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RESEARCH**

APPLICATION NUMBER:

203324Orig1s000

NON-CLINICAL REVIEW(S)

**DEPARTMENT OF HEALTH AND HUMAN SERVICES
PUBLIC HEALTH SERVICE
FOOD AND DRUG ADMINISTRATION
CENTER FOR DRUG EVALUATION AND RESEARCH**

PHARMACOLOGY/TOXICOLOGY NDA/BLA REVIEW AND EVALUATION

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Product: Riboflavin Ophthalmic Solution 0.12%/ KXL™
System
Indication:  (b) (4)
Applicant: Avedro Inc
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1 Executive Summary

1.1 Introduction

The subject of this NDA application is Riboflavin Ophthalmic Solution 0.12% (tentative trade name: Photrexa) as a topical ophthalmic crosslinking agent for use in combination with the KXL System, an ultraviolet A (UVA) emitting device which facilitates crosslinking of corneal collagen upon irradiation.

Upon administration of the riboflavin ophthalmic solution and exposure to UVA light, it is thought that cross-linking of corneal collagen fibrils in the stroma of the cornea results in increased tensile strength and diameter of the collagen fibrils. Such treatment may stabilize the corneal curvature in eyes with progressive ectatic conditions (e.g. keratoconus, ectasia following refractive surgery).

The applicant has submitted NDA 203324 as a 505(b)(2) application and will rely on published nonclinical data to support this application.

1.2 Brief Discussion of Nonclinical Findings

- All nonclinical safety and pharmacology data cited in the NDA are from published, publicly available research articles.
- The KXL System is not currently an FDA cleared device. The electronic system contains a light emitting diode (LED) used to deliver a dose of UVA light (370 nm; 3 mW/cm²).
- Riboflavin (Vitamin B2), an essential nutrient, is a water-soluble vitamin present in the cell as riboflavin phosphate (FMN; flavin mononucleotide) or flavin adenine dinucleotide (FAD). Approximately 70% of riboflavin is protein-bound. Free riboflavin forms the active site of electron transfer in a wide variety of redox reactions.
- Riboflavin is listed in the Inactive Ingredients Guide (IIG) for oral use and has been designated by FDA as Generally Recognized as Safe (GRAS). In addition, it has FDA approval as a component of multi-vitamins for intravenous therapeutic use for children and adults, e.g., M.V.I Pediatric® and M.V.I Adult™; Infuvite® Pediatric and Infuvite® Adult, at amounts of 1.4 and 3.6 mg/injection, respectively.
- Riboflavin increases the activity of UVA light to promote cross-linking and is to be used as a photosensitizer in the debrided cornea. Ocular riboflavin itself was not associated with any ocular toxicity though its inclusion in combination with UVA irradiation potentiated and increased the sensitivity of the corneal endothelium to cytotoxicity.
- The corneal endothelium, the most posterior layer of the cornea, is exposed to a lower UVA dose than what is applied to the surface of the debrided cornea due to absorption of UVA by the riboflavin saturated corneal stroma

- The surface UVA dose proposed for human use, 3 mW/cm², resulted in an endothelial dose found to cause endothelial toxicity in the rabbit. The applicant claims that the endothelial dose resulting from this surface dose will not cause endothelial toxicity in humans, because the resultant endothelial dose is lower in the human than in rabbit. This difference in the calculated endothelial doses between humans and rabbits is presumably based on differences in the absorption coefficient of the riboflavin treated corneas of humans and rabbits. No citation was provided (see Recommendations Section 1.3.1).
- A minimum corneal thickness of 400 μm was shown to be required for nontoxic effects on the corneal endothelium when exposed to the proposed UVA irradiation scheme.
- Photoactivated riboflavin (b) (4) showed genotoxic potential in the Ames and *umu* assays.

1.3 Recommendations

1.3.1 Approvability

- The application is approvable from a Pharmacology/Toxicology perspective.
- An Information Request (IR) was sent to the applicant on 2-19-2014 asking the following:
 - Regarding the nonclinical study cited to support the ocular safety of combined topical ocular riboflavin/UVA irradiation, the results suggest that a surface irradiation of 3 mW/cm² causes toxicity to the corneal endothelium in this rabbit model (Wollensak, G., et al., 2003, “Endothelial cell damage after riboflavin-ultraviolet-A treatment in the rabbit”, *J Cataract Surg*, 29: 1786). It appears that in the rabbit with cornea debridement and treated with topical riboflavin, a surface irradiation of 3 mW/cm² results in an endothelial dose of 0.36 mW/cm², which was shown to be cytotoxic. However the authors note that in the human eye, the 3 mW/cm² surface dose results in an endothelial dose of 0.18 mW/cm² which was not shown to cause endothelial toxicity. We are unable to use the formula provided to replicate the author’s calculations. Please provide line by line calculations that were used to estimate these endothelial doses. We realize these interspecies differences in corneal transmittance may be dependent on different absorption coefficients for the cornea. Please provide methods for how the absorbance coefficients were calculated, and the rationale regarding why they differ between species.
 - A brief review of the applicant’s response (eCTD024 dated 2-25-2014) to this IR is included in the discussion of the article by Wollensak discussed below.

