated and Amended Exclusive License Agreement between Dartmouth and ChromaDex ("the Restated Agreement") (Appx1510-1521), as the parties explained in a concurrently executed side letter (Appx1523-1524). The Agreement was entered into during the pendency of this case, but made retroactively effective to March 13, 2017, the date on which Healthspan first sold TRU NIAGEN®. (Appx1510; Appx1523.)

The Restated Agreement was similar to the Original Agreement, but granted the exclusive license to ChromaDex and its Affiliates, for the same Licensed Products, Field and Territory identified in the Original Agreement. (Appx1511 § 2.01.) "Affiliates" was defined to mean entities that control, are controlled by, or

are under common control with ChromaDex, and that were listed in Schedule 1 to the Agreement. (Appx1511 § 1.05.) At execution, Schedule 1 listed only Healthspan Research, LLC as an Affiliate, and the schedule was not subsequently amended. (Appx1521 Schedule I.) As with the Original Agreement, Dartmouth's exclusive license to ChromaDex and its Affiliates meant that Dartmouth promised not to grant licenses to any other entity (Appx1511 § 2.01), and ChromaDex and its Affiliates were entitled to enforce the Licensed Patents against third parties in patent infringement lawsuits (Appx1517 § 8.01).

The Restated Agreement also gave ChromaDex and its Affiliates the conditional right to grant sublicenses to third parties, but only "with the consent of Dartmouth …." (Appx1511 § 2.02.) Dartmouth did not provide its advance consent for a sublicense to any third party other than Opko Health, Inc. (*Id.*)

C. Elysium's Inability To Obtain A License And Infringement Of The Asserted Patents

By the time the present action was filed in September 2018, the relationship between ChromaDex and Elysium, which had previously purchased NIAGEN® brand NR from ChromaDex for incorporation into Elysium's BASIS® product, had deteriorated. Elysium had hired away ChromaDex's Vice President of Sales and Marketing, Mark Morris. This and other activities gave rise to trade secret misappropriation and breach of contract claims by ChromaDex against Elysium in the Central District of California. (Appx11 n.3, Appx1675, Appx1684-1694; *see*

ChromaDex, Inc. v. Elysium Health, Inc., No. 8:16-cv-02277 (C.D. Cal.).) ChromaDex and Elysium also were in litigation in the Southern District of New York in a subsequent suit involving mutual allegations of unfair business practices. (Appx11 n.3, Appx1591-1592 ¶ 7; Appx1569 n.2; *see In re Elysium Health-ChromaDex Litig.*, No. 1:17-cv-07394 (S.D.N.Y.).) Yet further, Elysium filed IPR petitions against Dartmouth's '807 and '086 Patents, which preceded the present action. (Appx1685 n.3; *Elysium Health, Inc. v. Trs. of Dartmouth Coll.*, IPR Nos. IPR 2017-1795, IPR 2017-1796.)

Mark Friedman, ChromaDex's General Counsel and Secretary and a member of ChromaDex's executive management team, submitted an unrebutted declaration in the district court, stating "[d]efinitively and without reservation" that in light of Elysium's interference with ChromaDex's business and the pending litigation in different forums, "Elysium would not have been able to obtain a license to the Asserted Patents from ChromaDex or Healthspan." (Appx1589-1592 ¶ 7; *see also* Appx1604, Appx1641 #4167 (Morris telling Elysium's co-founder and COO Dan Alminana "[w]e will make their worst nightmares come true!"), Appx1658 #4305 (Morris stating "I want to destroy them!").)

Furthermore, as Mr. Friedman explained, ChromaDex and Healthspan were under the common control of ChromaDex Corporation and managed by the same executive management team, consisting of Mr. Friedman, Rob Fried (CEO), and

Kevin Farr (CFO). (Appx1590-1592 ¶¶ 5-7, Appx11 n.3.) Therefore, all decisions for these companies were made in the interests of the ChromaDex organization as a whole, and Healthspan would not (and could not) act against ChromaDex's interests by extending a sublicense to Elysium, nor would ChromaDex act against Healthspan's interests by doing that either. (Appx1590-1592 ¶¶ 5-7.) Elysium never challenged the factual assertions in this declaration, nor did it submit any of its own evidence to establish that ChromaDex or Healthspan would have, or could have, extended a license to Elysium.

Elysium also submitted no evidence to establish that Dartmouth ever consented, or would give consent, to a sublicense to Elysium or that it would have been unreasonable for Dartmouth to withhold its consent to such a license. To the contrary, Elysium was actively challenging and attempting to invalidate Dartmouth's '807 and '086 Patents in IPR proceedings, and Dartmouth was forced to file suit against Elysium for infringement of those patents in 2018, further demonstrating that Dartmouth would not give consent to a sublicense under the asserted patents. (Appx1591-1592 ¶ 7.)

D. The Litigation

ChromaDex and Dartmouth together filed this action against Elysium on September 17, 2018, asserting that Elysium was infringing the '807 and '086 Patents through the manufacture, use, and sale of BASIS® products,

eventually asserting claims 1-3 of the '807 Patent and claim 2 of the '086 Patent.[2] The complaint alleged that ChromaDex was the exclusive licensee under the '807 and '086 Patents in the field of dietary supplements. (Appx76 ¶ 4.)

1. The District Court's Rulings Regarding Standing

On April 24, 2020, ChromaDex filed a motion to amend the complaint to add Healthspan as a plaintiff, and on May 5, 2020, Elysium filed a motion to dismiss claims brought by ChromaDex for lack of subject matter jurisdiction based on ChromaDex's purported lack of standing. Briefing on the two motions was consolidated, as both motions addressed common issues of standing.

Elysium's contention was that two parties could not both be exclusive licensees, and that ChromaDex lacked standing because its affiliate Healthspan also had a license and the right to sublicense Elysium after March 13, 2017, thereby destroying ChromaDex's exclusionary rights. (Appx1538-1539, 1544-1546, 1548-1549.) The district court agreed (Appx13-14; Appx19-20), even though: (1) there was no evidence that Elysium could obtain a license from any party outside of the exclusively licensed family of companies; (2) any sublicense to Elysium from ChromaDex or Healthspan required consent from Dartmouth (Appx1511 § 2.02), and there was no evidence that Dartmouth had authorized, or

[2] The two patents share a common specification. The asserted claims are reproduced on the inside cover of this brief. Citations herein are to the specification of the '807 Patent.

would authorize, Elysium as a sublicensee; and (3) by the district court's own acknowledgement, ChromaDex and Healthspan were under common management (Appx11 n.3), and "Healthspan would likely never have agreed to give Elysium a license to the asserted patents" (Appx11).

Based on its conclusion that ChromaDex lacked exclusionary rights, the district court concluded that ChromaDex did not have standing for accused infringement by Elysium occurring after March 12, 2017, when the Restated Agreement took retroactive effect (Appx14). *ChromaDex, Inc. v. Elysium Health, Inc.*, 570 F. Supp 3d 579 (D. Del 2020). The court likewise held that Healthspan did not have standing to sue for that period because ChromaDex had the right to extend a sublicense to Elysium, and thus the court denied Plaintiffs' motion to amend to add Healthspan as a plaintiff. (Appx19-20.)

For the period prior to March 13, 2017, the district court held that the Original Agreement (in place when Plaintiffs first commenced this lawsuit) applied. The court held that for that earlier period, ChromaDex had standing as an exclusive licensee, and that no other party had the ability to license Elysium. (Appx14.) Accordingly, the district court held that ChromaDex had standing to allege infringement by Elysium from the July 13, 2012 effective date of the Original Agreement until March 12, 2017. (*Id.*)

Following the district court's rulings, Dartmouth and ChromaDex further amended their agreement on December 29, 2020 ("Amended Agreement") to include recitals and an amended Section 2.02 reflecting that the parties to the license agreement always intended and understood that ChromaDex's and Healthspan's exclusive rights under the agreement required them to act in unison and not adversely to one another in connection with the conditional right to grant sublicenses. (Appx2622-2624, 4th, 6th, 7th, 10th WHEREAS clauses, § 2.02(b), Appx2626.) The amendment did not otherwise substantively alter the agreement. Reflecting the commercial reality as it existed at the time, the parties made the amendment effective retroactively as of March 13, 2017. (Appx2622.)

On December 29, 2020, Plaintiffs moved for reconsideration of the district court's rulings concerning standing in light of the Amended Agreement and the impending dissolution of Healthspan, contending that the amendment confirmed that Healthspan did not have the ability to sublicense Elysium without ChromaDex's prior consent, which ChromaDex would not have granted. (Appx2598, Appx2603-2604.) On April 27, 2021, the district court denied Plaintiffs' motion for reconsideration. The dissolution of Healthspan in January 2020 and the Amended Agreement, according to the court, did not constitute "newly available evidence," and Plaintiffs had not met the standard for

reconsideration. (Appx25-26.) The district court did not evaluate the merits of Plaintiffs' arguments for standing in light of the additional evidence presented.[3]

2. The District Court's Ruling Regarding Claim Construction

On January 5, 2021, the district court issued a claim construction order. (Appx21-23.) One construction, while not implicated in the § 101 ruling discussed next, warrants this Court's discretionary review. The district court construed "nicotinamide riboside" to mean "nicotinamide riboside or a derivative (*e.g.* L-valine or L-phenylalanine esters) of nicotinamide riboside." (Appx22.) The court's construction was based on a single sentence in the specification. (Appx2526 28:63-65.) The court's stated reasons included explicit and implicit lexicography and a "disclaimer," which *broadened* the claim.[4]

3. The District Court's Ruling Regarding § 101

On May 4, 2021, Elysium moved for summary judgment that the asserted claims of the '807 and '086 Patents were invalid under 35 U.S.C. § 101 for claiming unpatentable products of nature. In opposing the motion, Plaintiffs submitted what would remain unrebutted evidence regarding the inherent

[3] Plaintiffs do not appeal herein the district court's ruling on the motion for reconsideration. However, Plaintiffs reserve all rights and arguments concerning the impact of the Amended Agreement and the dissolution of Healthspan on standing, either in the present case or in any subsequently filed case.

[4] The district court's claim construction order referred to the December 17, 2020 *Markman* hearing. The pertinent portions of that transcript, discussed in detail, *infra*, appear at Appx11543-11565.

properties of a key ingredient recited in all the asserted claims—isolated nicotinamide riboside ("NR"). Plaintiffs marshalled that evidence to address this Court's recent holding regarding the patent-eligibility of compositions of naturally occurring compounds as set forth in *Natural Alternatives International, Inc. v. Creative Compounds, LLC*, 918 F.3d 1338 (Fed. Cir. 2019).

On September 21, 2021, the district court granted Elysium's motion for summary judgment. *ChromaDex, Inc. v. Elysium Health, Inc*., No. 18-1434-CFC, 2021 U.S. Dist. LEXIS 179771 (D. Del. Sept. 21, 2021) (Appx28-43; Appx44.) The decision was based entirely on the fact that NR is a naturally occurring substance (found in trace amounts in milk) and that the properties of *isolated* NR upon which Plaintiffs were relying (stability, bioavailability, sufficient purity, and therapeutic efficacy), as compared with naturally occurring (*i.e.*, nonisolated) NR, were not expressly recited in the claims. Accordingly, the court did not question or even address the substantial and unrefuted evidence of inherent properties submitted by Plaintiffs, concluding that the failure to expressly recite those properties in the asserted claims was dispositive to the § 101 analysis.

This appeal followed.

VI. SUMMARY OF THE ARGUMENT

1. With regard to § 101, the district court erred at *Alice* Step One in holding that the claims were directed to a naturally occurring substance by, first,

disregarding limitations, ignoring the claimed invention as a whole, and treating the claims as being solely directed to isolated NR in and of itself. The district court then failed to give credence to unrebutted evidence of inherent properties of the claimed combination, such as stability and bioavailability, which are not found with naturally occurring NR. Thus, while the claims incorporate a natural substance, the claimed combination *as a whole* has materially different characteristics and can be used in a manner that the natural substance NR itself cannot.

While the court's error regarding Step One should obviate the need to consider Step Two, there too, the district court failed to appreciate the "inventive steps" of discovering the untapped potential of NR, isolating the NR, and formulating it into a pharmaceutical composition for oral administration. This showing by Plaintiffs more than clears the bar for Step Two, but at a minimum created a genuine issue of material fact that should not be resolved on summary judgment.

2. With regard to standing, the district court held that only Dartmouth had standing to sue for infringement on or after March 13, 2017, and that ChromaDex and Healthspan did not. In reaching this conclusion, the district court committed several errors.

First, the district court concluded that both ChromaDex and Healthspan lacked exclusionary rights under the asserted patents because both entities had licenses, including the purported legal right to sublicense Elysium, and thus either entity could undercut the exclusionary rights of the other. As this Court's precedents demonstrate, however, two entities may share exclusionary rights. Here, the grant of exclusionary rights to ChromaDex and its Affiliates was express and unambiguous in a single agreement, including the right to sue and obtain remedies for infringement. In any suit brought to enforce such exclusionary rights, all holders of the shared rights possess standing and are only deprived of it if the defendant can obtain a license from a third party. *WiAV Solutions, LLC v. Motorola, Inc.*, 631 F.3d 1257, 1267 (Fed. Cir. 2010). Here, it is undisputed that Elysium could not.

Second, the express language of the contract shows that ChromaDex and Healthspan themselves did not have the unrestricted ability to sublicense Elysium. The contract states that any sublicense requires Dartmouth's consent, making the ability to grant sublicenses conditional and not permitted without factual predicates that are absent from the record below. Because neither ChromaDex nor Healthspan had an unrestricted legal right to grant sublicenses, the district court's resulting conclusion that ChromaDex and Healthspan lacked standing for infringing acts after March 12, 2017 was in error.

Third, the district court erred by disregarding the Court's holding in *WiAV* that an exclusive licensee's standing is not nullified by another licensee's sublicensing rights if the accused infringer is incapable of obtaining a license. *E.g.*, *WiAV*, 631 F.3d at 1266-67 ("[I]f an exclusive licensee has the right to exclude others from practicing a patent, and a party accused of infringement does not possess, ***and is incapable of obtaining, a license of those rights from any other party,*** the exclusive licensee's exclusionary right is violated.") (emphasis supplied). The record in the district court established unequivocally that Healthspan would not extend a sublicense to Elysium. Elysium provided no factual rebuttal. Yet, the district court deemed Plaintiffs' unrebutted evidence irrelevant and ignored this Court's guidance in *WiAV* that standing depends on the accused infringer's ability to obtain a license. Because it is undisputed that Elysium could not have obtained a license from either ChromaDex or Healthspan (or from Dartmouth), ChromaDex's and Healthspan's exclusionary rights against Elysium were preserved, and ChromaDex and Healthspan had standing to sue for injury to those rights caused by Elysium's infringement.

3. With regard to claim construction, the district court erred in adopting a circular construction, which includes in the construction the very term that is being construed. That alone is a compelling reason to reject it.

The district court further erred in finding an explicit definition to justify the addition of derivatives to the term "nicotinamide riboside," where the specification consistently followed a linguistic formula making clear when the inventor was acting as his own lexicographer, and that formula was not followed here. Nor was there implicit lexicography, where the context of the critical language in the specification made clear that the inventor was actually identifying one of his many inventions, which was not the invention claimed in the patents here in suit. And there could not have been a disclaimer because the inclusion of derivatives did not surrender claim scope but broadened it.

Although the subject claim construction did not figure in the other issues here under review, this Court should exercise its discretion to review the issue, as it will significantly benefit proceedings on remand.

VII. ARGUMENT

A. The District Court Erred In Invalidating The '807 And '086 Patents Under 35 U.S.C. § 101 On Summary Judgment

1. Statement Of The Standard Of Review

Issued patents are presumed to be valid. 35 U.S.C. § 282. Patent eligibility under § 101 presents an issue of law, which this Court reviews *de novo*. *Accenture Global Servs. v. Guidewire Software, Inc.*, 728 F.3d 1336, 1340-41 (Fed. Cir. 2013).

Where underlying facts in a § 101 analysis are at issue, the party asserting invalidity must prove them by clear and convincing evidence. *Berkheimer v. HP, Inc.*, 881 F.3d 1360, 1368 (Fed Cir. 2018). When viewed through the lens of the clear and convincing standard, "a moving party seeking to invalidate a patent at summary judgment must submit such clear and convincing evidence of invalidity so that no reasonable jury could find otherwise." *Eli Lilly & Co. v. Barr Labs., Inc.*, 251 F.3d 955, 962 (Fed. Cir. 2001).

And where, as here, factual issues were raised in the context of a summary judgment motion, this Court reviews the grant of summary judgment under the law of the regional circuit—here, the Third Circuit. As this Court has explained: "The Third Circuit employs plenary review of a district court's grant of summary judgment, viewing the facts in the light most favorable to the non-moving party." *Accenture,* 728 F.3d at 1340.

2. The District Court Erred In Its *Alice* Step One Analysis

As the district court correctly noted at the very outset (Appx35), "Under step one of *Mayo/Alice*, the claims are considered in their entirety to ascertain whether their character as a whole is directed to excluded subject matter." (Citing *Internet Patents Corp. v. Active Network, Inc.,* 790 F.3d 1343, 1346 (Fed. Cir. 2015)). Although correctly acknowledging this black-letter law, the district court failed to follow it.

In its opinion, the district court pointed out that Elysium had argued in its briefing that the asserted claims are directed to nothing but "compositions comprising isolated nicotinamide riboside ('NR')…a naturally-occurring vitamin present in cow milk." (Appx35 (citing Appx2709).) The district court then stated, "ChromaDex does not dispute this description of the asserted claims' subject matter." (Appx35.)

This was manifestly incorrect. Contrary to the district court's conclusion that Plaintiffs conceded the issue, in opposing Elysium's motion, Plaintiffs did indeed point out that the claims required more than simply isolated NR. (*See, e.g.*, Appx9689 (pointing out that claims require compositions capable of improving health and well-being or enhanced NAD+ biosynthesis); Appx9692 (pointing out that claims require oral administration).) Moreover, just three pages after Elysium's one-limitation mischaracterization of the claims, Elysium itself had more candidly explained, "[t]he claims of the '807 patent are 'directed to' compositions containing NR in combination with tryptophan, nicotinic acid, and/or nicotinamide, where the compositions increase NAD+ biosynthesis upon oral administration." (Appx2712.)

The district court's analysis ignored many of the express limitations in the claims of the patents-in-suit. And to the extent the court addressed the one limitation on which it focused—isolated NR—it erred in doing so by improperly

requiring that its inherent properties be spelled out in the claims to be considered in a § 101 analysis.

a. The District Court Fully Ignored Several Claim Limitations

All of the asserted claims require that the isolated NR be in an admixture with a carrier, several of which are enumerated in the '807 claims. Asserted claim 2 of the '086 Patent requires a *"pharmaceutical"* composition, construed by the district court to mean "a composition that can be used to improve or prolong the health or well-being of humans or other animals." All asserted claims require that the composition be "formulated for oral administration." Claim 1 of the '807 Patent and claims 2 and 3, which depend from it, further require that the isolated NR be in combination with tryptophan, nicotinic acid, or nicotinamide. The '807 claims as construed also include, as an affirmative limitation, that the composition "increases NAD+ biosynthesis upon oral administration" (as compared to the level if the composition were not administered). (*See* Appx22.)[5]

Nowhere in its four-page discussion of *Alice* Step One did the court so much as mention these limitations. In full effect, it treated the claims as though they read: "a composition comprising isolated NR"—full stop.

[5] "NAD+" (nicotinamide adenine dinucleotide) is necessary for maintaining cellular energy levels and is considered to have "immeasurable importance in cellular metabolism." (Appx2855.)

Addressing issues under § 101 does not and should not justify departing from foundational principles that permeate patent law. "It is a bedrock principle of patent law that the claims of a patent define the invention to which the patentee is entitled the right to exclude." *Innova/Pure Water, Inc. v. Safari Water Filtration Sys.*, 381 F.3d 1111, 1115 (Fed. Cir. 2004). Accordingly, any analysis of a patent claim "begins and ends in all cases with the actual words of the claim …." *Renishaw PLC v. Marposs Società Per Azioni*, 158 F.3d 1243, 1248 (Fed. Cir. 1998).

In the present case, it is of course true that isolated NR is an important element of the asserted claims. However, it is well settled that "there is no legally recognizable or protected essential element, gist or heart of the invention in a combination patent. Rather, the invention is defined by the claims." *Allen Eng'g Corp. v. Bartell Indus*., 299 F.3d 1336, 1345 (Fed. Cir. 2002) (internal quotations omitted) (quoting *Aro Mfg. Co. v. Convertible Top Replacement Co.*, 365 U.S. 336, 345 (1961)).

In fact, on the same day that Elysium was inviting the court to disregard claim limitations for § 101 purposes, in another of Elysium's four summary judgment motions, Elysium was touting the significance of those limitations in arguing that the claims were invalid under 35 U.S.C. § 112. (*See, e.g.*, Appx3113-3114 (describing as "relevant" for § 112 purposes the required increase in NAD+

biosynthesis, the need for a "pharmaceutical composition," and the capability of improving health and well-being); Appx3114 n.2 (describing "the composition as a whole" and the '807 Patent's requirement of tryptophan, nicotinic acid or nicotinamide); Appx3119 (the '807 composition "must increase NAD+ biosynthesis upon oral administration").)

This Court has repeatedly cautioned that courts must be careful in a § 101 analysis to avoid oversimplifying claims "by looking at them generally and failing to account for the specific requirements of the claims." *McRo, Inc v. Bandai Namco Games Am. Inc.*, 837 F.3d 1299, 1313 (Fed. Cir. 2016). And "describing the claims at such a high level of abstraction and untethered from the language of the claims all but ensures that the exceptions to § 101 swallow the rule." *Enfish LLC v. Microsoft Corp*., 822 F.3d 1327, 1337 (Fed. Cir. 2016). "The *Alice* inquiry does not myopically focus on a claim's novel features when the claim as a whole suggests a different focus." *Nevro Corp. v. Boston Sci. Corp.*, 21-258, 2021 U.S. Dist. LEXIS 242032, at *12 (D. Del. Dec. 20, 2021) (Connolly, C.J.).

The district court did cite *Synopsys, Inc. v. Mentor Graphics Corp.*, 839 F.3d 1138, 1149 (Fed. Cir. 2016), for its statement that "[t]he §101 inquiry must focus on the language of the Asserted Claims themselves." (Appx37.) In *Synopsys*, unlike in this case, the court was addressing the *absence* of claim limitations. The patentee tried to avoid a holding that its claim was directed to mental steps by

arguing that the invention was intended to be used on a computer; but the claims did not call for computer implementation. Here, the claims *have* significant limitations above and beyond isolated NR, but both Elysium and the district court chose to disregard them.

Thus, while the district court correctly cited this Court's directive to consider asserted claims *in their entirety* to ascertain whether *their character as a whole* is directed to ineligible subject matter, the district court manifestly failed to do so when tasked with deciding whether the claims are "directed to" a product of nature.

b. The District Court Erred As A Matter Of Law In Categorically Refusing To Consider Inherent Properties Of Claimed Components

This Court recently provided important guidance on how *Alice* Step 1 should be approached in the context of a claim to a composition of matter. As stated in *Natural Alternatives*, "[a] claim to a manufacture or composition of matter made from a natural product is not directed to the natural product where it has different characteristics and 'the potential for significant utility'" *Natural Alternatives*, 918 F.3d at 1378 (quoting *Diamond v. Chakrabarty*, 447 U.S. 303, 310 (1980)). Thus, such product claims satisfy the *Alice* Step 1 test for patent-eligible subject matter where such claims "are directed to specific treatment formulations and incorporate

natural products, but they have different characteristics and can be used in a manner that [the natural product] as it appears in nature cannot." *Id.*

In examining whether it was legally proper for the district court to disregard inherent properties that are not spelled out in a patent claim, it is instructive to go back to basics, and particularly the following observations made over 50 years ago by Judge Giles Rich:

> From the standpoint of patent law, a compound and all of its properties are inseparable; they are one and the same thing. The graphic formulae, and the chemical nomenclature, the systems of classification and study such as the concepts of homology, isomerism, etc., are mere symbols by which compounds can be identified, classified, and compared. But a formula is not a compound and while it may serve in a claim to identify what is being patented, as the metes and bounds of a deed identify a plot of land, the thing that is patented is not the formula but the compound identified by it. And the patentability of the thing does not depend on the similarity of its formula to that of another compound but of the similarity of the former compound to the latter. There is no basis in law for ignoring any property in making such a comparison.

In re Papesch, 315 F.2d 381, 391 (C.C.P.A. 1963).

This concept pervades numerous issues in patent law. For example, in the law of anticipation, "a prior art reference may anticipate without disclosing a feature of the claimed invention if that missing characteristic is necessarily present, or inherent, in the single anticipating reference." *Schering Corp. v. Geneva Pharms., Inc.*, 339 F.3d 1373, 1377 (Fed. Cir. 2003). Indeed, "inherent anticipation does not require a person of ordinary skill in the art to recognize the inherent

disclosure in the prior art at the time the prior art is created." *SmithKline Beecham Corp. v. Apotex Corp.*, 403 F.3d 1331, 1343 (Fed. Cir. 2005).

Similarly, the same principles have been applied by this Court in analyzing compliance with the written description requirement of 35 U.S.C. § 112. *See, e.g.*, *Yeda Rsch. & Dev. Co. v. Abbott GmbH & Co. KG*, 837 F.3d 1341, 1345 (Fed. Cir. 2016) ("Under the doctrine of inherent disclosure, when a specification describes an invention that has certain undisclosed but inherent properties, that specification serves as adequate written description to support a subsequent patent application that explicitly recites the invention's inherent properties."); *see also Kennecott Corp. v. Kyocera Int'l, Inc.*, 835 F.2d 1419, 1423 (Fed. Cir. 1987) ("The disclosure in a subsequent patent application of an inherent property of a product does not deprive that product of the benefit of an earlier filing date.").

There is simply no precedent or reason to single out patent eligibility under § 101 and remove it from the mainstream of patent jurisprudence by mandating that courts ignore inherent properties of components recited in claims. A claim that calls for a mixture of H_2O and NaCl need not expressly recite that it is wet and salty. Accordingly, this Court can and indeed should approach the question of whether a claim is "directed to" a product of nature under *Alice* Step 1 by not limiting itself to the actual words of a claim, but should consider any and all properties that are shown to be inherent in the claimed invention even if such

properties are not expressed in the claims. The district court erred as a matter of law in failing to do so.

c. The District Court Erroneously Disregarded Compelling And Unrefuted Evidence Of "Different Characteristics" And "The Potential For Significant Utility"

Having committed legal error by holding that inherent properties may not be considered in an *Alice* analysis, the district court compounded that error by ignoring the only evidence adduced on that issue. As the party challenging validity, Elysium had the burden of adducing clear and convincing evidence that the isolated NR in the claimed compositions did not manifest different characteristics or the potential for significant utility. *See Natural Alternatives*, 918 F.3d at 1348. Elysium adduced no such evidence. Plaintiffs, however, adduced compelling evidence of inherency, which Elysium did not even try to refute.

The district court stated that its construction of isolated NR "in no way requires that the NR in the claimed composition…have a therapeutic effect." (Appx38-39.) But the claims as a whole, as construed, most assuredly require a therapeutic effect. The asserted '807 claims require that the composition "increases NAD+ biosynthesis upon oral administration" (Appx2539), while the asserted '086 claim requires a "pharmaceutical composition" (Appx2571), construed to require that the composition "can be used to improve or prolong the health or well-being of humans or other animals." (Appx23).

At the outset, it is undisputed that NR is found in milk in only trace amounts: about one part per million—some three orders of magnitude less than the minimum taught in the patents to achieve effective uses and nowhere near enough to have therapeutic effect. (Appx10162-10163; Appx10166; Appx10174-10175; Appx10207-10208, at 28:19-29:11; Appx2527, at 30:23-29 (claimed compositions need at least 0.1% isolated NR to improve health and well-being).)

The district court declared it was "undisputed that NR in milk…enhanced NAD+ biosynthesis…." (Appx37.) But that assertion was neither undisputed nor correct. Elysium itself acknowledged that "while NR…can be found in trace amounts in various foods…[and] one cannot eat enough of anything to boost NAD+ levels." (Appx10245.) The district court disregarded this evidence.

And what little NR is found in milk is not bioavailable. Elysium's own expert explains that the NR in milk is bound to the lactalbumin whey protein such that it is not biologically available. (Appx10205, at 20:2-8.) In contrast, as reported in a 2017 article, "the molecules in [Elysium's] Basis have been demonstrated to be bioavailable, meaning they get into the body and do what they're intended to, including raising NAD+ levels." (Appx10249.)

As for whether the claims satisfy *Natural Alternatives*' requirement that the claimed compositions have the "the potential for significant utility," isolated NR has been proven (in a sufficient dose) to be uniquely effective, and its ability to

enhance NAD+ biosynthesis allows the enhancement of cellular health beyond anything achieved with milk. (Appx10192; Appx10195-10197.)

In response to Plaintiffs' argument that the isolation of the NR "in the claimed compositions" created its stability, bioavailability and purity, the district court cited *Association for Molecular Pathology v. Myriad Genetics, Inc*., 569 U.S. 576, 580 (2013), for its holding that "a naturally occurring DNA segment is a product of nature and not patent-eligible merely because it has been isolated." (Appx38.) But in *Myriad*, the claim—in its entirety—was directed to nothing more than an isolated DNA segment. Here, the "claimed compositions" are directed to far more than simply isolated NR. (*See* Section VII.A.2.a, *supra*.)

In declining "to import details not claimed" as a basis for avoiding a holding of invalidity, the district court cited *ChargePoint, Inc. v. SemaConnect, Inc*., 920 F.3d 759, 769 (Fed. Cir. 2019), *cert. denied*, 140 S.Ct. 983 (2020). (Appx39.) There, the court was examining "the claim language itself to consider the extent to which the claim would preempt building blocks of science and technology." *Id.* at 768. Here, the district court never undertook such a preemption analysis. But it was well aware of the limits of Plaintiffs' claims when it noted that claim 1 of the '086 Patent—which is *not* limited to *isolated* NR—was invalidated in an IPR proceeding. (*See* Appx31, at 2 n.1.)

Accordingly, this Court should conclude that the claims here at issue satisfy *Alice* Step 1 (making it unnecessary to advance to Step 2), or that there is at least a genuine issue of material fact that should have precluded the grant of summary judgment against Plaintiffs on § 101 grounds.

3. The District Court Erred In Its *Alice* Step Two Analysis

Inasmuch as Elysium failed to prove by clear and convincing evidence, and beyond any genuine issue of fact, that the asserted claims are directed to a product of nature, there is no need for this Court to reach Step Two. If it does so, however, it should conclude that the district court erred here as well.

The Step Two analysis again requires this Court to consider the asserted claims at issue—*in their entireties*. Doing so reveals that they spell out an "inventive step" of recognizing the utility of NR for enhancing health and well-being and the wisdom of *isolating* the NR to provide concentrations higher than what occur naturally. As claimed, such isolated NR is *formulated for oral administration* to achieve beneficial effects.

The district court rejected this argument by, unhelpfully, noting things Plaintiffs *did not* invent: the concept of isolating a compound or any specific technique for isolation; the specific carriers used in the claimed compositions; etc. (Appx40.) The court then stated: "Because NR's oral bioavailability is an inherent property of NR *and thus is itself a natural phenomenon*, ChromaDex did not alter

NR to create this property." (Appx41 (emphasis added).) But NR as it naturally exists in trace amounts in milk *is not* bioavailable, as demonstrated above. And while Plaintiffs did not "alter" the molecular structure of NR, Dr. Brenner *did* appreciate that NR could be isolated and formulated into orally administered pharmaceutical compositions where the NR *would* be bioavailable and where it *would* be a safe and effective vehicle to improve health.

As in *Rapid Litigation Management Ltd. v. CellzDirect, Inc.*, 827 F.3d 1042 (Fed. Cir. 2016), Dr. Brenner made a "discovery of something natural" (the untapped potential for NR to provide significant health benefits) that led him to do something that "can hardly be considered routine or conventional" (he isolated the NR and formulated it into a composition for oral administration). "To require something more at [*Alice*] step two would be to discount the human ingenuity that comes from applying a natural discovery in a way that achieves a new and useful end." *Id.* at 1051-52 (internal quotations omitted).

B. The District Court Erred In Holding That ChromaDex And Healthspan Lacked The Exclusionary Rights Necessary To Confer Standing

1. Statement Of The Standard Of Review

The issue of constitutional standing to sue for patent infringement is a question of law that this Court reviews *de novo*, applying Federal Circuit precedent. *Prima Tek II, LLC v. A-Roo Co.*, 222 F.3d 1372, 1376 (Fed. Cir. 2000).

2. ChromaDex And Healthspan Had Standing Based On Their Right To Exclude Elysium

a. The Touchstone Of Constitutional Standing Is A Licensee's Right To Exclude The Defendant From Practicing The Invention

For a party to establish constitutional standing, it must "show that the conduct of which [it] complains has caused [it] to suffer an 'injury in fact' that a favorable judgment will redress." *Elk Grove Unified Sch. Dist. v. Newdow*, 542 U.S. 1, 12 (2004). In patent cases, "the touchstone of constitutional standing … is whether a party can establish that it has an exclusionary right in a patent that, if violated by another, would cause the party having the exclusionary right to suffer legal injury." *WiAV*, 631 F.3d at 1265.

In cases involving an express exclusive license, the question is *not* whether the licensee "has established that it has the right to exclude *all* others from practicing the patent." *WiAV*, 631 F.3d at 1267 (emphasis in original). "The question is whether [the licensee] has shown that it has the right under the patents to exclude *the Defendants* from engaging in the alleged infringing activity and therefore is injured by the Defendants' conduct." *Id.* (emphasis in original).[6]

[6] Exclusive licensees that do not possess all substantial rights in a patent must generally join the patent owner in an infringement suit. *WiAV*, 631 F.3d at 1264-65; *Morrow v. Microsoft Corp.*, 499 F.3d 1332, 1340 (Fed. Cir. 2007). Whether ChomaDex and Healthspan were granted "all substantial rights" is not at issue here because patentee Dartmouth was named as a plaintiff.

The linchpin of Elysium's argument against standing in the district court—that there cannot be two entities that share exclusive rights under a patent (*e.g.*, Appx1539)—is false. "Exclusive licensee" does not mean, and is not limited to, one and only one licensee. Under this Court's precedents, exclusive licensees possess constitutional standing even if the patent is licensed to others in the same field (and even if other licensees have the right to sublicense), so long as the exclusive licensees have the right to exclude the particular defendant they have sued. *WiAV*, 631 F.3d at 1267 ("an exclusive licensee does not lack constitutional standing to assert its rights under the licensed patent merely because its license is subject not only to rights in existence at the time of the license, but also to future licenses that may be granted only to parties other than the accused"); *see also Intellectual Prop. Dev., Inc. v. TCI Cablevision of Cal., Inc.*, 248 F.3d 1333, 1337, 1345 (Fed. Cir. 2001) (plaintiff not deprived of standing as exclusive licensee by continuing prior license under the same patent).

In *WiAV*, plaintiff WiAV was expressly granted an "exclusive right" to practice the asserted patents, although those patents had been licensed to several licensees other than WiAV, including at least Conexant, Mindspeed, Skyworks, and Qualcomm in the same "Wireless Handset" field. *WiAV*, 631 F.3d at 1260-62. Some of those licensees, including Conexant, Mindspeed, and Qualcomm, themselves had the right to grant further sublicenses. *Id.* Table 1. Yet WiAV was

held to be an exclusive licensee with standing to sue because the defendants could not obtain a license in the Wireless Handset field from any of the licensees who were not parties to the suit. *Id.* at 1267. In so holding, this Court specifically rejected the defendants' argument that "a licensee cannot be an exclusive licensee of a patent if others retain the right to license the patent." *Id.* at 1263-64; *see also id.* at 1266 (rejecting argument that licensee, to be an exclusive licensee, must be "the *only* party with the ability to license the patent" (emphasis in original)).

Similarly, in *Sanofi-Aventis Deutschland GmbH v. Glenmark Pharms., Inc.*, 748 F.3d 1354 (Fed. Cir 2014), this Court affirmed a district court's finding that separate companies, Abbott Laboratories and Abbott Laboratories, Inc. (ALI) possessed standing because both had "fully exclusive rights in the United States." *Id.* at 1363-1365. Because the evidence supported an "exclusive license to [both] United States companies" under the asserted patent, *id.* at 1365, the companies shared exclusive rights and both had standing to sue for injury caused by the defendant's infringement, *id.* at 1364-65.

For these reasons, Elysium's suggestion that a patentee cannot license a company and its closely related corporate affiliates without destroying the exclusivity of the license—and the licensees' ability to sue for injury caused by infringement—is wrong.

b. ChromaDex And Its Affiliates Had An Exclusive License And The Right To Exclude Elysium From Practicing The Asserted Patents

Under the Restated Agreement, ChromaDex and its Affiliates received an exclusive license under the asserted patents, with the right to exclude third parties such as Elysium.[7] The license to ChromaDex and its Affiliates to practice the invention was expressly "exclusive," such that Dartmouth was prohibited from granting further licenses to anyone else. (Appx1511 § 2.01.) ChromaDex and its Affiliates also received the right to exclude infringers from the market via patent infringement suits and requests for injunctive relief. (Appx1517 § 8.01.) The agreement thus expressed the clear intention of the contracting parties to grant ChromaDex and its Affiliates exclusionary rights, and did so with express language. Such an agreement is the antithesis of a "bare" license that would deprive the licensees of standing.

Contrary to Elysium's argument in the district court, the fact that the agreement gave Healthspan, a ChromaDex affiliate, a license in addition to ChromaDex in the same Field and Territory, did not make the license "nonexclusive" to either party. *WiAV*, 631 F.3d at 1267; *Intellectual Prop. Dev.*, 248 F.3d at 1337, 1345; *Sanofi-Aventis*, 748 F.3d at 1363-1365 (Abbott

[7] Because the district court and Elysium relied on the Restated Agreement in denying ChromaDex and Healthspan standing after March 12, 2017, Plaintiffs discuss that agreement herein. Plaintiffs' arguments apply under the Amended Agreement as well.

Laboratories and ALI both had "fully exclusive rights in the United States"). As *WiAV* explains, a party can possess exclusionary rights sufficient to confer standing even if other licenses exist under the same patents, in the same field. *WiAV*, 631 F.3d at 1267.

Furthermore, because ChromaDex and Healthspan shared exclusionary rights, Elysium would be unable, in any suit by Dartmouth, ChromaDex, and Healthspan, to obtain a license from any third party. Dartmouth's grant of an "exclusive" license meant that no such third party could obtain a license under the patents or the right to sublicense. Although both ChromaDex and Healthspan possessed the conditional right to grant sublicenses, the issue is not whether anyone (such as a nonparty to the present suit) could obtain a sublicense from either of them, but rather whether *Elysium* could obtain a sublicense from any other entity. *Id.* It could not because of Dartmouth's promise of exclusivity to ChromaDex and its affiliates. As in *WiAV*, where plaintiff WiAV's ability to sublicense, *id.* at 1262, did not extinguish its standing to sue because defendants could not obtain a license from any third party to the suit, *id.* at 1267, here, Elysium would have no ability to obtain a license from any third party either.

But for the district court's erroneous denial of Plaintiffs' motion to amend to include Healthspan as a plaintiff and partial grant of Elysium's motion to dismiss, the Plaintiffs in the present case would be Dartmouth, ChromaDex, and

Healthspan. The district court's refusal to add Healthspan was based on the district court's erroneous conclusion that Healthspan lacked standing. (Appx19-20.) Elysium did not argue, and the district court did not set forth, any other grounds for denying Plaintiffs' motion to amend. (Appx19-20; Appx1553.) Because the district court's determination that Healthspan lacked standing was erroneous, its denial of Plaintiffs' motion to amend should be reversed. Likewise, the district court's partial grant of Elysium's motion to dismiss as to ChromaDex's infringement claims after March 12, 2017 for lack of standing should be reversed.

3. Neither ChromaDex Nor Healthspan Had Unfettered Rights To Sublicense Elysium Because Their Sublicensing Rights Were Subject To A Condition That Was Never Satisfied

A separate and independent basis for reversing the district court's rulings on standing is that neither ChromaDex nor Healthspan had the unrestricted ability to grant Elysium a sublicence even if they wanted to do so. The district court held that ChromaDex did not have exclusionary rights *vis-à-vis* Elysium on or after March 13, 2017, because Healthspan purportedly had the legal right to grant Elysium a license and Elysium therefore had "the ability to obtain … from another party with the right to grant it" a license, as contemplated by *WiAV*. (Appx11; Appx13-14.) But Plaintiffs had shown that neither Healthspan nor ChromaDex in fact had that unfettered right. (Appx1583.) As Plaintiffs explained, "any sublicense of the Asserted Patents requires Dartmouth's consent," and Plaintiffs further

explained that Elysium had not offered any evidence that Dartmouth ever consented to a sublicense or would have done so. (Appx1583.) Indeed, the record on this point was undisputed: Healthspan's right to sublicense was conditional on Dartmouth's consent, Dartmouth had never given consent, and there were simply no facts in the record below that Dartmouth would have even considered a sublicense for Elysium. Thus, the court's conclusion that Healthspan had the unconstrained legal right to grant Elysium a license was wrong. Setting aside that Healthspan and Plaintiffs were under common ownership and management and thus Healthspan would not grant Elysium a license (as discussed *infra* Section VII.B.4.), absent Dartmouth's consent, Healthspan was contractually prohibited from doing so.

WiAV is clear that an exclusive licensee's standing to sue is defeated by another entity's sublicensing rights only if that other entity has the *legal right* to extend a license:

- "an exclusive licensee lacks standing to sue a party who has the ability to obtain such a license from another party ***with the right to grant it,***" *WiAV*, 631 F.3d at 1266 (emphasis supplied);
- "neither Rockwell Science Center, MindSpeed, Conexant, Skyworks, Qualcomm, nor Sipro ***has the right to extend licenses*** to the Defendants," *id.* at 1267 (emphasis supplied);
- "the relevant question is whether Skyworks ***can license*** the Defendants to practice the patents…." *id.* (emphasis supplied).

Subsequent decisions are consistent with an interpretation of *WiAV* as requiring third-party sublicensing rights to be unconditional before they can extinguish an exclusive licensee's standing. For example, in *Uniloc USA, Inc. v. ADP, LLC*, 772 F. App'x 890 (Fed. Cir. 2019) (nonprecedential), the defendants contended that plaintiff Uniloc lost its exclusionary rights due to defendants' ability to obtain a sublicense from a licensed third party, IBM. *Id.* at 894. IBM's right to sublicense, however, was subject to a condition that had not yet been met—Uniloc's breach of its obligation to indemnify ADP, which would trigger IBM's legal right to license others. *Id.* Because the defendants had not shown that IBM even considered Uniloc to be in breach of its agreement with IBM, the condition necessary for IBM to have a right to sublicense had not yet occurred. *Id.* at 895. Accordingly, Uniloc did not lose standing by virtue of IBM's conditional license rights. *Id.* So too here. ChromaDex did not lose standing by virtue of what were only conditional rights for Elysium to obtain a license.

Similarly, in *Luminara Worldwide, LLC v. Liown Electronics Co.*, 814 F.3d 1343 (Fed. Cir. 2016), defendant Liown argued that exclusive licensee Candella forfeited its standing by virtue of rights retained by patent owner Disney to license the patents to affiliates of Disney or entities "under license" from Disney. *Id.* at 1347-1348. The court held, however, that there were conditions on Disney's ability to give licenses to parties such as Liown. *Id.* at 1349. For example, affiliates

had to be under operational control of Disney and any entity operated "under license" from Disney required a license that related to the operation of a Disney affiliate, such as through a franchise agreement. *Id.* Because Liown could not demonstrate satisfaction of the conditions necessary for a party such as Liown to obtain a license from Disney, Disney's licensing rights did not extinguish Candella's standing to sue as an exclusive licensee. *Id.* Again, so too here. Because Elysium has not and could not demonstrate fulfillment of the conditions necessary to obtain a license from Dartmouth or Healthspan, ChromaDex's standing to sue as an exclusive licensee has not been extinguished.

Given the conditional nature of Healthspan's ability to sublicense, the district court's conclusion that Healthspan could license Elysium was, at best, utter speculation and indeed impossible without Dartmouth's consent. When drafting the original Agreement, the parties recognized that Dartmouth might not consent to all requests for sublicensing. Illustrating that although Dartmouth's consent to a sublicense would not be unreasonably withheld, Dartmouth's consent to a sublicense was *not* a given, the parties specifically set forth companies for whom Dartmouth gave such consent in advance. (Appx1499 § 2.02; Appx1511 § 2.02.) There would have been no reason to list any precleared sublicensees if Dartmouth's consent to sublicensing was a mere formality.

Because Healthspan had no unfettered legal right to grant Elysium a license, and neither Dartmouth nor any third party held such legal rights either, ChromaDex's exclusive license gave it an exclusionary right against Elysium that supported constitutional standing.

For the same reasons, ChromaDex had no unrestricted legal right to sublicense Elysium and the district court therefore erred in finding that Healthspan lacked constitutional standing.

4. Elysium Could Not Obtain A License To The Asserted Patents From Dartmouth, ChromaDex, Healthspan Or Any Other Entity

The district court's conclusion that Healthspan could provide Elysium a sublicense was not merely speculation; it was contrary to the undisputed facts in the district court. ChromaDex advanced testimony and other evidence demonstrating—without rebuttal—that under no circumstances would Healthspan extend Elysium a license. (*E.g.*, Appx1590-1592 ¶¶ 5-7.) As part of the ChromaDex family of companies, ChromaDex and Healthspan were under common management and control. (Appx1590 ¶ 5.) In order to accept Elysium's premise that it could obtain a sublicense, one would have to conclude that Mr. Fried as CEO of ChromaDex would refuse a sublicense to Elysium after suing it, but that in his capacity as CEO of Healthspan, he would be willing to grant such a sublicense. This illogical and farfetched suggestion should be rejected.

Simply put, this Court's precedent permitted patentee Dartmouth to provide an exclusive license to a company and its affiliates. Here, because the affiliated companies were under common management and control and operated so as to further the interests of the corporate family against infringement by Elysium (Appx1591 ¶ 6), Elysium had no ability to obtain a sublicense from those companies. *See WiAV*, 631 F.3d at 1266-67.

The refusal by Healthspan and ChromaDex to give Elysium a license was also understandable given the adversarial posture between Elysium and the ChromaDex organization in multiple active lawsuits, Elysium's interference with ChromaDex's business, Elysium's filing of IPR proceedings against the Dartmouth patents, and Elysium's active infringement of the patents. (*E.g.*, Appx1591-1592 ¶ 7; Appx1684-1694, Appx11 n.3.)

As *WiAV* explains, standing depends on whether a defendant can obtain a license from any other entity. *WiAV*, 635 F.3d at 1266-67. "[I]f an exclusive licensee has the right to exclude others from practicing a patent, and a party accused of infringement does not possess, ***and is incapable of obtaining***, a license of those rights from any other party, the exclusive licensee's exclusionary right is violated." *Id.* (emphasis supplied). On the other hand, an exclusive licensee lacks standing to sue a party only if that party "***has the ability to obtain such a license*** from another party with the right to grant it." *Id.* at 1266 (emphasis supplied). The

district court considered the meaning of "ability to obtain a license from another party with a right to grant it" (Appx10), concluding that *WiAV* only required Elysium to demonstrate that a licensee had the legal right to grant Elysium a license (and not that Elysium actually had the ability to obtain one) (Appx12-13). The district court thus effectively read the language concerning a defendant's ability to obtain a license out of *WiAV*. The district court did so based on three reasons, none of which justifies its interpretation.