1.3.2 Applicant’s proposed labeling (sections relevant to nonclinical Pharmacology/Toxicology)

8. Use in Specific Populations

8.1. Pregnancy

(b) (4)



12.1. Mechanism of Action

(b) (4)



13. Nonclinical Toxicology

13.1. Carcinogenesis, Mutagenesis, Impairment of Fertility

(b) (4)



(b) (4)

1.3.3 FDA's proposed changes to labeling (Redline version of sections relevant to nonclinical Pharmacology/Toxicology). Details regarding proposed changes are included in this review in the relevant sections. Suggested deletions are notated as a strikethrough font and suggested additions are notated as a thick underlined font (blue). [Recommendations to the label are tentative, as inclusion of additional text will depend on the adequacy of data we receive from the Sponsor in response to a communicated IR sent to them on 2-19-2014]

8. Use in Specific Populations

8.1. Pregnancy

(b) (4)
Animal development and reproduction studies have not been conducted with the (b) (4) system.
(b) (4) Since it is not known whether the corneal collagen cross-linking procedure can cause fetal harm or affect reproduction capacity, it should not be performed on pregnant women.

(b) (4)

[Redacted] (b) (4)

12.1. Mechanism of Action

[Redacted] (b) (4) is the [Redacted] (b) (4) precursor of two coenzymes, flavin adenine dinucleotide and flavin mononucleotide, which catalyze oxidation/reduction reactions involved in a number of metabolic pathways [Redacted] (b) (4)

Under the conditions used for corneal collagen cross-linking, [Redacted] (b) (4) and generates singlet oxygen which is responsible for the cross-linking.

13. Nonclinical Toxicology

13.1. Carcinogenesis, Mutagenesis, Impairment of Fertility

Carcinogenicity data on photoexcited riboflavin are not available. [Redacted] (b) (4)

[Large redacted area]

Photoexcited riboflavin has been shown to be genotoxic in the Ames Salmonella reverse mutation assay and in the SOS/umu test system. The genotoxicity of riboflavin in the absence of photoexcitation, has been examined *in vitro* in bacterial reverse mutation assays, sister chromatid exchange assay [Redacted] (b) (4) chromosomal

aberration assays, and *in vivo* in ^{(b) (4)} mouse micronucleus study. The overall weight of evidence indicates that riboflavin in the absence of photoexcitation is not genotoxic.

Animal studies to determine the effects of the corneal collagen cross-linking procedure on fertility were not conducted.

(b) (4)

2 Drug Information

2.1 Drug

CAS Registry Number: 83-88-5

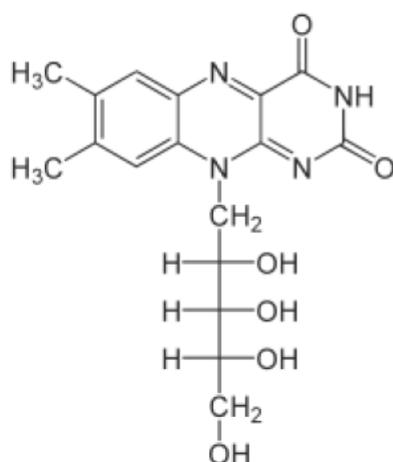
Generic Name: Riboflavin Ophthalmic Solution 0.12%

Code Name: Photrexa (proposed trade name)

Chemical Name: 7,8-dimethyl-10[(2S,3S,4R)-2,3,4,5-tetrahydroxypentyl]benzo[g]pteridine-2,4-dione

Molecular Formula/Molecular Weight: 376.36 g/mol

Structure :



Pharmacologic Class: B-vitamin; unestablished for ophthalmic use; potential photosensitizer

2.2 Relevant INDs, NDAs, BLAs and DMFs

Riboflavin has received FDA approval as a component of multi-vitamins for intravenous therapeutic use for children and adults, e.g., M.V.I Pediatric® and M.V.I Adult™; Infuvite® Pediatric and Infuvite® Adult, at amounts of 1.4 and 3.6 mg/injection, respectively.

2.3 Drug Formulation

| Ingredient | Formulation content | Function |
|--------------------------------|---------------------|-------------------|
| Riboflavin 5'-phosphate sodium | 0.12% | Active ingredient |
| Dextran 500 | | (b) (4) |
| Sodium chloride | | |
| Sodium phosphate, monobasic | | |
| Sodium phosphate, dibasic | | |
| Sterile water for injection | | |
| | | |

Another formulation of the drug product also may be used for the cross-linking procedure. The formulation is the same except that it does not contain the dextran 500 component and is currently referred to as Photrexa (b) (4). This solution is to be used at the end of the initial soaking period if corneal thickness is less than 400 microns. The prescriber is instructed to instill 2 drops of Photrexa (b) (4) every 5 to 10 seconds until the corneal thickness increases to at least 400 microns. Irradiation should not be performed unless this 400 micron threshold is met.

The average drop size for Photrexa is (b) (4) µL and for Photrexa (b) (4) it is (b) (4) µL (Amendment of 1/15/14). For the purpose of this review, the drop size will be assumed as 30 µL for Photrexa. If 31 drops are applied over the dosing period of 60 minutes, then the total volume applied is 0.93 mL. The total amount of riboflavin applied would be 1.12 mg based on 0.12% solution.*

***Reviewer's note:** For the purposes of this review, any calculations made concerning the riboflavin component of the drug product were made using the applicant's original calculation of 1.20 mg riboflavin 5' phosphate / mL of the drug product. However, the CMC reviewer noted in his review, "the active component is riboflavin 5'-phosphate sodium. The nonproprietary name should be "riboflavin phosphates ophthalmic solution 1.46 mg/g." Also the container label or PI should include the phrase "Each gram of solution contains 1.20 mg of riboflavin as a mixture of riboflavin, riboflavin phosphates, and riboflavin diphosphates".

2.4 Comments on Novel Excipients

Dextran 500 has not been previously qualified for topical ophthalmic use. Current over the counter formulations of artificial tears contain (b) (4) dextran (b) (4) (different polymer chain size). The ocular toxicity study provided by the applicant (Wollensak, G. *et al.*, 2003, "Endothelial cell damage after riboflavin-ultraviolet-A treatment in the

rabbit", *J Cataract Refract Surg*, 29: 1786 – 1790) while limited in scope to detect ocular toxicity endpoints reported no ocular toxicity to the cornea following treatment with a riboflavin solution containing 20% dextran (b) (4) at low UVA doses. At higher UVA doses, cytotoxic effects including corneal endothelial cell loss and apoptosis were attributed to photo-activated riboflavin. The published results of clinical trials of riboflavin/dextran (b) (4) submitted by the applicant (UVX-001, UVX-002, UVX-003) did not attribute any adverse events or toxicity to the dextran 500 component of the formulation at concentrations up to 20%. The excipient is considered qualified at 20% in the final formulation.

2.5 Comments on Impurities/Degradants of Concern

Information concerning residual solvents in the drug substance was presented by the Chemistry, Manufacturing, and Controls reviewer. In the DMF for the riboflavin drug substance (DMF (b) (4)), specification limits of (b) (4) ppm (parts per million) are set for two residual solvents, (b) (4)

Neither of these solvents is listed in the ICHQ3(c) tables/list to establish acceptable levels. Actual levels of these residual solvents were reported as (b) (4) ppm in the drug substance. For the drug product, containing 0.12% of the drug substance, concentrations of the residual solvents if present at the specification ((b) (4) ppm) would result in levels of the residual solvents in the drug product of up to (b) (4) ppm of the drug product. If the applied dose of the drug product is 1.12 mL (as 32 drops over 60 minutes assuming a 0.035 mL drop), then (b) (4) ppm of the drug product would calculate as (b) (4) µg of each residual solvent being applied over the dosing period of 60 minutes.

For comparative purposes, for those compounds which may be considered the most toxic Class 1 solvents in the ICHQ3(c) guidance (b) (4) residual levels considered acceptable when their use is unavoidable are (b) (4) ppm, respectively. Therefore, the specifications for the drug substance set by the DMF would, at maximum, result in levels lower than these Class 1 solvents in the final drug product. Acceptable residual solvent levels for Class 2 solvents considered inherently toxic range from (b) (4) ppm to (b) (4) ppm.