First, the district court reasoned that *WiAV* cannot be read to require that the accused infringer actually be able to obtain a license because the "focus of standing in patent cases is on the plaintiff's rights, not the defendant's rights or the defendant's abilities." (Appx12.) The court relied on *Morrow v. Microsoft Corp.*, 499 F.3d 1332 (Fed. Cir. 2007) and *Ortho Pharmaceutical Corp. v. Genetics Institute, Inc.*, 52 F3d 1026 (Fed. Cir. 1995), but in those cases, this Court did not need to consider whether the defendant could have obtained a license, as that issue was never presented. *Morrow*, 499 F.3d at 1385-86; *Ortho*, 52 F.3d at 1033-34. In *Ortho*, unlike here, the licensee was denied standing because it held a bare, nonexclusive license. *Ortho*, 52 F.3d at 1033-34. And in *Morrow*, the party holding the right to sue (GUCLT) did not have exclusionary rights at all, because they were retained by the patentee (AHLT). *Morrow*, 499 F.3d at 1338, 1384.

Second, the district court reasoned that in *WiAV*, the court never considered whether other parties were *willing* to license to the defendants (Appx13), but there was no need to reach this issue because the licenses themselves made clear that no existing licensee had the power to grant defendants a license. *WiAV*, 631 F.3d at 1267-68.

Third, the district court stated that whether Healthspan would have given Elysium a license was a "hypothetical question" and "ultimately conjecture." (Appx13.) But the facts were undisputed in the district court that Healthspan would *not* have done so. Accordingly, there was no need for the district court to speculate because unrebutted facts had been introduced on this issue that proved that a license was not an option for Elysium. These included the common management and control of ChromaDex and Healthspan, the fact that management acted in the interests of the ChromaDex corporate family as a whole, ChromaDex's view that Elysium had interfered with its business, Elysium's challenges to the Dartmouth '807 and '086 Patents, active litigation between the parties in different courts, and Elysium's active infringement of the patents. (*E.g.*, Appx11 n.3, Appx1590-1592, ¶ 7; Appx1684-1694.)

Because it was undisputed that Elysium could not obtain a license from Healthspan (or from ChromaDex), each of ChromaDex and Healthspan had

standing to sue for injury caused by Elysium's infringement. *WiAV*, 631 F.3d at 1266-67.

C. The District Court Erred In Construing "Nicotinamide Riboside"

1. Statement Of The Standard Of Review

Where, as here, the district court relied solely on intrinsic evidence to construe a claim term, this Court's review is *de novo*. *Acceleration Bay LLC v. 2k Sports, Inc.*, 15 F.4th 1069, 1075 (Fed. Cir. 2021).

2. This Court Can And Should Exercise Its Discretion To Review And Reverse The District Court's Erroneous Construction

Although the district court construed several claim terms, those constructions played no direct role in addressing the § 101 and standing issues. One of those terms was "nicotinamide riboside" (NR), which appears in all asserted claims. While Plaintiffs urged that NR is a chemical compound with a defined structure and a plain and ordinary meaning, Elysium convinced the court to significantly *broaden* the term to include not only NR *per se*, but also certain "derivatives" of NR, including certain exemplary esters, to wit: **"nicotinamide riboside or a derivative (e.g. L-valine or L-phenylalanine esters) of nicotinamide riboside."** (Appx22.)

To begin with, by defining NR to include NR itself, the construction became "circular." The very fact that a proposed construction is circular is itself a "compelling reason" to reject it. *Harris Corp. v. IXYS Corp.*, 114 F.3d 1149, 1152

(Fed. Cir. 1997).

In the event this Court reverses or vacates the holding of ineligibility under § 101 and remands, Plaintiffs respectfully ask this Court to review this one claim construction issue, which could vastly simplify future proceedings, such as addressing issues under 35 U.S.C. §112. *See, e.g.*, *Interval Licensing LLC v. AOL, Inc.*, 766 F.3d 1364, 1376 (Fed. Cir. 2014) (vacating judgment of noninfringement based on one construction, but exercising discretion to review another "[i]n the interest of judicial economy" because the construction "may become important on remand"); *Aspex Eyewear, Inc. v. Marchon Eyewear, Inc.*, 672 F.3d 1335, 1346-47 (Fed. Cir. 2012) (after vacating judgment of noninfringement, reaching additional claim construction disputes because, although "not compelled to address those rulings," failing to address them "would in all likelihood result in further proceedings in the district court followed by another appeal in which precisely the same claim construction issues would be presented"); *Thorner v. Sony Comput. Ent. Am. LLC*, 669 F.3d 1362, 1368-69 (Fed. Cir. 2012) (vacating and remanding judgment of infringement, and further deciding an additional claim construction issue because it was "fully briefed and argued," and "it would waste judicial resources to refuse to decide this issue on appeal").

3. The District Court Erred In Finding An Explicit Definition

The court's construction was based entirely on this one sentence in a 54-column specification: "[T]he nicotinamide riboside can be a derivative (e.g. L-valine or L-phenylalanine esters) of nicotinamide riboside." (Appx2526, at 28:63-65.) The district court's primary rationale was that Dr. Brenner was here serving as his own lexicographer and providing an explicit definition of NR. (Appx11560-11561, at 21:20-22:25; Appx11562-11563, 23:9-24:21.)

In so holding, the district court rejected Plaintiffs' showing that when Dr. Brenner chose to define a term, he used express definitional language: *see, e.g.*, Appx2517, at 9:23-25 (*As used herein*, an isolated molecule…means…"); *id.* at 9:51 ("*As used herein*, a functional polypeptide is …."); Appx2520, at 15:28-29 ("*As used herein*, the term viral vector or viral delivery vector can refer to…"); *id.* at 15:46-47 (The term adenovirus *as used herein* in intended to encompass…"); Appx2521, at 18:65-66 ("*As used herein*, the terms transformation and transfection refer to…."); Appx2522, at 20:16-20 ("*As used herein*, a nicotinamide riboside-related drug is …."); Appx2524, at 23:49-50 ("A patient, *as used herein*, is intended to include…"); Appx2525, at 26:43-46 ("The term primer, *as defined herein*, is meant to encompass ….). This is not how the specification of the patents-in-suit references NR, either in the sentence relied upon by the district court or anywhere else.

In *Medicines Co. v. Mylan, Inc.*, 853 F.3d 1296 (Fed. Cir. 2017), the patentees placed "the defined terms in quotation marks, followed by the term 'refers to' or 'as defined herein.'" But as to the disputed term, the patentees just stated what it "is characterized by." This Court held, "[a]lthough this statement is taken verbatim from the specification, it does not purport to be definitional because it does not accord with the linguistic formula used by the patentee to signal the designation of other defined terms …." *Id.* at 1306 (citations omitted). The same reasoning should apply here.

4. The District Court Erroneously Found A Broadening "Disclaimer"

The district court alternatively viewed the single sentence in question as an instance of "disclaimer" by the patentee. (*See* Appx11559-11560, at 20:19-21:2; Appx11561, at 22:7-12.) But we know of no such doctrine of "disclaimer" that results in the *broadening* of a claim, as occurred here. *See, e.g.*, *Epistar Corp. v. ITC*, 566 F.3d 1321, 1336 (Fed. Cir. 2009) ("[I]n certain cases, the specification may reveal an intentional disclaimer, or disavowal, of claim scope by the inventor….In such cases, this court interprets the claim *more narrowly* than it otherwise would to give effect to the inventor's intent to *disavow a broader claim scope*." (citations and internal quotes omitted, emphasis added)). It was legal error to apply the disclaimer doctrine to effectively hold that a patentee "disavowed" a relatively narrow claim scope in favor of a broader one.

5. The District Court Erred In Finding An Implicit Definition

Although not argued by Elysium, the district court appears to have concluded that its construction of NR was based on not just an explicit definition, but also "implicit lexicography." (Appx11563-11564, at 24:18-25:12.) While this Court has acknowledged that definition by implication is permissible, the district court gave no real explanation why it applied here, essentially concluding that if the single sentence it relied upon was not an explicit definition, it must be an implicit one. In fact, it was neither.

The court cited *AstraZeneca AB v. Mutual Pharm. Co*., 384 F.3d 1333, 1339 (Fed. Cir. 2004), for the proposition that "rigid formalism…is not required." (Appx11561, at 22:13-19.) There, the specification stated that "the solubilizers suitable according to the invention *are defined below* …," which was followed by a description of "the solubilizers suitable for the preparations according to the invention…." *AstraZeneca*, 384 F.3d at 1339. Thus, *AstraZeneca* was actually consistent with *Medicines Co.* The present case involves nothing similar.

The district court also cited *Innova/Pure Water, Inc. v. Safari Water Filtration Sys.*, 381 F.3d 1111 (Fed. Cir. 2004), characterizing its holding as follows: "a patentee who notifies the public that claim terms are to be understood *beyond their ordinary meaning* will be bound by that notification." (Appx11564-11565 (emphasis added).) But what this Court actually said in

Innova/Pure was that "a patentee who notifies the public that claim terms are to be *limited beyond their ordinary meaning* to one of skill in the art will be bound…." *Innova/Pure*, 381 F.3d at 1117 (emphasis added). In that case, the term in question had an ordinary meaning, but the district court narrowed it significantly based on examples in the specification. In reversing that ruling, this Court offered a reminder that while courts "may construe [claims] in view of the specifications and the state of the art, they may not add to or detract from the claim." *Id.* at 1116 (quoting *White v. Dunbar*, 119 U.S. 47, 52 (1886)).[8]

The district court also cited *In re Abbott Diabetes Care*, 696 F.3d 1142 (Fed. Cir. 2012), as a case where implicit lexicography was attempted in an effort to broaden a term to include cables and wires. (Appx11564, at 25:3-13.) But that effort failed because context was ignored: the references to cables and wires *disparaged* them and discussed their *failings* in prior art devices—in full effect a narrowing disclaimer.[9]

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[8] *See also Evolusion Concepts, Inc. v. HOC Events, Inc.*, Nos. 2021-1963, 2021-1987, ___F.4th___, 2022 U.S. App. LEXIS 1126 (Fed. Cir., Jan. 14, 2022) (reversing claim construction where the district court narrowed the plain meaning of a term primarily based on a single sentence in the specification which merely described certain embodiments).

[9] The district court described *Abbott* as a case where the written description included express definitions of 11 terms but found another term implicitly defined. (Appx11564, at 25:2-12.) The court seems to have intended to refer to another case, which counsel cannot locate.

Context provides a further reason why the district court erred here. The single sentence on which it relied was followed in the same paragraph with "[a]ccordingly, the present invention also encompasses derivatives of nicotinamide riboside …." (Appx2527, at 29:4-10.) It must be appreciated that the common specification discloses a substantial number of inventions. Indeed, the Summary of the Invention sets forth no fewer than a dozen inventions discussed later, repeatedly stating that "the invention" includes (*inter alia*): "an isolated nucleic acid," "an expression vector," "cultured cell" and "a method of treating cancer." (Appx2514, at 3:21-4:67.) The paragraph discussing derivatives of NR is not a definition of NR itself.[10] It is a discussion of one or more additional inventions disclosed in the specification—derivatives of NR—*but these are not the inventions claimed in the '807 and '086 Patents.*

6. Extrinsic Evidence Further Undermines The Court's Construction

Although relevant to the claim construction issue before this Court, the district court did not rely on any extrinsic evidence presented by the parties. But if extrinsic evidence is considered, all such evidence was notably provided by Elysium's expert, Dr. James Adams, and uniformly supports Plaintiffs

[10] The specification nowhere mentions the L-valine or L-phenylamine esters of NR other than in the one paragraph discussed herein.

During claim construction, Dr. Adams explained that compositions for oral administration must be stable and therefore electrically neutral. (Appx2278 ¶ 13.) But the NR molecule is ionic, with a positive charge. (Appx2277 ¶ 9.) The positively charged NR molecule can combine with a negatively charge ion to form a salt that would be stable for oral administration. (Appx2279 ¶ 15.) Creating such a salt, however, does not alter the structure of the NR molecule. (Appx2407-2408, at 84:4-86:10.)

On the other hand, and Dr. Adams explained, with an ester of the type singled out in the court's construction, the NR structure is altered. In the phenylalanine ester recited in the construction, an "OH" group on the NR molecule is replaced with phenylalanine, which makes it "doubly positively charged" and "[m]akes the molecule very different." (Appx2409, at 89:5-92:6.) Similarly, replacing the "OH" group in NR to create the valine ester cited in the construction will "have an enormous impact." (Appx2411, at 98:7-17.) The NR ceases to exist when these derivatives are created.

7. Elysium Convinced The District Court To Adopt A Construction That Needlessly Undermines Validity

Elysium wasn't subtle about its motivation for seeking a construction of NR that significantly broadened the scope of each asserted claim. As Elysium candidly argued in claim construction briefing, by broadening the claims as Elysium was

urging, Elysium could then argue that the full scope of the broadened claims was not enabled or described. (*See* Appx1909 n.6.)

And that is exactly what Elysium did after the district court adopted Elysium's construction. (*See* Appx3113.) Elysium argued that there were at least billions of derivatives of NR, such that it would require extensive, unpredictable brute force testing to determine which ones were effective. (Appx3114-3115.) Thus, argued Elysium, the claims were not enabled because a person of ordinary skill could not practice the full scope of the claims without undue experimentation. (Appx3118.)

This Court has acknowledged that where other tools of claim construction leave an issue unresolved, claims should be construed to preserve their validity. *Phillips v. AWH Corp.*, 415 F.3d 1303, 1336 (Fed. Cir. 2005) (en banc). This is especially true where—as here—the construction that preserves validity "does not revise or ignore the explicit language of the claims." *Id*. at 1337.

Here, Elysium expressly invited the district court to adopt a construction that would facilitate Elysium's ability to attack the validity of the asserted claims under § 112. The court erred in accepting that invitation. *See Medtronic Navigation, Inc. v. BrainLAB Medizinische Computersysteme GmbH,* 222 F. App'x 952, 956 (Fed. Cir. 2007) (nonprecedential) (rejecting claim construction supported only by

a "minimal one sentence reference," which would expand the claim to cover systems for which there is no enablement).

* * *

Accordingly, the district court's circular construction of nicotinamide riboside is supported by neither the intrinsic nor the extrinsic evidence. And none of that evidence overcomes "the heavy presumption that claim terms are to be given their ordinary and customary meaning." *Aventis Pharms., Inc. v. Amino Chems., Ltd.,* 715 F.3d 1363, 1373 (Fed. Cir. 2013). On *de novo* review, this Court can and should reverse and adopt the plain and ordinary meaning.

VIII. CONCLUSION

For all the foregoing reasons, this Court is respectfully urged to *reverse* the district court's holding of invalidity under 35 U.S.C. § 101; *reverse* the holding that ChromaDex and Healthspan lacked standing to sue Elysium for infringement occurring after March 12, 2017; *reverse* the district court's construction of "nicotinamide riboside" in favor of the ordinary and customary meaning; and *remand* the case to the district court for further proceedings.

Respectfully submitted,

Dated: February 2, 2022

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UNITED STATES COURT OF APPEALS
FOR THE FEDERAL CIRCUIT

CERTIFICATE OF COMPLIANCE WITH TYPE-VOLUME LIMITATIONS

Case Number: 2022-1116
ChromaDex, Inc. v. Elysium Health, Inc.

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Dated: February 2, 2022 By: /s/ Roy H. Wepner
Roy H. Wepner

Addendum

IN THE UNITED STATES DISTRICT COURT
FOR THE DISTRICT OF DELAWARE

CHROMADEX, INC. and TRUSTEES OF DARTMOUTH COLLEGE Plaintiffs, v. ELYSIUM HEALTH, INC. Defendant.	Civil Action No. 18-1434-CFC-JLH

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REVISED MEMORANDUM OPINION[†]

December 17, 2020
Wilmington, Delaware

[†] The Memorandum Opinion has been revised to correct typographical errors.

COLM F. CONNOLLY
UNITED STATES DISTRICT JUDGE

Plaintiffs ChromaDex, Inc. and Trustees of Dartmouth College have sued Defendant Elysium Health, Inc. for infringement of two patents. D.I. 1 ¶ 1. Elysium has moved pursuant to Federal Rule of Civil Procedure 12(b)(1) to dismiss the claims asserted by ChromaDex for lack of subject matter jurisdiction. D.I. 58.

I.

Dartmouth owns both of the asserted patents. In 2012, in what the parties call the Original License Agreement, Dartmouth granted ChromaDex and ChromaDex's subsidiaries an "exclusive" license to (1) "make, have made, use and/or sell" products or processes covered by the asserted patents; (2) sue others who infringed the patents; and (3) grant with Dartmouth's consent sublicenses to third parties to "make, have made, use and sell" products or processes covered by the asserted patents. D.I. 50, Ex. C §§ 1.02, 2.01, 2.02 and 8.01.

On March 12, 2017, ChromaDex's parent company acquired Healthspan Research LLC. Although this acquisition made Healthspan an affiliate and not a subsidiary of ChromaDex, Dartmouth and ChromaDex have said that they "treated" Healthspan as a licensed subsidiary under the Original License Agreement. D.I. 49 at 2.

Dartmouth and ChromaDex filed this lawsuit on September 17, 2018. They accuse Elysium of infringing the asserted patents beginning "no later than July 2017." D.I. 1 ¶ 23.

On September 10, 2019, Dartmouth and ChromaDex executed a so-called Restated License Agreement that "clarif[ied] that ChromaDex's 'Affiliates,' rather than just 'Subsidiaries,' were exclusive licensees to the patents-in-suit." D.I. 49 at 2. The Restated License Agreement was made effective as of March 13, 2017—the day after ChromaDex's parent company acquired Healthspan. D.I. 49 at 2.

Elysium treats the Restated License Agreement as effective as of March 13, 2017 and argues that because the Agreement granted Healthspan the ability to sublicense the asserted patents, ChromaDex was deprived of exclusionary rights in the patents as of that date. D.I. 59 at 4–6. Elysium argues that ChromaDex therefore lacked standing to bring this case in 2018 and this Court lacks subject matter jurisdiction to entertain ChromaDex's infringement claims against Elysium. *Id.* Dartmouth and ChromaDex argue that the Restated License Agreement gave ChromaDex standing as an exclusive licensee to the asserted patents. D.I. 61 at 18–19 (stating that "[t]he Restated Agreement expressly granted ChromaDex and Healthspan 'the right to exclude others from practicing' the Asserted Patents.").

II.

Article III of the Constitution limits the jurisdiction of federal courts to "Cases" and "Controversies." *Lujan v. Defs. of Wildlife*, 504 U.S. 555, 559 (1992). Standing is "an essential and unchanging part" of this case-or-controversy requirement. *Id.* at 560. "Only a party with standing can invoke the jurisdiction of the federal courts." *Constitution Party of Pa. v. Aichele*, 757 F.3d 347, 357 (3d Cir. 2014). When the court's subject matter jurisdiction is challenged pursuant to Rule 12(b)(1), the plaintiff bears the burden of demonstrating standing. *Ortho Pharm. Corp. v. Genetics Inst.*, 52 F.3d 1026, 1032–33 (Fed. Cir. 1995). To meet that burden, the plaintiff must allege a "personal injury fairly traceable to the defendant's allegedly unlawful conduct and likely to be redressed by the requested relief." *Allen v. Wright*, 468 U.S. 737, 751 (1984). The personal injury must be "an invasion of a legally protected interest which is (a) concrete and particularized and (b) actual or imminent, not 'conjectural' or 'hypothetical.'" *Lujan*, 504 U.S. at 560 (internal quotation marks and citations omitted).

Patents and the rights they confer are creatures of statute. Section 2 of the Patent Act empowers the United States Patent & Trademark Office (PTO) to grant and issue patents, 35 U.S.C. § 2; and § 154 of the Act provides that every patent issued by the PTO "grant[s] to the patentee, his heirs or assigns . . . the right to exclude others from making, using, offering for sale, or selling [an] invention," 35

U.S.C. § 154(a). *See also* 35 U.S.C. § 271 ("[W]hoever without authority makes, uses, offers to sell, or sells any patented invention . . . infringes the patent."). Thus, in a patent infringement case, the actual or threatened injury required by Article III exists solely by virtue of the Patent Act. *See Intell. Prop. Dev., Inc. v. TCI Cablevision of Cal., Inc.*, 248 F.3d 1333, 1345 (Fed. Cir. 2001) ("Standing in a patent infringement case is derived from the Patent Act."); *see also WiAV Solutions LLC v. Motorola, Inc.*, 631 F.3d 1257 (Fed. Cir. 2010) ("Because the Patent Act creates the legally protected interests in dispute [in an infringement case], the right to assert infringement of those interests comes from the Act itself."); *see generally Linda R.S. v. Richard D.*, 410 U.S. 614, 617 n.3 (1973) ("Congress may enact statutes creating legal rights, the invasion of which creates standing, even though no injury would exist without the statute.").[1]

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[1] The right to exclude that accompanies the issuance of a patent pursuant to § 154 is legally distinct from the cause of action created by § 281 of the Patent Act, which provides that "[a] patentee shall have remedy by civil action for infringement of his patent." *See generally Davis v. Passman*, 442 U.S. 228, 239 n.18 (1979) (explaining that a party's standing to sue is a separate question from whether the party has a cause of action). In *Lexmark International, Inc. v. Static Control Components, Inc.*, 572 U.S. 118, 128 n.4 (2014), the Supreme Court held that "the absence of a valid (as opposed to arguable) cause of action does not implicate subject-matter jurisdiction, i.e., the court's statutory or constitutional power to adjudicate the case." In *Lone Star Silicon Innovations LLC v. Nanya Technology Corp.*, 925 F.3d 1225, 1235 (Fed. Cir. 2019), the Federal Circuit held that "*Lexmark* is irreconcilable with our earlier authority treating § 281 as a jurisdictional requirement." Whether a party constitutes a patentee for purposes of § 281 is, at least in theory, a different question than whether a party is a patentee for purposes of § 154 or otherwise has a legally protected interest, the invasion of

The language of § 154 appear to be straightforward. The right that comes with a patent is the right to exclude others from making, using, offering for sale, and selling an invention. The right is granted to the patentee, his heirs, and his assigns.

But constitutional standing in a patent case is anything but straightforward. Courts long ago abandoned the text of the statute (and its predecessor statute) and expanded the list of potential grantees of a patent's exclusionary rights beyond the patentee and the patentee's heirs and assigns. Perhaps most notably, in *Independent Wireless Telegraph Co. v. Radio Corp. of America*, 269 U.S. 459 (1926), the Supreme Court confirmed "the rule" that "an exclusive licensee has a sufficient interest in [a] patent to have standing to sue under Article III of the Constitution." *Propat Int'l Corp. v. RPost US, Inc.*, 473 F.3d 1187, 1193 (Fed. Cir. 2007). As often happens when a statute's text is ignored, the case law bred by *Independent Wireless* has led to confusion about the requirements for and the rights that flow from exclusive licensee status.[2]

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which constitutes the injury required for Article III standing. There is no natural or common law right to exclude others from practicing a party's invention. A party alleging infringement has constitutional standing only because of statutory rights.

[2] *See, e.g.*, *Uniloc USA, Inc. v. Apple, Inc.*, No C 18-00358 WHA, 2020 WL 7122617, at *3 (N.D. Cal. Dec. 4, 2020) (noting that "confusion about the interplay between . . . [a plaintiff's] statutory right to sue [in a patent case] and our doctrine of standing has long persisted"); *Aspex Eyewear, Inc. v. Miracle Optics, Inc.*, No. CV01-10396 MMM (CWx), 2009 WL 10699035, at *9 (C.D. Cal. July 14, 2009)

In *Rite-Hite Corp. v. Kelley Co., Inc.*, 56 F.3d 1538 (Fed. Cir. 1995) (en banc), the Federal Circuit held that "[t]o be an exclusive licensee for standing purposes, a party must have received, not only the right to practice the invention within a given territory, but also the patentee's express or implied promise that *others* shall be excluded from practicing the invention within that territory as well." *Id.* at 1552 (emphasis added). That test left open the question of whether an exclusive licensee must receive from the patentee a promise to exclude *all* others from practicing the invention in the specified territory for the exclusive licensee to have constitutional standing.

In two cases decided after *Rite-Hite* the Federal Circuit appeared to answer yes to that question. In *Mars, Inc. v. Coin Acceptor, Inc.*, 527 F.3d 1359 (Fed. Cir. 2008), the court held that "if the patentee *allows* others to practice the patent in the licensee's territory, the licensee is *not* an exclusive licensee." *Id.* at 1368 (emphasis in original). And in *Textile Productions Inc. v. Mead Corp.*, 134 F.3d 1481 (Fed. Cir. 1998), the court held that "[t]o qualify as an exclusive license, an

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(noting "confusion [that] arises from the fact that, in the context of standing to sue for patent infringement, the term 'exclusive licensee' can refer to various types of contractual arrangements with different legal effects"); Roger D. Blair & Thomas F. Cotter, *The Elusive Logic of Standing Doctrine in Intellectual Property Law*, 74 Tul. L. Rev. 1323, 1328 (2000) ("[T]he standing rules in [patent] law appear to be as much a patchwork as Dr. Frankenstein's monster, and only marginally more coherent.").

agreement must clearly manifest the patentee's promise to refrain from granting *to anyone else* a license in the area of exclusivity." *Id.* at 1484 (emphasis added); *see also id.* ("[I]f a patentee-licensor is free to grant licenses to others, licensees under that patent are not exclusive licensees."). The court emphasized in *Textile Productions* that the contract at issue—an exclusive requirements contract—did not "confer a right to exclude *all others* from making an invention" and that the patent holder "did not promise that *all others*" shall be excluded from practicing the invention. *Id.* at 1484–85 (emphasis added).

The meaning of "exclusive licensee" endorsed by *Mars* and *Textile Products* is consistent with the general understanding of the term today, *see Exclusive License, Black's Law Dictionary* (11th ed. 2019) (defining an "exclusive license" as "[a] license that gives the licensee the sole right to perform the licensed act, often in a defined territory, and that prohibits the licensor from performing the licensed act and from granting the right to anyone else; esp., such a license of a copyright, patent, or trademark right"); and with the general understanding of the term at the time the Supreme Court decided *Independent Wireless*, *see Exclusive, Black's Law Dictionary* (2d ed. 1910) (defining "exclusive right" as a right "which only the grantee thereof can exercise, and from which all others are prohibited or shut out").

But in *WiAV* the Federal Circuit held that *Textile Products*'s and *Mars*'s holdings apply only where "a party [is] an *implied* exclusive licensee of the patents in suit in the absence of a written agreement explicitly granting the party exclusionary rights in the patents." *WiAV*, 631 F.3d at 1266 (emphasis in original). Under *WiAV*, when a contract expressly grants an exclusionary license,

> the key question in determining whether [a plaintiff] has standing to assert the [patents] against the Defendants is not . . . whether [the plaintiff] has established that it has the right to exclude *all* others from practicing the patent. The question is whether [the plaintiff] has shown that it has the right under the patents to exclude *the Defendants* from engaging in the alleged infringing activity and therefore is injured by the Defendants' conduct.

Id. at 1267 (emphasis in original). Applying this test, the court in *WiAV* held that in cases involving an express grant of an exclusive patent license, the "exclusive licensee lacks standing to sue a party who has the ability to obtain such a license from another party with the right to grant it." *Id.* at 1266; *see also id.* at 1266–67 ("[I]f an exclusive licensee has the right to exclude others from practicing a patent, and a party accused of infringement does not possess, and is incapable of obtaining, a license of those rights from any other party, the exclusive licensee's exclusionary right is violated.").

III.

The parties dispute what the *WiAV* court meant by the phrase "ability to obtain . . . a license from another party with a right to grant it." Elysium argues

that a defendant has the ability to obtain a license from another party if any other party had the legal right to grant the defendant a license. Dartmouth and ChromaDex argue that a defendant has the ability to obtain a license from another party only if that party had the legal right to grant the defendant a license *and* would have been willing to grant the defendant a license. It is undisputed that both ChromaDex and Healthspan had the legal right to grant Elysium a license; and I am persuaded by the record evidence submitted by Dartmouth and ChromaDex that Healthspan would likely never have agreed to give Elysium a license to the asserted patents.[3] Thus, the resolution of the pending motion turns on the meaning

[3] Curiously, Dartmouth and ChromaDex never expressly state in their briefing or supporting declarations that Healthspan would not have agreed to give a license to Elysium even though their entire argument is predicated on the assumption that Healthspan would have refused to grant Elysium a license. At oral argument, ChromaDex's counsel stated that ChromaDex and Healthspan were managed by the same person and that "[t]hat person is not going to allow ChromaDex to license the patents out from under Healthspan and vice-versa." Tr. 39:8–10. The record establishes that (1) Healthspan and Elysium have been competitors since March 2017, D.I. 62 ¶ 4; (2) ChromaDex and Elysium have been involved in litigation in the Central District of California since 2016 and in the Southern District of New York since 2017, D.I. 61 at 4 n.2; and (3) the same executive management team manages all entities within the ChromaDex corporate family. The General Counsel and Secretary of ChromaDex and Healthspan, Mark Friedman, stated in a sworn declaration that he believes that "Elysium . . . inten[ds] to destroy the ChromaDex organization, [has] conspired with a key ChromaDex employee to abscond with trade secrets and other confidential information, and attempted to financially ruin the ChromaDex organization . . . ." D.I. 62 ¶ 7. Friedman intimates, but does not state directly in his declaration, that ChromaDex and Healthspan would never have agreed to grant Elysium a license. *See id.* (stating

of "ability to obtain . . . a license from another party with a right to grant it" in *WiAV*. Although the disputed language by itself lends support to both parties' positions, I think Elysium has the better argument for three reasons.

First, the focus of standing in patent case is on the plaintiff's rights, not the defendant's rights or the defendant's abilities. As the Federal Circuit held in *Morrow v. Microsoft Corp.*, 499 F.3d 1332 (Fed. Cir. 2007), "[i]n determining whether a party holds the exclusionary rights, we determine *the substance of the rights* conferred *on that party* . . . ." *Id.* at 1340 n.7 (emphasis added). *See also Ortho*, 52 F.3d at 1032 ("[I]t is the licensee's beneficial ownership of *a right to prevent others* from making, using or selling the patented technology that provides the foundation for . . . standing." (emphasis added)).

The court emphasized this point in *WiAV*. It stated that "the touchstone of constitutional standing in a patent infringement suit is whether a party can establish that *it has an exclusionary right* in a patent that, if violated by another, would cause *the party holding the exclusionary right* to suffer legal injury." 631 F.3d at 1265 (emphasis added). And the court explained that "the key question" in determining standing is whether the plaintiff "has shown that *it has the right* under the patents to exclude" the defendant from practicing the invention covered by the

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that "Elysium would not have been able to obtain a license to the Asserted Patents from ChromaDex or Healthspan").

patents. *Id.* at 1267 (emphasis added). A right is "[a] legally enforceable claim that another will do or will not do a given act." *Right*, *Black's Law Dictionary* (11th ed. 2019). A plaintiff does not have the right to prevent a defendant from using a patent if another party has the right to allow the defendant to use the patent.

Second, in answering "the key question" to determine whether the plaintiff in *WiAV* had standing, the court did not consider whether other parties were willing to license the asserted patents to the defendants. Instead the court examined whether other parties "ha[d] the right to extend [a] license to the Defendants." 631 F.3d at 1267. In the court's words: "the relevant question is whether [another party] can license the Defendants to practice the patents in [the plaintiff's] field of exclusivity." *Id.*

Third, the injury necessary for constitutional standing cannot be "conjectural or hypothetical." *Lujan,* 504 U.S. at 560 (quotation marks and citation omitted). But whether Healthspan would have refused to give Elysium a license in the past is a hypothetical question. Even though it makes sense to me based on the current record that Healthspan would likely have refused to give Elysium a license, that conclusion is ultimately conjecture—an inference formed without proof.

Accordingly, I find that Elysium had "the ability to obtain . . . from another party with the right to grant it" a license to practice the asserted patents, as that phrase is used in *WiAV*. Because Healthspan had the right to give Elysium a

license to practice the asserted patents as of March 13, 2017, ChromaDex did not have the right to exclude Elysium from practicing the patents from that date forward. ChromaDex therefore lacks standing to allege that Elysium infringed the asserted patents after that date.

IV.

There remains the issue of whether ChromaDex has standing to allege infringement based on Elysium's actions that occurred before March 13, 2017. The parties did not address this issue in their briefing or at oral argument. Dartmouth and ChromaDex allege that Elysium's infringement began no later than July 2017. Thus, the Complaint allows for possible infringement before the Restated License Agreement took effect.

The Original License Agreement became effective July 13, 2012. Elysium has not suggested that the Original License Agreement did not grant ChromaDex exclusive licensee status for constitutional standing; nor is there anything to suggest that another party had the ability to license Elysium to practice the asserted patents during the term of the Original License Agreement. Accordingly, ChromaDex has standing to allege an infringement claim based on Elysium's conduct occurring between July 13, 2012 and March 12, 2017.

V.

For the reasons discussed above, ChromaDex lacks standing to bring a claim for infringement based on Elysium's conduct that occurred on or after March 13, 2017 but has standing to allege a claim based on Elysium's conduct that occurred before that date. Accordingly, I will grant in part and deny in part Elysium's motion to dismiss.

The Court will issue a Revised Order consistent with this Revised Memorandum Opinion.

IN THE UNITED STATES DISTRICT COURT
FOR THE DISTRICT OF DELAWARE

CHROMADEX, INC. and TRUSTEES OF DARTMOUTH COLLEGE Plaintiffs, v. ELYSIUM HEALTH, INC. Defendant.	Civil Action No. 18-1434-CFC-JLH

REVISED ORDER[†]

At Wilmington this Seventeenth day of December in 2020:

For the reasons set forth in the Revised Memorandum Opinion issued this day, **IT IS HEREBY ORDERED** that Defendant's Rule 12(b)(1) Motion to Dismiss ChormaDex, Inc.'s Claims (D.I. 58) is **GRANTED IN PART AND DENIED IN PART**:

1. The motion is **GRANTED** insofar as it seeks dismissal of claims of infringement brought by ChromaDex Inc. for activities alleged to have occurred on or after March 13, 2017; and

[†] The Order has been revised to correct typographical errors.

2. The motion is **DENIED** is insofar as it seeks dismissal of claims of infringement brought by ChromaDex for activities alleged to have occurred before March 13, 2017.

UNITED STATES DISTRICT JUDGE

IN THE UNITED STATES DISTRICT COURT
FOR THE DISTRICT OF DELAWARE

CHROMADEX, INC. and TRUSTEES OF DARTMOUTH COLLEGE Plaintiffs, v. ELYSIUM HEALTH, INC. Defendant.	Civil Action No. 18-1434-CFC-JLH

REVISED ORDER[†]

Pending before me is Plaintiffs' Motion for Leave to Amend Complaint (D.I. 49), which seeks to add Healthspan Research LLC as a plaintiff. Defendant argues that Healthspan lacks standing to bring suit and asks that I deny the motion as futile.

Defendant moved to dismiss Plaintiff ChromaDex, Inc. from the suit, arguing that ChromaDex lacked standing to bring claims for infringement. I granted in part and denied in part that motion and my reasoning is set out in a Revised Memorandum Opinion issued this date. D.I. 141.

[†] The Order has been revised to correct typographical errors.

"If the complaint, as amended, would not survive a motion to dismiss, leave to amend may be denied as futile." *Delaware Display Grp. LLC v. Lenovo Grp. Ltd., Lenovo Holding Co.*, 2016 WL 720977, at *7 (D. Del. Feb. 23, 2016) (citation omitted).

"Under [Federal Circuit] precedent, only parties with exclusionary rights to a patent may bring suit for patent infringement." *Luminara Worldwide, LLC v. Liown Elec. Co. Ltd.*, 814 F.3d 1343, 1347 (Fed. Cir. 2016). Plaintiffs argue that Healthspan has constitutional standing to be a plaintiff in this case by virtue of a Restated License Agreement that granted to both ChromaDex and Healthspan effective March 13, 2017 an "exclusive" license to (1) "make, have made, use and/or sell" products or processes covered by the asserted patents, (2) sue others who infringed the patents; and (3) grant with Dartmouth's consent sublicenses to third parties to "make, have made, use and sell" products or processes covered by the asserted patents. D.I. 50, Ex. D §§ 1.02, 2.01, 2.02 and 8.01.

For the reasons set forth in the Revised Memorandum Opinion, because the Restated License Agreement gave both Healthspan and ChromaDex the right to give Elysium a license to practice the asserted patents as of March 13, 2017, neither Healthspan nor ChromaDex had the right to exclude Elysium from practicing the patents from that date forward. Accordingly, Healthspan lacks

standing to allege that Elysium infringed the asserted patents on or after March 13, 2017 and granting leave to add Healthspan as a plaintiff would be futile.

NOW THEREFORE, at Wilmington this Seventeenth day of December in 2020, **IT IS HEREBY ORDERED** that Plaintiffs' Motion for Leave to Amend Complaint (D.I. 49) is **DENIED**.

UNITED STATES DISTRICT JUDGE

IN THE UNITED STATES DISTRICT COURT
FOR THE DISTRICT OF DELAWARE

CHROMADEX, INC. and TRUSTEES OF DARTMOUTH COLLEGE, Plaintiffs, v. ELYSIUM HEALTH, INC., Defendant.	C.A. No. 18-1434-CFC

~~PROPOSED~~ CLAIM CONSTRUCTION ORDER

As set forth by the Court at the claim construction hearing held on December 17, 2020, IT IS HEREBY ORDERED that the claim terms below are construed as follows:

TERM	PATENT(S) / CLAIM(S)	COURT'S CONSTRUCTION
"nicotinamide riboside"	'807 Patent: Claims 1 and 2 '086 Patent: Claim 2	**"nicotinamide riboside or a derivative (e.g., L-valine or L-phenylalanine esters) of nicotinamide riboside"**
"isolated nicotinamide riboside"	'807 Patent: Claim 1	**"nicotinamide riboside that is separated or substantially free from at least some of the other components associated with the source of the nicotinamide riboside"**
"the nicotinamide riboside is isolated from a natural or synthetic source"	'807 Patent: Claim 2 '086 Patent: Claim 2	**"the nicotinamide riboside is isolated from a natural source or synthetic source and is not chemically synthesized"**
"in combination with one or more of tryptophan, nicotinic acid, or nicotinamide"	'807 Patent: Claim 1	**"both isolated nicotinamide riboside and one or more of tryptophan, nicotinic acid, or nicotinamide are found in the composition"**
"increases NAD+ biosynthesis upon oral administration"	'807 Patent: Claim 1	**"increases NAD+ biosynthesis upon oral administration to an animal relative to the level of NAD+ biosynthesis if the composition were not administered to an animal"**

TERM	PATENT(S) / CLAIM(S)	COURT'S CONSTRUCTION
"pharmaceutical composition"	'086 Patent: Claim 2	**"a composition that can be used to improve or prolong the health or well-being of humans or other animals"**

SO ORDERED this 5th day of January, 2020.

The Honorable Colm F. Connolly
United States District Judge

IN THE UNITED STATES DISTRICT COURT
FOR THE DISTRICT OF DELAWARE

CHROMADEX, INC. and TRUSTEES OF DARTMOUTH COLLEGE Plaintiffs, v. ELYSIUM HEALTH, INC. Defendant.	Civil Action No. 18-1434-CFC-JLH

MEMORANDUM ORDER

Plaintiffs ChromaDex, Inc. and Trustees of Dartmouth College have moved pursuant to Local Rule 7.1.5 for reconsideration of the Revised Memorandum Opinion (D.I. 141) and related Revised Orders (D.I. 142 and D.I 143) I issued on December 17, 2020. D.I. 148. They seek reconsideration specifically of my dismissal pursuant to Federal Rule of Civil Procedure 12(b)(1) of certain claims asserted by ChromaDex for lack of subject matter jurisdiction and my denial of Plaintiffs' motion for leave to amend the complaint pursuant to Federal Rule of Civil Procedure 15(a). D.I. 142, D.I. 143.

A motion brought pursuant to Local Rule 7.1.5 is the "functional equivalent" of a motion brought pursuant to Federal Rule of Civil Procedure 59(e) to alter or

amend a judgment. *Jones v. Pittsburgh Nat'l Corp.*, 899 F.2d 1350, 1352 (3d Cir. 1990). Such a motion "must rely on one of three grounds: (1) an intervening change in controlling law; (2) the availability of new evidence; or (3) the need to correct a clear error of law or fact or to prevent manifest injustice." *Lazaridis v. Wehmer*, 591 F.3d 666, 669 (3d Cir. 2010) (citing *N. River Ins. Co. v. CIGNA Reinsurance Co.*, 52 F.3d 1194, 1218 (3d Cir. 1995)). Plaintiffs invoke the second ground. They argue that their motion "meets the reargument standard because it [presents] 'new factual matter[] not previously obtainable' that 'ha[s] been discovered since the issue was submitted to the Court.'" D.I. 162-1 at 1 (quoting *Brambles USA, Inc. v. Blocker*, 735 F. Supp. 1239, 1241 (D. Del. 1990)) (first alteration added). Plaintiffs identify two "new factual matters": (1) an amendment to a contract executed by Dartmouth and ChromaDex on December 29, 2020; and (2) the dissolution of non-party Healthspan Research, LLC on January 15, 2021. D.I. 148 at 1-2.

These matters are not newly available evidence for purposes of the pending motion because they did not exist at the time I issued the Revised Memorandum Opinion and Orders. *See Brown v. Pennsylvania R.R. Co.*, 282 F.2d 522, 526–27 (holding that "'newly discovered evidence' within the purview of Rule 60(b)(2) . . . refers to evidence of facts in existence at the time of [the decision] of which the aggrieved party was excusably ignorant"); *Compass Tech. v. Tensing Labs.*, 71

F.3d 1125, 1130 (3d Cir. 1995) (holding that "Rule 59 and Rule 60(b)(2) share the same standard for granting relief on the basis of newly discovered evidence"). The matters are also not fairly characterized as "not previously obtainable" because Plaintiffs point to no fact or circumstance that precluded Healthspan from dissolving or Dartmouth and ChromaDex from executing the cited amendment before December 17, 2020. Finally, the matters are not accurately described as "discovered" because ChromaDex played a role in their creation after December 17, 2020.

NOW THEREFORE, at Wilmington this Twenty-seventh day of April in 2021, **IT IS HEREBY ORDERED** that ChromaDex, Inc. and Trustees of Dartmouth College's Motion for Reargument or Reconsideration of the Revised Memorandum Opinion and Orders Issued December 17, 2020 (D.I. 148) is **DENIED**.

UNITED STATES DISTRICT JUDGE

IN THE UNITED STATES DISTRICT COURT

FOR THE DISTRICT OF DELAWARE

CHROMADEX, INC. and TRUSTEES OF DARTMOUTH COLLEGE,	)	
Plaintiffs,	)	
v.	)	Civil Action No. 18-1434-CFC
ELYSIUM HEALTH, INC.,	)	
Defendant.	)	

ORDER

At Wilmington this Fourteenth day of September in 2021, having reviewed the parties' summary judgment motion briefing and having decided to grant Defendant's motion for summary judgment of invalidity under 35 U.S.C. §101 for reasons that were shared today by the Court with the parties on the telephone and will be set forth more fully in a memorandum opinion to be issued on a later date;

IT IS HEREBY ORDERED that Defendant's Motion for Summary Judgment of Invalidity (D.I. 182) is granted and the September 27, 2021 jury trial is canceled.

Chief Judge

IN THE UNITED STATES DISTRICT COURT
FOR THE DISTRICT OF DELAWARE

CHROMADEX, INC. and TRUSTEES OF DARTMOUTH COLLEGE Plaintiffs, v. ELYSIUM HEALTH, INC. Defendant.	Civil Action No. 18-1434-CFC-JLH

Adam Poff, Pilar Kraman, YOUNG, CONWAY, STARGATT & TAYLOR LLP, Wilmington, Delaware; James Haley, HALEY GUILIANO LLP, New York, New York; Jason Fowler, COVINGTON & BURLING LLP, Washington, District of Columbia

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Counsel for Defendant

MEMORANDUM OPINION

September 21, 2021
Wilmington, Delaware

COLM F. CONNOLLY
CHIEF JUDGE

Plaintiffs ChromaDex, Inc. and Trustees of Dartmouth College (collectively, ChromaDex) have sued Defendant Elysium Health, Inc. for infringement of U.S. Patent Numbers 8,197,807 (the #807 patent) and 8,383,086 (the #086 patent). Pending before me is Elysium Health's Motion for Summary Judgment (No. 1) of Invalidity Under 35 U.S.C. § 101. D.I. 182. Elysium argues that claims 1, 2, and 3 of the #807 patent and claim 2 of the #086 patent are invalid under 35 U.S.C. § 101 for claiming patent-ineligible subject matter.

I. BACKGROUND

The asserted patents claim compositions containing isolated nicotinamide riboside (NR), a naturally occurring form of vitamin B3. Isolated NR facilitates production of "NAD^+," a coenzyme associated with various biological activities.

The asserted claims of the #807 patent read as follows:

> 1. A composition comprising isolated nicotinamide riboside in combination with one or more of tryptophan, nicotinic 60 acid, or nicotinamide, wherein said combination is in admixture with a carrier comprising a sugar, starch, cellulose, powdered tragacanth, malt, gelatin, talc, cocoa butter, suppository wax, oil, glycol, polyol, ester, agar, buffering agent, alginic acid, isotonic saline, Ringer's solution, ethyl alcohol, poly- 65 ester, polycarbonate, or polyanhydride, wherein said composition is formulated for oral administration and increases NAD+ biosynthesis upon oral administration.

> 2. The composition of claim 1, wherein the nicotinamide riboside is isolated from a natural or synthetic source.
>
> 3. The composition of claim 1, wherein the formulation comprises a tablet, troche, capsule, elixir, suspension, syrup, wafer, chewing gum, or food.

#807 patent at claims 1–3.

Asserted claim 2 of the #086 patent depends from independent claim 1, which is not asserted.[1] Those two claims read as follows:

> 1. A pharmaceutical composition comprising nicotinamide riboside in admixture with a carrier, wherein said composition is formulated for oral administration.
>
> 2. The pharmaceutical composition of claim 1, wherein the nicotinamide riboside is isolated from a natural or synthetic source.

#086 patent at claims 1, 2. I have construed the phrase "pharmaceutical composition" to mean "a composition that can be used to improve or prolong the health or well-being of humans or other animals." D.I. 152 at 3.

[1] The Patent Trial and Appeal Board has already held that claim 1 of the #086 patent is invalid. *See Elysium Health Inc. v. Trustees of Dartmouth College*, No. IPR2017-01795, Paper No. 39 (P.T.A.B. Jan. 16, 2019), *aff'd*, 796 Fed. App'x 745 (Fed. Cir. 2020).

II. LEGAL STANDARDS

A. Summary Judgment

A court must grant summary judgment "if the movant shows that there is no genuine dispute as to any material fact and the movant is entitled to judgment as a matter of law." Fed. R. Civ. P. 56(a). Material facts are those "that could affect the outcome" of the proceeding. *Lamont v. New Jersey*, 637 F.3d 177, 181 (3d Cir. 2011). "[A] dispute about a material fact is genuine if the evidence is sufficient to permit a reasonable jury to return a verdict for the non-moving party." *Id.* (internal quotation marks omitted). A non-moving party asserting that a fact is genuinely disputed must support such an assertion by: "(A) citing to particular parts of materials in the record, including depositions, documents, electronically stored information, affidavits or declarations, stipulations, . . . admissions, interrogatory answers, or other materials; or (B) showing that the materials cited [by the opposing party] do not establish the absence . . . of a genuine dispute . . . ." Fed. R. Civ. P. 56(c)(1). The non-moving party's evidence "must amount to more than a scintilla, but may amount to less (in the evaluation of the court) than a preponderance." *Williams v. Borough of West Chester, Pa.*, 891 F.2d 458, 460–61 (3d Cir. 1989).