(b) (4)
The highest recommended oral dose is (b) (4) grams per day which exceeds potential exposure to the residual solvent in the (b) (4) drug product by a factor (b) (4)

For (b) (4) the U.S. National Toxicology Program describes the toxicity (b) (4) {"Toxicology and Carcinogenic Study (b) (4)" [National Toxicology Program (NTP)

(b) (4). Toxicology and Carcinogenesis Studies (b) (4) in F344fN1 Rats and B6C3F Mice (Gavage Studies). Technical Report Series (b) (4) NIH Publication (b) (4) U.S. Department of Health and Human Services, Public Health Service, National Institutes of Health, Research Triangle Park, NC}. A 13 - week toxicity study was performed in rats (b) (4) was administered in corn oil by gavage. No lesions or toxicity related to the administration (b) (4) occurred at doses up to 225 mg/kg in the rat. This dose represents a safety factor > 1-million over the maximum dose (b) (4) administered at the proposed specification .

Administration (b) (4) to rats at oral doses of up to 1,000 mg/kg/day for 83 (males) or 104 (females) weeks resulted in no increase in tumors. No evidence of carcinogenic activity in male or female rats was seen in 2-year carcinogenic studies (b) (4)

2.6 Proposed Clinical Population and Dosing Regimen

Riboflavin ophthalmic solution/KXL™ System is indicated (b) (4)

The labeling describes the dosing regimen:

Using topical anesthesia, debride the epithelium using standard aseptic technique. Post epithelial debridement, instill 1 drop of (b) (4) topically in the eye every 2 minutes for 30 minutes. At the end of the 30 minute soaking period, examine the eye under the slit lamp for the presence of a yellow flare in the anterior chamber. If the yellow flare is not detected, instill 1 drop of (b) (4) every 2 minutes for an additional 2 to 3 drops and recheck for the presence of a yellow flare. This process can be repeated as necessary. Once the yellow flare is observed, perform ultrasound pachymetry. If the corneal thickness is less than 400 microns as measured by an ultrasound pachymeter, instill 2 drops of (b) (4) every 5 to 10 seconds until the corneal thickness increases to at least 400 microns. Irradiation should not be performed unless this 400 micron threshold is met. Irradiate the eye for 30 minutes at 3mW/cm² using the KXL System as per the instructions in the KXL manual. During irradiation, continue topical instillation of (b) (4) onto the eye every 2 minutes for the 30 minute irradiation period. Please refer to the KXL manual for specific device instructions.

3 Studies Submitted

3.1 Studies Reviewed

Pharmacology

- Spoerl, E., *et al.*, 1998, "Induction of cross-links in corneal tissue", *Exp Eye Res*, 66(1): 97 – 103.
- Wollensak, G., *et al.*, 2004, "Collagen fiber diameter in the rabbit cornea after collagen crosslinking by riboflavin/UVA", *Cornea*, 23: 503 – 507.

- Bottós, K., *et al.*, 2008, “Immunofluorescence confocal microscopy of porcine corneas following cross-linking treatment with riboflavin and ultraviolet A”, *J Refract Surg*, 24: S715 – S719
- Hayes, S., *et al.*, 2008, “Effect of complete epithelial debridement before riboflavin–ultraviolet-A corneal collagen crosslinking therapy”, *J Cataract Refract Surg*, 34: 657–661

Pharmacokinetics

- Akimoto, M., *et al.*, 2007, “Pharmacokinetic profile of flavin adenine dinucleotide, flavin mononucleotide and riboflavin following intravenous administration or its coenzymes in rats”, *J Health Sci*, 53(3): 332 – 338.
- Yang, C., and D. McCormick, 1967, “Degradation and excretion of riboflavin in the rat”, *J Nutr*, 93: 445 – 453.
- Ono, S., and H. Hirano, 1983, “Riboflavin metabolism in the single lens of rat”, *Ophthalmic Res*, 15: 140 – 145.
- Hirano, H., *et al.*, 1988, “Riboflavin metabolism in the optic nerve of albino rabbit”, *Int J Vitam Nutr Res*, 58(4): 470.
- Spoerl, E., *et al.*, “Safety of UVA-riboflavin cross-linking of the cornea”, *Cornea*, 26: 385 – 389.
- Ohkawa, H., *et al.*, 1983, “New metabolites of riboflavin appeared in rat urine”, *Biochem Int*, 6(2): 239 – 247.
- Unna, K. and J. Greslin, 1942, “Studies on the toxicity and pharmacology of riboflavin”, *J Pharmacol Exp Ther*, 76(1): 75 – 80.