B. Patent-Eligible Subject Matter

Section 101 of the Patent Act defines patent-eligible subject matter. It provides: "Whoever invents or discovers any new and useful process, machine,

manufacture, or composition of matter, or any new and useful improvement thereof, may obtain a patent therefor, subject to the conditions and requirements of this title." 35 U.S.C. § 101.

There are three judicially created limitations on the literal words of § 101. The Supreme Court has long held that laws of nature, natural phenomena, and abstract ideas are not patentable subject matter. *Alice Corp. Pty. v. CLS Bank Int'l*, 573 U.S. 208, 216 (2014). These exceptions to patentable subject matter arise from the concern that the monopolization of "these basic tools of scientific and technological work" "might tend to impede innovation more than it would tend to promote it." *Id.* (internal quotation marks and citations omitted).

"A claim to otherwise statutory subject matter does not become ineligible simply because it recites a natural law," *Cleveland Clinic Foundation v. True Health Diagnostics LLC*, 760 Fed. App'x 1013, 1018 (Fed. Cir. 2019), since "all inventions at some level embody, use, reflect, rest upon, or apply laws of nature, natural phenomena, or abstract ideas." *Mayo Collaborative Servs. v. Prometheus Lab'ys, Inc.*, 566 U.S. 66, 71 (2012). But in order "to transform an unpatentable law of nature [or natural phenomena] into a patent-eligible application of such law [or natural phenomena], one must do more than simply state the law of nature [or natural phenomena] while adding the words 'apply it.'" *Id.* (emphasis omitted).

In *Alice*, the Supreme Court established a two-step framework by which courts are to distinguish patents that claim eligible subject matter under § 101 from patents that do not claim eligible subject matter under § 101. The court must first determine whether the patent's claims are drawn to a patent-ineligible concept—i.e., are the claims directed to a law of nature, natural phenomenon, or abstract idea? *Alice*, 573 U.S. at 217. If the answer to this question is no, then the patent is not invalid for teaching ineligible subject matter. If the answer to this question is yes, then the court must proceed to step two, where it considers "the elements of each claim both individually and as an ordered combination" to determine if there is an "inventive concept—*i.e.*, an element or combination of elements that is sufficient to ensure that the patent in practice amounts to significantly more than a patent upon the [ineligible concept] itself." *Id.* at 217–18 (alteration in original) (internal quotations and citations omitted).[2]

[2] The Court in *Alice* literally said that this two-step framework is "for distinguishing patents that claim laws of nature, natural phenomena, and abstract ideas from those that claim patent-eligible applications of those concepts." 573 U.S. at 217. But as a matter of logic, I do not see how the first step of the *Alice/Mayo* framework can distinguish (or even help to distinguish) patents in terms of these two categories (i.e., the categories of (1) "patents that claim laws of nature, natural phenomena, and abstract ideas" and (2) patents "that claim patent-eligible applications of [laws of nature, natural phenomena, and abstract ideas]"). *Both* categories *by definition* claim laws of nature, natural phenomena, and abstract ideas; and only one of *Alice*'s steps (i.e., the second, "inventive concept" step) could distinguish the two categories. I therefore understand *Alice*'s two-step framework to be the framework by which courts are to distinguish patents that

Issued patents are presumed to be valid, but this presumption is rebuttable. *Microsoft Corp. v. i4i Ltd. Partnership*, 564 U.S. 91, 96 (2011). Subject-matter eligibility is a matter of law, but underlying facts must be shown by clear and convincing evidence. *Berkheimer v. HP Inc.*, 881 F.3d 1360, 1368 (Fed. Cir. 2018).

III. DISCUSSION

Applying the two-step framework from *Alice*, I find that the asserted patent claims are invalid under § 101.

A. *Alice* Step One

"[C]laims are considered in their entirety [at step one] to ascertain whether their character as a whole is directed to excluded subject matter." *Internet Patents Corp. v. Active Network, Inc.*, 790 F.3d 1343, 1346 (Fed. Cir. 2015). Elysium argues in its briefing that the asserted claims are directed to "compositions comprising isolated nicotinamide riboside ("NR")[,] . . . a naturally-occurring vitamin present in cow milk." D.I. 183 at 1. ChromaDex does not dispute this description of the asserted claims' subject matter. And Elysium's description of the claims' subject matter is entirely consistent with the language of the claims and

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claim eligible subject matter under § 101 from patents that do not claim eligible subject matter under § 101.

the patents' shared written description. Accordingly, the asserted claims are directed to a natural phenomenon.

ChromaDex counters that "the mere fact that NR is found in nature does not establish that the claimed compositions are directed to patent-ineligible subject matter." D.I. 278 at 2. Quoting language from *Natural Alternatives International, Inc. v. Creative Compounds, LLC*, 918 F.3d 1338 (Fed. Cir. 2019), ChromaDex argues that the "correct inquiry under *Alice* step 1 is . . . whether compositions of the Asserted Claims 'have different characteristics and can be used in a manner that [NR] as it appears in nature cannot.'" D.I. 278 at 3 (citing *Natural Alternatives*, 918 F.3d at 1348) (alterations in the original). According to ChromaDex:

> The characteristics of the claimed compositions dramatically distinguish those compositions from naturally occurring NR. The claimed compositions contain isolated NR that is stable, bioavailable, and sufficiently pure that the compositions can be administered orally to deliver NR to the cells of an animal and exert therapeutic effect. Elysium's motion contains no showing that the NR in milk even reaches the bloodstream after the milk is consumed, let alone enters cells and provides therapeutic effect.

D.I. 278 at 6.

But even if I were to apply the *Alice* step one test as framed by ChromaDex, its argument fails. As an initial matter, the characteristics of the isolated NR in the claimed compositions that ChromaDex has identified as being different from the

characteristics of NR in milk—i.e., stability, bioavailability, sufficient purity, and therapeutic efficacy—are immaterial to the *Alice* inquiry because none of these characteristics are required by the claims. *Synopsys, Inc. v. Mentor Graphics Corp.*, 839 F.3d 1138, 1149 (Fed. Cir. 2016) ("The § 101 inquiry must focus on the language of the Asserted Claims themselves."). Nothing in the language of the asserted claims or the patent's intrinsic evidence suggests that the claims require these characteristics. And, indeed, ChromaDex does not allege in its briefing that the claims impose such requirements. ChromaDex expressly states in its briefing that the asserted claims require that the recited compositions be capable of improving a patient's health and of enhancing NAD^+ synthesis. *See* D.I. 278 at 7 (stating that "the claims do require that the compositions have the capability to improve health and well-being (the [#]086 Patent) [and] enhance NAD^+ biosynthesis (the [#] 807 Patent)"). But those requirements have no bearing on the *Alice* step one test articulated by ChromaDex, since it is undisputed that NR in milk improves health and well-being and enhances NAD^+ biosynthesis, and thus those characteristics do not distinguish isolated NR in the claimed compositions from NR found in milk.

The crux of ChromaDex's position seems to be that stability, bioavailability, purity, and therapeutic efficacy are implicitly required by the claims' "isolation" limitation. ChromaDex states, for example, that "[t]he use of isolated NR in the

Asserted Claims requires that the NR in the claimed compositions be stable and bioavailable, allowing it to reach the bloodstream, enter the cell, and provide therapeutic effect." D.I. 278 at 4. And it argues that "[b]ecause the NR in the claimed compositions is isolated—and therefore stable, bioavailable, and pure—the claimed compositions can be used to deliver effective amounts of NR to cells." D.I. 278 at 6–7. But the Supreme Court unanimously rejected this line of argument in *Association for Molecular Pathology v. Myriad Genetics, Inc.*, 569 U.S. 576, 580 (2013). The Court held in *Myriad* that "a naturally occurring DNA segment is a product of nature and not patent-eligible merely because it has been isolated." *Id.* And it expressly rejected the argument that the asserted claims in that case were "saved by the fact that isolating DNA from the human genome severs chemical bonds and thereby creates a nonnaturally occurring molecule," because "Myriad's claims are simply not expressed in terms of chemical composition, nor do they rely in any way on the chemical changes that result from the isolation of a particular section of DNA." *Id.* at 593.

In this case, the asserted claims are simply not expressed in terms of stability, bioavailability, or purity; nor do they rely in any way on changes that result from the isolation of NR. ChromaDex consented to my construction of "isolated [NR]" as NR "that is separated or substantially free from at least some of the other components associated with the source of the [NR]." Tr. of Dec. 17,

2020 Hr'g at 32:1–6. And that construction in no way requires that the NR in the claimed composition be stable, bioavailable, sufficiently pure, or have a therapeutic effect.

Accordingly, I decline to import details not claimed and find that the asserted claims are directed to a natural product. *See ChargePoint, Inc. v. SemaConnect, Inc.*, 920 F.3d 759, 769 (Fed. Cir. 2019) (focusing § 101 analysis on the asserted claims because "the specification cannot be used to import details from the specification if those details are not claimed."), *cert. denied*, 140 S. Ct. 983 (2020).

B. *Alice* Step Two

Having found that the claims are directed to a product of nature, I consider next whether they contain an "'inventive concept' sufficient to 'transform' the claimed [ineligible concept] into a patent-eligible application." *Alice*, 573 U.S. at 221 (quoting *Mayo*, 566 U.S. at 77). It is insufficient for the patent to "simply state the law of nature while adding the words 'apply it.'" *Mayo*, 566 U.S. at 72. A claim directed towards a natural product must include "additional features to ensure that the claim is more than a drafting effort designed to monopolize the [natural product]." *Alice*, 573 U.S. at 221 (quotation marks and alterations omitted) (quoting *Mayo*, 566 U.S. at 77).

There are no such additional features here. The patents' shared written description acknowledges, and ChromaDex does not dispute, that compositions containing NR "can be prepared by methods and contain carriers which are well-known in the art." #807 patent at 29:24–35; #086 patent at 28:49–60. Nor does ChromaDex dispute that the physical act of isolating NR is not an inventive concept. *See* #807 patent at 27:45–54 ("Isolated extracts of the natural sources can be prepared using standard methods."); #086 patent at 27:3–12 (same); D.I. 292-1, Ex. 1 ¶ 164 (ChromaDex's expert stating that "[i]t is not the specific techniques of isolation that transform the Asserted Claims beyond a law of nature or natural phenomenon"); *see also Myriad*, 569 U.S. at 591, 595 (stating that "the processes used by Myriad to isolate DNA were well understood by geneticists at the time of Myriad's patents" and that "separating th[e] [BRCA1 or BRCA2] gene from its surrounding genetic material is not an act of invention").

ChromaDex argues initially in its briefing that the "inventive step" of the asserted claims is the "recogni[tion] [of] the utility of NR for enhancing health and well-being." D.I. 278 at 9. But "[t]he inventive concept necessary at step 2 of the *Mayo/Alice* analysis cannot be furnished by [an] unpatentable law of nature (or natural phenomenon or abstract idea)." *Genetic Techs. Ltd. v. Merial L.L.C.*, 818 F.3d 1369, 1376 (Fed. Cir. 2016). Perhaps because it realized the futility of its argument, ChromaDex abandoned it in the very next paragraph of its brief, stating

there that "[t]he inventive concept of the Asserted Claims is not the *discovery* of the NR vitamin pathway, but rather therapeutic *applications* of this discovery in inventive ways beyond that of the prior art." D.I. 278 at 9–10 (emphasis in the original). Its expert agrees with this latter position. In the expert's words:

> [T]he inventive concept is the pioneering decision to create a composition comprising isolated NR formulated for oral administration. This was not well-understood, routine, and conventional activity at the time of the invention; . . . it was not until [the inventor] Dr. Brenner's work in 2004 that the scientific community even became aware of the importance of NR as an orally available vitamin or what it would do in the body.

D.I. 292-1, Ex.1 ¶ 164.

This revised articulation of the putative inventive concept fails too. Because NR's oral bioavailability is an inherent property of NR and thus is itself a natural phenomenon, ChromaDex did not alter NR to create this property. It simply uncovered it. ChromaDex is essentially arguing that the idea of making an oral formulation of NR was inventive. But the decision to create an oral formulation of NR after discovering that NR is orally bioavailable is simply applying a patent-ineligible law of nature. And the Supreme Court has made clear that more than "apply it" is needed to "transform an unpatentable law of nature into a patent-eligible application of such a law." *Mayo,* 566 U.S. at 72.

ChromaDex disagrees and cites the Federal Circuit's decision in *Rapid Litigation Management, Ltd. v. CellzDirect, Inc.*, 827 F.3d 1042, 1050–51 (Fed.

Cir. 2016) for the proposition that "a claim that 'applies the discovery' to achieve something new and useful suffices to provide an inventive concept." D.I. 278 at 10 (citing *CellzDirect*, 827 F.3d at 1050–51). But the Court in *CellzDirect* stressed that the patent-eligible asserted claims at issue in that case were "directed to a new and useful method," as opposed to a product claim. 827 F.3d at 1048–49 (noting that the asserted claims "are like thousands of others that recite *processes* to achieve a desired outcome, e.g., *methods* of producing things or, *methods* of treating diseases") (emphasis added)); *id.* at 1049 (stating "the claims are directed to a new and useful process of creating [the] pool [of cells], not to the pool [of cells] itself"); *id.* at 1049 (stating that the method claims before it were "distinguishable from [the composition claims] held unpatentable in *Myriad*"). The asserted claims here are composition claims, and thus they are governed by *Myriad*. *See Myriad*, 569 U.S. at 595 (noting that the claims the Court found to be patent-ineligible were not method claims purporting to create an inventive method of manipulating genes).

IV. CONCLUSION

For the reasons discussed above, I find that claims 1, 2, and 3 of the #807 patent and claim 2 of the #086 patent are invalid under 35 U.S.C. § 101 for claiming patent-ineligible subject matter. Accordingly, I will grant Elysium's motion for summary judgment (D.I. 182).

The Court will issue an Order consistent with this Memorandum Opinion.

IN THE UNITED STATES DISTRICT COURT
FOR THE DISTRICT OF DELAWARE

CHROMADEX, INC. and TRUSTEES OF DARTMOUTH COLLEGE Plaintiffs, v. ELYSIUM HEALTH, INC. Defendant.	Civil Action No. 18-1434-CFC-JLH

ORDER

At Wilmington this Twenty-First day of September in 2021:

For the reasons set forth in the Memorandum Opinion issued this day, **IT IS HEREBY ORDERED** that Elysium Health's Motion for Summary Judgment (No. 1) of Invalidity Under 35 U.S.C. § 101 (D.I. 182) is **GRANTED** and that claims 1, 2, and 3 of U.S. Patent No. 8,197,807 and claim 2 of U.S. Patent No. 8,383,086 are **INVALID**.

United States District Judge

IN THE UNITED STATES DISTRICT COURT
FOR THE DISTRICT OF DELAWARE

CHROMADEX, INC. and TRUSTEES OF DARTMOUTH COLLEGE,	)	
Plaintiffs,	)	C.A. No. 18-1434-CFC
v.	)	
ELYSIUM HEALTH, INC.,	)	
Defendant.	)	

FINAL JUDGMENT

IT IS HEREBY ORDERED, ADJUDGED, AND DECREED that:

1. The Court having granted Elysium Health, Inc.'s Motion (D.I. 182) for Summary Judgment (No. 1) of Invalidity under 35 U.S.C. § 101, Final Judgment of invalidity of claims 1, 2, and 3 of U.S. Patent No. 8,197,807 and claim 2 of U.S. Patent No. 8,383,086 is hereby entered in favor of Defendant Elysium Health, Inc. and against Plaintiffs ChromaDex, Inc. and Trustees of Dartmouth College.

2. All other pending motions are denied as moot.

3. Any motion for attorney fees and/or costs—including any bill of costs under Fed. R. Civ. P. 54(d) and/or D. Del. LR 54.1, or motion pursuant to 35 U.S.C. § 285 or Fed. R. Civ. P. 54(d)(2)—shall be considered timely if filed and served (1) within 30 days after issuance of the mandate by the Federal Circuit in

any appeal; or (2) in the event that no appeal is filed, within 30 days after the expiration of the time for filing a notice of appeal from this judgment under Fed. R. App. P. 3 and 4.

This is a final, appealable judgment.

10.6.21

The Honorable Colm F. Connolly
Chief Judge

US008197807B2

(12) **United States Patent**
Brenner

(10) **Patent No.: US 8,197,807 B2**
(45) **Date of Patent: Jun. 12, 2012**

(54) **NICOTINAMIDE RIBOSIDE KINASE COMPOSITIONS AND METHODS FOR USING THE SAME**

(75) Inventor: **Charles M. Brenner**, Lyme, NH (US)

(73) Assignee: **Trustees of Dartmouth College**, Hanover, NH (US)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 213 days.

(21) Appl. No.: **11/912,400**

(22) PCT Filed: **Apr. 20, 2006**

(86) PCT No.: **PCT/US2006/015495**
§ 371 (c)(1),
(2), (4) Date: **Nov. 20, 2007**

(87) PCT Pub. No.: **WO2006/116322**

PCT Pub. Date: **Nov. 2, 2006**

(65) **Prior Publication Data**

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(51) **Int. Cl.**
A61K 38/45 (2006.01)
C07H 17/00 (2006.01)
A61P 35/00 (2006.01)

(52) **U.S. Cl.** **424/94.5**; 514/45; 514/25; 435/15

(58) **Field of Classification Search** ..... None
See application file for complete search history.

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(74) *Attorney, Agent, or Firm* — Licata & Tyrrell P.C.

(57) **ABSTRACT**

The present invention relates to isolated nicotinamide riboside kinase (Nrk) nucleic acid sequences, vectors and cultured cells containing the same, and Nrk polypeptides encoded thereby. Methods for identifying individuals or tumors susceptible to nicotinamide riboside-related prodrug treatment and methods for treating cancer by administering an Nrk nucleic acid sequence or polypeptide in combination with a nicotinamide riboside-related prodrug are also provided. The present invention further provides screening methods for isolating a nicotinamide riboside-related prodrug and identifying a natural source of nicotinamide riboside.

3 Claims, 1 Drawing Sheet

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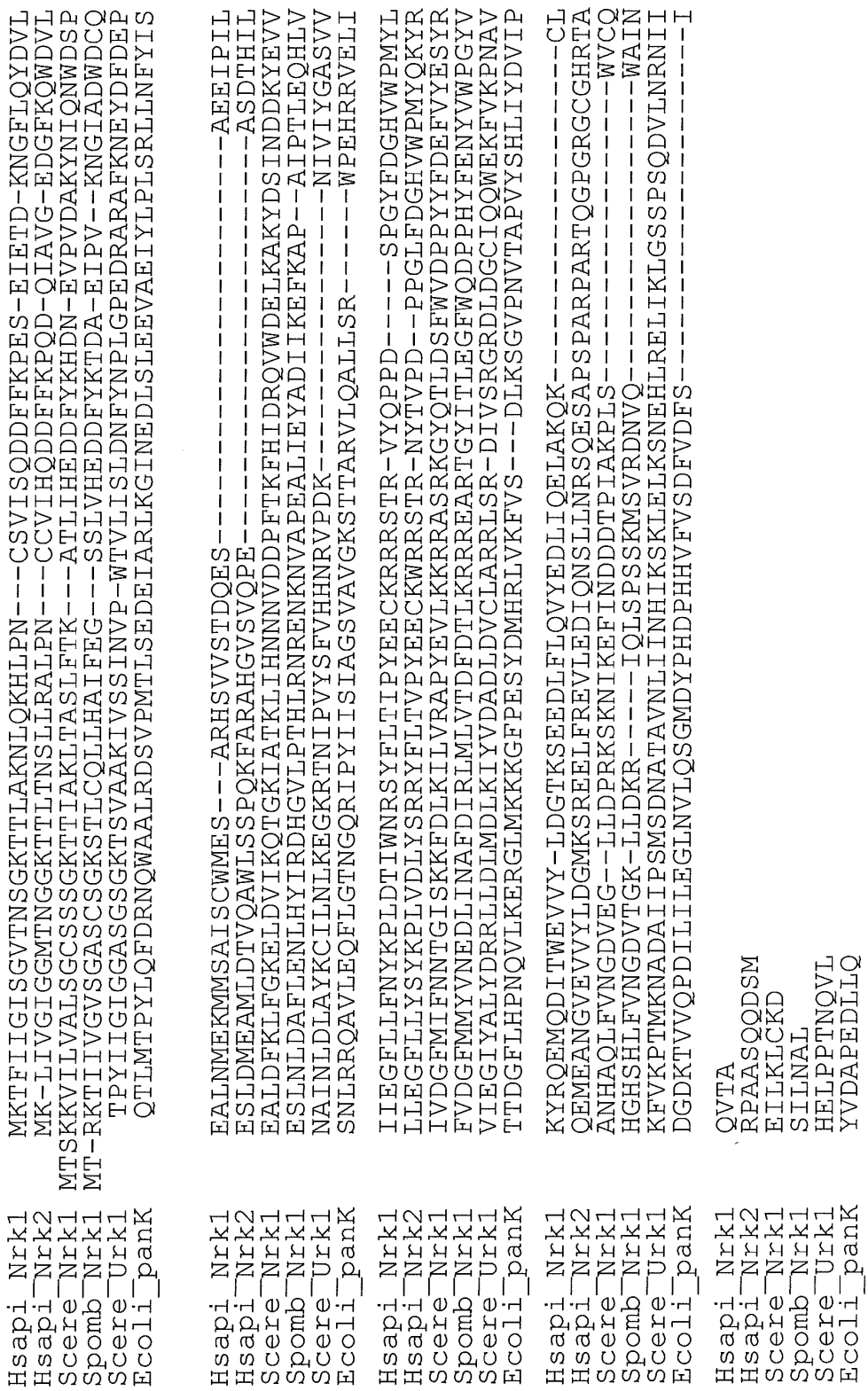

Hsapi_Nrk1 MKTFIIGISGVTNSGKTTLAKNLQKHLPN---CSVISQDDFFKPES-EIETD-KNGFLQYDVL
Hsapi_Nrk2 MK-LIVGIGGMTNGGKTTLTNSLLRALPN---CCVIHQDDFFKPQD-QIAVG-EDGFKQWDVL
Scere_Nrk1 MTSKKVILVALSGCSSSGKTTIAKLTASLFTK---ATLIHEDDFYKHDN-EVPVDAKYNIQNWDSP
Spomb_Nrk1 MT-RKTIIVGVSGASCSGKSTLCQLLHAIFEG---SSLVHEDDFYKTDA-EIPV--KNGIADWDCQ
Scere_Urk1 TPYIIGIGGASGSGKTSVAAKIVSSINVP-WTVLISLDNFYNPLGPEDRARAFKNEYDFDEP
Ecoli_panK QTLMTPYLQFDRNQWAALRDSVPMTLSEDEIARLKGINEDLSLEEVAEIYLPLSRLLNFYIS
Hsapi_Nrk1 EALNMEKMMSAISCWMES---ARHSVVSTDQES----------AEEIPIL
Hsapi_Nrk2 ESLDMEAMLDTVQAWLSSPQKFARAHGVSVQPE----------ASDTHIL
Scere_Nrk1 EALDFKLFGKELDVIKQTGKIATKLIHNNNVDDPFTKFHIDRQVWDELKAKYDSINDDKYEVV
Spomb_Nrk1 ESLNLDAFLENLHYIRDHGVLPTHLRNRENKNVAPEALIEYADIIKEFKAP--AIPTLEQHLV
Scere_Urk1 NAINLDLAYKCILNLKEGKRTNIPVYSFVHHNRVPDK----------NIVIYGASVV
Ecoli_panK SNLRRQAVLEQFLGTNGQRIPYIISIAGSVAVGKSTTARVLQALLSR----------WPEHRRVELI
Hsapi_Nrk1 IIEGFLLFNYKPLDTIWNRSYFLTIPYEECKRRRSTR-VYQPPD-----SPGYFDGHVWPMYL
Hsapi_Nrk2 LLEGFLLYSYKPLVDLYSRRYFLTVPYEECKWRRSTR-NYTVPD--PPGLFDGHVWPMYQKYR
Scere_Nrk1 IVDGFMIFNNTGISKKFDLKILVRAPYEVLKKRRASRKGYQTLDSFWVDPPYYFDEFVYESYR
Spomb_Nrk1 FVDGFMMYVNEDLINAFDIRLMLVTDFDTLKRRREARTGYITLEGFWQDPPHYFENYVWPGYV
Scere_Urk1 VIEGIYALYDRRLLDLMDLKIYVDADLDVCLARRLSR-DIVSRGRDLDGCIQQWEKFVKPNAV
Ecoli_panK TTDGFLHPNQVLKERGLMKKKGFPESYDMHRLVKFVS---DLKSGVPNVTAPVYSHLIYDVIP
Hsapi_Nrk1 KYRQEMQDITWEVVY-LDGTKSEEDLFLQVYEDLIQELAKQK----------CL
Hsapi_Nrk2 QEMEANGVEVVYLDGMKSREELFREVLEDIQNSLLNRSQESAPSPARPARTQGPGRGCGHRTA
Scere_Nrk1 ANHAQLFVNGDVEG--LLDPRKSKNIKEFINDDDTPIAKPLS----------WVCQ
Spomb_Nrk1 HGHSHLFVNGDVTGK-LLDKR-----IQLSPSSKMSVRDNVQ----------WAIN
Scere_Urk1 KFVKPTMKNADAIIPSMSDNATAVNLIINHIKSKLELKSNEHLRELIKLGSSPSQDVLNRNII
Ecoli_panK DGDKTVVQPDILILEGLNVLQSGMDYPHDPHHVFVSDFVDFS----------I
Hsapi_Nrk1 QVTA
Hsapi_Nrk2 RPAASQQDSM
Scere_Nrk1 EILKLCKD
Spomb_Nrk1 SILNAL
Scere_Urk1 HELPPTNQVL
Ecoli_panK YVDAPEDLLQ

NICOTINAMIDE RIBOSIDE KINASE COMPOSITIONS AND METHODS FOR USING THE SAME

INTRODUCTION

This invention was made in the course of research sponsored by the National Cancer Institute (Grant No. CA77738). The U.S. government may have certain rights in this invention.

This application claims benefit of priority to PCT/US2006/015495, filed Apr. 20, 2006, which claims benefit from U.S. patent application Ser. No. 11/113,701, filed Apr. 25, 2005, now abandoned which is a continuation-in-part of PCT application No. PCT/US2005/004337, filed Feb. 9, 2005, which claims benefit under 35 U.S.C. §119 to U.S. Provisional Patent Application Ser. No. 60/543,347, filed on Feb. 10, 2004, whose contents are incorporated herein by reference in their entireties.

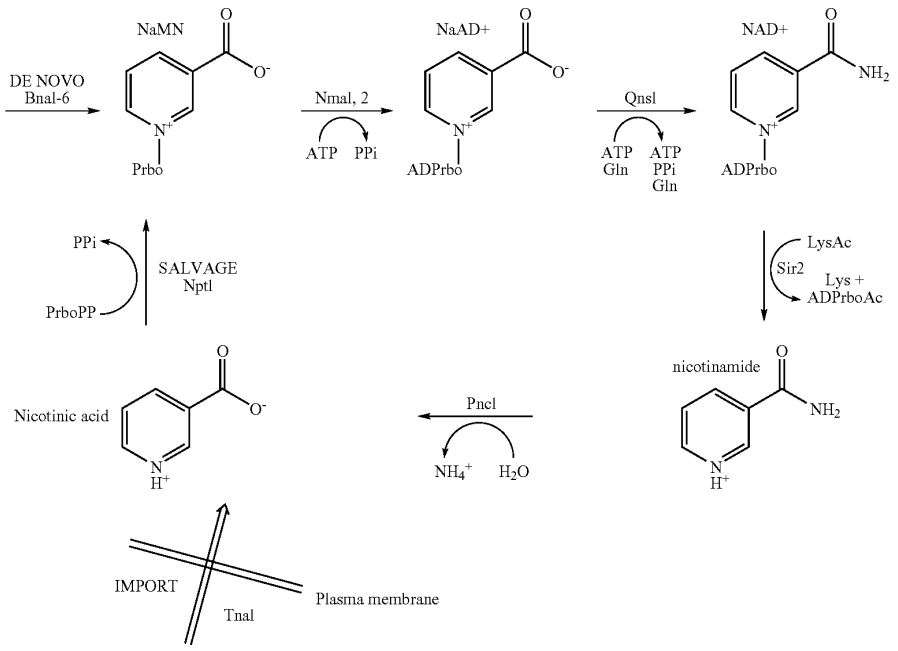
BACKGROUND OF THE INVENTION

Nicotinic acid and nicotinamide, collectively niacins, are the vitamin forms of nicotinamide adenine dinucleotide (NAD+). Eukaryotes can synthesize NAD+ de novo via the kynurenine pathway from tryptophan (Krehl, et al. (1945) *Science* 101:489-490; Schutz and Feigelson (1972) *J. Biol. Chem.* 247:5327-5332) and niacin supplementation prevents the pellagra that can occur in populations with a tryptophan-poor diet. It is well-established that nicotinic acid is phosphoribosylated to nicotinic acid mononucleotide (NaMN), which is then adenylylated to form nicotinic acid adenine dinucleotide (NaAD), which in turn is amidated to form NAD+ (Preiss and Handler (1958) *J. Biol. Chem.* 233:488-492; Preiss and Handler (1958b) *J. Biol. Chem.* 233:493-50).

NAD+ was initially characterized as a co-enzyme for oxidoreductases. Though conversions between NAD+, NADH, NADP and NADPH would not be accompanied by a loss of total co-enzyme, it was discovered that NAD+ is also turned over in cells for unknown purposes (Maayan (1964) *Nature* 204:1169-1170). Sirtuin enzymes such as Sir2 of *S. cerevisiae* and its homologs deacetylate lysine residues with consumption of an equivalent of NAD+ and this activity is required for Sir2 function as a transcriptional silencer (Imai, et al. (2000) *Cold Spring Harb. Symp. Quant. Biol.* 65:297-302). NAD^+-dependent deacetylation reactions are required not only for alterations in gene expression but also for repression of ribosomal DNA recombination and extension of lifespan in response to calorie restriction (Lin, et al. (2000) *Science* 289:2126-2128; Lin, et al. (2002) *Nature* 418:344-348). NAD+ is consumed by Sir2 to produce a mixture of 2'-and 3' O-acetylated ADP-ribose plus nicotinamide and the deacetylated polypeptide (Sauve, et al. (2001) *Biochemistry* 40:15456-15463). Additional enzymes, including poly(ADPribose) polymerases and cADPribose synthases are also NAD^+-dependent and produce nicotinamide and ADPribosyl products (Ziegler (2000) *Eur. J. Biochem.* 267:1550-1564; Burkle (2001) *Bioessays* 23:795-806).

The non-coenzymatic properties of NAD+ has renewed interest in NAD+ biosynthesis. Four recent publications have suggested what is considered to be all of the gene products and pathways to NAD+ in *S. cerevisiae* (Panozzo, et al. (2002) *FEBS Lett.* 517:97-102; Sandmeier, et al. (2002) *Genetics* 160:877-889; Bitterman, et al. (2002) J. Biol. Chem. 277:45099-45107; Anderson, et al. (2003) *Nature* 423:181-185) depicting convergence of the flux to NAD+ from de novo synthesis, nicotinic acid import, and nicotinamide salvage at NaMN (Scheme 1).

Scheme 1



SUMMARY OF THE INVENTION

It has now been shown that nicotinamide riboside, which was known to be an NAD+ precursor in bacteria such as *Haemophilus influenza* (Gingrich and Schlenk (1944) *J. Bacteriol.* 47:535-550; Leder and Handler (1951) *J. Biol. Chem.* 189:889-899; Shifrine and Biberstein (1960) *Nature* 187: 623) that lack the enzymes of the de novo and Preiss-Handler pathways (Fleischmann, et al. (1995) *Science* 269:496-512), is an NAD+ precursor in a previously unknown but conserved eukaryotic NAD+ biosynthetic pathway. Yeast nicotinamide riboside kinase, Nrk1, and human Nrk enzymes with specific functions in NAD+ metabolism are provided herein. The specificity of these enzymes indicates that they are the long-sought tiazofurin kinases that perform the first step in converting cancer drugs such as tiazofurin and benzamide riboside and their analogs into toxic NAD+ analogs. Further, yeast mutants of defined genotype were used to identify sources of nicotinamide riboside and it is shown that milk is a source of nicotinamide riboside.

Accordingly, the present invention is an isolated nucleic acid encoding a eukaryotic nicotinamide riboside kinase polypeptide. A eukaryotic nicotinamide riboside kinase nucleic acid encompasses (a) a nucleotide sequence of SEQ ID NO:1, SEQ ID NO:2 or SEQ ID NO:3; (b) a nucleotide sequence that hybridizes to a nucleotide sequence of SEQ ID NO:1, SEQ ID NO:2 or SEQ ID NO:3 or its complementary nucleotide sequence under stringent conditions, wherein said nucleotide sequence encodes a functional nicotinamide riboside kinase polypeptide; or (c) a nucleotide sequence encoding an amino acid sequence encoded by the nucleotide sequences of (a) or (b), but which has a different nucleotide sequence than the nucleotide sequences of (a) or (b) due to the degeneracy of the genetic code or the presence of non-translated nucleotide sequences.

The present invention is also an expression vector containing an isolated nucleic acid encoding a eukaryotic nicotinamide riboside kinase polypeptide. In one embodiment, the expression vector is part of a composition containing a pharmaceutically acceptable carrier. In another embodiment, the composition further contains a prodrug wherein the prodrug is a nicotinamide riboside-related analog that is phosphorylated by the expressed nicotinamide riboside kinase thereby performing the first step in activating said prodrug.

The present invention is also an isolated eukaryotic nicotinamide riboside kinase polypeptide. In one embodiment, the isolated nicotinamide riboside kinase polypeptide has an amino acid sequence having at least about 70% amino acid sequence similarity to an amino acid sequence of SEQ ID NO:4, SEQ ID NO:5 or SEQ ID NO:6 or a functional fragment thereof.

The present invention is further a cultured cell containing an isolated nucleic acid encoding a eukaryotic nicotinamide riboside kinase polypeptide or a polypeptide encoded thereby.

Still further, the present invention is a composition containing an isolated eukaryotic nicotinamide riboside kinase polypeptide and a pharmaceutically acceptable carrier. In one embodiment, the composition further contains a prodrug wherein said prodrug is a nicotinamide riboside-related analog that is phosphorylated by the nicotinamide riboside kinase thereby performing the first step in activating said prodrug.

The present invention is also a method for treating cancer by administering to a patient having or suspected of having cancer an effective amount of a nicotinamide riboside-related prodrug in combination with an isolated eukaryotic nicotinamide riboside kinase polypeptide or expression vector containing an isolated nucleic acid sequence encoding an eukaryotic nicotinamide riboside kinase polypeptide wherein the nicotinamide riboside kinase polypeptide phosphorylates the prodrug thereby performing the first step in activating the prodrug so that the signs or symptoms of said cancer are decreased or eliminated.

The present invention is further a method for identifying a natural or synthetic source for nicotinamide riboside. The method involves contacting a first cell lacking a functional glutamine-dependent NAD+ synthetase with an isolated extract from a natural source or synthetic; contacting a second cell lacking functional glutamine-dependent NAD+ synthetase and nicotinamide riboside kinase with the isolated extract; and detecting growth of the first cell compared to the growth of the second cell, wherein the presence of growth in the first cell and absence of growth in the second cell is indicative of the presence of nicotinamide riboside in the isolated extract. In one embodiment, the natural source is cow's milk.

Further, the present invention is a dietary supplement composition containing nicotinamide riboside identified in accordance with the methods of the present invention and a carrier.

Moreover, the present invention is a method for preventing or treating a disease or condition associated with the nicotinamide riboside kinase pathway of NAD+ biosynthesis. The method involves administering to a patient having a disease or condition associated with the nicotinamide riboside kinase pathway of NAD+ biosynthesis an effective amount of a nicotinamide riboside composition so that the signs or symptoms of the disease or condition are prevented or reduced. In one embodiment, the nicotinamide riboside is neuroprotective. In another embodiment the nicotinamide riboside is anti-fungal. In a further embodiment, the nicotinamide riboside is administered in combination with tryptophan, nicotinic acid or nicotinamide.

The present invention is also an in vitro method for identifying a nicotinamide riboside-related prodrug. The method involves contacting a nicotinamide riboside kinase polypeptide with a nicotinamide riboside-related test agent and determining whether said test agent is phosphorylated by said nicotinamide riboside kinase polypeptide wherein phosphorylation of said test agent is indicative of said test agent being a nicotinamide riboside-related prodrug. A nicotinamide riboside-related prodrug identified by this method is also encompassed within the present invention.

The present invention is further a cell-based method for identifying a nicotinamide riboside-related prodrug. This method involves contacting a first test cell which expresses a recombinant Nrk polypeptide with a nicotinamide riboside-related test agent; contacting a second test cell which lacks a functional Nrk polypeptide with the same test agent; and determining the viability of the first and second test cells, wherein sensitivity of the first cell and not the second cell is indicative of a nicotinamide riboside-related prodrug. A nicotinamide riboside-related prodrug identified by this method is also encompassed within the context of the present invention.

The present invention is also a method for identifying an individual or tumor which is susceptible to treatment with a nicotinamide riboside-related prodrug. This method involves detecting the presence of mutations in, or the level of expression of, a nicotinamide riboside kinase in an individual or tumor wherein the presence of a mutation or change in expression of nicotinamide riboside kinase in said individual or tumor compared to a control is indicative of said individual or tumor having an altered level of susceptibility to treatment with a nicotinamide riboside-related prodrug.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. **1** shows the amino acid sequence alignment and consensus sequence (SEQ ID NO:34) of human Nrk1 (SEQ ID NO:5), human Nrk2 (SEQ ID NO:6), *S. cerevisiae* Nrk1 (SEQ ID NO:4), *S. pombe* nrk1 (SEQ ID NO:7), as compared to portions of *S. cerevisiae* uridine/cytidine kinase Urk1 (SEQ ID NO:8) and *E. coli* pantothenate kinase (SEQ ID NO:9).

DETAILED DESCRIPTION OF THE INVENTION

A *Saccharomyces cerevisiae* QNS1 gene encoding glutamine-dependent NAD+synthetase has been characterized and mutation of either the glutaminase active site or the NAD+ synthetase active site resulted in inviable cells (Bieganowski, et al. (2003) *J. Biol. Chem.* 278:33049-33055). Possession of strains containing the qns1 deletion and a plasmid-borne QNS1 gene allowed a determination of whether the canonical de novo, import and salvage pathways for NAD+ of Scheme 1 (Panozzo, et al. (2002) supra; Sandmeier, et al. (2002) supra; Bitterman, et al. (2002) supra; Anderson, et al. (2003) supra) are a complete representation of the metabolic pathways to NAD+ in *S. cerevisiae*. The pathways depicted in scheme 1 suggest that: nicotinamide is deamidated to nicotinic acid before the pyridine ring is salvaged to make more NAD+, thus supplementation with nicotinamide may not rescue qns1 mutants by shunting nicotinamide-containing precursors through the pathway; and QNS1 is common to the three pathways, thus there may be no NAD+ precursor that rescues qns1 mutants. However, it has now been found that while nicotinamide does not rescue qns1 mutants even at 1 or 10 mM, nicotinamide riboside functions as a vitamin form of NAD+ at 10 μM.

Anticancer agents such as tiazofurin (Cooney, et al. (1983) *Adv. Enzyme Regul.* 21:271-303) and benzamide riboside (Krohn, et al. (1992) *J. Med. Chem.* 35:511-517) have been shown to be metabolized intracellularly to NAD+ analogs, taizofurin adenine dinucleotide and benzamide adenine dinucleotide, which inhibit IMP dehydrogenase the rate-limiting enzyme for de novo purine nucleotide biosynthesis.

Though an NMN/NaMN adenylyltransferase is thought to be the enzyme that converts the mononucleotide intermediates to NAD+ analogs and the structural basis for this is known (Zhou et al. (2002) supra), several different enzymes including adenosine kinase, 5' nucleotidase (Fridland, et al. (1986) *Cancer Res.* 46:532-537; Saunders, et al. (1990) *Cancer Res.* 50:5269-5274) and a specific nicotinamide riboside kinase (Saunders, et al. (1990) supra) have been proposed to be responsible for tiazofurin phosphorylation in vivo. A putative nicotinamide riboside kinase (Nrk) activity was purified, however no amino acid sequence information was obtained and, as a consequence, no genetic test was performed to assess its function (Sasiak and Saunders (1996) *Arch. Biochem. Biophys.* 333:414-418).

Using a qns1 deletion strain that was additionally deleted for yeast homologs of candidate genes encoding nucleoside kinases proposed to phosphorylate tiazofurin, i.e., adenosine kinase ado1 (Lecoq, et al. (2001) *Yeast* 18:335-342), uridine/cytidine kinase urk1 (Kern (1990) *Nucleic Acids Res.* 18:5279; Kurtz, et al. (1999) *Curr. Genet.* 36:130-136), and ribokinase rbk1 (Thierry, et al. (1990) *Yeast* 6:521-534), it was determined whether the nucleoside kinases are uniquely or collectively responsible for utilization of nicotinamide riboside. It was found that despite these deletions, the strain retained the ability to utilize nicotinamide riboside in an anabolic pathway independent of NAD+ synthetase.

Given that mammalian pharmacology provided no useful clue to the identity of a putative fungal Nrk, it was considered whether the gene might have been conserved with the Nrk of *Haemophilus influenza*. The Nrk domain of *H. influenza* is encoded by amino acids 225 to 421 of the NadR gene product (the amino terminus of which is NMN adenylyltransferase). Though this domain is structurally similar to yeast thymidylate kinase (Singh, et al. (2002) *J. Biol. Chem.* 277:33291-33299), sensitive sequence searches revealed that bacterial Nrk has no ortholog in yeast. Genomic searches with the Nrk domain of *H. influenza* NadR have identified a growing list of bacterial genomes predicted to utilize nicotinamide riboside as an NAD+ precursor (Kurnasov, et al. (2002) *J. Bacteriol.* 184:6906-6917). Thus, had fungi possessed NadR Nrk-homologous domains, comparative genomics would have already predicted that yeast can salvage nicotinamide riboside.

To identify the Nrk of *S. cerevisiae*, an HPLC assay for the enzymatic activity was established and used in combination with a biochemical genomics approach to screen for the gene encoding this activity (Martzen, et al. (1999) *Science* 286: 1153-1155). Sixty-four pools of 90-96 *S. cerevisiae* open reading frames fused to glutathione S-transferase (GST), expressed in *S. cerevisiae*, were purified as GST fusions and screened for the ability to convert nicotinamide riboside plus ATP to NMN plus ADP. Whereas most pools contained activities that consumed some of the input ATP, only pool 37 consumed nicotinamide riboside and produced NMN. In pool 37, approximately half of the 1 mM ATP was converted to ADP and the 500 μM nicotinamide riboside peak was almost entirely converted to NMN. Examination of the 94 open reading frames that were used to generate pool 37 revealed that YNL129W (SEQ ID NO:1) encodes a predicted 240 amino acid polypeptide with a 187 amino acid segment containing 23% identity with the 501 amino acid yeast uridine/cytidine kinase Urk1 and remote similarity with a segment of *E. coli* pantothenate kinase panK (Yun, et al. (2000) *J. Biol. Chem.* 275:28093-28099) (FIG. **1**). After cloning YNL129W into a bacterial expression vector it was ascertained whether this homolog of metabolite kinases was the eukaryotic Nrk. The specific activity of purified YNL129W was ~100-times that of pool 37, consistent with the idea that all the Nrk activity of pool 37 was encoded by this open reading frame. To test genetically whether this gene product phosphorylates nicotinamide riboside in vivo, a deletion of YNL129W was created in the qns1 background. It was found that nicotinamide riboside rescue of the qns1 deletion strain was entirely dependent on this gene product. Having shown biochemically and genetically that YNL129W encodes an authentic Nrk activity, the gene was designated NRK1.

A PSI-BLAST (Altschul, et al. (1997) *Nucleic Acids Res.* 25:3389-3402) comparison was conducted on the predicted *S. cerevisiae* Nrk1 polypeptide and an orthologous human protein Nrk1 (NP_060351; SEQ ID NO:5; FIG. **1**) was found. The human NP_060351 protein encoded at locus 9q21.31 is a polypeptide of 199 amino acids and is annotated as an uncharacterized protein of the uridine kinase family. In addition, a second human gene product Nrk2 (NP_733778; SEQ ID NO:6; FIG. **1**) was found that is 57% identical to human Nrk1. Nrk2 is a 230 amino acid splice form of what was described as a 186 amino acid muscle integrin beta 1 binding protein (ITGB1BP3) encoded at 19p13.3 (Li, et al. (1999) *J. Cell Biol.* 147:1391-1398; Li, et al. (2003) *Dev. Biol.* 261:209-219). Amino acid conservation between *S. cerevisiae, S. pombe* and human Nrk homologs and similarity with fragments of *S. cerevisiae* Urk1 and *E. coli* panK is shown in FIG. **1**. Fungal and human Nrk enzymes are members of a

metabolite kinase superfamily that includes pantothenate kinase but is unrelated to bacterial nicotinamide riboside kinase. Robust complementation of the failure of qns1 nrk1 to grow on nicotinamide riboside-supplemented media was provided by human NRK1 and human NRK2 cDNA even when expressed from the GAL1 promoter on glucose.

As shown in Table 1, purification of yeast Nrk1 and human Nrk1 and Nrk2 revealed high specificity for phosphorylation of nicotinamide riboside and tiazofurin.

TABLE 1

	Nicotinamide riboside	Tiazofurin	Uridine	Cytidine
Human Nrk1	275 ± 17	538 ± 27	19.3 ± 1.7	35.5 ± 6.4
Human Nrk2	2320 ± 20	2150 ± 210	2220 ± 170	222 ± 8
Yeast Nrk1	535 ± 60	1129 ± 134	15.2 ± 3.4	82.9 ± 4.4

Specific activity is expressed in nmole mg^{-1} min^{-1} for phosphorylation of nucleoside substrates.

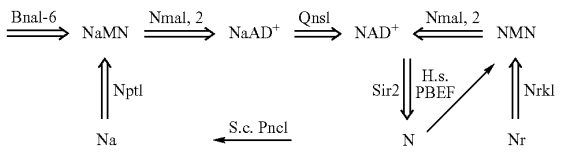
In the cases of yeast and human Nrk1 enzymes, the enzymes preferred tiazofurin to the natural substrate nicotinamide riboside by a factor of two and both enzymes retained less than 7% of their maximal specific activity on uridine and cytidine. In the case of human Nrk2, the 230 amino acid form was essentially equally active on nicotinamide riboside, tiazofurin and uridine with less than 10% of corresponding activity on cytidine. Conversely, the 186 amino acid integrin beta 1 binding protein form was devoid of enzymatic activity in this in vitro assay and was not functional as an Nrk in vivo. However, both the 186 and 230 amino acid isoforms function in vivo in a yeast nicotinamide riboside utilization assay. Thus, though Nrk2 may contribute additionally to formation of uridylate, these data demonstrate that fungi and mammals possess specific nicotinamide riboside kinases that function to synthesize NAD+ through NMN in addition to the well-known pathways through NaMN. Identification of Nrk enzymatic activities thus accounts for the dual specificity of fungal and mammalian NaMN/NMN adenylyltransferases.

On the basis of SAGE data, NRK1 is a rare message in many tissues examined while NRK2 is highly expressed in heart and skeletal muscle and has lower level expression in retinal epithelium and placenta (Boon, et al. (2002) *Proc. Natl. Acad. Sci. USA* 99:11287-11292). From cancer cell line to cancer cell line the expression levels are quite variable (Boon, et al. (2002) supra). Thus, in individuals whose tumors are NRK1, NRK2-low, tiazofurin conversion to NAD+ may occur more extensively in the patients hearts and muscles than in tumors. In tumors that are NRK1 and/or NRK2-high, a substantial amount of tiazofurin may be converted to tiazofurin adenine dinucleotide in tumors.

A yeast qns1 mutant was used to screen for natural sources of nicotinamide riboside wherein it was identified in an acid whey preparation of cowls milk. Unlike the original screen for vitamins in protein-depleted extracts of liver for reversal of black-tongue in starving dogs (Elvehjem, et al. (1938) *J. Biol. Chem.* 123:137-149), this assay is pathway-specific in identifying NAD+ precursors. Because of the qns1 deletion, nicotinic acid and nicotinamide do not score positively in this assay. As the factor from milk requires nicotinamide riboside kinase for growth, the nutrient is clearly nicotinamide riboside and not NMN or NAD+.

A revised metabolic scheme for NAD+, incorporating Nrk1 homologs and the nicotinamide riboside salvage pathway is shown in Scheme 2 wherein double arrows depict metabolic steps common to yeast and humans (with yeast gene names) and single arrows depict steps unique to humans (PBEF, nicotinamide phosphoribosyltransferase) and yeast (Pnc1, nicotinamidase).

Scheme 2



A difference between humans and yeasts concerns the organisms' uses of nicotinamide and nicotinic acid, the two niacins that were co-identified as anti-black tongue factor (Elvehjem, et al. (1938) supra). Humans encode a homolog of the *Haemophilus ducreyi* nadV gene, termed pre-B-cell colony enhancing factor, that may convert nicotinamide to NMN (Rongvaux, et al. (2002) *Eur. J. Immunol.* 32:3225-3234) and is highly induced during lymphocyte activation (Samal, et al. (1994) *Mol. Cell. Biol.* 14:1431-1437). In contrast, *S. cerevisiae* lacks a homolog of nadV and instead has a homolog of the *E. coli* pncA gene, termed PNC1, that converts nicotinamide to nicotinic acid for entry into the Preiss-Handler pathway (Ghislain, et al. (2002) *Yeast* 19:215-224; Sandmeier, et al. (2002) supra). Though the Preiss-Handler pathway is frequently considered a salvage pathway from nicotinamide, it technically refers to the steps from nicotinic acid to NAD+ (Preiss and Handler (1958) supra; Preiss and Handler (1958) supra). Reports that nicotinamidase had been purified from mammalian liver in the 1960s (Petrack, et al. (1965) *J. Biol. Chem.* 240:1725-1730) may have contributed to the sense that fungal and animal NAD+ biosynthesis is entirely conserved. However, animal genes for nicotinamidase have not been identified and there is no compelling evidence that nicotinamide and nicotinic acid are utilized as NAD+ precursors through the same route in mammals. The persistence of "niacin" as a mixture of nicotinamide and nicotinic acid may attest to the utility of utilizing multiple pathways to generate NAD+ and indicates that supplementation with nicotinamide riboside as third importable NAD+ precursor can be beneficial for certain conditions.

First reported in 1955, high doses of nicotinic acid are effective at reducing cholesterol levels (Altschul, et al. (1955) *Arch. Biochem. Biophys.* 54:558-559). Since the initial report, many controlled clinical studies have shown that nicotinic acid preparations, alone and in combination with HMG CoA reductase inhibitors, are effective in controlling low-density lipoprotein cholesterol, increasing high-density lipo-

protein cholesterol, and reducing triglyceride and lipoprotein a levels in humans (Pasternak, et al. (1996) *Ann. Intern. Med.* 125:529-540). Though nicotinic acid treatment effects all of the key lipids in the desirable direction and has been shown to reduce mortality in target populations (Pasternak, et al. (1996) supra), its use is limited because of a side effect of heat and redness termed "flushing," which is significantly effected by the nature of formulation (Capuzzi, et al. (2000) *Curr. Atheroscler. Rep.* 2:64-71). Thus, nicotinamide riboside supplementation could be one route to improve lipid profiles in humans. Further, nicotinamide is protective in animal models of stroke (Klaidman, et al. (2003) *Pharmacology* 69:150-157) and nicotinamide riboside could be an important supplement for acute conditions such as stroke. Additionally, regulation of NAD+ biosynthetic enzymes could be useful in sensitizing tumors to compounds such as tiazofurin, to protect normal tissues from the toxicity of compounds such as tiazofurin adenine dinucleotide, and to stratify patients for the most judicious use of tiazofurin chemotherapy.

The present invention is an isolated nucleic acid containing a eukaryotic nucleotide sequence encoding a nicotinamide riboside kinase polypeptide. As used herein, an isolated molecule (e.g., an isolated nucleic acid such as genomic DNA, RNA or cDNA or an isolated polypeptide) means a molecule separated or substantially free from at least some of the other components of the naturally occurring organism, such as for example, the cell structural components or other polypeptides or nucleic acids commonly found associated with the molecule. When the isolated molecule is a polypeptide, said polypeptide is at least about 25%, 50%, 60%, 70%, 75%, 80%, 85%, 90%, 95%, 97%, 98%, 99% or more pure (w/w).

In one embodiment, the eukaryotic nucleotide sequence encoding a nicotinamide riboside kinase polypeptide is a nucleotide sequence of SEQ ID NO:1, SEQ ID NO:2 or SEQ ID NO:3. In another embodiment, the eukaryotic nucleotide sequence encoding a nicotinamide riboside kinase polypeptide is a nucleotide sequence that hybridizes to a nucleotide sequence of SEQ ID NO:1, SEQ ID NO:2 or SEQ ID NO:3 or its complementary nucleotide sequence under stringent conditions, wherein said nucleotide sequence encodes a functional nicotinamide riboside kinase polypeptide. In a further embodiment, the eukaryotic nucleotide sequence encoding a nicotinamide riboside kinase polypeptide is a nucleotide sequence encoding a functional nicotinamide riboside kinase polypeptide but which has a different nucleotide sequence than the nucleotide sequences of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3 due to the degeneracy of the genetic code or the presence of non-translated nucleotide sequences.

As used herein, a functional polypeptide is one that retains at least one biological activity normally associated with that polypeptide. Alternatively, a functional polypeptide retains all of the activities possessed by the unmodified peptide. By retains biological activity, it is meant that the polypeptide retains at least about 50%, 60%, 75%, 85%, 90%, 95%, 97%, 98%, 99%, or more, of the biological activity of the native polypeptide (and can even have a higher level of activity than the native polypeptide). A non-functional polypeptide is one that exhibits essentially no detectable biological activity normally associated with the polypeptide (e.g., at most, only an insignificant amount, e.g., less than about 10% or even 5%).

As used herein, the term polypeptide encompasses both peptides and proteins, unless indicated otherwise.

A nicotinamide riboside kinase polypeptide or Nrk protein as used herein, is intended to be construed broadly and encompasses an enzyme capable of phosphorylating nicotinamide riboside. The term nicotinamide riboside kinase or Nrk also includes modified (e.g., mutated) Nrk that retains biological function (i.e., have at least one biological activity of the native Nrk protein, e.g., phosphorylating nicotinamide riboside), functional Nrk fragments including truncated molecules, alternatively spliced isoforms (e.g., the alternatively spliced isoforms of human Nrk2), and functional Nrk fusion polypeptides (e.g., an Nrk-GST protein fusion or Nrk-His tagged protein).

Any Nrk polypeptide or Nrk-encoding nucleic acid known in the art can be used according to the present invention. The Nrk polypeptide or Nrk-encoding nucleic acid can be derived from yeast, fungal (e.g., *Saccharomyces cerevisiae, Saccharomyces pombe, Pichia* sp., *Neurospora* sp., and the like) plant, animal (e.g., insect, avian (e.g., chicken), or mammalian (e.g., rat, mouse, bovine, porcine, ovine, caprine, equine, feline, canine, lagomorph, simian, human and the like) sources.

Representative cDNA and amino acid sequences of a *S. cerevisiae* Nrk1 are shown in SEQ ID NO:1 and SEQ ID NO:4 (FIG. **1**), respectively. Representative cDNA and amino acid sequences of a human Nrk1 are shown in SEQ ID NO:2 and SEQ ID NO:5 (FIG. **1**), respectively. Representative cDNA and amino acid sequences of a human Nrk2 are shown in SEQ ID NO:3 and SEQ ID NO:6 (FIG. **1**), respectively. Other Nrk sequences encompassed by the present invention include, but are not limited to, Nrk1 of GENBANK accession numbers NM_017881, AK000566, BC001366, BC036804, and BC026243 and Nrk2 of GENBANK accession number NM_170678. Moreover, locus CAG61927 from the *Candida glabrata* CBS138 genome project (Dujon, et al. (2004) *Nature* 430:35-44) is 54% identical to the *Saccharomyces cerevisiae* Nrk1 protein. Particular embodiments of the present invention embrace a Nrk polypeptide having the conserved amino acid sequence XXXXDDFXK (SEQ ID NO:34), wherein Xaa_1 and Xaa_2 are aliphatic amino acid residues, Xaa_3 is His or Ser, Xaa_4 is a hydrophilic amino acid residue, and Xaa_5 is an aromatic amino acid residue.

To illustrate, hybridization of such sequences can be carried out under conditions of reduced stringency, medium stringency or even stringent conditions (e.g., conditions represented by a wash stringency of 35-40% Formamide with 5×Denhardt's solution, 0.5% SDS and 1×SSPE at 37° C.; conditions represented by a wash stringency of 40-45% Formamide with 5×Denhardt's solution, 0.5% SDS, and 1×SSPE at 42° C.; and/or conditions represented by a wash stringency of 50% Formamide with 5×Denhardt's solution, 0.5% SDS and 1×SSPE at 42° C., respectively) to the sequences specifically disclosed herein. See, e.g., Sambrook et al., *Molecular Cloning, A Laboratory Manual* (2d Ed. 1989) (Cold Spring Harbor Laboratory).

Alternatively stated, isolated nucleic acids encoding Nrk of the invention have at least about 50%, 60%, 70%, 80%, 90%, 95%, 97%, 98% or higher sequence similarity with the isolated nucleic acid sequences specifically disclosed herein (or fragments thereof, as defined above) and encode a functional Nrk as defined herein.

It will be appreciated by those skilled in the art that there can be variability in the nucleic acids that encode the Nrk of the present invention due to the degeneracy of the genetic code. The degeneracy of the genetic code, which allows different nucleic acid sequences to code for the same polypeptide, is well known in the literature (see Table 2).

TABLE 2

Amino Acid	3-Letter Code	1-Letter Code	Codons
Alanine	Ala	A	GCA GCC GCG GCT
Cysteine	Cys	C	TGC TGT
Aspartic acid	Asp	D	GAC GAT
Glutamic acid	Glu	E	GAA GAG
Phenylalanine	Phe	F	TTC TTT
Glycine	Gly	G	GGA GGC GGG GGT
Histidine	His	H	CAC CAT
Isoleucine	Ile	I	ATA ATC ATT
Lysine	Lys	K	AAA AAG
Leucine	Leu	L	TTA TTG CTA CTC CTG CTT
Methionine	Met	M	ATG
Asparagine	Asn	N	AAC AAT
Proline	Pro	P	CCA CCC CCG CCT
Glutamine	Gln	Q	CAA CAG
Arginine	Arg	R	AGA AGG CGA CGC CGG CGT
Serine	Ser	S	AGC ACT TCA TCC TCG TCT
Threonine	Thr	T	ACA ACC ACG ACT
Valine	Val	V	GTA GTC GTG GTT
Tryptophan	Trp	W	TGG
Tyrosine	Tyr	Y	TAC TAT

Further variation in the nucleic acid sequence can be introduced by the presence (or absence) of non-translated sequences, such as intronic sequences and 5' and 3' untranslated sequences.

Moreover, the isolated nucleic acids of the invention encompass those nucleic acids encoding Nrk polypeptides that have at least about 60%, 70%, 80%, 90%, 95%, 97%, 98% or higher amino acid sequence similarity with the polypeptide sequences specifically disclosed herein (or fragments thereof) and further encode a functional Nrk as defined herein.

As is known in the art, a number of different programs can be used to identify whether a nucleic acid or polypeptide has sequence identity or similarity to a known sequence. Sequence identity and/or similarity can be determined using standard techniques known in the art, including, but not limited to, the local sequence identity algorithm of Smith & Waterman (1981) *Adv. Appl. Math.* 2:482, by the sequence identity alignment algorithm of Needleman & Wunsch (1970) *J. Mol. Biol.* 48:443, by the search for similarity method of Pearson & Lipman (1988) *Proc. Natl. Acad. Sci. USA* 85:2444, by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Drive, Madison, Wis.), the Best Fit sequence program described by Devereux, et al. (1984) *Nucl. Acid Res.* 12:387-395, either using the default settings, or by inspection.

An example of a useful algorithm is PILEUP. PILEUP creates a multiple sequence alignment from a group of related sequences using progressive, pairwise alignments. It can also plot a tree showing the clustering relationships used to create the alignment. PILEUP uses a simplification of the progressive alignment method of Feng & Doolittle (1987) *J. Mol. Evol.* 35:351-360; the method is similar to that described by Higgins & Sharp (1989) *CABIOS* 5:151-153.

Another example of a useful algorithm is the BLAST algorithm, described in Altschul, et al. (1990) *J. Mol. Biol.* 215:403-410 and Karlin, et al. (1993) *Proc. Natl. Acad. Sci. USA* 90:5873-5787. A particularly useful BLAST program is the WU-BLAST-2 program which was obtained from Altschul, et al. (1996) *Methods in Enzymology,* 266:460-480; http://blast.wustl/edu/blast/README.html. WU-BLAST-2 uses several search parameters, which can be set to the default values. The parameters are dynamic values and are established by the program itself depending upon the composition of the particular sequence and composition of the particular database against which the sequence of interest is being searched; however, the values can be adjusted to increase sensitivity.

An additional useful algorithm is gapped BLAST as reported by Altschul, et al. (1997) *Nucleic Acids Res.* 25:3389-3402.

A percentage amino acid sequence identity value can be determined by the number of matching identical residues divided by the total number of residues of the longer sequence in the aligned region. The longer sequence is the one having the most actual residues in the aligned region (gaps introduced by WU-Blast-2 to maximize the alignment score are ignored).

The alignment can include the introduction of gaps in the sequences to be aligned. In addition, for sequences which contain either more or fewer amino acids than the polypeptides specifically disclosed herein, it is understood that in one embodiment, the percentage of sequence identity will be determined based on the number of identical amino acids in relation to the total number of amino acids. Thus, for example, sequence identity of sequences shorter than a sequence specifically disclosed herein, will be determined using the number of amino acids in the shorter sequence, in one embodiment. In percent identity calculations relative weight is not assigned to various manifestations of sequence variation, such as, insertions, deletions, substitutions, etc.

In one embodiment, only identities are scored positively (+1) and all forms of sequence variation including gaps are assigned a value of "0", which obviates the need for a weighted scale or parameters as described below for sequence similarity calculations. Percent sequence identity can be calculated, for example, by dividing the number of matching identical residues by the total number of residues of the shorter sequence in the aligned region and multiplying by 100. The longer sequence is the one having the most actual residues in the aligned region.

To modify Nrk amino acid sequences specifically disclosed herein or otherwise known in the art, amino acid substitutions can be based on any characteristic known in the art, including the relative similarity or differences of the amino acid side-chain substituents, for example, their hydrophobicity, hydrophilicity, charge, size, and the like. In particular embodiments, conservative substitutions (i.e., substitution with an amino acid residue having similar properties) are made in the amino acid sequence encoding Nrk.

In making amino acid substitutions, the hydropathic index of amino acids may be considered. The importance of the hydropathic amino acid index in conferring interactive bio-

logic function on a protein is generally understood in the art (see, Kyte and Doolittle (1982) *J. Mol. Biol.* 157:105). It is accepted that the relative hydropathic character of the amino acid contributes to the secondary structure of the resultant protein, which in turn defines the interaction of the protein with other molecules, for example, enzymes, substrates, receptors, DNA, antibodies, antigens, and the like.

Each amino acid has been assigned a hydropathic index on the basis of its hydrophobicity and charge characteristics (Kyte and Doolittle (1982) supra), and these are: isoleucine (+4.5); valine (+4.2); leucine (+3.8); phenylalanine (+2.8); cysteine/cystine (+2.5); methionine (+1.9); alanine (+1.8); glycine (−0.4); threonine (−0.7); serine (−0.8); tryptophan (−0.9); tyrosine (−1.3); proline (−1.6); histidine (−3.2); glutamate (−3.5); glutamine (−3.5); aspartate (−3.5); asparagine (−3.5); lysine (−3.9); and arginine (−4.5).

It is also understood in the art that the substitution of amino acids can be made on the basis of hydrophilicity. U.S. Pat. No. 4,554,101 states that the greatest local average hydrophilicity of a protein, as governed by the hydrophilicity of its adjacent amino acids, correlates with a biological property of the protein.

As detailed in U.S. Pat. No. 4,554,101, the following hydrophilicity values have been assigned to amino acid residues: arginine (+3.0); lysine (+3.0); aspartate (+3.0±1); glutamate (+3.0±1); serine (+0.3); asparagine (+0.2); glutamine (+0.2); glycine (0); threonine (−0.4); proline (−0.5±1); alanine (−0.5); histidine (−0.5); cysteine (−1.0); methionine (−1.3); valine (−1.5); leucine (−1.8); isoleucine (−1.8); tyrosine (−2.3); phenylalanine (−2.5); tryptophan (−3.4).

Isolated nucleic acids of this invention include RNA, DNA (including cDNAs) and chimeras thereof. The isolated nucleic acids can further contain modified nucleotides or nucleotide analogs.

The isolated nucleic acids encoding Nrk can be associated with appropriate expression control sequences, e.g., transcription/translation control signals and polyadenylation signals.

It will be appreciated that a variety of promoter/enhancer elements can be used depending on the level and tissue-specific expression desired. The promoter can be constitutive or inducible (e.g., the metallothionein promoter or a hormone inducible promoter), depending on the pattern of expression desired. The promoter can be native or foreign and can be a natural or a synthetic sequence. By foreign, it is intended that the transcriptional initiation region is not found in the wild-type host into which the transcriptional initiation region is introduced. The promoter is chosen so that it will function in the target cell(s) of interest. In particular embodiments, the promoter functions in tumor cells or in cells that can be used to express nucleic acids encoding Nrk for the purposes of large-scale protein production. Likewise, the promoter can be specific for these cells and tissues (i.e., only show significant activity in the specific cell or tissue type).

To illustrate, an Nrk coding sequence can be operatively associated with a cytomegalovirus (CMV) major immediate-early promoter, an albumin promoter, an Elongation Factor 1-α (EF1-α) promoter, a PγK promoter, a MFG promoter, a Rous sarcoma virus promoter, or a glyceraldehyde-3-phosphate promoter.

Moreover, specific initiation signals are generally required for efficient translation of inserted protein coding sequences. These translational control sequences, which can include the ATG initiation codon and adjacent sequences, can be of a variety of origins, both natural and synthetic.

Nrk can be expressed not only directly, but also as a fusion protein with a heterologous polypeptide, i.e. a signal sequence for secretion and/or other polypeptide which will aid in the purification of Nrk. In one embodiment, the heterologous polypeptide has a specific cleavage site to remove the heterologous polypeptide from Nrk.

In general, a signal sequence can be a component of the vector and should be one that is recognized and processed (i.e., cleaved by a signal peptidase) by the host cell. For production in a prokaryote, a prokaryotic signal sequence from, for example, alkaline phosphatase, penicillinase, lpp, or heat-stable enterotoxin II leaders can be used. For yeast secretion, one can use, e.g., the yeast invertase, alpha factor, or acid phosphatase leaders, the *Candida albicans* glucoamylase leader (EP 362,179), or the like (see, for example WO 90/13646). In mammalian cell expression, signal sequences from secreted polypeptides of the same or related species, as well as viral secretory leaders, for example, the herpes simplex glycoprotein D signal can be used.

Other useful heterologous polypeptides which can be fused to Nrk include those which increase expression or solubility of the fusion protein or aid in the purification of the fusion protein by acting as a ligand in affinity purification. Typical fusion expression vectors include those exemplified herein as well as pMAL (New England Biolabs, Beverly, Mass.) and pRIT5 (Pharmacia, Piscataway, N.J.) which fuse maltose E binding protein or protein A, respectively, to the target recombinant protein.

The isolated nucleic acids encoding Nrk can be incorporated into a vector, e.g., for the purposes of cloning or other laboratory manipulations, recombinant protein production, or gene delivery. In particular embodiments, the vector is an expression vector. Exemplary vectors include bacterial artificial chromosomes, cosmids, yeast artificial chromosomes, phage, plasmids, lipid vectors and viral vectors. By the term express, expresses or expression of a nucleic acid coding sequence, in particular an Nrk coding sequence, it is meant that the sequence is transcribed, and optionally, translated. Typically, according to the present invention, transcription and translation of the coding sequence will result in production of Nrk polypeptide.

The methods of the present invention provide a means for delivering, and optionally expressing, nucleic acids encoding Nrk in a broad range of host cells, including both dividing and non-dividing cells in vitro (e.g., for large-scale recombinant protein production or for use in screening assays) or in vivo (e.g., for recombinant large-scale protein production, for creating an animal model for disease, or for therapeutic purposes). In embodiments of the invention, the nucleic acid can be expressed transiently in the target cell or the nucleic acid can be stably incorporated into the target cell, for example, by integration into the genome of the cell or by persistent expression from stably maintained episomes (e.g., derived from Epstein Barr Virus).

The isolated nucleic acids, vectors, methods and pharmaceutical formulations of the present invention find use in a method of administering a nucleic acid encoding Nrk to a subject. In this manner, Nrk can thus be produced in vivo in the subject. The subject can have a deficiency of Nrk, or the production of a foreign Nrk in the subject can impart some therapeutic effect. Pharmaceutical formulations and methods of delivering nucleic acids encoding Nrk for therapeutic purposes are described herein.

Alternatively, an isolated nucleic acid encoding Nrk can be administered to a subject so that the nucleic acid is expressed by the subject and Nrk is produced and purified therefrom, i.e., as a source of recombinant Nrk protein. According to this

embodiment, the Nrk is secreted into the systemic circulation or into another body fluid (e.g., milk, lymph, spinal fluid, urine) that is easily collected and from which the Nrk can be further purified. As a further alternative, Nrk protein can be produced in avian species and deposited in, and conveniently isolated from, egg proteins.

Likewise, Nrk-encoding nucleic acids can be expressed transiently or stably in a cell culture system for the purpose of screening assays or for large-scale recombinant protein production. The cell can be a bacterial, protozoan, plant, yeast, fungus, or animal cell. In one embodiment, the cell is an animal cell (e.g., insect, avian or mammalian), and in another embodiment a mammalian cell (e.g., a fibroblast).

It will be apparent to those skilled in the art that any suitable vector can be used to deliver the isolated nucleic acids of this invention to the target cell(s) or subject of interest. The choice of delivery vector can be made based on a number of factors known in the art, including age and species of the target host, in vitro vs. in vivo delivery, level and persistence of expression desired, intended purpose (e.g., for therapy or drug screening), the target cell or organ, route of delivery, size of the isolated nucleic acid, safety concerns, and the like.

Suitable vectors include virus vectors (e.g., retrovirus, alphavirus; vaccinia virus; adenovirus, adeno-associated virus, or herpes simplex virus), lipid vectors, poly-lysine vectors, synthetic polyamino polymer vectors that are used with nucleic acid molecules, such as plasmids, and the like.

As used herein, the term viral vector or viral delivery vector can refer to a virus particle that functions as a nucleic acid delivery vehicle, and which contains the vector genome packaged within a virion. Alternatively, these terms can be used to refer to the vector genome when used as a nucleic acid delivery vehicle in the absence of the virion.

Protocols for producing recombinant viral vectors and for using viral vectors for nucleic acid delivery can be found in *Current Protocols in Molecular Biology*, Ausubel, F. M. et al. (eds.) Greene Publishing Associates, (1989) and other standard laboratory manuals (e.g., Vectors for Gene Therapy. In: *Current Protocols in Human Genetics*. John Wiley and Sons, Inc.: 1997).

Particular examples of viral vectors are those previously employed for the delivery of nucleic acids including, for example, retrovirus, adenovirus, AAV, herpes virus, and poxvirus vectors.

In certain embodiments of the present invention, the delivery vector is an adenovirus vector. The term adenovirus as used herein is intended to encompass all adenoviruses, including the Mastadenovirus and Aviadenovirus genera. To date, at least forty-seven human serotypes of adenoviruses have been identified (see, e.g., Fields, et al., Virology, volume 2, chapter 67 (3d ed., Lippincott-Raven Publishers). In one embodiment, the adenovirus is a human serogroup C adenovirus, in another embodiment the adenovirus is serotype 2 (Ad2) or serotype 5 (Ad5) or simian adenovirus such as AdC68.

Those skilled in the art will appreciate that vectors can be modified or targeted as described in Douglas, et al. (1996) *Nature Biotechnology* 14:1574 and U.S. Pat. Nos. 5,922,315; 5,770,442 and/or 5,712,136.

An adenovirus genome can be manipulated such that it encodes and expresses a nucleic acid of interest but is inactivated in terms of its ability to replicate in a normal lytic viral life cycle. See, for example, Berkner, et al. (1988) *BioTechniques* 6:616; Rosenfeld, et al. (1991) *Science* 252:431-434; and Rosenfeld et al. (1992) *Cell* 68:143-155.

Recombinant adenoviruses can be advantageous in certain circumstances in that they are not capable of infecting non-dividing cells and can be used to infect a wide variety of cell types, including epithelial cells. Furthermore, the virus particle is relatively stable and amenable to purification and concentration, and can be modified so as to affect the spectrum of infectivity. Additionally, introduced adenoviral DNA (and foreign DNA contained therein) is not integrated into the genome of a host cell but remains episomal, thereby avoiding potential problems that can occur as a result of insertional mutagenesis in situations where introduced DNA becomes integrated into the host genome (e.g., as occurs with retroviral DNA). Moreover, the carrying capacity of the adenoviral genome for foreign DNA is large relative to other delivery vectors (Haj-Ahmand and Graham (1986) *J. Virol.* 57:267).

In particular embodiments, the adenovirus genome contains a deletion therein, so that at least one of the adenovirus genomic regions does not encode a functional protein. For example, an adenovirus vectors can have E1 genes and packaged using a cell that expresses the E1 proteins (e.g., 293 cells). The E3 region is also frequently deleted as well, as there is no need for complementation of this deletion. In addition, deletions in the E4, E2a, protein IX, and fiber protein regions have been described, e.g., by Armentano, et al. (1997) *J. Virology* 71:2408; Gao, et al. (1996) *J. Virology* 70:8934; Dedieu, et al. (1997) *J. Virology* 71:4626; Wang, et al. (1997) *Gene Therapy* 4:393; U.S. Pat. No. 5,882,877. In general, the deletions are selected to avoid toxicity to the packaging cell. Combinations of deletions that avoid toxicity or other deleterious effects on the host cell can be routinely selected by those skilled in the art.

Those skilled in the art will appreciate that typically, with the exception of the E3 genes, any deletions will need to be complemented in order to propagate (replicate and package) additional virus, e.g., by transcomplementation with a packaging cell.

The present invention can also be practiced with gutted adenovirus vectors (as that term is understood in the art, see e.g., Lieber, et al. (1996) *J. Virol.* 70:8944-60) in which essentially all of the adenovirus genomic sequences are deleted.

Adeno-associated viruses (AAV) have also been employed as nucleic acid delivery vectors. For a review, see Muzyczka et al. *Curr. Topics in Micro. and Immunol.* (1992) 158:97-129). AAV are among the few viruses that can integrate their DNA into non-dividing cells, and exhibit a high frequency of stable integration into human chromosome 19 (see, for example, Flotte, et al. (1992) *Am. J. Respir. Cell. Mol. Biol.* 7:349-356; Samulski, et al., (1989) *J Virol.* 63:3822-3828; McLaughlin, et al. (1989) *J. Virol.* 62:1963-1973). A variety of nucleic acids have been introduced into different cell types using AAV vectors (see, for example, Hermonat, et al. (1984) *Proc. Natl. Acad. Sci. USA* 81:6466-6470; Tratschin, et al. (1985) *Mol. Cell. Biol.* 4:2072-2081; Wondisford, et al. (1988) *Mol. Endocrinol.* 2:32-39; Tratschin, et al. (1984) *J. Virol.* 51:611-619; and Flotte, et al. (1993) *J. Biol. Chem.* 268:3781-3790).

Any suitable method known in the art can be used to produce AAV vectors expressing the nucleic acids encoding Nrk of this invention (see, e.g., U.S. Pat. Nos. 5,139,941; 5,858,775; 6,146,874 for illustrative methods). In one particular method, AAV stocks can be produced by co-transfection of a rep/cap vector encoding AAV packaging functions and the template encoding the AAV vDNA into human cells infected with the helper adenovirus (Samulski, et al. (1989) *J. Virology* 63:3822). The AAV rep and/or cap genes can alternatively be provided by a packaging cell that stably expresses the genes (see, e.g., Gao, et al. (1998) *Human Gene Therapy*

9:2353; Inoue, et al. (1998) *J. Virol.* 72:7024; U.S. Pat. No. 5,837,484; WO 98/27207; U.S. Pat. No. 5,658,785; WO 96/17947).

Another vector for use in the present invention is Herpes Simplex Virus (HSV). HSV can be modified for the delivery of nucleic acids to cells by producing a vector that exhibits only the latent function for long-term gene maintenance. HSV vectors are useful for nucleic acid delivery because they allow for a large DNA insert of up to or greater than 20 kilobases; they can be produced with extremely high titers; and they have been shown to express nucleic acids for a long period of time in the central nervous system as long as the lytic cycle does not occur.

In other particular embodiments of the present invention, the delivery vector of interest is a retrovirus. The development of specialized cell lines (termed packaging cells) which produce only replication-defective retroviruses has increased the utility of retroviruses for gene therapy, and defective retroviruses are characterized for use in gene transfer for gene therapy purposes (for a review, see Miller (1990) *Blood* 76:271). A replication-defective retrovirus can be packaged into virions which can be used to infect a target cell through the use of a helper virus by standard techniques.

In addition to viral transfer methods, such as those illustrated above, non-viral methods can also be employed. Many non-viral methods of nucleic acid transfer rely on normal mechanisms used by mammalian cells for the uptake and intracellular transport of macromolecules. In particular embodiments, non-viral nucleic acid delivery systems rely on endocytic pathways for the uptake of the nucleic acid molecule by the targeted cell. Exemplary nucleic acid delivery systems of this type include liposomal derived systems, polylysine conjugates, and artificial viral envelopes.

In particular embodiments, plasmid vectors are used in the practice of the present invention. Naked plasmids can be introduced into muscle cells by injection into the tissue. Expression can extend over many months, although the number of positive cells is typically low (Wolff, et al. (1989) *Science* 247:247). Cationic lipids have been demonstrated to aid in introduction of nucleic acids into some cells in culture (Felgner and Ringold (1989) *Nature* 337:387). Injection of cationic lipid plasmid DNA complexes into the circulation of mice has been shown to result in expression of the DNA in lung (Brigham, et al. (1989) *Am. J. Med. Sci.* 298:278). One advantage of plasmid DNA is that it can be introduced into non-replicating cells.

In a representative embodiment, a nucleic acid molecule (e.g., a plasmid) can be entrapped in a lipid particle bearing positive charges on its surface and, optionally, tagged with antibodies against cell-surface antigens of the target tissue (Mizuno, et al. (1992) *No Shinkei Geka* 20:547; WO 91/06309; Japanese patent application 1047381; and European patent publication EP-A-43075).

Liposomes that consist of amphiphilic cationic molecules are useful non-viral vectors for nucleic acid delivery in vitro and in vivo (reviewed in Crystal (1995) *Science* 270:404-410; Blaese, et al. (1995) *Cancer Gene Ther.* 2:291-297; Behr, et al. (1994) *Bioconjugate Chem.* 5:382-389; Remy, et al. (1994) *Bioconjugate Chem.* 5:647-654; and Gao, et al. (1995) *Gene Therapy* 2:710-722). The positively charged liposomes are believed to complex with negatively charged nucleic acids via electrostatic interactions to form lipid:nucleic acid complexes. The lipid:nucleic acid complexes have several advantages as nucleic acid transfer vectors. Unlike viral vectors, the lipid:nucleic acid complexes can be used to transfer expression cassettes of essentially unlimited size. Since the complexes lack proteins, they can evoke fewer immunogenic and inflammatory responses. Moreover, they cannot replicate or recombine to form an infectious agent and have low integration frequency. A number of publications have demonstrated that amphiphilic cationic lipids can mediate nucleic acid delivery in vivo and in vitro (Felgner, et al. (1987) *Proc. Natl. Acad. Sci. USA* 84:7413-17; Loeffler, et al. (1993) *Methods in Enzymology* 217:599-618; Felgner, et al. (1994) *J. Biol. Chem.* 269:2550-2561).

As indicated above, Nrk polypeptide can be produced in, and optionally purified from, cultured cells or organisms expressing a nucleic acid encoding Nrk for a variety of purposes (e.g., screening assays, large-scale protein production, therapeutic methods based on delivery of purified Nrk).

In particular embodiments, an isolated nucleic acid encoding Nrk can be introduced into a cultured cell, e.g., a cell of a primary or immortalized cell line for recombinant protein production. The recombinant cells can be used to produce the Nrk polypeptide, which is collected from the cells or cell culture medium. Likewise, recombinant protein can be produced in, and optionally purified from an organism (e.g., a microorganism, animal or plant) being used essentially as a bioreactor.

Generally, the isolated nucleic acid is incorporated into an expression vector (viral or nonviral as described herein). Expression vectors compatible with various host cells are well-known in the art and contain suitable elements for transcription and translation of nucleic acids. Typically, an expression vector contains an expression cassette, which includes, in the 5' to 3' direction, a promoter, a coding sequence encoding an Nrk operatively associated with the promoter, and, optionally, a termination sequence including a stop signal for RNA polymerase and a polyadenylation signal for polyadenylase.

Expression vectors can be designed for expression of polypeptides in prokaryotic or eukaryotic cells. For example, polypeptides can be expressed in bacterial cells such as *E. coli*, insect cells (e.g., in the baculovirus expression system), yeast cells or mammalian cells. Some suitable host cells are discussed further in Goeddel (1990) Gene Expression Technology: Methods in Enzymology 185, Academic Press, San Diego, Calif. Examples of vectors for expression in yeast *S. cerevisiae* include pYepSec1 (Baldari, et al. (1987) *EMBO J.* 6:229-234), pMFa (Kurjan and Herskowitz (1982) *Cell* 30:933-943), pJRY88 (Schultz, et al. (1987) *Gene* 54:113-123), and pYES2 (INVITROGEN Corporation, San Diego, Calif.). Baculovirus vectors available for expression of nucleic acids to produce proteins in cultured insect cells (e.g., Sf 9 cells) include the pAc series (Smith, et al. (1983) *Mol. Cell. Biol.* 3:2156-2165) and the pVL series (Lucklow and Summers (1989) *Virology* 170:31-39).

Examples of mammalian expression vectors include pCDM8 (Seed (1987) *Nature* 329:840) and pMT2PC (Kaufman, et al. (1987) *EMBO J.* 6:187-195). When used in mammalian cells, the expression vector's control functions are often provided by viral regulatory elements. For example, commonly used promoters are derived from polyoma, adenovirus 2, cytomegalovirus and Simian Virus 40.

In addition to the regulatory control sequences discussed herein, the recombinant expression vector can contain additional nucleotide sequences. For example, the recombinant expression vector can encode a selectable marker gene to identify host cells that have incorporated the vector.

Vectors can be introduced into prokaryotic or eukaryotic cells via conventional transformation or transfection techniques. As used herein, the terms transformation and transfection refer to a variety of art-recognized techniques for introducing foreign nucleic acids (e.g., DNA) into a host cell,

including calcium phosphate or calcium chloride co-precipitation, DEAE-dextran-mediated transfection, lipofection, electroporation, microinjection, DNA-loaded liposomes, lipofectamine-DNA complexes, cell sonication, gene bombardment using high velocity microprojectiles, and viral-mediated transfection. Suitable methods for transforming or transfecting host cells can be found in Sambrook, et al. (Molecular Cloning: A Laboratory Manual, 2nd Edition, Cold Spring Harbor Laboratory press (1989)), and other laboratory manuals.

Often only a small fraction of cells (in particular, mammalian cells) integrate the foreign DNA into their genome. In order to identify and select these integrants, a nucleic acid that encodes a selectable marker (e.g., resistance to antibiotics) can be introduced into the host cells along with the nucleic acid of interest. In particular embodiments, selectable markers include those that confer resistance to drugs, such as G418, hygromycin and methotrexate. Nucleic acids encoding a selectable marker can be introduced into a host cell on the same vector as that comprising the nucleic acid of interest or can be introduced on a separate vector. Cells stably transfected with the introduced nucleic acid can be identified by drug selection (e.g., cells that have incorporated the selectable marker gene will survive, while the other cells die).

Recombinant proteins can also be produced in a transgenic plant in which the isolated nucleic acid encoding the protein is inserted into the nuclear or plastidic genome. Plant transformation is known as the art. See, in general, *Methods in Enzymology* Vol. 153 (Recombinant DNA Part D) 1987, Wu and Grossman Eds., Academic Press and European Patent Application EP 693554.

The present invention further provides cultured or recombinant cells containing the isolated nucleic acids encoding Nrk for use in the screening methods and large-scale protein production methods of the invention (e.g., Nrk is produced and collected from the cells and, optionally, purified). In one particular embodiment, the invention provides a cultured cell containing an isolated nucleic acid encoding Nrk as described above for use in a screening assay for identifying a nicotinamide riboside-related prodrug. Also provided is a cell in vivo produced by a method comprising administering an isolated nucleic acid encoding Nrk to a subject in a therapeutically effective amount.

For in vitro screening assays and therapeutic administration, Nrk polypeptides can be purified from cultured cells. Typically, the polypeptide is recovered from the culture medium as a secreted polypeptide, although it also can be recovered from host cell lysates when directly expressed without a secretory signal. When Nrk is expressed in a recombinant cell other than one of human origin, the Nrk is completely free of proteins or polypeptides of human origin. However, it is necessary to purify Nrk from recombinant cell proteins or polypeptides to obtain preparations that are substantially homogeneous as to Nrk. As a first step, the culture medium or lysate is centrifuged to remove particulate cell debris. The membrane and soluble protein fractions are then separated. The Nrk can then be purified from the soluble protein fraction. Nrk thereafter can then be purified from contaminant soluble proteins and polypeptides with, for example, the following suitable purification procedures: by fractionation on immunoaffinity or ion-exchange columns; ethanol precipitation; reverse phase HPLC; chromatography on silica or on a cation-exchange resin such as DEAE; chromatofocusing; SDS-PAGE; ammonium sulfate precipitation; gel filtration using, for example, SEPHADEX G-75; ligand affinity chromatography, and protein A SEPHAROSE columns to remove contaminants such as IgG.

As Nrk phosphorylates tiazofurin, thereby performing the first step in activating it, Nrk is a useful target for identifying compounds which upon phosphorylation by Nrk and subsequent adenylylation inhibit IMPDH. As it has been shown that inhibitors of the IMPDH enzyme function as anti-bovine viral diarrhoea virus agents (Stuyver, et al. (2002) *Antivir. Chem. Chemother.* 13(6):345-52); inhibitors of IMPDH block hepatitis B replicon colony-forming efficiency (Zhou, et al. (2003) *Virology* 310(2):333-42); and tiazofurin (Cooney, et al. (1983) *Adv. Enzyme Regul.* 21:271-303) and benzamide riboside (Krohn, et al. (1992) *J. Med. Chem.* 35:511-517), when activated, inhibit IMP dehydrogenase; it is contemplated by using Nrk and the nicotinamide riboside pathway for drug screening, anticancer and antiviral agents will be identified. Accordingly, the present invention provides methods for identifying a nicotinamide riboside-related prodrug. As used herein, a nicotinamide riboside-related prodrug is any analog of nicotinamide riboside (e.g., tiazofurin and benzamide riboside) that, when phosphorylated by Nrk, ultimately can result in cell death or antiviral activity.

In one embodiment, a nicotinamide riboside-related prodrug is identified in a cell-free assay using isolated Nrk polypeptide. The steps involved in a this screening assay of the invention include, isolating or purifying an Nrk polypeptide; contacting or adding at least one nicotinamide riboside-related test agent to a point of application, such as a well, in the plate containing the isolated Nrk and a suitable phosphate donor such as ATP, Mg-ATP, Mn-ATP, Mg-GTP or Mn-GTP; and determining whether said test agent is phosphorylated by said Nrk polypeptide wherein phosphorylation of said test agent is indicative of a nicotinamide riboside-related prodrug. The phosphate donor can be added with or after the agent and the assay can be carried out under suitable assay conditions for phosphorylation, such as those exemplified herein.

With respect to the cell-free assay, test agents can be synthesized or otherwise affixed to a solid substrate, such as plastic pins, glass slides, plastic wells, and the like. Further, isolated Nrk can be free in solution, affixed to a solid support, or expressed on a cell surface.

Alternatively, an Nrk fusion protein can be provided to facilitate binding of Nrk to a matrix. For example, glutathione-S-transferase fusion proteins can be adsorbed onto glutathione SEPHAROSE beads (Sigma Chemical, St. Louis, Mo.) or glutathione derivatized microtitre plates, which are then combined with the test agent, and the mixture incubated under conditions conducive to complex formation (e.g., at physiological conditions for salt and pH) and phosphorylation as described above.

In another embodiment, a nicotinamide riboside-related prodrug is identified in a cell-based assay. The steps involved in a this screening assay of the invention include, contacting a first test cell which expresses a recombinant Nrk polypeptide with a nicotinamide riboside-related test agent; contacting a second test cell which lacks a functional Nrk polypeptide with the same test agent; and determining the viability of the first and second test cells wherein sensitivity or cell death of the first cell and not the second cell is indicative of a nicotinamide riboside-related prodrug. While the cell-based assay can be carried out using any suitable cell including bacteria, yeast, insect cells (e.g., with a baculovirus expression system), avian cells, mammalian cells, or plant cells, in particular embodiments, the test cell is a mammalian cell. In a further embodiment, said cell lacks a functional endogenous Nrk (e.g., the endogenous Nrk has been deleted or mutated or the cell does not express an Nrk). Said first test cell is transformed or transfected with an expression vector containing an exogenous Nrk so that upon exposure to a test agent, viability

of the transformed cell can be compared to a second test cell lacking any Nrk activity. Thus, it can be ascertained whether the test agent is being activated in an Nrk-dependent manner. Cells modified to express a recombinant Nrk can be transiently or stably transformed with the nucleic acid encoding Nrk. Stably transformed cells can be generated by stable integration into the genome of the organism or by expression from a stably maintained episome (e.g., Epstein Barr Virus derived episomes).

Suitable methods for determining cell viability are well-established in the art. One such method uses non-permeant dyes (e.g., propidium iodide, 7-Amino Actinomycin D) that do not enter cells with intact cell membranes or active cell metabolism. Cells with damaged plasma membranes or with impaired/no cell metabolism are unable to prevent the dye from entering the cell. Once inside the cell, the dyes bind to intracellular structures producing highly fluorescent adducts which identify the cells as non-viable. Alternatively, cell viability can be determined by assaying for active cell metabolism which results in the conversion of a non-fluorescent substrate into a highly fluorescent product (e.g., fluorescein diacetate).

The test cells of the screening method of the invention can be cultured under standard conditions of temperature, incubation time, optical density, plating density and media composition corresponding to the nutritional and physiological requirements of the cells. However, conditions for maintenance and growth of the test cell can be different from those for assaying candidate agents in the screening methods of the invention. Any techniques known in the art can be applied to establish the optimal conditions.

Screening assays of the invention can be performed in any format that allows rapid preparation and processing of multiple reactions such as in, for example, multi-well plates of the 96-well variety. Stock solutions of the agents as well as assay components are prepared manually and all subsequent pipetting, diluting, mixing, washing, incubating, sample readout and data collecting is done using commercially available robotic pipetting equipment, automated work stations, and analytical instruments for detecting the output of the assay.

In addition to the reagents provided above, a variety of other reagents can be included in the screening assays of the invention. These include reagents like salts, neutral proteins, e.g., albumin, detergents, etc. Also, reagents that otherwise improve the efficiency of the assay, such as protease inhibitors, nuclease inhibitors, anti-microbial agents, and the like can be used.

Screening assays can also be carried out in vivo in animals. Thus, the present invention provides a transgenic non-human animal containing an isolated nucleic acid encoding Nrk, which can be produced according to methods well-known in the art. The transgenic non-human animal can be any species, including avians and non-human mammals. IN accordance with the invention, suitable non-human mammals include mice, rats, rabbits, guinea pigs, goats, sheep, pigs and cattle. Mammalian models for cancer, bovine diarrhoea viral infection or hepatitis C viral infection can also be used.

A nucleic acid encoding Nrk is stably incorporated into cells within the transgenic animal (typically, by stable integration into the genome or by stably maintained episomal constructs). It is not necessary that every cell contain the transgene, and the animal can be a chimera of modified and unmodified cells, as long as a sufficient number of cells contain and express the Nrk transgene so that the animal is a useful screening tool (e.g., so that administration of test agents give rise to detectable cell death or anti-viral activity).

Methods of making transgenic animals are known in the art. DNA constructs can be introduced into the germ line of an avian or mammal to make a transgenic animal. For example, one or several copies of the construct can be incorporated into the genome of an embryo by standard transgenic techniques.

In an exemplary embodiment, a transgenic non-human animal is produced by introducing a transgene into the germ line of the non-human animal. Transgenes can be introduced into embryonal target cells at various developmental stages. Different methods are used depending on the stage of development of the embryonal target cell. The specific line(s) of any animal used should, if possible, be selected for general good health, good embryo yields, good pronuclear visibility in the embryo, and good reproductive fitness.

Introduction of the transgene into the embryo can be accomplished by any of a variety of means known in the art such as microinjection, electroporation, lipofection or a viral vector. For example, the transgene can be introduced into a mammal by microinjection of the construct into the pronuclei of the fertilized mammalian egg(s) to cause one or more copies of the construct to be retained in the cells of the developing mammal(s). Following introduction of the transgenic construct into the fertilized egg, the egg can be incubated in vitro for varying amounts of time, or reimplanted into the surrogate host, or both. One common method is to incubate the embryos in vitro for about 1-7 days, depending on the species, and then reimplant them into the surrogate host.

The progeny of the transgenically manipulated embryos can be tested for the presence of the construct (e.g., by Southern blot analysis) of a segment of tissue. An embryo having one or more copies of the exogenous cloned construct stably integrated into the genome can be used to establish a permanent transgenic animal line carrying the transgenically added construct.

Transgenically altered animals can be assayed after birth for the incorporation of the construct into the genome of the offspring. This can be done by hybridizing a probe corresponding to the DNA sequence coding for the polypeptide or a segment thereof onto chromosomal material from the progeny. Those progeny found to contain at least one copy of the construct in their genome are grown to maturity.

Methods of producing transgenic avians are also known in the art, see, e.g., U.S. Pat. No. 5,162,215.

Nicotinamide riboside-related test agents can be obtained from a wide variety of sources including libraries of synthetic or natural compounds. Such agents can include analogs or derivatives of nicotinamide riboside as well as tiazofurin and benzamide riboside and analogs or derivatives thereof.

Alternatively, the isolated Nrk polypeptide can be used to generate a crystal structure of Nrk and synthetic nicotinamide riboside analogs can be designed. Based on the crystal structure of *E. coli* panK, Asp127 appears to play a key role in transition-state stabilization of the transferring phosphoryl group of a pantothenate kinase (Yun, et al. (2000) *J. Biol. Chem.* 275:28093-28099). Accordingly, it is contemplated the corresponding Nrk mutant, e.g., NRK2-E100Q, can be used to generate a stable complex between an Nrk and a nucleotides (i.e., Nrk2-E100Q+nicotinamide riboside+ATP can be stable enough to crystallize). Alternatively, Nrk can produce a stable complex in the presence of an inhibitor such as an ATP-mimetic compound (e.g., AMP-PNHP and AMP-PCH_2P). For metabolite kinases, bisubstrate inhibitors have been very successfully employed. For example, thymidylate kinase, which performs the reaction, dTMP+ATP->dTDP+AMP, is strongly inhibited by dTpppppA (Bone, et al. (1986)

J. Biol. Chem. 261:16410-16413) and crystal structures were obtained with this inhibitor (Lavie, et al. (1998) *Biochemistry* 37:3677-3686).

It has been shown that the best inhibitors typically contain one or two more phosphates than the two substrates combined (i.e., dTppppA is not as good a substrate as dTpppppA). On the basis of the same types of results with adenosine kinase (Bone, et al. (1986) supra), it is contemplated that NrppppA (i.e., an NAD+ analog with two extra phosphates) will be a better inhibitor than NrpppA (i.e., an NAD+ analog with an extra phosphate, or, indeed, nicotinamide riboside+AppNHp). NAD+ analogs with extra phosphates can be generated using standard enzymatic methods (see, e.g., Guranowski, et al. (1990) *FEBS Lett.* 271:215-218) optimized for making a wide variety of adenylylated dinucleoside polyphosphates (Fraga, et al. (2003) *FEBS Lett.* 543:37-41), namely reaction of Nrpp (nicotinamide riboside diphosphate) and Nrppp (nicotinamide riboside triphosphate) with firefly luciferase-AMP. The diphosphorylated form of NMN (Nrpp) is prepared with either uridylate kinase or cytidylate kinase (NMN+ ATP->Nrpp). The triphosphorylated form of NMN (Nrppp) is subsequently prepared with nucleoside diphosphate kinase (Nrpp+ATP->Nrppp). The resulting inhibitors are then used in crystallization trials and/or are soaked into Nrk crystals.

Once the three-dimensional structure of Nrk is determined, a potential test agent can be examined through the use of computer modeling using a docking program such as GRAM, DOCK, or AUTODOCK (Dunbrack, et al. (1997) *Folding & Design* 2:27-42). This procedure can include computer fitting of potential agents to Nrk to ascertain how well the shape and the chemical structure of the potential ligand will interact with Nrk. Computer programs can also be employed to estimate the attraction, repulsion, and steric hindrance of the test agent. Generally the tighter the fit (e.g., the lower the steric hindrance, and/or the greater the attractive force) the better substrate the agent will be since these properties are consistent with a tighter binding constraint. Furthermore, the more specificity in the design of a potential test agent the more likely that the agent will not interfere with related mammalian proteins. This will minimize potential side-effects due to unwanted interactions with other proteins.

The invention is also a method of treating cancer in a patient, having or suspected of having cancer, with an isolated nucleic acid, delivery vector, or polypeptide of the invention in combination with a nicotinamide riboside-related prodrug. Administration of the nucleic acid, delivery vector, or polypeptide of the present invention to a human subject or an animal can be by any means known in the art for administering nucleic acids, vectors, or polypeptides. A patient, as used herein, is intended to include any mammal such as a human, agriculturally-important animal, pet or zoological animal. A patient having or suspected of having a cancer is a patient who exhibits signs or symptoms of a cancer or because of inheritance, environmental or natural reasons is suspected of having cancer. Nucleic acids encoding Nrk, vectors containing the same, or Nrk polypeptides can be administered to the subject in an amount effective to decrease, alleviate or eliminate the signs or symptoms of a cancer (e.g., tumor size, feelings of weakness, and pain perception). The amount of the agent required to achieve the desired outcome of decreasing, eliminating or alleviating a sign or symptom of a cancer will be dependent on the pharmaceutical composition of the agent, the patient and the condition of the patient, the mode of administration, the type of condition or disease being prevented or treated, age and species of the patient, the particular vector, and the nucleic acid to be delivered, and can be determined in a routine manner.

While the prodrug and the Nrk nucleic acid, delivery vector, or polypeptide can be delivered concomitantly, in an alternative embodiment the Nrk nucleic acid, delivery vector, or polypeptide is provided first, followed by administration of the prodrug to precondition the cells to generate the activated or toxic drug.

Types of cancers which can be treated in accordance with the method of the invention include, but are not limited to, pancreatic cancer, endometrial cancer, small cell and non-small cell cancer of the lung (including squamous, adenocarcinoma and large cell types), squamous cell cancer of the head and neck, bladder, ovarian, cervical, breast, renal, CNS, and colon cancers, myeloid and lymphocytic leukemia, lymphoma, hepatic tumors, medullary thyroid carcinoma, multiple myeloma, melanoma, retinoblastoma, and sarcomas of the soft tissue and bone.

Typically, with respect to viral vectors, at least about 10^3 virus particles, at least about 10^5 virus particles, at least about 10^7 virus particles, at least about 10^9 virus particles, at least about 10^{11} virus particles, at least about 10^{12} virus particles, or at least about 10^{13} virus particles are administered to the patient per treatment. Exemplary doses are virus titers of about 10^7 to about 10^{15} particles, about 10^7 to about 10^{14} particles, about 10^8 to about 10^{13} particles, about 10^{10} to about 10^{15} particles, about 10^{11} to about 10^{15} particles, about 10^{12} to about 10^{14} particles, or about 10^{12} to about 10^{13} particles.

In particular embodiments of the invention, more than one administration (e.g., two, three, four, or more administrations) can be employed over a variety of time intervals (e.g., hourly, daily, weekly, monthly, etc.) to achieve therapeutic levels of nucleic acid expression.

Tiazofurin is a nucleoside analog initially synthesized to be a cytidine deaminase inhibitor. Tiazofurin was shown to be a prodrug that is converted by cellular enzymes to TAD, an analog of NAD+, that inhibits IMP dehydrogenase, the rate limiting enzyme in producing GTP and dGTP (Cooney, et al. (1983) supra). In phase I/II trials of acute leukemia, tiazofurin produced response rates as high as 85% and was granted orphan drug status for treatment of CML in accelerated phase or blast crisis. Treatment of cultured cells has shown that tiazofurin selectively kills cancer cells by induction of apoptosis: the activity has been attributed both to the increased dependence of actively replicating cells on dGTP and to the addiction of many transformed genotypes to signaling through low molecular weight G proteins (Jayaram, et al. (2002) *Curr. Med. Chem.* 9:787-792). Examination of the sensitivity of the NCI-60 panel of cancer cell lines and the literature on tiazofurin indicates that particular breast, renal, CNS, colon and non-small cell lung-derived tumors are among the most sensitive while others from the same organ sites are among the most resistant (Johnson, et al. (2001) *Br. J. Cancer* 84:1424-1431). As was demonstrated herein, the function of nicotinamide riboside as an NAD+ precursor is entirely dependent on Nrk1 and human Nrks have at least as high specific activity in tiazofurin phosphorylation as in nicotinamide riboside phosphorylation. Because Nrk2 expression is muscle-specific (Li, et al. (1999) supra), and Nrk1 is expressed at a very low level (Boon, et al. (2002) supra), while NMN/NaMNAT is not restricted, it is contemplated that stratification of tumors by Nrk gene expression will largely predict and account for tiazofurin sensitivity.

Accordingly, the present invention is further a method for identifying an individual or tumor which is susceptible to treatment with a nicotinamide riboside-related prodrug. In one embodiment, the level of Nrk protein in an individual or tumor is detected by binding of a Nrk-specific antibody in an

immunoassay. In another embodiment, the level of Nrk enzyme activity is determined using, for example, the nicotinamide riboside phosphorylation assay disclosed herein. In another embodiment, the level of Nrk RNA transcript is determined using any number of well-known RNA-based assays for detecting levels of RNA. Once detected, the levels of Nrk are compared to a known standard. A change in the level of Nrk, as compared to the standard, is indicative of an altered level of susceptibility to treatment with a nicotinamide riboside-related prodrug. In a still further embodiment, mutations or polymorphisms in the Nrk gene can be identified which result in an altered level of susceptibility to treatment with a nicotinamide riboside-related prodrug.

Optimized treatments for cancer and other diseases with nicotinamide riboside-related prodrugs are directed toward cells with naturally high levels of an Nrk provided herein or toward cells which have been recombinantly engineered to express elevated levels of an Nrk. Safety, specificity and efficacy of these treatments can be modulated by supplementation with or restriction of the amounts of any of the NAD+ precursors, namely tryptophan, nicotinic acid, nicotinamide, or nicotinamide riboside.

For the detection of Nrk protein levels, antibodies which specifically recognize Nrk are generated. These antibodies can be either polyclonal or monoclonal. Moreover, such antibodies can be natural or partially or wholly synthetically produced. All fragments or derivatives thereof (e.g., Fab, Fab', $F(ab')_2$, scFv, Fv, or Fd fragments) which maintain the ability to specifically bind to and recognize Nrk are also included. The antibodies can be a member of any immunoglobulin class, including any of the human classes: IgG, IgM, IgA, IgD, and IgE.

The Nrk-specific antibodies can be generated using classical cloning and cell fusion techniques. See, for example, Kohler and Milstein (1975) *Nature* 256:495-497; Harlow and Lane (1988) Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory, New York. Alternatively, antibodies which specifically bind Nrk are derived by a phage display method. Methods of producing phage display antibodies are well-known in the art (e.g., Huse, et al. (1989) *Science* 246 (4935):1275-81).

Selection of Nrk-specific antibodies is based on binding affinity and can be determined by various well-known immunoassays including, enzyme-linked immunosorbent, immunodiffusion chemiluminescent, immunofluorescent, immunohistochemical, radioimmunoassay, agglutination, complement fixation, immunoelectrophoresis, and immunoprecipitation assays and the like which can be performed in vitro, in vivo or in situ. Such standard techniques are well-known to those of skill in the art (see, e.g., "Methods in Immunodiagnosis", 2nd Edition, Rose and Bigazzi, eds. John Wiley & Sons, 1980; Campbell et al., "Methods and Immunology", W.A. Benjamin, Inc., 1964; and Oellerich, M. (1984) *J. Clin. Chem. Clin. Biochem.* 22:895-904).

Once fully characterized for specificity, the antibodies can be used in diagnostic or predictive methods to evaluate the levels of Nrk in healthy and diseased tissues (i.e., tumors) via techniques such as ELISA, western blotting, or immunohistochemistry.

The general method for detecting levels of Nrk protein provides contacting a sample with an antibody which specifically binds Nrk, washing the sample to remove non-specific interactions, and detecting the antibody-antigen complex using any one of the immunoassays described above as well a number of well-known immunoassays used to detect and/or quantitate antigens (see, for example, Harlow and Lane (1988) supra). Such well-known immunoassays include antibody capture assays, antigen capture assays, and two-antibody sandwich assays.

For the detection of nucleic acid sequences encoding Nrk, either a DNA-based or RNA-based method can be employed. DNA-based methods for detecting mutations in an Nrk locus (i.e., frameshift mutations, point mutations, missense mutations, nonsense mutations, splice mutations, deletions or insertions of induced, natural or inherited origin) include, but are not limited to, DNA microarray technologies, oligonucleotide hybridization (mutant and wild-type), PCR-based sequencing, single-strand conformational polymorphism (SSCP) analysis, heteroduplex analysis (HET), PCR, or denaturing gradient gel electrophoresis. Mutations can appear, for example, as a dual base call on sequencing chromatograms. Potential mutations are confirmed by multiple, independent PCR reactions. Exemplary single nucleotide polymorphisms which can be identified in accordance with the diagnostic method of the invention include, but are not limited to, NCBI SNP Cluster ID Nos. rs3752955, rs1045882, rs11519, and rs3185880 for human Nrk1 and Cluster ID Nos. rs2304190, rs4807536, and rs1055767 for human Nrk2.

To detect the levels of RNA transcript encoding the Nrk, nucleic acids are isolated from cells of the individual or tumor, according to standard methodologies (e.g., Sambrook et al. (1989) *Molecular Cloning, a Laboratory Manual*, Cold Spring Harbor Laboratories, New York). The nucleic acid can be whole cell RNA or fractionated to Poly-A+. It may be desirable to convert the RNA to a complementary DNA (cDNA). Normally, the nucleic acid is amplified.

A variety of methods can be used to evaluate or quantitate the level of Nrk RNA transcript present in the nucleic acids isolated from an individual or tumor. For example, levels of Nrk RNA transcript can be evaluated using well-known methods such as northern blot analysis (see, e.g., Sambrook et al. (1989) *Molecular Cloning, a Laboratory Manual*, Cold Spring Harbor Laboratories, New York); oligonucleotide or cDNA fragment hybridization wherein the oligonucleotide or cDNA is configured in an array on a chip or wafer; real-time PCR analysis, or RT-PCR analysis.

Suitable primers, probes, or oligonucleotides useful for such detection methods can be generated by the skilled artisan from the Nrk nucleic acid sequences provided herein. The term primer, as defined herein, is meant to encompass any nucleic acid that is capable of priming the synthesis of a nascent nucleic acid in a template-dependent process. Typically, primers are oligonucleotides from ten to twenty base pairs in length, but longer sequences can be employed. Primers can be provided in double-stranded or single-stranded form. Probes are defined differently, although they can act as primers. Probes, while perhaps capable of priming, are designed for binding to the target DNA or RNA and need not be used in an amplification process. In one embodiment, the probes or primers are labeled with, for example, radioactive species (^{32}P, ^{14}C, ^{35}S, ^{3}H, or other label) or a fluorophore (rhodamine, fluorescein). Depending on the application, the probes or primers can be used cold, i.e., unlabeled, and the RNA or cDNA molecules are labeled.

Depending on the format, detection can be performed by visual means (e.g., ethidium bromide staining of a gel). Alternatively, the detection can involve indirect identification of the product via chemiluminescence, radiolabel or fluorescent label or even via a system using electrical or thermal impulse signals (Bellus (1994) *J. Macromol. Sci. Pure Appl. Chem.* A311:1355-1376).

After detecting mutations in Nrk or the levels of Nrk present in an individual or tumor, said mutations or levels are

compared with a known control or standard. A known control can be a statistically significant reference group of individuals that are susceptible or lack susceptibility to treatment with a nicotinamide riboside-related prodrug to provide diagnostic or predictive information pertaining to the individual or tumor upon which the analysis was conducted.

As described herein, nicotinamide riboside isolated from deproteinized whey fraction of cow's milk was sufficient to support NRK1-dependent growth in a qns1 mutant. Accordingly, mutant strains generated herein will be useful in identifying other natural or synthetic sources for nicotinamide riboside for use in dietary supplements. Thus, the present invention also encompasses is a method for identifying such natural or synthetic sources. As a first step of the method, a first cell lacking a functional glutamine-dependent NAD+ synthetase is contacted with an isolated extract from a natural or synthetic source. In one embodiment, the first cell is a qns1 mutant (i.e., having no NAD+ synthetase) carrying the QNS1 gene on a URA3 plasmid. While any cell can be used, in particular embodiments a yeast cell is used in this method of the invention. A qns1 mutant strain has normal growth on 5-fluoroorotic acid (i.e., cured of the URA3 QNS1 plasmid) as long as it is supplied with nicotinamide riboside.

As a second step of the method, a second cell lacking a functional glutamine-dependent NAD+ synthetase and a functional nicotinamide riboside kinase is contacted with the same isolated extract from the natural or synthetic source of the prior step. Using a qns1 and nrk1 double mutant, it was demonstrated herein that the NRK1 gene is necessary for growth on nicotinamide riboside: qns1 and nrk1 are synthetically lethal even with nicotinamide riboside. This deletion strain is useful in this screening assay of the invention as it allows one to distinguish between nicotinamide riboside, NMN and NAD+ as the effective nutrient.

As a subsequent step of the method, the growth of the first cell and second cell are compared. If the isolated extract contains a nicotinamide riboside, the first cell will grow and the second cell will not.

Synthetic sources of nicotinamide riboside can include any library of chemicals commercially available from most large chemical companies including Merck, Glaxo, Bristol Meyers Squibb, Monsanto/Searle, Eli Lilly and Pharmacia. Natural sources which can be tested for the presence of a nicotinamide riboside include, but are not limited to, cow's milk, serum, meats, eggs, fruit and cereals. Isolated extracts of the natural sources can be prepared using standard methods. For example, the natural source can be ground or homogenized in a buffered solution, centrifuged to remove cellular debris, and fractionated to remove salts, carbohydrates, polypeptides, nucleic acids, fats and the like before being tested on the mutants strains of the invention. Any source of nicotinamide riboside that scores positively in the assay of the invention can be further fractionated and confirmed by standard methods of HPLC and mass spectrometry.

Nicotinic acid is an effective agent in controlling low-density lipoprotein cholesterol, increasing high-density lipoprotein cholesterol, and reducing triglyceride and lipoprotein (a) levels in humans (see, e.g., Miller (2003) *Mayo Clin. Proc.* 78(6):735-42). Though nicotinic acid treatment effects all of the key lipids in the desirable direction and has been shown to reduce mortality in target populations, its use is limited because of a side effect of heat and redness termed flushing, which is significantly effected by the nature of formulation. Further, nicotinamide protects against stroke injury in model systems, due to multiple mechanisms including increasing mitochondrial NAD+ levels and inhibiting PARP (Klaidman, et al. (2003) *Pharmacology* 69(3):150-7). Altered levels of NAD+ precursors have been shown to effect the regulation of a number of genes and lifespan in yeast (Anderson, et al. (2003) *Nature* 423(6936):181-5).

NAD+ administration and NMN adenylyltransferase (Nmnat1) expression have also been shown to protect neurons from axonal degeneration (Araki, et al. (2004) *Science* 305: 1010-1013). Because nicotinamide riboside is a soluble, transportable nucleoside precursor of NAD+, nicotinamide riboside can be used to protect against axonopathies such as those that occur in Alzheimer's Disease, Parkinson's Disease and Multiple Sclerosis. Expression of the NRK1 or NRK2 genes, or direct administration of nicotinamide riboside or a stable nicotinamide riboside prodrug, could also protect against axonal degeneration.

NMN adenylytransferase overexpression has been shown to protect neurons from the axonopathies that develop with ischemia and toxin exposure, including vincristine treatment (Araki, et al. (2004) *Science* 305:1010-1013). Vincristine is one of many chemotherapeutic agents whose use is limited by neurotoxicity. Thus, administration of nicotinamide riboside or an effective nicotinamide riboside prodrug derivative could be used to protect against neurotoxicity before, during or after cytotoxic chemotherapy.

Further, conversion of benign *Candida glabrata* to the adhesive, infective form is dependent upon the expression of EPA genes encoding adhesins whose expression is mediated by NAD+ limitation, which leads to defective Sir2-dependent silencing of these genes (Domergue, et al. (March 2005) *Science,* 10.1126/science.1108640). Treatment with nicotinic acid reduces expression of adhesins and increasing nicotinic acid in mouse chow reduces urinary tract infection by *Candida glabrata*. Thus, nicotinamide riboside can be used in the treatment of fungal infections, in particular, those of *Candida* species by preventing expression of adhesins.

Accordingly, agents (e.g., nicotinamide riboside) that work through the discovered nicotinamide riboside kinase pathway of NAD+ biosynthesis could have therapeutic value in improving plasma lipid profiles, preventing stroke, providing neuroprotection with chemotherapy treatment, treating fungal infections, preventing or reducing neurodegeneration, or in prolonging health and well-being. Thus, the present invention is further a method for preventing or treating a disease or condition associated with the nicotinamide riboside kinase pathway of NAD+ biosynthesis by administering an effective amount of a nicotinamide riboside composition. Diseases or conditions which typically have altered levels of NAD+ or NAD+ precursors or could benefit from increased NAD+ biosynthesis by treatment with nicotinamide riboside include, but are not limited to, lipid disorders (e.g., dyslipidemia, hypercholesterolaemia or hyperlipidemia), stroke, neurodegenerative diseases (e.g., Alzheimer's, Parkinsons and Multiple Sclerosis), neurotoxicity as observed with chemotherapies, *Candida glabrata* infection, and the general health declines associated with aging. Such diseases and conditions can be prevented or treated by supplementing a diet or a therapeutic treatment regime with a nicotinamide riboside composition.

The source of nicotinamide riboside can be from a natural or synthetic source identified by the method of the instant invention, or can be chemically synthesized using established methods (Tanimori (2002) *Bioorg. Med. Chem. Lett.* 12:1135-1137; Franchetti (2004) *Bioorg. Med. Chem. Lett.* 14:4655-4658). In addition, the nicotinamide riboside can be a derivative (e.g., L-valine or L-phenylalanine esters) of nicotinamide riboside. For example, an L-valyl (valine) ester on the 5' O of acyclovir (valacyclovir) improved the pharmacokinetic properties of the drug by promoting transport and

allowing cellular delivery of the nucleoside after hydrolysis by an abundant butyryl esterase (Han, et al. (1998) *Pharm. Res.* 15:1382-1386; Kim, et al. (2003) *J. Biol. Chem.* 278: 25348-25356). Accordingly, the present invention also encompasses derivatives of nicotinamide riboside, in particular L-valine or L-phenylalanine esters of nicotinamide riboside, which are contemplated as having improved pharmacokinetic properties (e.g., transport and delivery). Such derivatives can be used alone or formulated with a pharmaceutically acceptable carrier as disclosed herein.

An effective amount of nicotinamide riboside is one which prevents, reduces, alleviates or eliminates the signs or symptoms of the disease or condition being prevented or treated and will vary with the disease or condition. Such signs or symptoms can be evaluated by the skilled clinician before and after treatment with the nicotinamide riboside to evaluate the effectiveness of the treatment regime and dosages can be adjusted accordingly.

As alterations of NAD+ metabolism may need to be optimized for particular conditions, it is contemplated that nicotinamide riboside treatments can further be used in combination with other NAD+ precursors, e.g., tryptophan, nicotinic acid and/or nicotinamide.

Polypeptides, nucleic acids, vectors, dietary supplements (i.e. nicotinamide riboside), and nicotinamide riboside-related prodrugs produced or identified in accordance with the methods of the invention can be conveniently used or administered in a composition containing the active agent in combination with a pharmaceutically acceptable carrier. Such compositions can be prepared by methods and contain carriers which are well-known in the art. A generally recognized compendium of such methods and ingredients is Remington: The Science and Practice of Pharmacy, Alfonso R. Gennaro, editor, 20th ed. Lippincott Williams & Wilkins: Philadelphia, Pa., 2000. A carrier, pharmaceutically acceptable carrier, or vehicle, such as a liquid or solid filler, diluent, excipient, or solvent encapsulating material, is involved in carrying or transporting the subject compound from one organ, or portion of the body, to another organ, or portion of the body. Each carrier must be acceptable in the sense of being compatible with the other ingredients of the formulation and not injurious to the patient.

Examples of materials which can serve as carriers include sugars, such as lactose, glucose and sucrose; starches, such as corn starch and potato starch; cellulose, and its derivatives, such as sodium carboxymethyl cellulose, ethyl cellulose and cellulose acetate; powdered tragacanth; malt; gelatin; talc; excipients, such as cocoa butter and suppository waxes; oils, such as peanut oil, cottonseed oil, safflower oil, sesame oil, olive oil, corn oil and soybean oil; glycols, such as propylene glycol; polyols, such as glycerin, sorbitol, mannitol and polyethylene glycol; esters, such as ethyl oleate and ethyl laurate; agar; buffering agents, such as magnesium hydroxide and aluminum hydroxide; alginic acid; pyrogen-free water; isotonic saline; Ringer's solution; ethyl alcohol; pH buffered solutions; polyesters, polycarbonates and/or polyanhydrides; and other non-toxic compatible substances employed in formulations. Wetting agents, emulsifiers and lubricants, such as sodium lauryl sulfate and magnesium stearate, as well as coloring agents, release agents, coating agents, sweetening, flavoring and perfuming agents, preservatives and antioxidants can also be present in the compositions.

Polypeptides, nucleic acids, vectors, dietary supplements, and nicotinamide riboside-related prodrugs produced or identified in accordance with the methods of the invention, hereafter referred to as compounds, can be administered via any route include, but not limited to, oral, rectal, topical, buccal (e.g., sub-lingual), vaginal, parenteral (e.g., subcutaneous, intramuscular including skeletal muscle, cardiac muscle, diaphragm muscle and smooth muscle, intradermal, intravenous, intraperitoneal), topical (i.e., both skin and mucosal surfaces, including airway surfaces), intranasal, transdermal, intraarticular, intrathecal and inhalation administration, administration to the liver by intraportal delivery, as well as direct organ injection (e.g., into the liver, into the brain for delivery to the central nervous system). The most suitable route in any given case will depend on the nature and severity of the condition being treated and on the nature of the particular compound which is being used.

For injection, the carrier will typically be a liquid, such as sterile pyrogen-free water, pyrogen-free phosphate-buffered saline solution, bacteriostatic water, or Cremophor (BASF, Parsippany, N.J.). For other methods of administration, the carrier can be either solid or liquid.

For oral therapeutic administration, the compound can be combined with one or more carriers and used in the form of ingestible tablets, buccal tablets, troches, capsules, elixirs, suspensions, syrups, wafers, chewing gums, foods and the like. Such compositions and preparations should contain at least 0.1% of active compound. The percentage of the compound and preparations can, of course, be varied and can conveniently be between about 0.1 to about 100% of the weight of a given unit dosage form. The amount of active compound in such compositions is such that an effective dosage level will be obtained.

The tablets, troches, pills, capsules, and the like can also contain the following: binders such as gum tragacanth, acacia, corn starch or gelatin; excipients such as dicalcium phosphate; a disintegrating agent such as corn starch, potato starch, alginic acid and the like; a lubricant such as magnesium stearate; and a sweetening agent such as sucrose, fructose, lactose or aspartame or a flavoring agent such as peppermint, oil of wintergreen, or cherry flavoring. The above listing is merely representative and one skilled in the art could envision other binders, excipients, sweetening agents and the like. When the unit dosage form is a capsule, it can contain, in addition to materials of the above type, a liquid carrier, such as a vegetable oil or a polyethylene glycol. Various other materials can be present as coatings or to otherwise modify the physical form of the solid unit dosage form. For instance, tablets, pills, or capsules can be coated with gelatin, wax, shellac or sugar and the like.

A syrup or elixir can contain the active agent, sucrose or fructose as a sweetening agent, methyl and propylparabens as preservatives, a dye and flavoring such as cherry or orange flavor. Of course, any material used in preparing any unit dosage form should be substantially non-toxic in the amounts employed. In addition, the active compounds can be incorporated into sustained-release preparations and devices including, but not limited to, those relying on osmotic pressures to obtain a desired release profile.

Formulations of the present invention suitable for parenteral administration contain sterile aqueous and non-aqueous injection solutions of the compound, which preparations are generally isotonic with the blood of the intended recipient. These preparations can contain anti-oxidants, buffers, bacteriostats and solutes which render the formulation isotonic with the blood of the intended recipient. Aqueous and non-aqueous sterile suspensions can include suspending agents and thickening agents. The formulations can be presented in unit\dose or multi-dose containers, for example sealed ampoules and vials, and can be stored in a freeze-dried

(lyophilized) condition requiring only the addition of the sterile liquid carrier, for example, saline or water-for-injection immediately prior to use.

Formulations suitable for topical application to the skin can take the form of an ointment, cream, lotion, paste, gel, spray, aerosol, or oil. Carriers which can be used include petroleum jelly, lanoline, polyethylene glycols, alcohols, transdermal enhancers, and combinations of two or more thereof.

Formulations suitable for transdermal administration can be presented as discrete patches adapted to remain in intimate contact with the epidermis of the recipient for a prolonged period of time. Formulations suitable for transdermal administration can also be delivered by iontophoresis (see, for example, *Pharmaceutical Research* 3 (6):318 (1986)) and typically take the form of an optionally buffered aqueous solution of the compound. Suitable formulations contain citrate or bis\tris buffer (pH 6) or ethanol/water and contain from 0.1 to 0.2 M of the compound.

A compound can alternatively be formulated for nasal administration or otherwise administered to the lungs of a subject by any suitable means. In particular embodiments, the compound is administered by an aerosol suspension of respirable particles containing the compound, which the subject inhales. The respirable particles can be liquid or solid. The term aerosol includes any gas-borne suspended phase, which is capable of being inhaled into the bronchioles or nasal passages. Specifically, aerosol includes a gas-borne suspension of droplets, as can be produced in a metered dose inhaler or nebulizer, or in a mist sprayer. Aerosol also includes a dry powder composition suspended in air or other carrier gas, which can be delivered by insufflation from an inhaler device, for example. See Ganderton & Jones, *Drug Delivery to the Respiratory Tract*, Ellis Horwood (1987); Gonda (1990) *Critical Reviews in Therapeutic Drug Carrier Systems* 6:273-313; and Raeburn, et al. (1992) *J. Pharmacol. Toxicol. Methods* 27:143-159. Aerosols of liquid particles containing the compound can be produced by any suitable means, such as with a pressure-driven aerosol nebulizer or an ultrasonic nebulizer, as is known to those of skill in the art. See, e.g., U.S. Pat. No. 4,501,729. Aerosols of solid particles containing the compound can likewise be produced with any solid particulate medicament aerosol generator, by techniques known in the pharmaceutical art.

Alternatively, one can administer the compound in a local rather than systemic manner, for example, in a depot or sustained-release formulation.

Further, the present invention provides liposomal formulations of the compounds disclosed herein and salts thereof. The technology for forming liposomal suspensions is well-known in the art. When the compound or salt thereof is an aqueous-soluble salt, using conventional liposome technology, the same can be incorporated into lipid vesicles. In such an instance, due to the water solubility of the compound or salt, the compound or salt will be substantially entrained within the hydrophilic center or core of the liposomes. The lipid layer employed can be of any conventional composition and can either contain cholesterol or can be cholesterol-free. When the compound or salt of interest is water-insoluble, again employing conventional liposome formation technology, the salt can be substantially entrained within the hydrophobic lipid bilayer which forms the structure of the liposome. In either instance, the liposomes which are produced can be reduced in size, as through the use of standard sonication and homogenization techniques.

A liposomal formulation containing a compound disclosed herein or salt thereof, can be lyophilized to produce a lyophilizate which can be reconstituted with a carrier, such as water, to regenerate a liposomal suspension.

In particular embodiments, the compound is administered to the subject in an effective amount, as that term is defined herein. Dosages of active compounds can be determined by methods known in the art, see, e.g., Remington: The Science and Practice of Pharmacy, Alfonso R. Gennaro, editor, 20th ed. Lippincott Williams & Wilkins: Philadelphia, Pa., 2000. The selected effective dosage level will depend upon a variety of factors including the activity of the particular compound of the present invention employed, the route of administration, the time of administration, the rate of excretion or metabolism of the particular compound being employed, the duration of the treatment, other drugs, compounds and/or materials used in combination with the particular compound employed, the age, sex, weight, condition, general health and prior medical history of the patient being treated, and like factors well-known in the medical arts.

A physician or veterinarian having ordinary skill in the art can readily determine and prescribe the effective amount of the pharmaceutical composition required for prevention or treatment in an animal subject such as a human, agriculturally-important animal, pet or zoological animal.

The invention is described in greater detail by the following non-limiting examples.

EXAMPLE 1

S. cerevisiae Strains

Yeast diploid strain BY165, heterozygous for qns1 deletion and haploid BY165-1d carrying a chromosomal deletion of qns1 gene, transformed with plasmid pB175 containing QNS1 and URA3 is known in the art (Bieganowski, et al. (2003) supra). Genetic deletions were introduced by direct transformation with PCR products (Brachmann, et al. (1998) *Yeast* 14:115-132) generated from primers. After 24 hours of growth on complete media, cells were plated on media containing 5-fluoroorotic acid (Boeke, et al. (1987) *Methods Enzymol.* 154:164-175). The ado1 disruption cassette was constructed by PCR with primers 7041 (5'-CTA TTT AGA GTA AGG ATA TTT TTT CGG AAG GGT AAG AGG GAC CAA CTT CTT CTG TGC GGT ATT TCA CAC CG-3'; SEQ ID NO:10) and 7044 (5'-ATG ACC GCA CCA TTG GTA GTA TTG GGT AAC CCA CTT TTA GAT TTC CAA GCA GAT TGT ACT GAG AGT GCA C-3'; SEQ ID NO:11) and plasmid pRS413 as a template. Yeast strain BY165 was transformed with this PCR product, and homologous recombination in histidine prototrophic transformants was confirmed by PCR with primers 7042 (5'-AAG CTA GAG GGA ACA CGT AGA G-3'; SEQ ID NO:12) and 7043 (5'-TTA TCT TGT GCA GGG TAG AAC C-3'; SEQ ID NO:13). This strain was transformed with plasmid pB175 and subjected to sporulation and tetrad dissection. Haploid strain BY237, carrying qns1 and ado1 deletions and plasmid, was selected for further experiments. The urk1 deletion was introduced into strain BY237 by transformation with the product of the PCR amplification that used pRS415 as a template and PCR primers 7051 (5'-CGA TCT TCA TCA TTT ATT TCA ATT TTA GAC GAT GAA ACA AGA GAC ACA TTA GAT TGT ACT GAG AGT GCA C-3'; SEQ ID NO:14) and 7052 (5'-AAA ATA CTT TGA ATC AAA AAA TCT GGT CAA TGC CCA TTT GTA TTG ATG ATC TGT GCG GTA TTT CAC ACC G-3'; SEQ ID NO:15). Disruption was confirmed by PCR with primers 7053 (5'-ATG TCC CAT CGT ATA GCA CCT TCC-3'; SEQ ID NO:16) and 7054 (5'-GCC TCT AAT TAT TCT CAA TCA CAA CC-3'; SEQ ID NO:17), and the result-

ing strain was designated BY247. The rbk1 disruption cassette was constructed by PCR with primers 7063 (5'-AAA CTT TCA GGG CTA ACC ACT TCG AAA CAC ATG CTG GTG GTA AGG GAT TGA GAT TGT ACT GAG AGT GCA C-3'; SEQ ID NO:18) and 7065 (5'-GAA CAG AAA AGC ACC CCT CTC GAA CCC AAA GTC ATA ACC ACA ATT CCT CTC TGT GCG GTA TTT CAC ACC G-3'; SEQ ID NO:19) and plasmid pRS411 as a template. Disruption was introduced into strain BY242 by transformation with the product of this reaction and confirmed by PCR with primers 7062 (5'-GGA TAG ATT ACC TAA CGC TGG AG-3'; SEQ ID NO:20) and 7064 (5'-TTG TAC TTC AGG GCT TTC GTG C-3'; SEQ ID NO:21). The resulting strain, carrying deletions of qns1, ado1, urk1 and rbk1 genes was designated BY252. A yeast strain carrying disruption of the NRK1 locus was made by transformation of the strain BY165-1d with the HIS3 marker introduced into disruption cassette by PCR with primers 4750 (5'-AAT AGC GTG CAA AAG CTA TCG AAG TGT GAG CTA GAG TAG AAC CTC AAA ATA GAT TGT ACT GAG AGT GCA C-3'; SEQ ID NO:22) and 4751 (5'-CTA ATC CTT ACA AAG CTT TAG AAT CTC TTG GCA CAC CCA GCT TAA AGG TCT GTG CGG TAT TTC ACA CCG-3'; SEQ ID NO:23). Correct integration of the HIS3 marker into NRK1 locus was confirmed by PCR with primers 4752 (5'-ACC AAC TTG CAT TTT AGG CTG TTC-3'; SEQ ID NO:24) and 4753 (5'-TAA GTT ATC TAT CGA GGT ACA CAT TC-3'; SEQ ID NO:25).

EXAMPLE 2

Nicotinamide Riboside and Whey Preparations

NMN (39.9 mg; Sigma, St. Louis, Mo.) was treated with 1250 units of calf intestinal alkaline phosphatase (Sigma) for 1 hour at 37° C. in 1 mL 100 mM NaCl, 20 mM Tris pH 8.0, 5 mM $MgCl_2$. Hydrolysis of NMN to nicotinamide riboside was verified by HPLC and phosphatase was removed by centrifuging the reaction through a 5,000 Da filter (Millipore, Billerica, Mass.). A whey vitamin fraction of commercial nonfat cow's milk was prepared by adjusting the pH to 4 with HCl, stirring at 55° C. for 10 minutes, removal of denatured casein by centrifugation, and passage through a 5,000 Da filter. In yeast media, nicotinamide riboside was used at 10 μM and whey vitamin fraction at 50% by volume.

EXAMPLE 3

Yeast GST-ORF Library

Preparation of the fusion protein library was in accordance with well-established methods (Martzen, et al. (1999) supra; Phizicky, et al. (2002) *Methods Enzymol.* 350:546-559) at a 0.5 liter culture scale for each of the 64 pools of 90-96 protein constructs. Ten percent of each pool preparation was assayed for Nrk activity in overnight incubations.

EXAMPLE 4

Nicotinamide Riboside Phosphorylation Assays

Reactions (0.2 mL) containing 100 mM NaCl, 20 mM NaHEPES pH 7.2, 5 mM β-mercaptoethanol, 1 mM ATP, 5 mM $MgCl_2$, and 500 μM nicotinamide riboside or alternate nucleoside, were incubated at 30° C. and terminated by addition of EDTA to 20 mM and heating for 2 minutes at 100° C. Specific activity assays, containing 50 ng to 6 μg enzyme depending on the enzyme and substrate, were incubated for 30 minutes at 30° C. to maintain initial rate conditions. Reaction products were analyzed by HPLC on a strong anion exchange column with a 10 mM to 750 mM gradient of KPO_4 pH 2.6.

EXAMPLE 5

NRK Gene and cDNA Cloning and Enzyme Purification

The *S. cerevisiae* NRK1 gene was amplified from total yeast DNA with primers 7448 (5'-CGC TGC ACA TAT GAC TTC GAA AAA AGT GAT ATT AGT TGC-3'; SEQ ID NO:26) and 7449 (5'-CCG TCT CGA GCT AAT CCT TAC AAA GCT TTA GAA TCT CTT GG-3'; SEQ ID NO:27). The amplified DNA fragment was cloned in vector pSG04 (Ghosh and Lowenstein (1997) *Gene* 176:249-255) for *E. coli* expression using restriction sites for NdeI and XhoI included in primer sequences and the resulting plasmid was designated pB446. Samples of cDNA made from human lymphocytes and spleen were used as a template for amplification of human NRK1 using primers 4754 (5'-CCG GCC CAT GGC GCA CCA CCA TCA CCA CCA TCA TAT GAA AAC ATT TAT CAT TGG AAT CAG TGG-3'; SEQ ID NO:28) and 4755 (5'-GCG GGG ATC CTT ATG CTG TCA CTT GCA AAC ACT TTT GC-3'; SEQ ID NO:29). For *E. coli* expression, PCR amplicons from this reaction were cloned into restriction sites NcoI and BamHI of vector pMR103 (Munson, et al. (1994) *Gene* 144:59-62) resulting in plasmid pB449. Subsequently, plasmid pB449 was used as a template for PCR with primers 7769 (5'-CCG CGG ATC CAT GAA AAC ATT TAT CAT TGG AAT CAG TGG-3'; SEQ ID NO:30) and 7770 (5'-GCC GCT CGA GTT ATG CTG TCA CTT GCA AAC ACT T-3'; SEQ ID NO:31). The product of this amplification was cloned between BamHI and XhoI sites of vector p425GAL1 (Mumberg, et al. (1994) *Nucleic Acids Res.* 22:5767-5768) and the resulting plasmid carrying human NRK1 gene under GAL1 promoter control was designated pB450. Human NRK2 cDNA was amplified with primers 7777 (5'-GGC AGG CAT ATG AAG CTC ATC GTG GGC ATC G-3'; SEQ ID NO:32) and 7776 (5'-GCT CGC TCG AGT CAC ATG CTG TCC TGC TGG GAC-3'; SEQ ID NO:33). The amplified fragment was digested with NdeI and XhoI enzymes and cloned in plasmid pSGA04 for *E. coli* expression. His-tagged enzymes were purified with Ni-NTA agarose.

SEQUENCE LISTING

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<213> ORGANISM: Saccharomyces cerevisiae

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atactcgaag atttgcattg aacgatcgtt gccgttgact catttgaaac agaaaaacaa 180

tacacgcagg aaaggaacgg cagttggtct gagaaacaaa accaacttgc attttaggct 240

gttccgatag tttatcagag taagggaaaa aatagcgtgc aaaagctatc gaagtgtgag 300

ctagagtaga acctcaaaat atgacttcga aaaaagtgat attagttgca ttgagtggat 360

gctcctccag tggtaagacg acaattgcga aacttacagc aagtttattc acgaaggcta 420

cattaattca tgaagatgac ttttacaaac atgataatga agtgccagta gatgctaaat 480

ataacattca aaattgggat tcgccagaag ctcttgattt taaacttttc ggtaaagaat 540

tagatgtgat caaacaaact ggtaaaatag ccaccaaact tatacacaat aacaacgtag 600

atgatccctt tacaaagttc cacattgata gacaagtttg ggacgagtta aaggctaagt 660

atgactctat taatgacgac aaatatgaag ttgtaattgt agatgggttt atgattttca 720

ataatactgg aatatcaaaa aaatttgatt tgaagatatt agtgcgtgct ccctatgaag 780

tactaaaaaa aaggagggct tccagaaaag gataccagac tttggattct ttctgggtgg 840

atccgccgta ttatttcgac gaatttgtgt atgaatctta tcgtgcaaat catgcgcagt 900

tatttgttaa tggagacgta gaaggtttac tagacccaag gaagtcaaag aatataaaag 960

agttcataaa tgatgatgac actccaattg cgaaaccttt aagctgggtg tgccaagaga 1020

ttctaaagct ttgtaaggat taggaaagcg ccacaaaatc gatgagaagt ataaaaaaaa 1080

aaaagtaaaa acaataaaaa taagaatgtg tacctcgata gataacttaa ataagacaat 1140

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<213> ORGANISM: Homo sapiens

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cgcgcttggc tgtcgcctct tccgctgtgt ttgggaggac tcgaactggc gccaggaaat 180

attaggaagc tgtgattttc aaagctaatt atgaaaacat ttatcattgg aatcagtggt 240

gtgacaaaca gtggcaaaac aacactggct aagaatttgc agaaacacct cccaaattgc 300

agtgtcatat ctcaggatga tttcttcaag ccagagtctg agatagagac agataaaaat 360

ggatttttgc agtacgatgt gcttgaagca cttaacatgg aaaaaatgat gtcagccatt 420

tcctgctgga tggaaagcgc aagacactct gtggtatcaa cagaccagga aagtgctgag 480

gaaattccca ttttaatcat cgaaggtttt cttctttta attataagcc ccttgacact 540

atatggaata gaagctattt cctgactatt ccatatgaag aatgtaaaag gaggaggagt 600

acaagggtct atcagcctcc agactctccg ggatactttg atggccatgt gtggcccatg 660

tatctaaagt acagacaaga aatgcaggac atcacatggg aagttgtgta cctggatgga 720

acaaaatctg aagaggacct ctttttgcaa gtatatgaag atctaataca agaactagca 780

aagcaaaagt gtttgcaagt gacagcataa agacggaaca caacaaatcc ttcctgaagt 840

gaattaggaa actccaagga gtaatttaag aaccttcacc aagatacaat gtatactgtg 900

gtacaatgac agccattgtt tcatatgttt gatttttatt gcacatggtt ttcccaacat 960

gtggaacaat aaatatccat gccaatggac aggactgtac cttagcaagt tgctccctct 1020

ccagggagcg catagataca gcagagctca cagtgagtca gaaagtctcc actttctgaa 1080

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gattacatca caatccaaaa aaaaaaaaaa aa 1172

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<213> ORGANISM: Homo sapiens

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tgccggcgcc tccgccccat ccccaggggc cgcctccccc ggggcggcct ccaggctgcc 180

gagacctata aaggcgccag gttttctcaa tgaagccggg acgcactccg gagcgcactg 240

cgtggtcgca ccctacccgg gctgccttgg aagtcgtccc cgccgcccct ccgcaccggc 300

atgaagctca tcgtgggcat cggaggcatg accaacggcg gcaagaccac gctgaccaac 360

agcctgctca gagccctgcc caactgctgc gtgatccatc aggatgactt cttcaagccc 420

caagaccaaa tagcagttgg ggaagacggc ttcaaacagt gggacgtgct ggagtctctg 480

gacatggagg ccatgctgga caccgtgcag gcctggctga gcagcccgca gaagtttgcc 540

cgtgcccacg gggtcagcgt ccagccagag gcctcggaca cccacatcct cctcctggaa 600

ggcttcctgc tctacagcta caagcccctg gtggacttgt acagccgccg gtacttcctg 660

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gacgcctctg taacctcgct gcagcttcag tagtaaactg ggtcctgttt tttt 1134

<210> SEQ ID NO 4
<211> LENGTH: 240
<212> TYPE: PRT
<213> ORGANISM: Saccharomyces cerevisiae

<400> SEQUENCE: 4

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Ser Gly Lys Thr Thr Ile Ala Lys Leu Thr Ala Ser Leu Phe Thr Lys
20 25 30

Ala Thr Leu Ile His Glu Asp Asp Phe Tyr Lys His Asp Asn Glu Val
35 40 45

Pro Val Asp Ala Lys Tyr Asn Ile Gln Asn Trp Asp Ser Pro Glu Ala
50 55 60

Leu Asp Phe Lys Leu Phe Gly Lys Glu Leu Asp Val Ile Lys Gln Thr
65 70 75 80

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Gly Lys Ile Ala Thr Lys Leu Ile His Asn Asn Asn Val Asp Asp Pro
                85                  90                  95

Phe Thr Lys Phe His Ile Asp Arg Gln Val Trp Asp Glu Leu Lys Ala
            100                 105                 110

Lys Tyr Asp Ser Ile Asn Asp Asp Lys Tyr Glu Val Val Ile Val Asp
        115                 120                 125

Gly Phe Met Ile Phe Asn Asn Thr Gly Ile Ser Lys Lys Phe Asp Leu
    130                 135                 140

Lys Ile Leu Val Arg Ala Pro Tyr Glu Val Leu Lys Lys Arg Arg Ala
145                 150                 155                 160

Ser Arg Lys Gly Tyr Gln Thr Leu Asp Ser Phe Trp Val Asp Pro Pro
                165                 170                 175

Tyr Tyr Phe Asp Glu Phe Val Tyr Glu Ser Tyr Arg Ala Asn His Ala
            180                 185                 190

Gln Leu Phe Val Asn Gly Asp Val Glu Gly Leu Leu Asp Pro Arg Lys
        195                 200                 205

Ser Lys Asn Ile Lys Glu Phe Ile Asn Asp Asp Asp Thr Pro Ile Ala
    210                 215                 220

Lys Pro Leu Ser Trp Val Cys Gln Glu Ile Leu Lys Leu Cys Lys Asp
225                 230                 235                 240


<210> SEQ ID NO 5
<211> LENGTH: 199
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

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Met Lys Thr Phe Ile Ile Gly Ile Ser Gly Val Thr Asn Ser Gly Lys
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Thr Thr Leu Ala Lys Asn Leu Gln Lys His Leu Pro Asn Cys Ser Val
            20                  25                  30

Ile Ser Gln Asp Asp Phe Phe Lys Pro Glu Ser Glu Ile Glu Thr Asp
        35                  40                  45

Lys Asn Gly Phe Leu Gln Tyr Asp Val Leu Glu Ala Leu Asn Met Glu
    50                  55                  60

Lys Met Met Ser Ala Ile Ser Cys Trp Met Glu Ser Ala Arg His Ser
65                  70                  75                  80

Val Val Ser Thr Asp Gln Glu Ser Ala Glu Glu Ile Pro Ile Leu Ile
                85                  90                  95

Ile Glu Gly Phe Leu Leu Phe Asn Tyr Lys Pro Leu Asp Thr Ile Trp
            100                 105                 110

Asn Arg Ser Tyr Phe Leu Thr Ile Pro Tyr Glu Glu Cys Lys Arg Arg
        115                 120                 125

Arg Ser Thr Arg Val Tyr Gln Pro Pro Asp Ser Pro Gly Tyr Phe Asp
    130                 135                 140

Gly His Val Trp Pro Met Tyr Leu Lys Tyr Arg Gln Glu Met Gln Asp
145                 150                 155                 160

Ile Thr Trp Glu Val Val Tyr Leu Asp Gly Thr Lys Ser Glu Glu Asp
                165                 170                 175

Leu Phe Leu Gln Val Tyr Glu Asp Leu Ile Gln Glu Leu Ala Lys Gln
            180                 185                 190

Lys Cys Leu Gln Val Thr Ala
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<210> SEQ ID NO 6
<211> LENGTH: 230
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<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 6

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Thr Leu Thr Asn Ser Leu Leu Arg Ala Leu Pro Asn Cys Cys Val Ile
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His Gln Asp Asp Phe Phe Lys Pro Gln Asp Gln Ile Ala Val Gly Glu
35 40 45

Asp Gly Phe Lys Gln Trp Asp Val Leu Glu Ser Leu Asp Met Glu Ala
50 55 60

Met Leu Asp Thr Val Gln Ala Trp Leu Ser Ser Pro Gln Lys Phe Ala
65 70 75 80

Arg Ala His Gly Val Ser Val Gln Pro Glu Ala Ser Asp Thr His Ile
85 90 95

Leu Leu Leu Glu Gly Phe Leu Leu Tyr Ser Tyr Lys Pro Leu Val Asp
100 105 110

Leu Tyr Ser Arg Arg Tyr Phe Leu Thr Val Pro Tyr Glu Glu Cys Lys
115 120 125

Trp Arg Arg Ser Thr Arg Asn Tyr Thr Val Pro Asp Pro Pro Gly Leu
130 135 140

Phe Asp Gly His Val Trp Pro Met Tyr Gln Lys Tyr Arg Gln Glu Met
145 150 155 160

Glu Ala Asn Gly Val Glu Val Val Tyr Leu Asp Gly Met Lys Ser Arg
165 170 175

Glu Glu Leu Phe Arg Glu Val Leu Glu Asp Ile Gln Asn Ser Leu Leu
180 185 190

Asn Arg Ser Gln Glu Ser Ala Pro Ser Pro Ala Arg Pro Ala Arg Thr
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Gln Gly Pro Gly Arg Gly Cys Gly His Arg Thr Ala Arg Pro Ala Ala
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Ser Gln Gln Asp Ser Met
225 230

<210> SEQ ID NO 7
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<213> ORGANISM: Schizosaccharomyces pombe

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20 25 30

Ser Leu Val His Glu Asp Asp Phe Tyr Lys Thr Asp Ala Glu Ile Pro
35 40 45

Val Lys Asn Gly Ile Ala Asp Trp Asp Cys Gln Glu Ser Leu Asn Leu
50 55 60

Asp Ala Phe Leu Glu Asn Leu His Tyr Ile Arg Asp His Gly Val Leu
65 70 75 80

Pro Thr His Leu Arg Asn Arg Glu Asn Lys Asn Val Ala Pro Glu Ala
85 90 95

Leu Ile Glu Tyr Ala Asp Ile Ile Lys Glu Phe Lys Ala Pro Ala Ile
100 105 110

Pro Thr Leu Glu Gln His Leu Val Phe Val Asp Gly Phe Met Met Tyr

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        115                 120                 125

Val Asn Glu Asp Leu Ile Asn Ala Phe Asp Ile Arg Leu Met Leu Val
    130                 135                 140

Thr Asp Phe Asp Thr Leu Lys Arg Arg Arg Glu Ala Arg Thr Gly Tyr
145                 150                 155                 160

Ile Thr Leu Glu Gly Phe Trp Gln Asp Pro Pro His Tyr Phe Glu Asn
                165                 170                 175

Tyr Val Trp Pro Gly Tyr Val His Gly His Ser His Leu Phe Val Asn
            180                 185                 190

Gly Asp Val Thr Gly Lys Leu Leu Asp Lys Arg Ile Gln Leu Ser Pro
        195                 200                 205

Ser Ser Lys Met Ser Val Arg Asp Asn Val Gln Trp Ala Ile Asn Ser
    210                 215                 220

Ile Leu Asn Ala Leu
225


<210> SEQ ID NO 8
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<400> SEQUENCE: 8

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Ser Val Ala Ala Lys Ile Val Ser Ser Ile Asn Val Pro Trp Thr Val
            20                  25                  30

Leu Ile Ser Leu Asp Asn Phe Tyr Asn Pro Leu Gly Pro Glu Asp Arg
        35                  40                  45

Ala Arg Ala Phe Lys Asn Glu Tyr Asp Phe Asp Glu Pro Asn Ala Ile
    50                  55                  60

Asn Leu Asp Leu Ala Tyr Lys Cys Ile Leu Asn Leu Lys Glu Gly Lys
65                  70                  75                  80

Arg Thr Asn Ile Pro Val Tyr Ser Phe Val His His Asn Arg Val Pro
                85                  90                  95

Asp Lys Asn Ile Val Ile Tyr Gly Ala Ser Val Val Val Ile Glu Gly
            100                 105                 110

Ile Tyr Ala Leu Tyr Asp Arg Arg Leu Leu Asp Leu Met Asp Leu Lys
        115                 120                 125

Ile Tyr Val Asp Ala Asp Leu Asp Val Cys Leu Ala Arg Arg Leu Ser
    130                 135                 140

Arg Asp Ile Val Ser Arg Gly Arg Asp Leu Asp Gly Cys Ile Gln Gln
145                 150                 155                 160

Trp Glu Lys Phe Val Lys Pro Asn Ala Val Lys Phe Val Lys Pro Thr
                165                 170                 175

Met Lys Asn Ala Asp Ala Ile Ile Pro Ser Met Ser Asp Asn Ala Thr
            180                 185                 190

Ala Val Asn Leu Ile Ile Asn His Ile Lys Ser Lys Leu Glu Leu Lys
        195                 200                 205

Ser Asn Glu His Leu Arg Glu Leu Ile Lys Leu Gly Ser Ser Pro Ser
    210                 215                 220

Gln Asp Val Leu Asn Arg Asn Ile Ile His Glu Leu Pro Pro Thr Asn
225                 230                 235                 240

Gln Val Leu


<210> SEQ ID NO 9
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<211> LENGTH: 232
<212> TYPE: PRT
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20 25 30

Arg Leu Lys Gly Ile Asn Glu Asp Leu Ser Leu Glu Glu Val Ala Glu
35 40 45

Ile Tyr Leu Pro Leu Ser Arg Leu Leu Asn Phe Tyr Ile Ser Ser Asn
50 55 60

Leu Arg Arg Gln Ala Val Leu Glu Gln Phe Leu Gly Thr Asn Gly Gln
65 70 75 80

Arg Ile Pro Tyr Ile Ile Ser Ile Ala Gly Ser Val Ala Val Gly Lys
85 90 95

Ser Thr Thr Ala Arg Val Leu Gln Ala Leu Leu Ser Arg Trp Pro Glu
100 105 110

His Arg Arg Val Glu Leu Ile Thr Thr Asp Gly Phe Leu His Pro Asn
115 120 125

Gln Val Leu Lys Glu Arg Gly Leu Met Lys Lys Lys Gly Phe Pro Glu
130 135 140

Ser Tyr Asp Met His Arg Leu Val Lys Phe Val Ser Asp Leu Lys Ser
145 150 155 160

Gly Val Pro Asn Val Thr Ala Pro Val Tyr Ser His Leu Ile Tyr Asp
165 170 175

Val Ile Pro Asp Gly Asp Lys Thr Val Val Gln Pro Asp Ile Leu Ile
180 185 190

Leu Glu Gly Leu Asn Val Leu Gln Ser Gly Met Asp Tyr Pro His Asp
195 200 205

Pro His His Val Phe Val Ser Asp Phe Val Asp Phe Ser Ile Tyr Val
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Asp Ala Pro Glu Asp Leu Leu Gln
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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic primer

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<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic primer

<400> SEQUENCE: 11

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gagagtgcac 70

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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic primer

<400> SEQUENCE: 12

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<210> SEQ ID NO 13
<211> LENGTH: 22
<212> TYPE: DNA
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<220> FEATURE:
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<210> SEQ ID NO 14
<211> LENGTH: 70
<212> TYPE: DNA
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<223> OTHER INFORMATION: Synthetic primer

<400> SEQUENCE: 14

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gagagtgcac 70

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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic primer

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tttcacaccg 70

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<213> ORGANISM: Artificial Sequence
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<212> TYPE: DNA
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<210> SEQ ID NO 18
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<212> TYPE: DNA
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Xaa Xaa Xaa Xaa Asp Asp Phe Xaa Lys
1 5

What is claimed is:

1. A composition comprising isolated nicotinamide riboside in combination with one or more of tryptophan, nicotinic acid, or nicotinamide, wherein said combination is in admixture with a carrier comprising a sugar, starch, cellulose, powdered tragacanth, malt, gelatin, talc, cocoa butter, suppository wax, oil, glycol, polyol, ester, agar, buffering agent, alginic acid, isotonic saline, Ringer's solution, ethyl alcohol, polyester, polycarbonate, or polyanhydride, wherein said composition is formulated for oral administration and increases NAD+ biosynthesis upon oral administration.

2. The composition of claim **1**, wherein the nicotinamide riboside is isolated from a natural or synthetic source.

3. The composition of claim **1**, wherein the formulation comprises a tablet, troche, capsule, elixir, suspension, syrup, wafer, chewing gum, or food.

* * * * *

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 8,197,807 B2
APPLICATION NO. : 11/912400
DATED : June 12, 2012
INVENTOR(S) : Charles M. Brenner

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

In the Specification

At Column 1, please delete Lines 7-10 and insert in its place the following:
--This invention was made with government support under grant number CA077738 awarded by the National Institutes of Health. The government has certain rights in the invention.--

Signed and Sealed this
Twenty-eighth Day of January, 2020

Andrei Iancu
Director of the United States Patent and Trademark Office


US008383086B2

(12) **United States Patent**
Brenner

(10) **Patent No.: US 8,383,086 B2**
(45) **Date of Patent: *Feb. 26, 2013**

(54) **NICOTINAMIDE RIBOSIDE KINASE COMPOSITIONS AND METHODS FOR USING THE SAME**

(75) Inventor: **Charles M. Brenner**, Lyme, NH (US)

(73) Assignee: **Trustees of Dartmouth College**, Hanover, NH (US)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

This patent is subject to a terminal disclaimer.

(21) Appl. No.: **13/445,289**

(22) Filed: **Apr. 12, 2012**

(65) **Prior Publication Data**

US 2012/0251463 A1 Oct. 4, 2012

Related U.S. Application Data

(63) Continuation of application No. 11/912,400, filed as application No. PCT/US2006/015495 on Apr. 20, 2006, now Pat. No. 8,197,807.

(51) **Int. Cl.**

A61K 38/45	(2006.01)
A61K 31/7088	(2006.01)
C07H 17/00	(2006.01)
A61P 35/00	(2006.01)

(52) **U.S. Cl.** ..... **424/48**; 424/94.5; 435/15; 435/194; 514/25; 514/44 R

(58) **Field of Classification Search** ..... None
See application file for complete search history.

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(Continued)

Primary Examiner — Kagnew H Gebreyesus

(74) *Attorney, Agent, or Firm* — Licata & Tyrrell P.C.

(57) **ABSTRACT**

The present invention relates to isolated nicotinamide riboside kinase (Nrk) nucleic acid sequences, vectors and cultured cells containing the same, and Nrk polypeptides encoded thereby. Methods for identifying individuals or tumors susceptible to nicotinamide riboside-related prodrug treatment and methods for treating cancer by administering an Nrk nucleic acid sequence or polypeptide in combination with a nicotinamide riboside-related prodrug are also prvided. The present invention further provides screening methods for isolating a nicotinamide riboside-related prodrug and identifying a natural source of nicotinamide riboside.

5 Claims, 1 Drawing Sheet o-

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Hsapi_Nrk1     MKTFIIGISGVTNSGKTTLAKNLQKHLPN---CSVISQDDFFKPES-EIETD-KNGFLQYDVL
Hsapi_Nrk2      MK-LIVGIGGMTNGGKTTLTNSLLRALPN---CCVIHQDDFFKPQD-QIAVG-EDGFKQWDVL
Scere_Nrk1  MTSKKVILVALSGCSSSGKTTIAKLTASLFTK---ATLIHEDDFYKHDN-EVPVDAKYNIQNWDSP
Spomb_Nrk1  MT-RKTIIVGVSGASCSGKSTLCQLLHAIFEG---SSLVHEDDFYKTDA-EIPV--KNGIADWDCQ
Scere_Urk1       TPYIIGIGGASGSGKTSVAAKIVSSINVP-WTVLISLDNFYNPLGPEDRARAFKNEYDFDEP
Ecoli_panK       QTLMTPYLQFDRNQWAALRDSVPMTLSEDEIARLKGINEDLSLEEVAEIYLPLSRLLNFYIS

Hsapi_Nrk1     EALNMEKMMSAISCWMES---ARHSVVSTDQES-----------------------AEEIPIL
Hsapi_Nrk2     ESLDMEAMLDTVQAWLSSPQKFARAHGVSVQPE-----------------------ASDTHIL
Scere_Nrk1     EALDFKLFGKELDVIKQTGKIATKLIHNNNVDDPFTKFHIDRQVWDELKAKYDSINDDKYEVV
Spomb_Nrk1     ESLNLDAFLENLHYIRDHGVLPTHILRNRENKNVAPEALIEYADIIKEFKAP--AIPTLEQHLV
Scere_Urk1     NAINLDLAYKCILNLKEGKRTNIPVYSFVHHNRVPDK-----------------NIVIYGASVV
Ecoli_panK     SNLRRQAVLEQFLGTNGQRIPYIISIAGSVAVGKSTTARVLQALLSR-------WPEHRRVELI

Hsapi_Nrk1     IIEGFLLFNYKPLDTIWNRSYFLTIPYEECKRRRSTR-VYQPPD-----SPGYFDGHVWPMYL
Hsapi_Nrk2     LLEGFLLYSYKPLVDLYSRRYFLTVPYEECKWRRSTR-NYTVPD--PPGLFDGHVWPMYQKYR
Scere_Nrk1     IVDGFMIFNNTGISKKFDLKILVRAPYEVLKKRRASRKGYQTLDSFWVDPPYYFDEFVYESYR
Spomb_Nrk1     FVDGFMMYVNEDLINAFDIRLMLVTDFDTLKRRREARTGYITLEGFWQDPPHYFENYVWPGYV
Scere_Urk1     VIEGIYALYDRRLLDLMDLKIYVDADLDVCLARRLSR-DIVSRGRDLDGCIQQWEKFVKPNAV
Ecoli_panK     TTDGFLHPNQVLKERGLMKKKGFPESYDMHRLVKFVS---DLKSGVPNVTAPVYSHLIYDVIP

Hsapi_Nrk1     KYRQEMQDITWEVVY-LDGTKSEEDLFLQVYEDLIQELAKQK-----------------CL
Hsapi_Nrk2     QEMEANGVEVVYLDGMKSREELFREVLEDIQNSLLNRSQESAPSPARPARTQGPGRGCGHRTA
Scere_Nrk1     ANHAQLFVNGDVEG--LLDPRKSKNIKEFINDDDTPIAKPLS-----------------WVCQ
Spomb_Nrk1     HGHSHLFVNGDVTGK-LLDKR-----IQLSPSSKMSVRDNVQ-----------------WAIN
Scere_Urk1     KFVKPTMKNADAIIPSMSDNATAVNLIINHIKSKLELKSNEHLRELIKLGSSPSQDVLNRNII
Ecoli_panK     DGDKTVVQPDILILEGLNVLQSGMDYPHDPHHVFVSDFVDFS-----------------I

Hsapi_Nrk1     QVTA
Hsapi_Nrk2     RPAASQQDSM
Scere_Nrk1     EILKLCKD
Spomb_Nrk1     SILNAL
Scere_Urk1     HELPPTNQVL
Ecoli_panK     YVDAPEDILQ
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NICOTINAMIDE RIBOSIDE KINASE COMPOSITIONS AND METHODS FOR USING THE SAME

INTRODUCTION

This application is a continuation of U.S. patent application Ser. No. 11/912,400 filed Nov. 20, 2007 now U.S. Pat. No. 8,197,807, which is the National Stage of International Application No. PCT/US2006/015495 filed Apr. 20, 2006, which claims benefit of priority to U.S. patent application Ser. No. 11/113,701 filed Apr. 25, 2005, the teachings of which are incorporated herein by reference in their entireties.

This invention was made with government support under grant number CA77738 awarded by the National Cancer Institute. The government has certain rights in the invention.

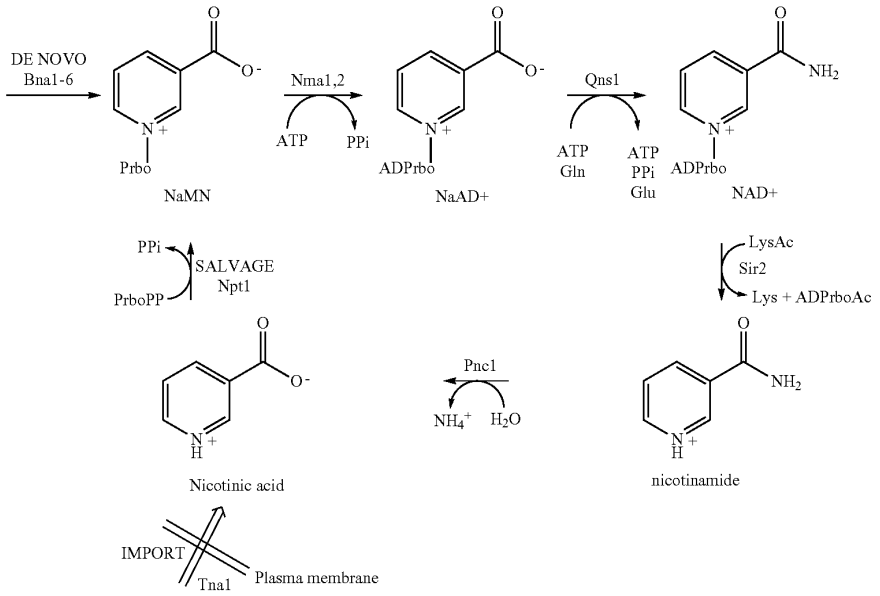
BACKGROUND OF THE INVENTION

Nicotinic acid and nicotinamide, collectively niacins, are the vitamin forms of nicotinamide adenine dinucleotide (NAD+). Eukaryotes can synthesize NAD+ de novo via the kynurenine pathway from tryptophan (Krehl, et al. (1945) *Science* 101:489-490; Schutz and Feigelson (1972) *J. Biol. Chem.* 247:5327-5332) and niacin supplementation prevents the pellagra that can occur in populations with a tryptophan-poor diet. It is well-established that nicotinic acid is phosphoribosylated to nicotinic acid mononucleotide (NaMN), which is then adenylylated to form nicotinic acid adenine dinucleotide (NaAD), which in turn is amidated to form NAD+ (Preiss and Handler (1958) *J. Biol. Chem.* 233:488-492; Preiss and Handler (1958b) *J. Biol. Chem.* 233:493-50).

NAD+ was initially characterized as a co-enzyme for oxidoreductases. Though conversions between NAD+, NADH, NADP and NADPH would not be accompanied by a loss of total co-enzyme, it was discovered that NAD+ is also turned over in cells for unknown purposes (Maayan (1964) *Nature* 204:1169-1170). Sirtuin enzymes such as Sir2 of *S. cerevisiae* and its homologs deacetylate lysine residues with consumption of an equivalent of NAD+ and this activity is required for Sir2 function as a transcriptional silencer (Imai, et al. (2000) *Cold Spring Harb. Symp. Quant. Biol.* 65:297-302). NAD^+-dependent deacetylation reactions are required not only for alterations in gene expression but also for repression of ribosomal DNA recombination and extension of lifespan in response to calorie restriction (Lin, et al. (2000) *Science* 289:2126-2128; Lin, et al. (2002) *Nature* 418:344-348). NAD+ is consumed by Sir2 to produce a mixture of 2'- and 3' O-acetylated ADP-ribose plus nicotinamide and the deacetylated polypeptide (Sauve, et al. (2001) *Biochemistry* 40:15456-15463). Additional enzymes, including poly(ADPribose) polymerases and cADPribose synthases are also NAD^+-dependent and produce nicotinamide and ADPribosyl products (Ziegler (2000) *Eur. J. Biochem.* 267:1550-1564; Burkle (2001) *Bioessays* 23:795-806).

The non-coenzymatic properties of NAD+ has renewed interest in NAD+ biosynthesis. Four recent publications have suggested what is considered to be all of the gene products and pathways to NAD+ in *S. cerevisiae* (Panozzo, et al. (2002) *FEBS Lett.* 517:97-102; Sandmeier, et al. (2002) *Genetics* 160:877-889; Bitterman, et al. (2002) *J. Biol. Chem.* 277:45099-45107; Anderson, et al. (2003) *Nature* 423:181-185) depicting convergence of the flux to NAD+ from de novo synthesis, nicotinic acid import, and nicotinamide salvage at NaMN (Scheme 1).

Scheme 1



SUMMARY OF THE INVENTION

It has now been shown that nicotinamide riboside, which was known to be an NAD+ precursor in bacteria such as *Haemophilus influenza* (Gingrich and Schlenk (1944) *J. Bacteriol.* 47:535-550; Leder and Handler (1951) *J. Biol. Chem.* 189:889-899; Shifrine and Biberstein (1960) *Nature* 187:623) that lack the enzymes of the de novo and Preiss-Handler

pathways (Fleischmann, et al. (1995) *Science* 269:496-512), is an NAD+ precursor in a previously unknown but conserved eukaryotic NAD+ biosynthetic pathway. Yeast nicotinamide riboside kinase, Nrk1, and human Nrk enzymes with specific functions in NAD+ metabolism are provided herein. The specificity of these enzymes indicates that they are the long-sought tiazofurin kinases that perform the first step in converting cancer drugs such as tiazofurin and benzamide riboside and their analogs into toxic NAD+ analogs. Further, yeast mutants of defined genotype were used to identify sources of nicotinamide riboside and it is shown that milk is a source of nicotinamide riboside.

Accordingly, the present invention is an isolated nucleic acid encoding a eukaryotic nicotinamide riboside kinase polypeptide. A eukaryotic nicotinamide riboside kinase nucleic acid encompasses (a) a nucleotide sequence of SEQ ID NO:1, SEQ ID NO:2 or SEQ ID NO:3; (b) a nucleotide sequence that hybridizes to a nucleotide sequence of SEQ ID NO:1, SEQ ID NO:2 or SEQ ID NO:3 or its complementary nucleotide sequence under stringent conditions, wherein said nucleotide sequence encodes a functional nicotinamide riboside kinase polypeptide; or (c) a nucleotide sequence encoding an amino acid sequence encoded by the nucleotide sequences of (a) or (b), but which has a different nucleotide sequence than the nucleotide sequences of (a) or (b) due to the degeneracy of the genetic code or the presence of non-translated nucleotide sequences.

The present invention is also an expression vector containing an isolated nucleic acid encoding a eukaryotic nicotinamide riboside kinase polypeptide. In one embodiment, the expression vector is part of a composition containing a pharmaceutically acceptable carrier. In another embodiment, the composition further contains a prodrug wherein the prodrug is a nicotinamide riboside-related analog that is phosphorylated by the expressed nicotinamide riboside kinase thereby performing the first step in activating said prodrug.

The present invention is also an isolated eukaryotic nicotinamide riboside kinase polypeptide. In one embodiment, the isolated nicotinamide riboside kinase polypeptide has an amino acid sequence having at least about 70% amino acid sequence similarity to an amino acid sequence of SEQ ID NO:4, SEQ ID NO:5 or SEQ ID NO:6 or a functional fragment thereof.

The present invention is further a cultured cell containing an isolated nucleic acid encoding a eukaryotic nicotinamide riboside kinase polypeptide or a polypeptide encoded thereby.

Still further, the present invention is a composition containing an isolated eukaryotic nicotinamide riboside kinase polypeptide and a pharmaceutically acceptable carrier. In one embodiment, the composition further contains a prodrug wherein said prodrug is a nicotinamide riboside-related analog that is phosphorylated by the nicotinamide riboside kinase thereby performing the first step in activating said prodrug.

The present invention is also a method for treating cancer by administering to a patient having or suspected of having cancer an effective amount of a nicotinamide riboside-related prodrug in combination with an isolated eukaryotic nicotinamide riboside kinase polypeptide or expression vector containing an isolated nucleic acid sequence encoding an eukaryotic nicotinamide riboside kinase polypeptide wherein the nicotinamide riboside kinase polypeptide phosphorylates the prodrug thereby performing the first step in activating the prodrug so that the signs or symptoms of said cancer are decreased or eliminated.

The present invention is further a method for identifying a natural or synthetic source for nicotinamide riboside. The method involves contacting a first cell lacking a functional glutamine-dependent NAD+ synthetase with an isolated extract from a natural source or synthetic; contacting a second cell lacking functional glutamine-dependent NAD+ synthetase and nicotinamide riboside kinase with the isolated extract; and detecting growth of the first cell compared to the growth of the second cell, wherein the presence of growth in the first cell and absence of growth in the second cell is indicative of the presence of nicotinamide riboside in the isolated extract. In one embodiment, the natural source is cow's milk.

Further, the present invention is a dietary supplement composition containing nicotinamide riboside identified in accordance with the methods of the present invention and a carrier.

Moreover, the present invention is a method for preventing or treating a disease or condition associated with the nicotinamide riboside kinase pathway of NAD+ biosynthesis. The method involves administering to a patient having a disease or condition associated with the nicotinamide riboside kinase pathway of NAD+ biosynthesis an effective amount of a nicotinamide riboside composition so that the signs or symptoms of the disease or condition are prevented or reduced. In one embodiment, the nicotinamide riboside is neuroprotective. In another embodiment the nicotinamide riboside is anti-fungal. In a further embodiment, the nicotinamide riboside is administered in combination with tryptophan, nicotinic acid or nicotinamide.

The present invention is also an in vitro method for identifying a nicotinamide riboside-related prodrug. The method involves contacting a nicotinamide riboside kinase polypeptide with a nicotinamide riboside-related test agent and determining whether said test agent is phosphorylated by said nicotinamide riboside kinase polypeptide wherein phosphorylation of said test agent is indicative of said test agent being a nicotinamide riboside-related prodrug. A nicotinamide riboside-related prodrug identified by this method is also encompassed within the present invention.

The present invention is further a cell-based method for identifying a nicotinamide riboside-related prodrug. This method involves contacting a first test cell which expresses a recombinant Nrk polypeptide with a nicotinamide riboside-related test agent; contacting a second test cell which lacks a functional Nrk polypeptide with the same test agent; and determining the viability of the first and second test cells, wherein sensitivity of the first cell and not the second cell is indicative of a nicotinamide riboside-related prodrug. A nicotinamide riboside-related prodrug identified by this method is also encompassed within the context of the present invention.

The present invention is also a method for identifying an individual or tumor which is susceptible to treatment with a nicotinamide riboside-related prodrug. This method involves detecting the presence of mutations in, or the level of expression of, a nicotinamide riboside kinase in an individual or tumor wherein the presence of a mutation or change in expression of nicotinamide riboside kinase in said individual or tumor compared to a control is indicative of said individual or tumor having an altered level of susceptibility to treatment with a nicotinamide riboside-related prodrug.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. **1** shows the amino acid sequence alignment and consensus sequence (SEQ ID NO:34) of human Nrk1 (SEQ ID NO:5), human Nrk2 (SEQ ID NO:6), *S. cerevisiae* Nrk1 (SEQ ID NO:4), *S. pombe* nrk1 (SEQ ID NO:7), as compared

to portions of *S. cerevisiae* uridine/cytidine kinase Urk1 (SEQ ID NO:8) and *E. coli* pantothenate kinase (SEQ ID NO:9).

DETAILED DESCRIPTION OF THE INVENTION

A *Saccharomyces cerevisiae* QNS1 gene encoding glutamine-dependent NAD+ synthetase has been characterized and mutation of either the glutaminase active site or the NAD+ synthetase active site resulted in inviable cells (Bieganowski, et al. (2003) *J. Biol. Chem.* 278:33049-33055). Possession of strains containing the qns1 deletion and a plasmid-borne QNS1 gene allowed a determination of whether the canonical de novo, import and salvage pathways for NAD+ of Scheme 1 (Panozzo, et al. (2002) supra; Sandmeier, et al. (2002) supra; Bitterman, et al. (2002) supra; Anderson, et al. (2003) supra) are a complete representation of the metabolic pathways to NAD+ in *S. cerevisiae*. The pathways depicted in scheme 1 suggest that: nicotinamide is deamidated to nicotinic acid before the pyridine ring is salvaged to make more NAD+, thus supplementation with nicotinamide may not rescue qns1 mutants by shunting nicotinamide-containing precursors through the pathway; and QNS1 is common to the three pathways, thus there may be no NAD+ precursor that rescues qns1 mutants. However, it has now been found that while nicotinamide does not rescue qns1 mutants even at 1 or 10 mM, nicotinamide riboside functions as a vitamin form of NAD+ at 10 μM.

Anticancer agents such as tiazofurin (Cooney, et al. (1983) *Adv. Enzyme Regul.* 21:271-303) and benzamide riboside (Krohn, et al. (1992) *J. Med. Chem.* 35:511-517) have been shown to be metabolized intracellularly to NAD+ analogs, taizofurin adenine dinucleotide and benzamide adenine dinucleotide, which inhibit IMP dehydrogenase the rate-limiting enzyme for de novo purine nucleotide biosynthesis.

Though an NMN/NaMN adenylyltransferase is thought to be the enzyme that converts the mononucleotide intermediates to NAD+ analogs and the structural basis for this is known (Zhou et al. (2002) supra), several different enzymes including adenosine kinase, 5' nucleotidase (Fridland, et al. (1986) *Cancer Res.* 46:532-537; Saunders, et al. (1990) *Cancer Res.* 50:5269-5274) and a specific nicotinamide riboside kinase (Saunders, et al. (1990) supra) have been proposed to be responsible for tiazofurin phosphorylation in vivo. A putative nicotinamide riboside kinase (Nrk) activity was purified, however no amino acid sequence information was obtained and, as a consequence, no genetic test was performed to assess its function (Sasiak and Saunders (1996) *Arch. Biochem. Biophys.* 333:414-418).

Using a qns1 deletion strain that was additionally deleted for yeast homologs of candidate genes encoding nucleoside kinases proposed to phosphorylate tiazofurin, i.e., adenosine kinase ado1 (Lecoq, et al. (2001) *Yeast* 18:335-342), uridine/cytidine kinase urk1 (Kern (1990) *Nucleic Acids Res.* 18:5279; Kurtz, et al. (1999) *Curr. Genet.* 36:130-136), and ribokinase rbk1 (Thierry, et al. (1990) *Yeast* 6:521-534), it was determined whether the nucleoside kinases are uniquely or collectively responsible for utilization of nicotinamide riboside. It was found that despite these deletions, the strain retained the ability to utilize nicotinamide riboside in an anabolic pathway independent of NAD+ synthetase.

Given that mammalian pharmacology provided no useful clue to the identity of a putative fungal Nrk, it was considered whether the gene might have been conserved with the Nrk of *Haemophilus influenza*. The Nrk domain of *H. influenza* is encoded by amino acids 225 to 421 of the NadR gene product (the amino terminus of which is NMN adenylyltransferase). Though this domain is structurally similar to yeast thymidylate kinase (Singh, et al. (2002) *J. Biol. Chem.* 277:33291-33299), sensitive sequence searches revealed that bacterial Nrk has no ortholog in yeast. Genomic searches with the Nrk domain of *H. influenza* NadR have identified a growing list of bacterial genomes predicted to utilize nicotinamide riboside as an NAD+ precursor (Kurnasov, et al. (2002) *J. Bacteriol.* 184:6906-6917). Thus, had fungi possessed NadR Nrk-homologous domains, comparative genomics would have already predicted that yeast can salvage nicotinamide riboside.

To identify the Nrk of *S. cerevisiae*, an HPLC assay for the enzymatic activity was established and used in combination with a biochemical genomics approach to screen for the gene encoding this activity (Martzen, et al. (1999) *Science* 286: 1153-1155). Sixty-four pools of 90-96 *S. cerevisiae* open reading frames fused to glutathione S-transferase (GST), expressed in *S. cerevisiae*, were purified as GST fusions and screened for the ability to convert nicotinamide riboside plus ATP to NMN plus ADP. Whereas most pools contained activities that consumed some of the input ATP, only pool 37 consumed nicotinamide riboside and produced NMN. In pool 37, approximately half of the 1 mM ATP was converted to ADP and the 500 μM nicotinamide riboside peak was almost entirely converted to NMN. Examination of the 94 open reading frames that were used to generate pool 37 revealed that YNL129W (SEQ ID NO:1) encodes a predicted 240 amino acid polypeptide with a 187 amino acid segment containing 23% identity with the 501 amino acid yeast uridine/cytidine kinase Urk1 and remote similarity with a segment of *E. coli* pantothenate kinase panK (Yun, et al. (2000) *J. Biol. Chem.* 275:28093-28099) (FIG. **1**). After cloning YNL129W into a bacterial expression vector it was ascertained whether this homolog of metabolite kinases was the eukaryotic Nrk. The specific activity of purified YNL129W was ~100-times that of pool 37, consistent with the idea that all the Nrk activity of pool 37 was encoded by this open reading frame. To test genetically whether this gene product phosphorylates nicotinamide riboside in vivo, a deletion of YNL129W was created in the qns1 background. It was found that nicotinamide riboside rescue of the qns1 deletion strain was entirely dependent on this gene product. Having shown biochemically and genetically that YNL129W encodes an authentic Nrk activity, the gene was designated NRK1.

A PSI-BLAST (Altschul, et al. (1997) *Nucleic Acids Res.* 25:3389-3402) comparison was conducted on the predicted *S. cerevisiae* Nrk1 polypeptide and an orthologous human protein Nrk1 (NP_060351; SEQ ID NO:5; FIG. **1**) was found. The human NP_060351 protein encoded at locus 9q21.31 is a polypeptide of 199 amino acids and is annotated as an uncharacterized protein of the uridine kinase family. In addition, a second human gene product Nrk2 (NP_733778; SEQ ID NO:6; FIG. **1**) was found that is 57% identical to human Nrk1. Nrk2 is a 230 amino acid splice form of what was described as a 186 amino acid muscle integrin beta 1 binding protein (ITGB1BP3) encoded at 19p13.3 (Li, et al. (1999) *J. Cell Biol.* 147:1391-1398; Li, et al. (2003) *Dev. Biol.* 261:209-219). Amino acid conservation between *S. cerevisiae, S. pombe* and human Nrk homologs and similarity with fragments of *S. cerevisiae* Urk1 and *E. coli* panK is shown in FIG. **1**. Fungal and human Nrk enzymes are members of a metabolite kinase superfamily that includes pantothenate kinase but is unrelated to bacterial nicotinamide riboside kinase. Robust complementation of the failure of qns1 nrk1 to grow on nicotinamide riboside-supplemented media was provided by human NRK1 and human NRK2 cDNA even when expressed from the GAL1 promoter on glucose.

As shown in Table 1, purification of yeast Nrk1 and human Nrk1 and Nrk2 revealed high specificity for phosphorylation of nicotinamide riboside and tiazofurin.

TABLE 1

	Nicotinamide riboside	Tiazofurin	Uridine	Cytidine
Human Nrk1	275 ± 17	538 ± 27	19.3 ± 1.7	35.5 ± 6.4
Human Nrk2	2320 ± 20	2150 ± 210	2220 ± 170	222 ± 8
Yeast Nrk1	535 ± 60	1129 ± 134	15.2 ± 3.4	82.9 ± 4.4

Specific activity is expressed in nmole mg^{-1} min^{-1} for phosphorylation of nucleoside substrates.

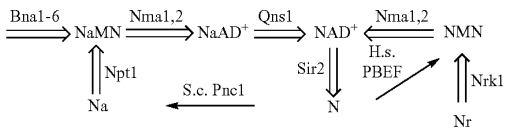
In the cases of yeast and human Nrk1 enzymes, the enzymes preferred tiazofurin to the natural substrate nicotinamide riboside by a factor of two and both enzymes retained less than 7% of their maximal specific activity on uridine and cytidine. In the case of human Nrk2, the 230 amino acid form was essentially equally active on nicotinamide riboside, tiazofurin and uridine with less than 10% of corresponding activity on cytidine. Conversely, the 186 amino acid integrin beta 1 binding protein form was devoid of enzymatic activity in this in vitro assay and was not functional as an Nrk in vivo. However, both the 186 and 230 amino acid isoforms function in vivo in a yeast nicotinamide riboside utilization assay. Thus, though Nrk2 may contribute additionally to formation of uridylate, these data demonstrate that fungi and mammals possess specific nicotinamide riboside kinases that function to synthesize NAD+ through NMN in addition to the well-known pathways through NaMN. Identification of Nrk enzymatic activities thus accounts for the dual specificity of fungal and mammalian NaMN/NMN adenylyltransferases.

On the basis of SAGE data, NRK1 is a rare message in many tissues examined while NRK2 is highly expressed in heart and skeletal muscle and has lower level expression in retinal epithelium and placenta (Boon, et al. (2002) *Proc. Natl. Acad. Sci. USA* 99:11287-11292). From cancer cell line to cancer cell line the expression levels are quite variable (Boon, et al. (2002) supra). Thus, in individuals whose tumors are NRK1, NRK2-low, tiazofurin conversion to NAD+ may occur more extensively in the patients hearts and muscles than in tumors. In tumors that are NRK1 and/or NRK2-high, a substantial amount of tiazofurin may be converted to tiazofurin adenine dinucleotide in tumors.

A yeast qns1 mutant was used to screen for natural sources of nicotinamide riboside wherein it was identified in an acid whey preparation of cow's milk. Unlike the original screen for vitamins in protein-depleted extracts of liver for reversal of black-tongue in starving dogs (Elvehjem, et al. (1938) *J. Biol. Chem.* 123:137-149), this assay is pathway-specific in identifying NAD+ precursors. Because of the qns1 deletion, nicotinic acid and nicotinamide do not score positively in this assay. As the factor from milk requires nicotinamide riboside kinase for growth, the nutrient is clearly nicotinamide riboside and not NMN or NAD+.

A revised metabolic scheme for NAD+, incorporating Nrk1 homologs and the nicotinamide riboside salvage pathway is shown in Scheme 2 wherein double arrows depict metabolic steps common to yeast and humans (with yeast gene names) and single arrows depict steps unique to humans (PBEF, nicotinamide phosphoribosyltransferase) and yeast (Pnc1, nicotinamidase).

Scheme 2



A difference between humans and yeasts concerns the organisms' uses of nicotinamide and nicotinic acid, the two niacins that were co-identified as anti-black tongue factor (Elvehjem, et al. (1938) supra). Humans encode a homolog of the *Haemophilus ducreyi* nadV gene, termed pre-B-cell colony enhancing factor, that may convert nicotinamide to NMN (Rongvaux, et al. (2002) *Eur. J. Immunol.* 32:3225-3234) and is highly induced during lymphocyte activation (Samal, et al. (1994) *Mol. Cell. Biol.* 14:1431-1437). In contrast, *S. cerevisiae* lacks a homolog of nadV and instead has a homolog of the *E. coli* pncA gene, termed PNC1, that converts nicotinamide to nicotinic acid for entry into the Preiss-Handler pathway (Ghislain, et al. (2002) *Yeast* 19:215-224; Sandmeier, et al. (2002) supra). Though the Preiss-Handler pathway is frequently considered a salvage pathway from nicotinamide, it technically refers to the steps from nicotinic acid to NAD+ (Preiss and Handler (1958) supra; Preiss and Handler (1958) supra). Reports that nicotinamidase had been purified from mammalian liver in the 1960s (Petrack, et al. (1965) *J. Biol. Chem.* 240:1725-1730) may have contributed to the sense that fungal and animal NAD+ biosynthesis is entirely conserved. However, animal genes for nicotinamidase have not been identified and there is no compelling evidence that nicotinamide and nicotinic acid are utilized as NAD+ precursors through the same route in mammals. The persistence of "niacin" as a mixture of nicotinamide and nicotinic acid may attest to the utility of utilizing multiple pathways to generate NAD+ and indicates that supplementation with nicotinamide riboside as third importable NAD+ precursor can be beneficial for certain conditions.

First reported in 1955, high doses of nicotinic acid are effective at reducing cholesterol levels (Altschul, et al. (1955) *Arch. Biochem. Biophys.* 54:558-559). Since the initial report, many controlled clinical studies have shown that nicotinic acid preparations, alone and in combination with HMG CoA reductase inhibitors, are effective in controlling low-density lipoprotein cholesterol, increasing high-density lipoprotein cholesterol, and reducing triglyceride and lipoprotein a levels in humans (Pasternak, et al. (1996) *Ann. Intern. Med.* 125:529-540). Though nicotinic acid treatment effects all of the key lipids in the desirable direction and has been shown to reduce mortality in target populations (Pasternak, et al. (1996) supra), its use is limited because of a side effect of heat and redness termed "flushing," which is significantly effected by the nature of formulation (Capuzzi, et al. (2000) *Curr. Atheroscler. Rep.* 2:64-71). Thus, nicotinamide riboside supplementation could be one route to improve lipid profiles in humans. Further, nicotinamide is protective in animal models of stroke (Klaidman, et al. (2003) *Pharmacology* 69:150-157) and nicotinamide riboside could be an important supplement for acute conditions such as stroke. Additionally, regulation of NAD+ biosynthetic enzymes could be useful in sensitizing tumors to compounds such as tiazofurin, to protect normal tissues from the toxicity of compounds such as tiazofurin adenine dinucleotide, and to stratify patients for the most judicious use of tiazofurin chemotherapy.

The present invention is an isolated nucleic acid containing a eukaryotic nucleotide sequence encoding a nicotinamide riboside kinase polypeptide. As used herein, an isolated molecule (e.g., an isolated nucleic acid such as genomic DNA, RNA or cDNA or an isolated polypeptide) means a molecule separated or substantially free from at least some of the other components of the naturally occurring organism, such as for example, the cell structural components or other polypeptides or nucleic acids commonly found associated with the molecule. When the isolated molecule is a polypeptide, said polypeptide is at least about 25%, 50%, 60%, 70%, 75%, 80%, 85%, 90%, 95%, 97%, 98%, 99% or more pure (w/w).

In one embodiment, the eukaryotic nucleotide sequence encoding a nicotinamide riboside kinase polypeptide is a nucleotide sequence of SEQ ID NO:1, SEQ ID NO:2 or SEQ ID NO:3. In another embodiment, the eukaryotic nucleotide sequence encoding a nicotinamide riboside kinase polypeptide is a nucleotide sequence that hybridizes to a nucleotide sequence of SEQ ID NO:1, SEQ ID NO:2 or SEQ ID NO:3 or its complementary nucleotide sequence under stringent conditions, wherein said nucleotide sequence encodes a functional nicotinamide riboside kinase polypeptide. In a further embodiment, the eukaryotic nucleotide sequence encoding a nicotinamide riboside kinase polypeptide is a nucleotide sequence encoding a functional nicotinamide riboside kinase polypeptide but which has a different nucleotide sequence than the nucleotide sequences of SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3 due to the degeneracy of the genetic code or the presence of non-translated nucleotide sequences.

As used herein, a functional polypeptide is one that retains at least one biological activity normally associated with that polypeptide. Alternatively, a functional polypeptide retains all of the activities possessed by the unmodified peptide. By retains biological activity, it is meant that the polypeptide retains at least about 50%, 60%, 75%, 85%, 90%, 95%, 97%, 98%, 99%, or more, of the biological activity of the native polypeptide (and can even have a higher level of activity than the native polypeptide). A non-functional polypeptide is one that exhibits essentially no detectable biological activity normally associated with the polypeptide (e.g., at most, only an insignificant amount, e.g., less than about 10% or even 5%).

As used herein, the term polypeptide encompasses both peptides and proteins, unless indicated otherwise.

A nicotinamide riboside kinase polypeptide or Nrk protein as used herein, is intended to be construed broadly and encompasses an enzyme capable of phosphorylating nicotinamide riboside. The term nicotinamide riboside kinase or Nrk also includes modified (e.g., mutated) Nrk that retains biological function (i.e., have at least one biological activity of the native Nrk protein, e.g., phosphorylating nicotinamide riboside), functional Nrk fragments including truncated molecules, alternatively spliced isoforms (e.g., the alternatively spliced isoforms of human Nrk2), and functional Nrk fusion polypeptides (e.g., an Nrk-GST protein fusion or Nrk-His tagged protein).

Any Nrk polypeptide or Nrk-encoding nucleic acid known in the art can be used according to the present invention. The Nrk polypeptide or Nrk-encoding nucleic acid can be derived from yeast, fungal (e.g., *Saccharomyces cerevisiae, Saccharomyces pombe, Pichia* sp., *Neurospora* sp., and the like) plant, animal (e.g., insect, avian (e.g., chicken), or mammalian (e.g., rat, mouse, bovine, porcine, ovine, caprine, equine, feline, canine, lagomorph, simian, human and the like) sources.

Representative cDNA and amino acid sequences of a *S. cerevisiae* Nrk1 are shown in SEQ ID NO:1 and SEQ ID NO:4 (FIG. **1**), respectively. Representative cDNA and amino acid sequences of a human Nrk1 are shown in SEQ ID NO:2 and SEQ ID NO:5 (FIG. **1**), respectively. Representative cDNA and amino acid sequences of a human Nrk2 are shown in SEQ ID NO:3 and SEQ ID NO:6 (FIG. **1**), respectively. Other Nrk sequences encompassed by the present invention include, but are not limited to, Nrk1 of GENBANK accession numbers NM_017881, AK000566, BC001366, BC036804, and BC026243 and Nrk2 of GENBANK accession number NM_170678. Moreover, locus CAG61927 from the *Candida glabrata* CBS138 genome project (Dujon, et al. (2004) *Nature* 430:35-44) is 54% identical to the *Saccharomyces cerevisiae* Nrk1 protein. Particular embodiments of the present invention embrace a Nrk polypeptide having the conserved amino acid sequence XXXXDDFXK (SEQ ID NO:34), wherein Xaa_1 and Xaa_2 are aliphatic amino acid residues, Xaa_3 is His or Ser, Xaa_4 is a hydrophilic amino acid residue, and Xaa_5 is an aromatic amino acid residue.

To illustrate, hybridization of such sequences can be carried out under conditions of reduced stringency, medium stringency or even stringent conditions (e.g., conditions represented by a wash stringency of 35-40% Formamide with 5×Denhardt's solution, 0.5% SDS and 1×SSPE at 37° C.; conditions represented by a wash stringency of 40-45% Formamide with 5×Denhardt's solution, 0.5% SDS, and 1×SSPE at 42° C.; and/or conditions represented by a wash stringency of 50% Formamide with 5×Denhardt's solution, 0.5% SDS and 1×SSPE at 42° C., respectively) to the sequences specifically disclosed herein. See, e.g., Sambrook et al., *Molecular Cloning, A Laboratory Manual* (2d Ed. 1989) (Cold Spring Harbor Laboratory).

Alternatively stated, isolated nucleic acids encoding Nrk of the invention have at least about 50%, 60%, 70%, 80%, 90%, 95%, 97%, 98% or higher sequence similarity with the isolated nucleic acid sequences specifically disclosed herein (or fragments thereof, as defined above) and encode a functional Nrk as defined herein.

It will be appreciated by those skilled in the art that there can be variability in the nucleic acids that encode the Nrk of the present invention due to the degeneracy of the genetic code. The degeneracy of the genetic code, which allows different nucleic acid sequences to code for the same polypeptide, is well known in the literature (see Table 2).

TABLE 2

Amino Acid	3-Letter Code	1-Letter Code	Codons
Alanine	Ala	A	GCA GCC GCG GCT
Cysteine	Cys	C	TGC TGT
Aspartic acid	Asp	D	GAC GAT
Glutamic acid	Glu	E	GAA GAG
Phenylalanine	Phe	F	TTC TTT
Glycine	Gly	G	GGA GGC GGG GGT
Histidine	His	H	CAC CAT
Isoleucine	Ile	I	ATA ATC ATT
Lysine	Lys	K	AAA AAG
Leucine	Leu	L	TTA TTG CTA CTC CTG CTT
Methionine	Met	M	ATG
Asparagine	Asn	N	AAC AAT
Proline	Pro	P	CCA CCC CCG CCT
Glutamine	Gln	Q	CAA CAG
Arginine	Arg	R	AGA AGG CGA CGC CGG CGT
Serine	Ser	S	AGC ACT TCA TCC TCG TCT
Threonine	Thr	T	ACA ACC ACG ACT
Valine	Val	V	GTA GTC GTG GTT
Tryptophan	Trp	W	TGG
Tyrosine	Tyr	Y	TAC TAT

Further variation in the nucleic acid sequence can be introduced by the presence (or absence) of non-translated sequences, such as intronic sequences and 5' and 3' untranslated sequences.

Moreover, the isolated nucleic acids of the invention encompass those nucleic acids encoding Nrk polypeptides that have at least about 60%, 70%, 80%, 90%, 95%, 97%, 98% or higher amino acid sequence similarity with the polypeptide sequences specifically disclosed herein (or fragments thereof) and further encode a functional Nrk as defined herein.

As is known in the art, a number of different programs can be used to identify whether a nucleic acid or polypeptide has sequence identity or similarity to a known sequence. Sequence identity and/or similarity can be determined using standard techniques known in the art, including, but not limited to, the local sequence identity algorithm of Smith & Waterman (1981) *Adv. Appl. Math.* 2:482, by the sequence identity alignment algorithm of Needleman & Wunsch (1970) *J. Mol. Biol.* 48:443, by the search for similarity method of Pearson & Lipman (1988) *Proc. Natl. Acad. Sci. USA* 85:2444, by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Drive, Madison, Wis.), the Best Fit sequence program described by Devereux, et al. (1984) *Nucl. Acid Res.* 12:387-395, either using the default settings, or by inspection.

An example of a useful algorithm is PILEUP. PILEUP creates a multiple sequence alignment from a group of related sequences using progressive, pairwise alignments. It can also plot a tree showing the clustering relationships used to create the alignment. PILEUP uses a simplification of the progressive alignment method of Feng & Doolittle (1987) *J. Mol. Evol.* 35:351-360; the method is similar to that described by Higgins & Sharp (1989) *CABIOS* 5:151-153.

Another example of a useful algorithm is the BLAST algorithm, described in Altschul, et al. (1990) *J. Mol. Biol.* 215: 403-410 and Karlin, et al. (1993) *Proc. Natl. Acad. Sci. USA* 90:5873-5787. A particularly useful BLAST program is the WU-BLAST-2 program which was obtained from Altschul, et al. (1996) *Methods in Enzymology,* 266:460-480; http://blast.wustl/edu/blast/README.html. WU-BLAST-2 uses several search parameters, which can be set to the default values. The parameters are dynamic values and are established by the program itself depending upon the composition of the particular sequence and composition of the particular database against which the sequence of interest is being searched; however, the values can be adjusted to increase sensitivity.

An additional useful algorithm is gapped BLAST as reported by Altschul, et al. (1997) *Nucleic Acids Res.* 25:3389-3402.

A percentage amino acid sequence identity value can be determined by the number of matching identical residues divided by the total number of residues of the longer sequence in the aligned region. The longer sequence is the one having the most actual residues in the aligned region (gaps introduced by WU-Blast-2 to maximize the alignment score are ignored).

The alignment can include the introduction of gaps in the sequences to be aligned. In addition, for sequences which contain either more or fewer amino acids than the polypeptides specifically disclosed herein, it is understood that in one embodiment, the percentage of sequence identity will be determined based on the number of identical amino acids in relation to the total number of amino acids. Thus, for example, sequence identity of sequences shorter than a sequence specifically disclosed herein, will be determined using the number of amino acids in the shorter sequence, in one embodiment. In percent identity calculations relative weight is not assigned to various manifestations of sequence variation, such as, insertions, deletions, substitutions, etc.

In one embodiment, only identities are scored positively (+1) and all forms of sequence variation including gaps are assigned a value of "0", which obviates the need for a weighted scale or parameters as described below for sequence similarity calculations. Percent sequence identity can be calculated, for example, by dividing the number of matching identical residues by the total number of residues of the shorter sequence in the aligned region and multiplying by 100. The longer sequence is the one having the most actual residues in the aligned region.

To modify Nrk amino acid sequences specifically disclosed herein or otherwise known in the art, amino acid substitutions can be based on any characteristic known in the art, including the relative similarity or differences of the amino acid side-chain substituents, for example, their hydrophobicity, hydrophilicity, charge, size, and the like. In particular embodiments, conservative substitutions (i.e., substitution with an amino acid residue having similar properties) are made in the amino acid sequence encoding Nrk.

In making amino acid substitutions, the hydropathic index of amino acids may be considered. The importance of the hydropathic amino acid index in conferring interactive biologic function on a protein is generally understood in the art (see, Kyte and Doolittle (1982) *J. Mol. Biol.* 157:105). It is accepted that the relative hydropathic character of the amino acid contributes to the secondary structure of the resultant protein, which in turn defines the interaction of the protein with other molecules, for example, enzymes, substrates, receptors, DNA, antibodies, antigens, and the like.

Each amino acid has been assigned a hydropathic index on the basis of its hydrophobicity and charge characteristics (Kyte and Doolittle (1982) supra), and these are: isoleucine (+4.5); valine (+4.2); leucine (+3.8); phenylalanine (+2.8); cysteine/cystine (+2.5); methionine (+1.9); alanine (+1.8); glycine (−0.4); threonine (−0.7); serine (−0.8); tryptophan (−0.9); tyrosine (−1.3); proline (−1.6); histidine (−3.2); glutamate (−3.5); glutamine (−3.5); aspartate (−3.5); asparagine (−3.5); lysine (−3.9); and arginine (−4.5).

It is also understood in the art that the substitution of amino acids can be made on the basis of hydrophilicity. U.S. Pat. No. 4,554,101 states that the greatest local average hydrophilicity of a protein, as governed by the hydrophilicity of its adjacent amino acids, correlates with a biological property of the protein.

As detailed in U.S. Pat. No. 4,554,101, the following hydrophilicity values have been assigned to amino acid residues: arginine (+3.0); lysine (±3.0); aspartate (+3.0±1); glutamate (+3.0±1); serine (+0.3); asparagine (+0.2); glutamine (+0.2); glycine (0); threonine (−0.4); proline (−0.5±1); alanine (−0.5); histidine (−0.5); cysteine (−1.0); methionine (−1.3); valine (−1.5); leucine (−1.8); isoleucine (−1.8); tyrosine (−2.3); phenylalanine (−2.5); tryptophan (−3.4).

Isolated nucleic acids of this invention include RNA, DNA (including cDNAs) and chimeras thereof. The isolated nucleic acids can further contain modified nucleotides or nucleotide analogs.

The isolated nucleic acids encoding Nrk can be associated with appropriate expression control sequences, e.g., transcription/translation control signals and polyadenylation signals.

It will be appreciated that a variety of promoter/enhancer elements can be used depending on the level and tissue-specific expression desired. The promoter can be constitutive or inducible (e.g., the metallothionein promoter or a hormone inducible promoter), depending on the pattern of expression desired. The promoter can be native or foreign and can be a natural or a synthetic sequence. By foreign, it is intended that the transcriptional initiation region is not found in the wild-type host into which the transcriptional initiation region is introduced. The promoter is chosen so that it will function in the target cell(s) of interest. In particular embodiments, the promoter functions in tumor cells or in cells that can be used to express nucleic acids encoding Nrk for the purposes of large-scale protein production. Likewise, the promoter can be specific for these cells and tissues (i.e., only show significant activity in the specific cell or tissue type).

To illustrate, an Nrk coding sequence can be operatively associated with a cytomegalovirus (CMV) major immediate-early promoter, an albumin promoter, an Elongation Factor 1-α (EF1-α) promoter, a PγK promoter, a MFG promoter, a Rous sarcoma virus promoter, or a glyceraldehyde-3-phosphate promoter.

Moreover, specific initiation signals are generally required for efficient translation of inserted protein coding sequences. These translational control sequences, which can include the ATG initiation codon and adjacent sequences, can be of a variety of origins, both natural and synthetic.

Nrk can be expressed not only directly, but also as a fusion protein with a heterologous polypeptide, i.e. a signal sequence for secretion and/or other polypeptide which will aid in the purification of Nrk. In one embodiment, the heterologous polypeptide has a specific cleavage site to remove the heterologous polypeptide from Nrk.

In general, a signal sequence can be a component of the vector and should be one that is recognized and processed (i.e., cleaved by a signal peptidase) by the host cell. For production in a prokaryote, a prokaryotic signal sequence from, for example, alkaline phosphatase, penicillinase, lpp, or heat-stable enterotoxin II leaders can be used. For yeast secretion, one can use, e.g., the yeast invertase, alpha factor, or acid phosphatase leaders, the *Candida albicans* glucoamylase leader (EP 362,179), or the like (see, for example WO 90/13646). In mammalian cell expression, signal sequences from secreted polypeptides of the same or related species, as well as viral secretory leaders, for example, the herpes simplex glycoprotein D signal can be used.

Other useful heterologous polypeptides which can be fused to Nrk include those which increase expression or solubility of the fusion protein or aid in the purification of the fusion protein by acting as a ligand in affinity purification. Typical fusion expression vectors include those exemplified herein as well as pMAL (New England Biolabs, Beverly, Mass.) and pRIT5 (Pharmacia, Piscataway, N.J.) which fuse maltose E binding protein or protein A, respectively, to the target recombinant protein.

The isolated nucleic acids encoding Nrk can be incorporated into a vector, e.g., for the purposes of cloning or other laboratory manipulations, recombinant protein production, or gene delivery. In particular embodiments, the vector is an expression vector. Exemplary vectors include bacterial artificial chromosomes, cosmids, yeast artificial chromosomes, phage, plasmids, lipid vectors and viral vectors. By the term express, expresses or expression of a nucleic acid coding sequence, in particular an Nrk coding sequence, it is meant that the sequence is transcribed, and optionally, translated. Typically, according to the present invention, transcription and translation of the coding sequence will result in production of Nrk polypeptide.

The methods of the present invention provide a means for delivering, and optionally expressing, nucleic acids encoding Nrk in a broad range of host cells, including both dividing and non-dividing cells in vitro (e.g., for large-scale recombinant protein production or for use in screening assays) or in vivo (e.g., for recombinant large-scale protein production, for creating an animal model for disease, or for therapeutic purposes). In embodiments of the invention, the nucleic acid can be expressed transiently in the target cell or the nucleic acid can be stably incorporated into the target cell, for example, by integration into the genome of the cell or by persistent expression from stably maintained episomes (e.g., derived from Epstein Barr Virus).

The isolated nucleic acids, vectors, methods and pharmaceutical formulations of the present invention find use in a method of administering a nucleic acid encoding Nrk to a subject. In this manner, Nrk can thus be produced in vivo in the subject. The subject can have a deficiency of Nrk, or the production of a foreign Nrk in the subject can impart some therapeutic effect. Pharmaceutical formulations and methods of delivering nucleic acids encoding Nrk for therapeutic purposes are described herein.

Alternatively, an isolated nucleic acid encoding Nrk can be administered to a subject so that the nucleic acid is expressed by the subject and Nrk is produced and purified therefrom, i.e., as a source of recombinant Nrk protein. According to this embodiment, the Nrk is secreted into the systemic circulation or into another body fluid (e.g., milk, lymph, spinal fluid, urine) that is easily collected and from which the Nrk can be further purified. As a further alternative, Nrk protein can be produced in avian species and deposited in, and conveniently isolated from, egg proteins.

Likewise, Nrk-encoding nucleic acids can be expressed transiently or stably in a cell culture system for the purpose of screening assays or for large-scale recombinant protein production. The cell can be a bacterial, protozoan, plant, yeast, fungus, or animal cell. In one embodiment, the cell is an animal cell (e.g., insect, avian or mammalian), and in another embodiment a mammalian cell (e.g., a fibroblast).

It will be apparent to those skilled in the art that any suitable vector can be used to deliver the isolated nucleic acids of this invention to the target cell(s) or subject of interest. The choice of delivery vector can be made based on a number of factors known in the art, including age and species of the target host, in vitro vs. in vivo delivery, level and persistence of expression desired, intended purpose (e.g., for therapy or drug screening), the target cell or organ, route of delivery, size of the isolated nucleic acid, safety concerns, and the like.

Suitable vectors include virus vectors (e.g., retrovirus, alphavirus; vaccinia virus; adenovirus, adeno-associated virus, or herpes simplex virus), lipid vectors, poly-lysine vectors, synthetic polyamino polymer vectors that are used with nucleic acid molecules, such as plasmids, and the like.

As used herein, the term viral vector or viral delivery vector can refer to a virus particle that functions as a nucleic acid delivery vehicle, and which contains the vector genome packaged within a virion. Alternatively, these terms can be used to refer to the vector genome when used as a nucleic acid delivery vehicle in the absence of the virion.

Protocols for producing recombinant viral vectors and for using viral vectors for nucleic acid delivery can be found in *Current Protocols in Molecular Biology*, Ausubel, F. M. et al. (eds.) Greene Publishing Associates, (1989) and other stan-

dard laboratory manuals (e.g., Vectors for Gene Therapy. In: *Current Protocols in Human Genetics*. John Wiley and Sons, Inc.: 1997).

Particular examples of viral vectors are those previously employed for the delivery of nucleic acids including, for example, retrovirus, adenovirus, AAV, herpes virus, and pox-virus vectors.

In certain embodiments of the present invention, the delivery vector is an adenovirus vector. The term adenovirus as used herein is intended to encompass all adenoviruses, including the Mastadenovirus and Aviadenovirus genera. To date, at least forty-seven human serotypes of adenoviruses have been identified (see, e.g., Fields, et al., Virology, volume 2, chapter 67 (3d ed., Lippincott-Raven Publishers). In one embodiment, the adenovirus is a human serogroup C adenovirus, in another embodiment the adenovirus is serotype 2 (Ad2) or serotype 5 (Ad5) or simian adenovirus such as AdC68.

Those skilled in the art will appreciate that vectors can be modified or targeted as described in Douglas, et al. (1996) *Nature Biotechnology* 14:1574 and U.S. Pat. Nos. 5,922,315; 5,770,442 and/or 5,712,136.

An adenovirus genome can be manipulated such that it encodes and expresses a nucleic acid of interest but is inactivated in terms of its ability to replicate in a normal lytic viral life cycle. See, for example, Berkner, et al. (1988) *BioTechniques* 6:616; Rosenfeld, et al. (1991) *Science* 252:431-434; and Rosenfeld et al. (1992) *Cell* 68:143-155.

Recombinant adenoviruses can be advantageous in certain circumstances in that they are not capable of infecting non-dividing cells and can be used to infect a wide variety of cell types, including epithelial cells. Furthermore, the virus particle is relatively stable and amenable to purification and concentration, and can be modified so as to affect the spectrum of infectivity. Additionally, introduced adenoviral DNA (and foreign DNA contained therein) is not integrated into the genome of a host cell but remains episomal, thereby avoiding potential problems that can occur as a result of insertional mutagenesis in situations where introduced DNA becomes integrated into the host genome (e.g., as occurs with retroviral DNA). Moreover, the carrying capacity of the adenoviral genome for foreign DNA is large relative to other delivery vectors (Haj-Ahmand and Graham (1986) *J. Virol.* 57:267).

In particular embodiments, the adenovirus genome contains a deletion therein, so that at least one of the adenovirus genomic regions does not encode a functional protein. For example, an adenovirus vectors can have E1 genes and packaged using a cell that expresses the E1 proteins (e.g., 293 cells). The E3 region is also frequently deleted as well, as there is no need for complementation of this deletion. In addition, deletions in the E4, E2a, protein IX, and fiber protein regions have been described, e.g., by Armentano, et al. (1997) *J. Virology* 71:2408; Gao, et al. (1996) *J. Virology* 70:8934; Dedieu, et al. (1997) *J. Virology* 71:4626; Wang, et al. (1997) *Gene Therapy* 4:393; U.S. Pat. No. 5,882,877. In general, the deletions are selected to avoid toxicity to the packaging cell. Combinations of deletions that avoid toxicity or other deleterious effects on the host cell can be routinely selected by those skilled in the art.

Those skilled in the art will appreciate that typically, with the exception of the E3 genes, any deletions will need to be complemented in order to propagate (replicate and package) additional virus, e.g., by transcomplementation with a packaging cell.

The present invention can also be practiced with gutted adenovirus vectors (as that term is understood in the art, see e.g., Lieber, et al. (1996) *J. Virol.* 70:8944-60) in which essentially all of the adenovirus genomic sequences are deleted.

Adeno-associated viruses (AAV) have also been employed as nucleic acid delivery vectors. For a review, see Muzyczka et al. *Curr. Topics in Micro. and Immunol.* (1992) 158:97-129). AAV are among the few viruses that can integrate their DNA into non-dividing cells, and exhibit a high frequency of stable integration into human chromosome (see, for example, Flotte, et al. (1992) *Am. J. Respir. Cell. Mol. Biol.* 7:349-356; Samulski, et al., (1989) *J. Virol.* 63:3822-3828; McLaughlin, et al. (1989) *J. Virol.* 62:1963-1973). A variety of nucleic acids have been introduced into different cell types using AAV vectors (see, for example, Hermonat, et al. (1984) *Proc. Natl. Acad. Sci. USA* 81:6466-6470; Tratschin, et al. (1985) *Mol. Cell. Biol.* 4:2072-2081; Wondisford, et al. (1988) *Mol. Endocrinol.* 2:32-39; Tratschin, et al. (1984) *J. Virol.* 51:611-619; and Flotte, et al. (1993) *J. Biol. Chem.* 268:3781-3790).

Any suitable method known in the art can be used to produce AAV vectors expressing the nucleic acids encoding Nrk of this invention (see, e.g., U.S. Pat. Nos. 5,139,941; 5,858,775; 6,146,874 for illustrative methods). In one particular method, AAV stocks can be produced by co-transfection of a rep/cap vector encoding AAV packaging functions and the template encoding the AAV vDNA into human cells infected with the helper adenovirus (Samulski, et al. (1989) *J. Virology* 63:3822). The AAV rep and/or cap genes can alternatively be provided by a packaging cell that stably expresses the genes (see, e.g., Gao, et al. (1998) *Human Gene Therapy* 9:2353; Inoue, et al. (1998) *J. Virol.* 72:7024; U.S. Pat. No. 5,837,484; WO 98/27207; U.S. Pat. No. 5,658,785; WO 96/17947).

Another vector for use in the present invention is Herpes Simplex Virus (HSV). HSV can be modified for the delivery of nucleic acids to cells by producing a vector that exhibits only the latent function for long-term gene maintenance. HSV vectors are useful for nucleic acid delivery because they allow for a large DNA insert of up to or greater than 20 kilobases; they can be produced with extremely high titers; and they have been shown to express nucleic acids for a long period of time in the central nervous system as long as the lytic cycle does not occur.

In other particular embodiments of the present invention, the delivery vector of interest is a retrovirus. The development of specialized cell lines (termed packaging cells) which produce only replication-defective retroviruses has increased the utility of retroviruses for gene therapy, and defective retroviruses are characterized for use in gene transfer for gene therapy purposes (for a review, see Miller (1990) *Blood* 76:271). A replication-defective retrovirus can be packaged into virions which can be used to infect a target cell through the use of a helper virus by standard techniques.

In addition to viral transfer methods, such as those illustrated above, non-viral methods can also be employed. Many non-viral methods of nucleic acid transfer rely on normal mechanisms used by mammalian cells for the uptake and intracellular transport of macromolecules. In particular embodiments, non-viral nucleic acid delivery systems rely on endocytic pathways for the uptake of the nucleic acid molecule by the targeted cell. Exemplary nucleic acid delivery systems of this type include liposomal derived systems, poly-lysine conjugates, and artificial viral envelopes.

In particular embodiments, plasmid vectors are used in the practice of the present invention. Naked plasmids can be introduced into muscle cells by injection into the tissue. Expression can extend over many months, although the number of positive cells is typically low (Wolff, et al. (1989) *Science* 247:247). Cationic lipids have been demonstrated to

aid in introduction of nucleic acids into some cells in culture (Feigner and Ringold (1989) *Nature* 337:387). Injection of cationic lipid plasmid DNA complexes into the circulation of mice has been shown to result in expression of the DNA in lung (Brigham, et al. (1989) *Am. J. Med. Sci.* 298:278). One advantage of plasmid DNA is that it can be introduced into non-replicating cells.

In a representative embodiment, a nucleic acid molecule (e.g., a plasmid) can be entrapped in a lipid particle bearing positive charges on its surface and, optionally, tagged with antibodies against cell-surface antigens of the target tissue (Mizuno, et al. (1992) *No Shinkei Geka* 20:547; WO 91/06309; Japanese patent application 1047381; and European patent publication EP-A-43075).

Liposomes that consist of amphiphilic cationic molecules are useful non-viral vectors for nucleic acid delivery in vitro and in vivo (reviewed in Crystal (1995) *Science* 270:404-410; Blaese, et al. (1995) *Cancer Gene Ther.* 2:291-297; Behr, et al. (1994) *Bioconjugate Chem.* 5:382-389; Remy, et al. (1994) *Bioconjugate Chem.* 5:647-654; and Gao, et al. (1995) *Gene Therapy* 2:710-722). The positively charged liposomes are believed to complex with negatively charged nucleic acids via electrostatic interactions to form lipid:nucleic acid complexes. The lipid:nucleic acid complexes have several advantages as nucleic acid transfer vectors. Unlike viral vectors, the lipid:nucleic acid complexes can be used to transfer expression cassettes of essentially unlimited size. Since the complexes lack proteins, they can evoke fewer immunogenic and inflammatory responses. Moreover, they cannot replicate or recombine to form an infectious agent and have low integration frequency. A number of publications have demonstrated that amphiphilic cationic lipids can mediate nucleic acid delivery in vivo and in vitro (Felgner, et al. (1987) *Proc. Natl. Acad. Sci. USA* 84:7413-17; Loeffler, et al. (1993) *Methods in Enzymology* 217:599-618; Feigner, et al. (1994) *J. Biol. Chem.* 269:2550-2561).

As indicated above, Nrk polypeptide can be produced in, and optionally purified from, cultured cells or organisms expressing a nucleic acid encoding Nrk for a variety of purposes (e.g., screening assays, large-scale protein production, therapeutic methods based on delivery of purified Nrk).

In particular embodiments, an isolated nucleic acid encoding Nrk can be introduced into a cultured cell, e.g., a cell of a primary or immortalized cell line for recombinant protein production. The recombinant cells can be used to produce the Nrk polypeptide, which is collected from the cells or cell culture medium. Likewise, recombinant protein can be produced in, and optionally purified from an organism (e.g., a microorganism, animal or plant) being used essentially as a bioreactor.

Generally, the isolated nucleic acid is incorporated into an expression vector (viral or nonviral as described herein). Expression vectors compatible with various host cells are well-known in the art and contain suitable elements for transcription and translation of nucleic acids. Typically, an expression vector contains an expression cassette, which includes, in the 5' to 3' direction, a promoter, a coding sequence encoding an Nrk operatively associated with the promoter, and, optionally, a termination sequence including a stop signal for RNA polymerase and a polyadenylation signal for polyadenylase.

Expression vectors can be designed for expression of polypeptides in prokaryotic or eukaryotic cells. For example, polypeptides can be expressed in bacterial cells such as *E. coli*, insect cells (e.g., in the baculovirus expression system), yeast cells or mammalian cells. Some suitable host cells are discussed further in Goeddel (1990) Gene Expression Technology: Methods in Enzymology 185, Academic Press, San Diego, Calif. Examples of vectors for expression in yeast *S. cerevisiae* include pYepSec1 (Baldari, et al. (1987) *EMBO J.* 6:229-234), pMFa (Kurjan and Herskowitz (1982) *Cell* 30:933-943), pJRY88 (Schultz, et al. (1987) *Gene* 54:113-123), and pYES2 (INVITROGEN Corporation, San Diego, Calif.). Baculovirus vectors available for expression of nucleic acids to produce proteins in cultured insect cells (e.g., Sf 9 cells) include the pAc series (Smith, et al. (1983) *Mol. Cell. Biol.* 3:2156-2165) and the pVL series (Lucklow and Summers (1989) *Virology* 170:31-39).

Examples of mammalian expression vectors include pCDM8 (Seed (1987) *Nature* 329:840) and pMT2PC (Kaufman, et al. (1987) *EMBO J.* 6:187-195). When used in mammalian cells, the expression vector's control functions are often provided by viral regulatory elements. For example, commonly used promoters are derived from polyoma, adenovirus 2, cytomegalovirus and Simian Virus 40.

In addition to the regulatory control sequences discussed herein, the recombinant expression vector can contain additional nucleotide sequences. For example, the recombinant expression vector can encode a selectable marker gene to identify host cells that have incorporated the vector.

Vectors can be introduced into prokaryotic or eukaryotic cells via conventional transformation or transfection techniques. As used herein, the terms transformation and transfection refer to a variety of art-recognized techniques for introducing foreign nucleic acids (e.g., DNA) into a host cell, including calcium phosphate or calcium chloride co-precipitation, DEAE-dextran-mediated transfection, lipofection, electroporation, microinjection, DNA-loaded liposomes, lipofectamine-DNA complexes, cell sonication, gene bombardment using high velocity microprojectiles, and viral-mediated transfection. Suitable methods for transforming or transfecting host cells can be found in Sambrook, et al. (Molecular Cloning: A Laboratory Manual, 2nd Edition, Cold Spring Harbor Laboratory press (1989)), and other laboratory manuals.

Often only a small fraction of cells (in particular, mammalian cells) integrate the foreign DNA into their genome. In order to identify and select these integrants, a nucleic acid that encodes a selectable marker (e.g., resistance to antibiotics) can be introduced into the host cells along with the nucleic acid of interest. In particular embodiments, selectable markers include those that confer resistance to drugs, such as G418, hygromycin and methotrexate. Nucleic acids encoding a selectable marker can be introduced into a host cell on the same vector as that comprising the nucleic acid of interest or can be introduced on a separate vector. Cells stably transfected with the introduced nucleic acid can be identified by drug selection (e.g., cells that have incorporated the selectable marker gene will survive, while the other cells die).

Recombinant proteins can also be produced in a transgenic plant in which the isolated nucleic acid encoding the protein is inserted into the nuclear or plastidic genome. Plant transformation is known as the art. See, in general, *Methods in Enzymology* Vol. 153 (Recombinant DNA Part D) 1987, Wu and Grossman Eds., Academic Press and European Patent Application EP 693554.

The present invention further provides cultured or recombinant cells containing the isolated nucleic acids encoding Nrk for use in the screening methods and large-scale protein production methods of the invention (e.g., Nrk is produced and collected from the cells and, optionally, purified). In one particular embodiment, the invention provides a cultured cell containing an isolated nucleic acid encoding Nrk as described above for use in a screening assay for identifying a nicotina-

mide riboside-related prodrug. Also provided is a cell in vivo produced by a method comprising administering an isolated nucleic acid encoding Nrk to a subject in a therapeutically effective amount.

For in vitro screening assays and therapeutic administration, Nrk polypeptides can be purified from cultured cells. Typically, the polypeptide is recovered from the culture medium as a secreted polypeptide, although it also can be recovered from host cell lysates when directly expressed without a secretory signal. When Nrk is expressed in a recombinant cell other than one of human origin, the Nrk is completely free of proteins or polypeptides of human origin. However, it is necessary to purify Nrk from recombinant cell proteins or polypeptides to obtain preparations that are substantially homogeneous as to Nrk. As a first step, the culture medium or lysate is centrifuged to remove particulate cell debris. The membrane and soluble protein fractions are then separated. The Nrk can then be purified from the soluble protein fraction. Nrk thereafter can then be purified from contaminant soluble proteins and polypeptides with, for example, the following suitable purification procedures: by fractionation on immunoaffinity or ion-exchange columns; ethanol precipitation; reverse phase HPLC; chromatography on silica or on a cation-exchange resin such as DEAE; chromatofocusing; SDS-PAGE; ammonium sulfate precipitation; gel filtration using, for example, SEPHADEX G-75; ligand affinity chromatography, and protein A SEPHAROSE columns to remove contaminants such as IgG.

As Nrk phosphorylates tiazofurin, thereby performing the first step in activating it, Nrk is a useful target for identifying compounds which upon phosphorylation by Nrk and subsequent adenylylation inhibit IMPDH. As it has been shown that inhibitors of the IMPDH enzyme function as anti-bovine viral diarrhoea virus agents (Stuyver, et al. (2002) *Antivir. Chem. Chemother.* 13(6):345-52); inhibitors of IMPDH block hepatitis B replicon colony-forming efficiency (Zhou, et al. (2003) *Virology* 310(2):333-42); and tiazofurin (Cooney, et al. (1983) *Adv. Enzyme Regul.* 21:271-303) and benzamide riboside (Krohn, et al. (1992) *J. Med. Chem.* 35:511-517), when activated, inhibit IMP dehydrogenase; it is contemplated by using Nrk and the nicotinamide riboside pathway for drug screening, anticancer and antiviral agents will be identified. Accordingly, the present invention provides methods for identifying a nicotinamide riboside-related prodrug. As used herein, a nicotinamide riboside-related prodrug is any analog of nicotinamide riboside (e.g., tiazofurin and benzamide riboside) that, when phosphorylated by Nrk, ultimately can result in cell death or antiviral activity.

In one embodiment, a nicotinamide riboside-related prodrug is identified in a cell-free assay using isolated Nrk polypeptide. The steps involved in a this screening assay of the invention include, isolating or purifying an Nrk polypeptide; contacting or adding at least one nicotinamide riboside-related test agent to a point of application, such as a well, in the plate containing the isolated Nrk and a suitable phosphate donor such as ATP, Mg-ATP, Mn-ATP, Mg-GTP or Mn-GTP; and determining whether said test agent is phosphorylated by said Nrk polypeptide wherein phosphorylation of said test agent is indicative of a nicotinamide riboside-related prodrug. The phosphate donor can be added with or after the agent and the assay can be carried out under suitable assay conditions for phosphorylation, such as those exemplified herein.

With respect to the cell-free assay, test agents can be synthesized or otherwise affixed to a solid substrate, such as plastic pins, glass slides, plastic wells, and the like. Further, isolated Nrk can be free in solution, affixed to a solid support, or expressed on a cell surface.

Alternatively, an Nrk fusion protein can be provided to facilitate binding of Nrk to a matrix. For example, glutathione-S-transferase fusion proteins can be adsorbed onto glutathione SEPHAROSE beads (Sigma Chemical, St. Louis, Mo.) or glutathione derivatized microtitre plates, which are then combined with the test agent, and the mixture incubated under conditions conducive to complex formation (e.g., at physiological conditions for salt and pH) and phosphorylation as described above.

In another embodiment, a nicotinamide riboside-related prodrug is identified in a cell-based assay. The steps involved in a this screening assay of the invention include, contacting a first test cell which expresses a recombinant Nrk polypeptide with a nicotinamide riboside-related test agent; contacting a second test cell which lacks a functional Nrk polypeptide with the same test agent; and determining the viability of the first and second test cells wherein sensitivity or cell death of the first cell and not the second cell is indicative of a nicotinamide riboside-related prodrug. While the cell-based assay can be carried out using any suitable cell including bacteria, yeast, insect cells (e.g., with a baculovirus expression system), avian cells, mammalian cells, or plant cells, in particular embodiments, the test cell is a mammalian cell. In a further embodiment, said cell lacks a functional endogenous Nrk (e.g., the endogenous Nrk has been deleted or mutated or the cell does not express an Nrk). Said first test cell is transformed or transfected with an expression vector containing an exogenous Nrk so that upon exposure to a test agent, viability of the transformed cell can be compared to a second test cell lacking any Nrk activity. Thus, it can be ascertained whether the test agent is being activated in an Nrk-dependent manner. Cells modified to express a recombinant Nrk can be transiently or stably transformed with the nucleic acid encoding Nrk. Stably transformed cells can be generated by stable integration into the genome of the organism or by expression from a stably maintained episome (e.g., Epstein Barr Virus derived episomes).

Suitable methods for determining cell viability are well-established in the art. One such method uses non-permeant dyes (e.g., propidium iodide, 7-Amino Actinomycin D) that do not enter cells with intact cell membranes or active cell metabolism. Cells with damaged plasma membranes or with impaired/no cell metabolism are unable to prevent the dye from entering the cell. Once inside the cell, the dyes bind to intracellular structures producing highly fluorescent adducts which identify the cells as non-viable. Alternatively, cell viability can be determined by assaying for active cell metabolism which results in the conversion of a non-fluorescent substrate into a highly fluorescent product (e.g., fluorescein diacetate).

The test cells of the screening method of the invention can be cultured under standard conditions of temperature, incubation time, optical density, plating density and media composition corresponding to the nutritional and physiological requirements of the cells. However, conditions for maintenance and growth of the test cell can be different from those for assaying candidate agents in the screening methods of the invention. Any techniques known in the art can be applied to establish the optimal conditions.

Screening assays of the invention can be performed in any format that allows rapid preparation and processing of multiple reactions such as in, for example, multi-well plates of the 96-well variety. Stock solutions of the agents as well as assay components are prepared manually and all subsequent pipetting, diluting, mixing, washing, incubating, sample readout and data collecting is done using commercially available

robotic pipetting equipment, automated work stations, and analytical instruments for detecting the output of the assay.

In addition to the reagents provided above, a variety of other reagents can be included in the screening assays of the invention. These include reagents like salts, neutral proteins, e.g., albumin, detergents, etc. Also, reagents that otherwise improve the efficiency of the assay, such as protease inhibitors, nuclease inhibitors, anti-microbial agents, and the like can be used.

Screening assays can also be carried out in vivo in animals. Thus, the present invention provides a transgenic non-human animal containing an isolated nucleic acid encoding Nrk, which can be produced according to methods well-known in the art. The transgenic non-human animal can be any species, including avians and non-human mammals. IN accordance with the invention, suitable non-human mammals include mice, rats, rabbits, guinea pigs, goats, sheep, pigs and cattle. Mammalian models for cancer, bovine diarrhoea viral infection or hepatitis C viral infection can also be used.

A nucleic acid encoding Nrk is stably incorporated into cells within the transgenic animal (typically, by stable integration into the genome or by stably maintained episomal constructs). It is not necessary that every cell contain the transgene, and the animal can be a chimera of modified and unmodified cells, as long as a sufficient number of cells contain and express the Nrk transgene so that the animal is a useful screening tool (e.g., so that administration of test agents give rise to detectable cell death or anti-viral activity).

Methods of making transgenic animals are known in the art. DNA constructs can be introduced into the germ line of an avian or mammal to make a transgenic animal. For example, one or several copies of the construct can be incorporated into the genome of an embryo by standard transgenic techniques.

In an exemplary embodiment, a transgenic non-human animal is produced by introducing a transgene into the germ line of the non-human animal. Transgenes can be introduced into embryonal target cells at various developmental stages. Different methods are used depending on the stage of development of the embryonal target cell. The specific line(s) of any animal used should, if possible, be selected for general good health, good embryo yields, good pronuclear visibility in the embryo, and good reproductive fitness.

Introduction of the transgene into the embryo can be accomplished by any of a variety of means known in the art such as microinjection, electroporation, lipofection or a viral vector. For example, the transgene can be introduced into a mammal by microinjection of the construct into the pronuclei of the fertilized mammalian egg(s) to cause one or more copies of the construct to be retained in the cells of the developing mammal(s). Following introduction of the transgenic construct into the fertilized egg, the egg can be incubated in vitro for varying amounts of time, or reimplanted into the surrogate host, or both. One common method is to incubate the embryos in vitro for about 1-7 days, depending on the species, and then reimplant them into the surrogate host.

The progeny of the transgenically manipulated embryos can be tested for the presence of the construct (e.g., by Southern blot analysis) of a segment of tissue. An embryo having one or more copies of the exogenous cloned construct stably integrated into the genome can be used to establish a permanent transgenic animal line carrying the transgenically added construct.

Transgenically altered animals can be assayed after birth for the incorporation of the construct into the genome of the offspring. This can be done by hybridizing a probe corresponding to the DNA sequence coding for the polypeptide or a segment thereof onto chromosomal material from the progeny. Those progeny found to contain at least one copy of the construct in their genome are grown to maturity.

Methods of producing transgenic avians are also known in the art, see, e.g., U.S. Pat. No. 5,162,215.

Nicotinamide riboside-related test agents can be obtained from a wide variety of sources including libraries of synthetic or natural compounds. Such agents can include analogs or derivatives of nicotinamide riboside as well as tiazofurin and benzamide riboside and analogs or derivatives thereof.

Alternatively, the isolated Nrk polypeptide can be used to generate a crystal structure of Nrk and synthetic nicotinamide riboside analogs can be designed. Based on the crystal structure of *E. coli* panK, Asp127 appears to play a key role in transition-state stabilization of the transferring phosphoryl group of a pantothenate kinase (Yun, et al. (2000) *J. Biol. Chem.* 275:28093-28099). Accordingly, it is contemplated the corresponding Nrk mutant, e.g., NRK2-E100Q, can be used to generate a stable complex between an Nrk and a nucleotides (i.e., Nrk2-E100Q+nicotinamide riboside+ATP can be stable enough to crystallize). Alternatively, Nrk can produce a stable complex in the presence of an inhibitor such as an ATP-mimetic compound (e.g., AMP-PNHP and AMP-PCH_2P). For metabolite kinases, bisubstrate inhibitors have been very successfully employed. For example, thymidylate kinase, which performs the reaction, dTMP+ATP->dTDP+AMP, is strongly inhibited by dTpppppA (Bone, et al. (1986) *J. Biol. Chem.* 261:16410-16413) and crystal structures were obtained with this inhibitor (Lavie, et al. (1998) *Biochemistry* 37:3677-3686).

It has been shown that the best inhibitors typically contain one or two more phosphates than the two substrates combined (i.e., dTppppA is not as good a substrate as dTppppA). On the basis of the same types of results with adenosine kinase (Bone, et al. (1986) supra), it is contemplated that NrppppA (i.e., an NAD+ analog with two extra phosphates) will be a better inhibitor than NrpppA (i.e., an NAD+ analog with an extra phosphate, or, indeed, nicotinamide riboside+AppNHp). NAD+ analogs with extra phosphates can be generated using standard enzymatic methods (see, e.g., Guranowski, et al. (1990) *FEBS Lett.* 271:215-218) optimized for making a wide variety of adenylylated dinucleoside polyphosphates (Fraga, et al. (2003) *FEBS Lett.* 543:37-41), namely reaction of Nrpp (nicotinamide riboside diphosphate) and Nrppp (nicotinamide riboside triphosphate) with firefly luciferase-AMP. The diphosphorylated form of NMN (Nrpp) is prepared with either uridylate kinase or cytidylate kinase (NMN+ATP->Nrpp). The triphosphorylated form of NMN (Nrppp) is subsequently prepared with nucleoside diphosphate kinase (Nrpp+ATP->Nrppp). The resulting inhibitors are then used in crystallization trials and/or are soaked into Nrk crystals.

Once the three-dimensional structure of Nrk is determined, a potential test agent can be examined through the use of computer modeling using a docking program such as GRAM, DOCK, or AUTODOCK (Dunbrack, et al. (1997) *Folding & Design* 2:27-42). This procedure can include computer fitting of potential agents to Nrk to ascertain how well the shape and the chemical structure of the potential ligand will interact with Nrk. Computer programs can also be employed to estimate the attraction, repulsion, and steric hindrance of the test agent. Generally the tighter the fit (e.g., the lower the steric hindrance, and/or the greater the attractive force) the better substrate the agent will be since these properties are consistent with a tighter binding constraint. Furthermore, the more specificity in the design of a potential test agent the more likely that the agent will not interfere with related mammalian proteins. This will minimize potential side-effects due to unwanted interactions with other proteins.

The invention is also a method of treating cancer in a patient, having or suspected of having cancer, with an isolated nucleic acid, delivery vector, or polypeptide of the invention in combination with a nicotinamide riboside-related prodrug. Administration of the nucleic acid, delivery vector, or polypeptide of the present invention to a human subject or an animal can be by any means known in the art for administering nucleic acids, vectors, or polypeptides. A patient, as used herein, is intended to include any mammal such as a human, agriculturally-important animal, pet or zoological animal. A patient having or suspected of having a cancer is a patient who exhibits signs or symptoms of a cancer or because of inheritance, environmental or natural reasons is suspected of having cancer. Nucleic acids encoding Nrk, vectors containing the same, or Nrk polypeptides can be administered to the subject in an amount effective to decrease, alleviate or eliminate the signs or symptoms of a cancer (e.g., tumor size, feelings of weakness, and pain perception). The amount of the agent required to achieve the desired outcome of decreasing, eliminating or alleviating a sign or symptom of a cancer will be dependent on the pharmaceutical composition of the agent, the patient and the condition of the patient, the mode of administration, the type of condition or disease being prevented or treated, age and species of the patient, the particular vector, and the nucleic acid to be delivered, and can be determined in a routine manner.

While the prodrug and the Nrk nucleic acid, delivery vector, or polypeptide can be delivered concomitantly, in an alternative embodiment the Nrk nucleic acid, delivery vector, or polypeptide is provided first, followed by administration of the prodrug to precondition the cells to generate the activated or toxic drug.

Types of cancers which can be treated in accordance with the method of the invention include, but are not limited to, pancreatic cancer, endometrial cancer, small cell and non-small cell cancer of the lung (including squamous, adneocarcinoma and large cell types), squamous cell cancer of the head and neck, bladder, ovarian, cervical, breast, renal, CNS, and colon cancers, myeloid and lymphocyltic leukemia, lymphoma, hepatic tumors, medullary thyroid carcinoma, multiple myeloma, melanoma, retinoblastoma, and sarcomas of the soft tissue and bone.

Typically, with respect to viral vectors, at least about 10^3 virus particles, at least about 10^5 virus particles, at least about 10^7 virus particles, at least about 10^9 virus particles, at least about 10^{11} virus particles, at least about 10^{12} virus particles, or at least about 10^{13} virus particles are administered to the patient per treatment. Exemplary doses are virus titers of about 10^7 to about 10^{15} particles, about 10^7 to about 10^{14} particles, about 10^8 to about 10^{13} particles, about 10^{10} to about 10^{15} particles, about 10^{11} to about 10^{15} particles, about 10^{12} to about 10^{14} particles, or about 10^{12} to about 10^{13} particles.

In particular embodiments of the invention, more than one administration (e.g., two, three, four, or more administrations) can be employed over a variety of time intervals (e.g., hourly, daily, weekly, monthly, etc.) to achieve therapeutic levels of nucleic acid expression.

Tiazofurin is a nucleoside analog initially synthesized to be a cytidine deaminase inhibitor. Tiazofurin was shown to be a prodrug that is converted by cellular enzymes to TAD, an analog of NAD+, that inhibits IMP dehydrogenase, the rate limiting enzyme in producing GTP and dGTP (Cooney, et al. (1983) supra). In phase I/II trials of acute leukemia, tiazofurin produced response rates as high as 85% and was granted orphan drug status for treatment of CML in accelerated phase or blast crisis. Treatment of cultured cells has shown that tiazofurin selectively kills cancer cells by induction of apoptosis: the activity has been attributed both to the increased dependence of actively replicating cells on dGTP and to the addiction of many transformed genotypes to signaling through low molecular weight G proteins (Jayaram, et al. (2002) *Curr. Med. Chem.* 9:787-792). Examination of the sensitivity of the NCl-60 panel of cancer cell lines and the literature on tiazofurin indicates that particular breast, renal, CNS, colon and non-small cell lung-derived tumors are among the most sensitive while others from the same organ sites are among the most resistant (Johnson, et al. (2001) *Br. J. Cancer* 84:1424-1431). As was demonstrated herein, the function of nicotinamide riboside as an NAD+ precursor is entirely dependent on Nrk1 and human Nrks have at least as high specific activity in tiazofurin phosphorylation as in nicotinamide riboside phosphorylation. Because Nrk2 expression is muscle-specific (Li, et al. (1999) supra), and Nrk1 is expressed at a very low level (Boon, et al. (2002) supra), while NMN/NaMNAT is not restricted, it is contemplated that stratification of tumors by Nrk gene expression will largely predict and account for tiazofurin sensitivity.

Accordingly, the present invention is further a method for identifying an individual or tumor which is susceptible to treatment with a nicotinamide riboside-related prodrug. In one embodiment, the level of Nrk protein in an individual or tumor is detected by binding of a Nrk-specific antibody in an immunoassay. In another embodiment, the level of Nrk enzyme activity is determined using, for example, the nicotinamide riboside phosphorylation assay disclosed herein. In another embodiment, the level of Nrk RNA transcript is determined using any number of well-known RNA-based assays for detecting levels of RNA. Once detected, the levels of Nrk are compared to a known standard. A change in the level of Nrk, as compared to the standard, is indicative of an altered level of susceptibility to treatment with a nicotinamide riboside-related prodrug. In a still further embodiment, mutations or polymorphisms in the Nrk gene can be identified which result in an altered level of susceptibility to treatment with a nicotinamide riboside-related prodrug.

Optimized treatments for cancer and other diseases with nicotinamide riboside-related prodrugs are directed toward cells with naturally high levels of an Nrk provided herein or toward cells which have been recombinantly engineered to express elevated levels of an Nrk. Safety, specificity and efficacy of these treatments can be modulated by supplementation with or restriction of the amounts of any of the NAD+ precursors, namely tryptophan, nicotinic acid, nicotinamide, or nicotinamide riboside.

For the detection of Nrk protein levels, antibodies which specifically recognize Nrk are generated. These antibodies can be either polyclonal or monoclonal. Moreover, such antibodies can be natural or partially or wholly synthetically produced. All fragments or derivatives thereof (e.g., Fab, Fab', $F(ab')_2$, scFv, Fv, or Fd fragments) which maintain the ability to specifically bind to and recognize Nrk are also included. The antibodies can be a member of any immunoglobulin class, including any of the human classes: IgG, IgM, IgA, IgD, and IgE.

The Nrk-specific antibodies can be generated using classical cloning and cell fusion techniques. See, for example, Kohler and Milstein (1975) *Nature* 256:495-497; Harlow and Lane (1988) Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory, New York. Alternatively, antibodies which specifically bind Nrk are derived by a phage display method. Methods of producing phage display antibodies are well-known in the art (e.g., Huse, et al. (1989) *Science* 246 (4935):1275-81).

Selection of Nrk-specific antibodies is based on binding affinity and can be determined by various well-known immunoassays including, enzyme-linked immunosorbent, immunodiffusion chemiluminescent, immunofluorescent, immunohistochemical, radioimmunoassay, agglutination, complement fixation, immunoelectrophoresis, and immunoprecipitation assays and the like which can be performed in vitro, in vivo or in situ. Such standard techniques are well-known to those of skill in the art (see, e.g., "Methods in Immunodiagnosis", 2nd Edition, Rose and Bigazzi, eds. John Wiley & Sons, 1980; Campbell et al., "Methods and Immunology", W. A. Benjamin, Inc., 1964; and Oellerich, M. (1984) *J. Clin. Chem. Clin. Biochem.* 22:895-904).

Once fully characterized for specificity, the antibodies can be used in diagnostic or predictive methods to evaluate the levels of Nrk in healthy and diseased tissues (i.e., tumors) via techniques such as ELISA, western blotting, or immunohistochemistry.

The general method for detecting levels of Nrk protein provides contacting a sample with an antibody which specifically binds Nrk, washing the sample to remove non-specific interactions, and detecting the antibody-antigen complex using any one of the immunoassays described above as well a number of well-known immunoassays used to detect and/or quantitate antigens (see, for example, Harlow and Lane (1988) supra). Such well-known immunoassays include antibody capture assays, antigen capture assays, and two-antibody sandwich assays.

For the detection of nucleic acid sequences encoding Nrk, either a DNA-based or RNA-based method can be employed. DNA-based methods for detecting mutations in an Nrk locus (i.e., frameshift mutations, point mutations, missense mutations, nonsense mutations, splice mutations, deletions or insertions of induced, natural or inherited origin) include, but are not limited to, DNA microarray technologies, oligonucleotide hybridization (mutant and wild-type), PCR-based sequencing, single-strand conformational polymorphism (SSCP) analysis, heteroduplex analysis (HET), PCR, or denaturing gradient gel electrophoresis. Mutations can appear, for example, as a dual base call on sequencing chromatograms. Potential mutations are confirmed by multiple, independent PCR reactions. Exemplary single nucleotide polymorphisms which can be identified in accordance with the diagnostic method of the invention include, but are not limited to, NCBI SNP Cluster ID Nos. rs3752955, rs1045882, rs11519, and rs3185880 for human Nrk1 and Cluster ID Nos. rs2304190, rs4807536, and rs1055767 for human Nrk2.

To detect the levels of RNA transcript encoding the Nrk, nucleic acids are isolated from cells of the individual or tumor, according to standard methodologies (e.g., Sambrook et al. (1989) *Molecular Cloning, a Laboratory Manual*, Cold Spring Harbor Laboratories, New York). The nucleic acid can be whole cell RNA or fractionated to Poly-A+. It may be desirable to convert the RNA to a complementary DNA (cDNA). Normally, the nucleic acid is amplified.

A variety of methods can be used to evaluate or quantitate the level of Nrk RNA transcript present in the nucleic acids isolated from an individual or tumor. For example, levels of Nrk RNA transcript can be evaluated using well-known methods such as northern blot analysis (see, e.g., Sambrook et al. (1989) *Molecular Cloning, a Laboratory Manual*, Cold Spring Harbor Laboratories, New York); oligonucleotide or cDNA fragment hybridization wherein the oligonucleotide or cDNA is configured in an array on a chip or wafer; real-time PCR analysis, or RT-PCR analysis.

Suitable primers, probes, or oligonucleotides useful for such detection methods can be generated by the skilled artisan from the Nrk nucleic acid sequences provided herein. The term primer, as defined herein, is meant to encompass any nucleic acid that is capable of priming the synthesis of a nascent nucleic acid in a template-dependent process. Typically, primers are oligonucleotides from ten to twenty base pairs in length, but longer sequences can be employed. Primers can be provided in double-stranded or single-stranded form. Probes are defined differently, although they can act as primers. Probes, while perhaps capable of priming, are designed for binding to the target DNA or RNA and need not be used in an amplification process. In one embodiment, the probes or primers are labeled with, for example, radioactive species (^{32}P, ^{14}C, ^{35}S, ^{3}H, or other label) or a fluorophore (rhodamine, fluorescein). Depending on the application, the probes or primers can be used cold, i.e., unlabeled, and the RNA or cDNA molecules are labeled.

Depending on the format, detection can be performed by visual means (e.g., ethidium bromide staining of a gel). Alternatively, the detection can involve indirect identification of the product via chemiluminescence, radiolabel or fluorescent label or even via a system using electrical or thermal impulse signals (Bellus (1994) *J. Macromol. Sci. Pure Appl. Chem. A*311:1355-1376).

After detecting mutations in Nrk or the levels of Nrk present in an individual or tumor, said mutations or levels are compared with a known control or standard. A known control can be a statistically significant reference group of individuals that are susceptible or lack susceptibility to treatment with a nicotinamide riboside-related prodrug to provide diagnostic or predictive information pertaining to the individual or tumor upon which the analysis was conducted.

As described herein, nicotinamide riboside isolated from deproteinized whey fraction of cow's milk was sufficient to support NRK1-dependent growth in a qns1 mutant. Accordingly, mutant strains generated herein will be useful in identifying other natural or synthetic sources for nicotinamide riboside for use in dietary supplements. Thus, the present invention also encompasses is a method for identifying such natural or synthetic sources. As a first step of the method, a first cell lacking a functional glutamine-dependent NAD+ synthetase is contacted with an isolated extract from a natural or synthetic source. In one embodiment, the first cell is a qns1 mutant (i.e., having no NAD+ synthetase) carrying the QNS1 gene on a URA3 plasmid. While any cell can be used, in particular embodiments a yeast cell is used in this method of the invention. A qns1 mutant strain has normal growth on 5-fluoroorotic acid (i.e., cured of the URA3 QNS1 plasmid) as long as it is supplied with nicotinamide riboside.

As a second step of the method, a second cell lacking a functional glutamine-dependent NAD+ synthetase and a functional nicotinamide riboside kinase is contacted with the same isolated extract from the natural or synthetic source of the prior step. Using a qns1 and nrk1 double mutant, it was demonstrated herein that the NRK1 gene is necessary for growth on nicotinamide riboside: qns1 and nrk1 are synthetically lethal even with nicotinamide riboside. This deletion strain is useful in this screening assay of the invention as it allows one to distinguish between nicotinamide riboside, NMN and NAD+ as the effective nutrient.

As a subsequent step of the method, the growth of the first cell and second cell are compared. If the isolated extract contains a nicotinamide riboside, the first cell will grow and the second cell will not.

Synthetic sources of nicotinamide riboside can include any library of chemicals commercially available from most large chemical companies including Merck, Glaxo, Bristol Meyers Squibb, Monsanto/Searle, Eli Lilly and Pharmacia. Natural

sources which can be tested for the presence of a nicotinamide riboside include, but are not limited to, cow's milk, serum, meats, eggs, fruit and cereals. Isolated extracts of the natural sources can be prepared using standard methods. For example, the natural source can be ground or homogenized in a buffered solution, centrifuged to remove cellular debris, and fractionated to remove salts, carbohydrates, polypeptides, nucleic acids, fats and the like before being tested on the mutants strains of the invention. Any source of nicotinamide riboside that scores positively in the assay of the invention can be further fractionated and confirmed by standard methods of HPLC and mass spectrometry.

Nicotinic acid is an effective agent in controlling low-density lipoprotein cholesterol, increasing high-density lipoprotein cholesterol, and reducing triglyceride and lipoprotein (a) levels in humans (see, e.g., Miller (2003) *Mayo Clin. Proc.* 78(6):735-42). Though nicotinic acid treatment effects all of the key lipids in the desirable direction and has been shown to reduce mortality in target populations, its use is limited because of a side effect of heat and redness termed flushing, which is significantly effected by the nature of formulation. Further, nicotinamide protects against stroke injury in model systems, due to multiple mechanisms including increasing mitochondrial NAD+ levels and inhibiting PARP (Klaidman, et al. (2003) *Pharmacology* 69(3):150-7). Altered levels of NAD+ precursors have been shown to effect the regulation of a number of genes and lifespan in yeast (Anderson, et al. (2003) *Nature* 423(6936):181-5).

NAD+ administration and NMN adenylyltransferase (Nmnat1) expression have also been shown to protect neurons from axonal degeneration (Araki, et al. (2004) *Science* 305: 1010-1013). Because nicotinamide riboside is a soluble, transportable nucleoside precursor of NAD+, nicotinamide riboside can be used to protect against axonopathies such as those that occur in Alzheimer's Disease, Parkinson's Disease and Multiple Sclerosis. Expression of the NRK1 or NRK2 genes, or direct administration of nicotinamide riboside or a stable nicotinamide riboside prodrug, could also protect against axonal degeneration.

NMN adenylytransferase overexpression has been shown to protect neurons from the axonopathies that develop with ischemia and toxin exposure, including vincristine treatment (Araki, et al. (2004) *Science* 305:1010-1013). Vincristine is one of many chemotherapeutic agents whose use is limited by neurotoxicity. Thus, administration of nicotinamide riboside or an effective nicotinamide riboside prodrug derivative could be used to protect against neurotoxicity before, during or after cytotoxic chemotherapy.

Further, conversion of benign *Candida glabrata* to the adhesive, infective form is dependent upon the expression of EPA genes encoding adhesins whose expression is mediated by NAD+ limitation, which leads to defective Sir2-dependent silencing of these genes (Domergue, et al. (March 2005) *Science,* 10.1126/science.1108640). Treatment with nicotinic acid reduces expression of adhesins and increasing nicotinic acid in mouse chow reduces urinary tract infection by *Candida glabrata*. Thus, nicotinamide riboside can be used in the treatment of fungal infections, in particular, those of *Candida* species by preventing expression of adhesins.

Accordingly, agents (e.g., nicotinamide riboside) that work through the discovered nicotinamide riboside kinase pathway of NAD+ biosynthesis could have therapeutic value in improving plasma lipid profiles, preventing stroke, providing neuroprotection with chemotherapy treatment, treating fungal infections, preventing or reducing neurodegeneration, or in prolonging health and well-being. Thus, the present invention is further a method for preventing or treating a disease or condition associated with the nicotinamide riboside kinase pathway of NAD+ biosynthesis by administering an effective amount of a nicotinamide riboside composition. Diseases or conditions which typically have altered levels of NAD+ or NAD+ precursors or could benefit from increased NAD+ biosynthesis by treatment with nicotinamide riboside include, but are not limited to, lipid disorders (e.g., dyslipidemia, hypercholesterolaemia or hyperlipidemia), stroke, neurodegenerative diseases (e.g., Alzheimer's, Parkinsons and Multiple Sclerosis), neurotoxicity as observed with chemotherapies, *Candida glabrata* infection, and the general health declines associated with aging. Such diseases and conditions can be prevented or treated by supplementing a diet or a therapeutic treatment regime with a nicotinamide riboside composition.

The source of nicotinamide riboside can be from a natural or synthetic source identified by the method of the instant invention, or can be chemically synthesized using established methods (Tanimori (2002) *Bioorg. Med. Chem. Lett.* 12:1135-1137; Franchetti (2004) *Bioorg. Med. Chem. Lett.* 14:4655-4658). In addition, the nicotinamide riboside can be a derivative (e.g., L-valine or L-phenylalanine esters) of nicotinamide riboside. For example, an L-valyl (valine) ester on the 5' O of acyclovir (valacyclovir) improved the pharmacokinetic properties of the drug by promoting transport and allowing cellular delivery of the nucleoside after hydrolysis by an abundant butyryl esterase (Han, et al. (1998) *Pharm. Res.* 15:1382-1386; Kim, et al. (2003) *J. Biol. Chem.* 278: 25348-25356). Accordingly, the present invention also encompasses derivatives of nicotinamide riboside, in particular L-valine or L-phenylalanine esters of nicotinamide riboside, which are contemplated as having improved pharmacokinetic properties (e.g., transport and delivery). Such derivatives can be used alone or formulated with a pharmaceutically acceptable carrier as disclosed herein.

An effective amount of nicotinamide riboside is one which prevents, reduces, alleviates or eliminates the signs or symptoms of the disease or condition being prevented or treated and will vary with the disease or condition. Such signs or symptoms can be evaluated by the skilled clinician before and after treatment with the nicotinamide riboside to evaluate the effectiveness of the treatment regime and dosages can be adjusted accordingly.

As alterations of NAD+ metabolism may need to be optimized for particular conditions, it is contemplated that nicotinamide riboside treatments can further be used in combination with other NAD+ precursors, e.g., tryptophan, nicotinic acid and/or nicotinamide.

Polypeptides, nucleic acids, vectors, dietary supplements (i.e. nicotinamide riboside), and nicotinamide riboside-related prodrugs produced or identified in accordance with the methods of the invention can be conveniently used or administered in a composition containing the active agent in combination with a pharmaceutically acceptable carrier. Such compositions can be prepared by methods and contain carriers which are well-known in the art. A generally recognized compendium of such methods and ingredients is Remington: The Science and Practice of Pharmacy, Alfonso R. Gennaro, editor, 20th ed. Lippingcott Williams & Wilkins: Philadelphia, Pa., 2000. A carrier, pharmaceutically acceptable carrier, or vehicle, such as a liquid or solid filler, diluent, excipient, or solvent encapsulating material, is involved in carrying or transporting the subject compound from one organ, or portion of the body, to another organ, or portion of the body. Each carrier must be acceptable in the sense of being compatible with the other ingredients of the formulation and not injurious to the patient.

Examples of materials which can serve as carriers include sugars, such as lactose, glucose and sucrose; starches, such as corn starch and potato starch; cellulose, and its derivatives, such as sodium carboxymethyl cellulose, ethyl cellulose and cellulose acetate; powdered tragacanth; malt; gelatin; talc; excipients, such as cocoa butter and suppository waxes; oils, such as peanut oil, cottonseed oil, safflower oil, sesame oil, olive oil, corn oil and soybean oil; glycols, such as propylene glycol; polyols, such as glycerin, sorbitol, mannitol and polyethylene glycol; esters, such as ethyl oleate and ethyl laurate; agar; buffering agents, such as magnesium hydroxide and aluminum hydroxide; alginic acid; pyrogen-free water; isotonic saline; Ringer's solution; ethyl alcohol; pH buffered solutions; polyesters, polycarbonates and/or polyanhydrides; and other non-toxic compatible substances employed in formulations. Wetting agents, emulsifiers and lubricants, such as sodium lauryl sulfate and magnesium stearate, as well as coloring agents, release agents, coating agents, sweetening, flavoring and perfuming agents, preservatives and antioxidants can also be present in the compositions.

Polypeptides, nucleic acids, vectors, dietary supplements, and nicotinamide riboside-related prodrugs produced or identified in accordance with the methods of the invention, hereafter referred to as compounds, can be administered via any route include, but not limited to, oral, rectal, topical, buccal (e.g., sub-lingual), vaginal, parenteral (e.g., subcutaneous, intramuscular including skeletal muscle, cardiac muscle, diaphragm muscle and smooth muscle, intradermal, intravenous, intraperitoneal), topical (i.e., both skin and mucosal surfaces, including airway surfaces), intranasal, transdermal, intraarticular, intrathecal and inhalation administration, administration to the liver by intraportal delivery, as well as direct organ injection (e.g., into the liver, into the brain for delivery to the central nervous system). The most suitable route in any given case will depend on the nature and severity of the condition being treated and on the nature of the particular compound which is being used.

For injection, the carrier will typically be a liquid, such as sterile pyrogen-free water, pyrogen-free phosphate-buffered saline solution, bacteriostatic water, or Cremophor (BASF, Parsippany, N.J.). For other methods of administration, the carrier can be either solid or liquid.

For oral therapeutic administration, the compound can be combined with one or more carriers and used in the form of ingestible tablets, buccal tablets, troches, capsules, elixirs, suspensions, syrups, wafers, chewing gums, foods and the like. Such compositions and preparations should contain at least 0.1% of active compound. The percentage of the compound and preparations can, of course, be varied and can conveniently be between about 0.1 to about 100% of the weight of a given unit dosage form. The amount of active compound in such compositions is such that an effective dosage level will be obtained.

The tablets, troches, pills, capsules, and the like can also contain the following: binders such as gum tragacanth, acacia, corn starch or gelatin; excipients such as dicalcium phosphate; a disintegrating agent such as corn starch, potato starch, alginic acid and the like; a lubricant such as magnesium stearate; and a sweetening agent such as sucrose, fructose, lactose or aspartame or a flavoring agent such as peppermint, oil of wintergreen, or cherry flavoring. The above listing is merely representative and one skilled in the art could envision other binders, excipients, sweetening agents and the like. When the unit dosage form is a capsule, it can contain, in addition to materials of the above type, a liquid carrier, such as a vegetable oil or a polyethylene glycol. Various other materials can be present as coatings or to otherwise modify the physical form of the solid unit dosage form. For instance, tablets, pills, or capsules can be coated with gelatin, wax, shellac or sugar and the like.

A syrup or elixir can contain the active agent, sucrose or fructose as a sweetening agent, methyl and propylparabens as preservatives, a dye and flavoring such as cherry or orange flavor. Of course, any material used in preparing any unit dosage form should be substantially non-toxic in the amounts employed. In addition, the active compounds can be incorporated into sustained-release preparations and devices including, but not limited to, those relying on osmotic pressures to obtain a desired release profile.

Formulations of the present invention suitable for parenteral administration contain sterile aqueous and non-aqueous injection solutions of the compound, which preparations are generally isotonic with the blood of the intended recipient. These preparations can contain anti-oxidants, buffers, bacteriostats and solutes which render the formulation isotonic with the blood of the intended recipient. Aqueous and non-aqueous sterile suspensions can include suspending agents and thickening agents. The formulations can be presented in unit\dose or multi-dose containers, for example sealed ampoules and vials, and can be stored in a freeze-dried (lyophilized) condition requiring only the addition of the sterile liquid carrier, for example, saline or water-for-injection immediately prior to use.

Formulations suitable for topical application to the skin can take the form of an ointment, cream, lotion, paste, gel, spray, aerosol, or oil. Carriers which can be used include petroleum jelly, lanoline, polyethylene glycols, alcohols, transdermal enhancers, and combinations of two or more thereof.

Formulations suitable for transdermal administration can be presented as discrete patches adapted to remain in intimate contact with the epidermis of the recipient for a prolonged period of time. Formulations suitable for transdermal administration can also be delivered by iontophoresis (see, for example, *Pharmaceutical Research* 3 (6):318 (1986)) and typically take the form of an optionally buffered aqueous solution of the compound. Suitable formulations contain citrate or bis\tris buffer (pH 6) or ethanol/water and contain from 0.1 to 0.2 M of the compound.

A compound can alternatively be formulated for nasal administration or otherwise administered to the lungs of a subject by any suitable means. In particular embodiments, the compound is administered by an aerosol suspension of respirable particles containing the compound, which the subject inhales. The respirable particles can be liquid or solid. The term aerosol includes any gas-borne suspended phase, which is capable of being inhaled into the bronchioles or nasal passages. Specifically, aerosol includes a gas-borne suspension of droplets, as can be produced in a metered dose inhaler or nebulizer, or in a mist sprayer. Aerosol also includes a dry powder composition suspended in air or other carrier gas, which can be delivered by insufflation from an inhaler device, for example. See Ganderton & Jones, *Drug Delivery to the Respiratory Tract*, Ellis Horwood (1987); Gonda (1990) *Critical Reviews in Therapeutic Drug Carrier Systems* 6:273-313; and Raeburn, et al. (1992) *J. Pharmacol. Toxicol. Methods* 27:143-159. Aerosols of liquid particles containing the compound can be produced by any suitable means, such as with a pressure-driven aerosol nebulizer or an ultrasonic nebulizer, as is known to those of skill in the art. See, e.g., U.S. Pat. No. 4,501,729. Aerosols of solid particles containing the compound can likewise be produced with any solid particulate medicament aerosol generator, by techniques known in the pharmaceutical art.

Alternatively, one can administer the compound in a local rather than systemic manner, for example, in a depot or sustained-release formulation.

Further, the present invention provides liposomal formulations of the compounds disclosed herein and salts thereof. The technology for forming liposomal suspensions is well-known in the art. When the compound or salt thereof is an aqueous-soluble salt, using conventional liposome technology, the same can be incorporated into lipid vesicles. In such an instance, due to the water solubility of the compound or salt, the compound or salt will be substantially entrained within the hydrophilic center or core of the liposomes. The lipid layer employed can be of any conventional composition and can either contain cholesterol or can be cholesterol-free. When the compound or salt of interest is water-insoluble, again employing conventional liposome formation technology, the salt can be substantially entrained within the hydrophobic lipid bilayer which forms the structure of the liposome. In either instance, the liposomes which are produced can be reduced in size, as through the use of standard sonication and homogenization techniques.

A liposomal formulation containing a compound disclosed herein or salt thereof, can be lyophilized to produce a lyophilizate which can be reconstituted with a carrier, such as water, to regenerate a liposomal suspension.

In particular embodiments, the compound is administered to the subject in an effective amount, as that term is defined herein. Dosages of active compounds can be determined by methods known in the art, see, e.g., Remington: The Science and Practice of Pharmacy, Alfonso R. Gennaro, editor, 20th ed. Lippingcott Williams & Wilkins: Philadelphia, Pa., 2000. The selected effective dosage level will depend upon a variety of factors including the activity of the particular compound of the present invention employed, the route of administration, the time of administration, the rate of excretion or metabolism of the particular compound being employed, the duration of the treatment, other drugs, compounds and/or materials used in combination with the particular compound employed, the age, sex, weight, condition, general health and prior medical history of the patient being treated, and like factors well-known in the medical arts.

A physician or veterinarian having ordinary skill in the art can readily determine and prescribe the effective amount of the pharmaceutical composition required for prevention or treatment in an animal subject such as a human, agriculturally-important animal, pet or zoological animal.

The invention is described in greater detail by the following non-limiting examples.

EXAMPLE 1

S. cerevisiae Strains

Yeast diploid strain BY165, heterozygous for qns1 deletion and haploid BY165-1d carrying a chromosomal deletion of qns1 gene, transformed with plasmid pB175 containing QNS1 and URA3 is known in the art (Bieganowski, et al. (2003) supra). Genetic deletions were introduced by direct transformation with PCR products (Brachmann, et al. (1998) *Yeast* 14:115-132) generated from primers. After 24 hours of growth on complete media, cells were plated on media containing 5-fluoroorotic acid (Boeke, et al. (1987) *Methods Enzymol.* 154:164-175). The ado1 disruption cassette was constructed by PCR with primers 7041 (5'-CTA TTT AGA GTA AGG ATA TTT TTT CGG AAG GGT AAG AGG GAC CAA CTT CTT CTG TGC GGT ATT TCA CAC CG-3'; SEQ ID NO:10) and 7044 (5'-ATG ACC GCA CCA TTG GTA GTA TTG GGT AAC CCA CTT TTA GAT TTC CAA GCA GAT TGT ACT GAG AGT GCA C-3'; SEQ ID NO:11) and plasmid pRS413 as a template. Yeast strain BY165 was transformed with this PCR product, and homologous recombination in histidine prototrophic transformants was confirmed by PCR with primers 7042 (5'-AAG CTA GAG GGA ACA CGT AGA G-3'; SEQ ID NO:12) and 7043 (5'-TTA TCT TGT GCA GGG TAG AAC C-3'; SEQ ID NO:13). This strain was transformed with plasmid pB175 and subjected to sporulation and tetrad dissection. Haploid strain BY237, carrying qns1 and ado1 deletions and plasmid, was selected for further experiments. The urk1 deletion was introduced into strain BY237 by transformation with the product of the PCR amplification that used pRS415 as a template and PCR primers 7051 (5'-CGA TCT TCA TCA TTT ATT TCA ATT TTA GAC GAT GAA ACA AGA GAC ACA TTA GAT TGT ACT GAG AGT GCA C-3'; SEQ ID NO:14) and 7052 (5'-AAA ATA CTT TGA ATC AAA AAA TCT GGT CAA TGC CCA TTT GTA TTG ATG ATC TGT GCG GTA TTT CAC ACC G-3'; SEQ ID NO:15). Disruption was confirmed by PCR with primers 7053 (5'-ATG TCC CAT CGT ATA GCA CCT TCC-3'; SEQ ID NO:16) and 7054 (5'-GCC TCT AAT TAT TCT CAA TCA CAA CC-3'; SEQ ID NO:17), and the resulting strain was designated BY247. The rbk1 disruption cassette was constructed by PCR with primers 7063 (5'-AAA CTT TCA GGG CTA ACC ACT TCG AAA CAC ATG CTG GTG GTA AGG GAT TGA GAT TGT ACT GAG AGT GCA C-3'; SEQ ID NO:18) and 7065 (5'-GAA CAG AAA AGC ACC CCT CTC GAA CCC AAA GTC ATA ACC ACA ATT CCT CTC TGT GCG GTA TTT CAC ACC G-3'; SEQ ID NO:19) and plasmid pRS411 as a template. Disruption was introduced into strain BY242 by transformation with the product of this reaction and confirmed by PCR with primers 7062 (5'-GGA TAG ATT ACC TAA CGC TGG AG-3'; SEQ ID NO:20) and 7064 (5'-TTG TAC TTC AGG GCT TTC GTG C-3'; SEQ ID NO:21). The resulting strain, carrying deletions of qns1, ado1, urk1 and rbk1 genes was designated BY252. A yeast strain carrying disruption of the NRK1 locus was made by transformation of the strain BY165-1d with the HIS3 marker introduced into disruption cassette by PCR with primers 4750 (5'-AAT AGC GTG CAA AAG CTA TCG AAG TGT GAG CTA GAG TAG AAC CTC AAA ATA GAT TGT ACT GAG AGT GCA C-3'; SEQ ID NO:22) and 4751 (5'-CTA ATC CTT ACA AAG CTT TAG AAT CTC TTG GCA CAC CCA GCT TAA AGG TCT GTG CGG TAT TTC ACA CCG-3'; SEQ ID NO:23). Correct integration of the HIS3 marker into NRK1 locus was confirmed by PCR with primers 4752 (5'-ACC AAC TTG CAT TTT AGG CTG TTC-3'; SEQ ID NO:24) and 4753 (5'-TAA GTT ATC TAT CGA GGT ACA CAT TC-3'; SEQ ID NO:25).

EXAMPLE 2

Nicotinamide Riboside and Whey Preparations

NMN (39.9 mg; Sigma, St. Louis, Mo.) was treated with 1250 units of calf intestinal alkaline phosphatase (Sigma) for 1 hour at 37° C. in 1 mL 100 mM NaCl, 20 mM Tris pH 8.0, 5 mM $MgCl_2$. Hydrolysis of NMN to nicotinamide riboside was verified by HPLC and phosphatase was removed by centrifuging the reaction through a 5,000 Da filter (Millipore, Billerica, Mass.). A whey vitamin fraction of commercial nonfat cow's milk was prepared by adjusting the pH to 4 with HCl, stirring at 55° C. for 10 minutes, removal of denatured casein by centrifugation, and passage through a 5,000 Da

filter. In yeast media, nicotinamide riboside was used at 10 μM and whey vitamin fraction at 50% by volume.

EXAMPLE 3

Yeast GST-ORF Library

Preparation of the fusion protein library was in accordance with well-established methods (Martzen, et al. (1999) supra; Phizicky, et al. (2002) *Methods Enzymol.* 350:546-559) at a 0.5 liter culture scale for each of the 64 pools of 90-96 protein constructs. Ten percent of each pool preparation was assayed for Nrk activity in overnight incubations.

EXAMPLE 4

Nicotinamide Riboside Phosphorylation Assays

Reactions (0.2 mL) containing 100 mM NaCl, 20 mM NaHEPES pH 7.2, 5 mM β-mercaptoethanol, 1 mM ATP, 5 mM $MgCl_2$, and 500 μM nicotinamide riboside or alternate nucleoside, were incubated at 30° C. and terminated by addition of EDTA to 20 mM and heating for 2 minutes at 100° C. Specific activity assays, containing 50 ng to 6 μg enzyme depending on the enzyme and substrate, were incubated for 30 minutes at 30° C. to maintain initial rate conditions. Reaction products were analyzed by HPLC on a strong anion exchange column with a 10 mM to 750 mM gradient of KPO_4 pH 2.6.

EXAMPLE 5

NRK Gene and cDNA Cloning and Enzyme Purification

The *S. cerevisiae* NRK1 gene was amplified from total yeast DNA with primers 7448 (5'-CGC TGC ACA TAT GAC TTC GAA AAA AGT GAT ATT AGT TGC-3'; SEQ ID NO:26) and 7449 (5'-CCG TCT CGA GCT AAT CCT TAC AAA GCT TTA GAA TCT CTT GG-3'; SEQ ID NO:27). The amplified DNA fragment was cloned in vector pSGO4 (Ghosh and Lowenstein (1997) *Gene* 176:249-255) for *E. coli* expression using restriction sites for NdeI and XhoI included in primer sequences and the resulting plasmid was designated pB446. Samples of cDNA made from human lymphocytes and spleen were used as a template for amplification of human NRK1 using primers 4754 (5'-CCG GCC CAT GGC GCA CCA CCA TCA CCA CCA TCA TAT GAA AAC ATT TAT CAT TGG AAT CAG TGG-3'; SEQ ID NO:28) and 4755 (5'-GCG GGG ATC CTT ATG CTG TCA CTT GCA AAC ACT TTT GC-3'; SEQ ID NO:29). For *E. coli* expression, PCR amplicons from this reaction were cloned into restriction sites NcoI and BamHI of vector pMR103 (Munson, et al. (1994) *Gene* 144:59-62) resulting in plasmid pB449. Subsequently, plasmid pB449 was used as a template for PCR with primers 7769 (5'-CCG CGG ATC CAT GAA AAC ATT TAT CAT TGG AAT CAG TGG-3'; SEQ ID NO:30) and 7770 (5'-GCC GCT CGA GTT ATG CTG TCA CTT GCA AAC ACT T-3'; SEQ ID NO:31). The product of this amplification was cloned between BamHI and XhoI sites of vector p425GAL1 (Mumberg, et al. (1994) *Nucleic Acids Res.* 22:5767-5768) and the resulting plasmid carrying human NRK1 gene under GAL1 promoter control was designated pB450. Human NRK2 cDNA was amplified with primers 7777 (5'-GGC AGG CAT ATG AAG CTC ATC GTG GGC ATC G-3'; SEQ ID NO:32) and 7776 (5'-GCT CGC TCG AGT CAC ATG CTG TCC TGC TGG GAC-3'; SEQ ID NO:33). The amplified fragment was digested with NdeI and XhoI enzymes and cloned in plasmid pSGA04 for *E. coli* expression. His-tagged enzymes were purified with Ni-NTA agarose.

SEQUENCE LISTING

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<212> TYPE: DNA
<213> ORGANISM: Saccharomyces cerevisiae

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atactcgaag atttgcattg aacgatcgtt gccgttgact catttgaaac agaaaaacaa 180

tacacgcagg aaaggaacgg cagttggtct gagaaacaaa accaacttgc attttaggct 240

gttccgatag tttatcagag taagggaaaa aatagcgtgc aaaagctatc gaagtgtgag 300

ctagagtaga acctcaaaat atgacttcga aaaaagtgat attagttgca ttgagtggat 360

gctcctccag tggtaagacg acaattgcga aacttacagc aagtttattc acgaaggcta 420

cattaattca tgaagatgac ttttacaaac atgataatga agtgccagta gatgctaaat 480

ataacattca aaattgggat tcgccagaag ctcttgattt taaacttttc ggtaaagaat 540

tagatgtgat caaacaaact ggtaaaatag ccaccaaact tatacacaat aacaacgtag 600

atgatccctt tacaaagttc cacattgata gacaagtttg ggacgagtta aaggctaagt 660

atgactctat taatgacgac aaatatgaag ttgtaattgt agatgggttt atgattttca 720

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ataatactgg aatatcaaaa aaatttgatt tgaagatatt agtgcgtgct ccctatgaag 780

tactaaaaaa aaggagggct tccagaaaag gataccagac tttggattct ttctgggtgg 840

atccgccgta ttatttcgac gaatttgtgt atgaatctta tcgtgcaaat catgcgcagt 900

tatttgttaa tggagacgta gaaggtttac tagacccaag gaagtcaaag aatataaaag 960

agttcataaa tgatgatgac actccaattg cgaaaccttt aagctgggtg tgccaagaga 1020

ttctaaagct ttgtaaggat taggaaagcg ccacaaaatc gatgagaagt ataaaaaaaa 1080

aaaagtaaaa acaataaaaa taagaatgtg tacctcgata gataacttaa ataagacaat 1140

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cgcgcttggc tgtcgcctct tccgctgtgt ttgggaggac tcgaactggc gccaggaaat 180

attaggaagc tgtgattttc aaagctaatt atgaaaacat ttatcattgg aatcagtggt 240

gtgacaaaca gtggcaaaac aacactggct aagaatttgc agaaacacct cccaaattgc 300

agtgtcatat ctcaggatga tttcttcaag ccagagtctg agatagagac agataaaaat 360

ggatttttgc agtacgatgt gcttgaagca cttaacatgg aaaaaatgat gtcagccatt 420

tcctgctgga tggaaagcgc aagacactct gtggtatcaa cagaccagga aagtgctgag 480

gaaattccca ttttaatcat cgaaggtttt cttcttttta attataagcc ccttgacact 540

atatggaata gaagctattt cctgactatt ccatatgaag aatgtaaaag gaggaggagt 600

acaagggtct atcagcctcc agactctccg ggatactttg atggccatgt gtggcccatg 660

tatctaaagt acagacaaga aatgcaggac atcacatggg aagttgtgta cctggatgga 720

acaaaatctg aagaggacct ctttttgcaa gtatatgaag atctaataca agaactagca 780

aagcaaaagt gtttgcaagt gacagcataa agacggaaca caacaaatcc ttcctgaagt 840

gaattaggaa actccaagga gtaatttaag aaccttcacc aagatacaat gtatactgtg 900

gtacaatgac agccattgtt tcatatgttt gatttttatt gcacatggtt ttcccaacat 960

gtggaacaat aaatatccat gccaatggac aggactgtac cttagcaagt tgctccctct 1020

ccagggagcg catagataca gcagagctca cagtgagtca gaaagtctcc actttctgaa 1080

catagctcta taacaatgat tgtcaaactt ttctaactgg agctcagagt aagaaataaa 1140

gattacatca caatccaaaa aaaaaaaaaa aa 1172

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tgccggcgcc tccgccccat ccccaggggc cgcctccccc ggggcggcct ccaggctgcc 180

gagacctata aaggcgccag gttttctcaa tgaagccggg acgcactccg gagcgcactg 240

cgtggtcgca ccctacccgg gctgccttgg aagtcgtccc cgccgcccct ccgcaccggc 300

atgaagctca tcgtgggcat cggaggcatg accaacggcg gcaagaccac gctgaccaac 360

agcctgctca gagccctgcc caactgctgc gtgatccatc aggatgactt cttcaagccc 420

caagaccaaa tagcagttgg ggaagacggc ttcaaacagt gggacgtgct ggagtctctg 480

gacatggagg ccatgctgga caccgtgcag gcctggctga gcagcccgca gaagtttgcc 540

cgtgcccacg gggtcagcgt ccagccagag gcctcggaca cccacatcct cctcctggaa 600

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<211> LENGTH: 240
<212> TYPE: PRT
<213> ORGANISM: Saccharomyces cerevisiae

<400> SEQUENCE: 4

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Ser Gly Lys Thr Thr Ile Ala Lys Leu Thr Ala Ser Leu Phe Thr Lys
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Ala Thr Leu Ile His Glu Asp Asp Phe Tyr Lys His Asp Asn Glu Val
35 40 45

Pro Val Asp Ala Lys Tyr Asn Ile Gln Asn Trp Asp Ser Pro Glu Ala
50 55 60

Leu Asp Phe Lys Leu Phe Gly Lys Glu Leu Asp Val Ile Lys Gln Thr
65 70 75 80

Gly Lys Ile Ala Thr Lys Leu Ile His Asn Asn Asn Val Asp Asp Pro
85 90 95

Phe Thr Lys Phe His Ile Asp Arg Gln Val Trp Asp Glu Leu Lys Ala
100 105 110

Lys Tyr Asp Ser Ile Asn Asp Asp Lys Tyr Glu Val Val Ile Val Asp
115 120 125

Gly Phe Met Ile Phe Asn Asn Thr Gly Ile Ser Lys Lys Phe Asp Leu
130 135 140

Lys Ile Leu Val Arg Ala Pro Tyr Glu Val Leu Lys Lys Arg Arg Ala
145 150 155 160

Ser Arg Lys Gly Tyr Gln Thr Leu Asp Ser Phe Trp Val Asp Pro Pro
165 170 175

Tyr Tyr Phe Asp Glu Phe Val Tyr Glu Ser Tyr Arg Ala Asn His Ala
180 185 190

Gln Leu Phe Val Asn Gly Asp Val Glu Gly Leu Leu Asp Pro Arg Lys
195 200 205

Ser Lys Asn Ile Lys Glu Phe Ile Asn Asp Asp Asp Thr Pro Ile Ala
210 215 220

Lys Pro Leu Ser Trp Val Cys Gln Glu Ile Leu Lys Leu Cys Lys Asp
225 230 235 240

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<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 5

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Thr Thr Leu Ala Lys Asn Leu Gln Lys His Leu Pro Asn Cys Ser Val
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Ile Ser Gln Asp Asp Phe Phe Lys Pro Glu Ser Glu Ile Glu Thr Asp
35 40 45

Lys Asn Gly Phe Leu Gln Tyr Asp Val Leu Glu Ala Leu Asn Met Glu
50 55 60

Lys Met Met Ser Ala Ile Ser Cys Trp Met Glu Ser Ala Arg His Ser
65 70 75 80

Val Val Ser Thr Asp Gln Glu Ser Ala Glu Glu Ile Pro Ile Leu Ile
85 90 95

Ile Glu Gly Phe Leu Leu Phe Asn Tyr Lys Pro Leu Asp Thr Ile Trp
100 105 110

Asn Arg Ser Tyr Phe Leu Thr Ile Pro Tyr Glu Glu Cys Lys Arg Arg
115 120 125

Arg Ser Thr Arg Val Tyr Gln Pro Pro Asp Ser Pro Gly Tyr Phe Asp
130 135 140

Gly His Val Trp Pro Met Tyr Leu Lys Tyr Arg Gln Glu Met Gln Asp
145 150 155 160

Ile Thr Trp Glu Val Val Tyr Leu Asp Gly Thr Lys Ser Glu Glu Asp
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Leu Phe Leu Gln Val Tyr Glu Asp Leu Ile Gln Glu Leu Ala Lys Gln
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Lys Cys Leu Gln Val Thr Ala
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<210> SEQ ID NO 6
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<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 6

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Thr Leu Thr Asn Ser Leu Leu Arg Ala Leu Pro Asn Cys Cys Val Ile
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His Gln Asp Asp Phe Phe Lys Pro Gln Asp Gln Ile Ala Val Gly Glu
35 40 45

Asp Gly Phe Lys Gln Trp Asp Val Leu Glu Ser Leu Asp Met Glu Ala
50 55 60

Met Leu Asp Thr Val Gln Ala Trp Leu Ser Ser Pro Gln Lys Phe Ala
65 70 75 80

Arg Ala His Gly Val Ser Val Gln Pro Glu Ala Ser Asp Thr His Ile
85 90 95

Leu Leu Leu Glu Gly Phe Leu Leu Tyr Ser Tyr Lys Pro Leu Val Asp
100 105 110

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Leu Tyr Ser Arg Arg Tyr Phe Leu Thr Val Pro Tyr Glu Glu Cys Lys
        115                 120                 125

Trp Arg Arg Ser Thr Arg Asn Tyr Thr Val Pro Asp Pro Pro Gly Leu
    130                 135                 140

Phe Asp Gly His Val Trp Pro Met Tyr Gln Lys Tyr Arg Gln Glu Met
145                 150                 155                 160

Glu Ala Asn Gly Val Glu Val Val Tyr Leu Asp Gly Met Lys Ser Arg
                165                 170                 175

Glu Glu Leu Phe Arg Glu Val Leu Glu Asp Ile Gln Asn Ser Leu Leu
            180                 185                 190

Asn Arg Ser Gln Glu Ser Ala Pro Ser Pro Ala Arg Pro Ala Arg Thr
        195                 200                 205

Gln Gly Pro Gly Arg Gly Cys Gly His Arg Thr Ala Arg Pro Ala Ala
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Ser Gln Gln Asp Ser Met
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Gly Lys Ser Thr Leu Cys Gln Leu Leu His Ala Ile Phe Glu Gly Ser
            20                  25                  30

Ser Leu Val His Glu Asp Asp Phe Tyr Lys Thr Asp Ala Glu Ile Pro
        35                  40                  45

Val Lys Asn Gly Ile Ala Asp Trp Asp Cys Gln Glu Ser Leu Asn Leu
    50                  55                  60

Asp Ala Phe Leu Glu Asn Leu His Tyr Ile Arg Asp His Gly Val Leu
65                  70                  75                  80

Pro Thr His Leu Arg Asn Arg Glu Asn Lys Asn Val Ala Pro Glu Ala
                85                  90                  95

Leu Ile Glu Tyr Ala Asp Ile Ile Lys Glu Phe Lys Ala Pro Ala Ile
            100                 105                 110

Pro Thr Leu Glu Gln His Leu Val Phe Val Asp Gly Phe Met Met Tyr
        115                 120                 125

Val Asn Glu Asp Leu Ile Asn Ala Phe Asp Ile Arg Leu Met Leu Val
    130                 135                 140

Thr Asp Phe Asp Thr Leu Lys Arg Arg Arg Glu Ala Arg Thr Gly Tyr
145                 150                 155                 160

Ile Thr Leu Glu Gly Phe Trp Gln Asp Pro Pro His Tyr Phe Glu Asn
                165                 170                 175

Tyr Val Trp Pro Gly Tyr Val His Gly His Ser His Leu Phe Val Asn
            180                 185                 190

Gly Asp Val Thr Gly Lys Leu Leu Asp Lys Arg Ile Gln Leu Ser Pro
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Ser Ser Lys Met Ser Val Arg Asp Asn Val Gln Trp Ala Ile Asn Ser
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Ile Leu Asn Ala Leu
225


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<213> ORGANISM: Saccharomyces cerevisiae

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Leu Ile Ser Leu Asp Asn Phe Tyr Asn Pro Leu Gly Pro Glu Asp Arg
35 40 45

Ala Arg Ala Phe Lys Asn Glu Tyr Asp Phe Asp Glu Pro Asn Ala Ile
50 55 60

Asn Leu Asp Leu Ala Tyr Lys Cys Ile Leu Asn Leu Lys Glu Gly Lys
65 70 75 80

Arg Thr Asn Ile Pro Val Tyr Ser Phe Val His His Asn Arg Val Pro
85 90 95

Asp Lys Asn Ile Val Ile Tyr Gly Ala Ser Val Val Val Ile Glu Gly
100 105 110

Ile Tyr Ala Leu Tyr Asp Arg Arg Leu Leu Asp Leu Met Asp Leu Lys
115 120 125

Ile Tyr Val Asp Ala Asp Leu Asp Val Cys Leu Ala Arg Arg Leu Ser
130 135 140

Arg Asp Ile Val Ser Arg Gly Arg Asp Leu Asp Gly Cys Ile Gln Gln
145 150 155 160

Trp Glu Lys Phe Val Lys Pro Asn Ala Val Lys Phe Val Lys Pro Thr
165 170 175

Met Lys Asn Ala Asp Ala Ile Ile Pro Ser Met Ser Asp Asn Ala Thr
180 185 190

Ala Val Asn Leu Ile Ile Asn His Ile Lys Ser Lys Leu Glu Leu Lys
195 200 205

Ser Asn Glu His Leu Arg Glu Leu Ile Lys Leu Gly Ser Ser Pro Ser
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Gln Asp Val Leu Asn Arg Asn Ile Ile His Glu Leu Pro Pro Thr Asn
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Gln Val Leu

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<212> TYPE: PRT
<213> ORGANISM: Escherichia coli

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Arg Leu Lys Gly Ile Asn Glu Asp Leu Ser Leu Glu Glu Val Ala Glu
35 40 45

Ile Tyr Leu Pro Leu Ser Arg Leu Leu Asn Phe Tyr Ile Ser Ser Asn
50 55 60

Leu Arg Arg Gln Ala Val Leu Glu Gln Phe Leu Gly Thr Asn Gly Gln
65 70 75 80

Arg Ile Pro Tyr Ile Ile Ser Ile Ala Gly Ser Val Ala Val Gly Lys
85 90 95

Ser Thr Thr Ala Arg Val Leu Gln Ala Leu Leu Ser Arg Trp Pro Glu
100 105 110

```
His Arg Arg Val Glu Leu Ile Thr Thr Asp Gly Phe Leu His Pro Asn
        115                 120                 125

Gln Val Leu Lys Glu Arg Gly Leu Met Lys Lys Lys Gly Phe Pro Glu
    130                 135                 140

Ser Tyr Asp Met His Arg Leu Val Lys Phe Val Ser Asp Leu Lys Ser
145                 150                 155                 160

Gly Val Pro Asn Val Thr Ala Pro Val Tyr Ser His Leu Ile Tyr Asp
                165                 170                 175

Val Ile Pro Asp Gly Asp Lys Thr Val Val Gln Pro Asp Ile Leu Ile
            180                 185                 190

Leu Glu Gly Leu Asn Val Leu Gln Ser Gly Met Asp Tyr Pro His Asp
        195                 200                 205

Pro His His Val Phe Val Ser Asp Phe Val Asp Phe Ser Ile Tyr Val
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<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic primer

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<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic primer

<400> SEQUENCE: 11

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gagagtgcac                                                            70


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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic primer

<400> SEQUENCE: 12

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<210> SEQ ID NO 13
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<212> TYPE: DNA
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<220> FEATURE:
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<400> SEQUENCE: 13

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<210> SEQ ID NO 14
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<212> TYPE: DNA
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<220> FEATURE:
<223> OTHER INFORMATION: Synthetic primer

<400> SEQUENCE: 14

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gagagtgcac 70

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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic primer

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tttcacaccg 70

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<220> FEATURE:
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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic primer

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<220> FEATURE:
<223> OTHER INFORMATION: Synthetic primer

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<220> FEATURE:
<223> OTHER INFORMATION: Synthetic primer

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<213> ORGANISM: Artificial Sequence
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tgg 63

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<400> SEQUENCE: 33

gctcgctcga gtcacatgct gtcctgctgg gac 33

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<220> FEATURE:
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<222> LOCATION: (1)..(2)
<223> OTHER INFORMATION: "Xaa" denotes an aliphatic amino acid residue
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<223> OTHER INFORMATION: "Xaa" denotes His or Ser
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<222> LOCATION: (4)..(4)
<223> OTHER INFORMATION: "Xaa" denotes a hydrophilic amino acid residue
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<223> OTHER INFORMATION: "Xaa" denotes an aromatic amino acid residue

<400> SEQUENCE: 34

Xaa Xaa Xaa Xaa Asp Asp Phe Xaa Lys
1 5

What is claimed is:

1. A pharmaceutical composition comprising nicotinamide riboside in admixture with a carrier, wherein said composition is formulated for oral administration.

2. The pharmaceutical composition of claim **1**, wherein the nicotinamide riboside is isolated from a natural or synthetic source.

3. The pharmaceutical composition of claim **1**, wherein the formulation comprises a tablet, troche, capsule, elixir, suspension, syrup, wafer, chewing gum, or food.

4. The pharmaceutical composition of claim **1**, further comprising one or more of tryptophan, nicotinic acid, or nicotinamide.

5. The pharmaceutical composition of claim **1** which increase NAD+ biosynthesis upon oral administration.

* * * * *

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 8,383,086 B2
APPLICATION NO. : 13/445289
DATED : February 26, 2013
INVENTOR(S) : Charles M. Brenner

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

On the Title Page:

At Item (57) in the Abstract:

Please delete "prvided."
Please insert --provided.--

In the claims:

In column 54, line 42, please delete "increase"
In column 54, line 42, please insert --increases--

Signed and Sealed this
Twenty-fourth Day of September, 2013

Teresa Stanek Rea
Deputy Director of the United States Patent and Trademark Office

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 8,383,086 B2
APPLICATION NO. : 13/445289
DATED : February 26, 2013
INVENTOR(S) : Charles M. Brenner

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

In the Specification

At Column 1, please delete Lines 14-16 and insert in its place the following:
--This invention was made with government support under grant number CA077738 awarded by the National Institutes of Health. The government has certain rights in the invention.--

Signed and Sealed this
Eleventh Day of February, 2020

Andrei Iancu
Director of the United States Patent and Trademark Office