Toxicology

Systemic toxicity

- Unna, K., and J. Greslin, 1942, “Studies on the toxicity and pharmacology of riboflavin”, *J Pharmacol Exp Ther*, 76(1): 75 – 80.
- Apeldoorn, M., *et al.*, 1999, “WHO Food Additive Series 42: Safety evaluation of certain food additives”, Prepared by the Fifty-first meeting of the Joint FAO/WHO Expert Committee on Food Additives (JECFA).

Ocular Toxicity

- Wollensak, G. *et al.*, 2003, “Endothelial cell damage after riboflavin-ultraviolet-A treatment in the rabbit”, *J Cataract Refract Surg*, 29: 1786 – 1790.
- Wollensak, G. *et al.*, 2004, “Keratocyte cytotoxicity of riboflavin/UVA-treatment *in vitro*”, *Eye*, 18: 718 – 722.

Genotoxicity

(b) (4)

- Ishidate, M., *et al.*, 1988, “A comparative analysis of data on the clastogenicity of 951 chemical substances tested in mammalian cell cultures”, *Mutat Res*, 195: 151 – 213.
- Hayashi, M., *et al.*, 1988, “Micronucleus tests in mice on 39 food additives and eight miscellaneous chemicals”, *Food Chem Toxicol*, 26(6): 487 – 500.

Carcinogenicity

- Pacernick, L., *et al.*, 1975, “The inefficacy of riboflavin against ultraviolet-induced carcinogenesis”, *J Invest Dermatol*, 65: 547 – 548.
- Roe, F., 1962, “Effect of massive doses of riboflavin, and other vitamins of the B group, on skin carcinogenesis in mice”, *Br J Cancer*, 16(2): 252 – 257.

Reproductive Toxicology

- Unna, K., and J. Greslin, 1942, “Studies on the toxicity and pharmacology of riboflavin”, *J Pharmacol Exp Ther*, 76(1): 75 – 80.

3.2 Studies Not Reviewed

In the nonclinical overview and list of nonclinical references, the applicant described or included many published articles which were found to be irrelevant to characterizing the nonclinical safety or nonclinical pharmacokinetics of topical ophthalmic riboflavin.

These studies are listed below with a short explanation describing reason for irrelevance to nonclinical safety and pharmacology.

Pharmacology

- Abalain, J., *et al.*, 2000, “Levels of collagen degradation products (telopeptides) in the tear film of patients with keratoconus”, *Cornea*, 19(4): 474 – 476.
 - Human proof of concept data
- Anderson, K., *et al.*, 2004, “Application of structural analysis to the mechanical behavior of the cornea”, *J R Soc Interface*, 1(1): 3 – 15.
 - Proof of concept describing models of corneal biomechanics (no riboflavin/UVA treatment)
- Anderson, O., *et al.*, 2008, “Human transscleral albumin permeability and the effect of topographical location and donor age”, *Invest Ophthalmol Vis Sci*, 49(9): 4041 – 4045)
 - Human proof of concept regarding scleral permeability (no riboflavin/UVA treatment)
- Hayes, S., *et al.*, 2007, “A study of corneal thickness, shape and collagen organisation (sic) in keratoconus using videokeratography and X-ray scattering techniques”, *Exp Eye Res*, 84: 423 – 434
 - Human pathology characterization

- Holmes, D., *et al.*, 2001, “Corneal collagen fibril structure in three dimensions: Structural insights into fibril assembly, mechanical properties, and tissue organization”, *PNAS*, 98(13): 7307 – 7312.
 - Proof of concept regarding corneal collagen structure; no riboflavin/UVA treatment
- Krueger, R. *et al.*, 2008, “Rapid vs Standard Collagen CXL with Equivalent Energy Dosing”
 - Powerpoint presentation of techniques and data
- Lee, J., *et al.*, 2001, “Cure depth in photopolymerization: Experiments and theory”, *J Mater Res*, 16(12): 3536 – 3544
 - Proof of concept; photocuring in a model resin system, no ophthalmology, riboflavin/UVA treatment
- Massad, W., *et al.*, 2004, “Kinetics and mechanism of vitamin B₂-sensitized photooxidation of isoproterenol”, *Photochem Photobiol*, 79(5): 428 – 433
 - Proof of concept: Kinetics of riboflavin mediated oxidation (not ophthalmic)
- Maurice, D., 1960, “The movement of fluorescein and water in the cornea”, *Am J Ophthalmol*, 49: 1011 – 1016.
 - Human physiology; no UVA/riboflavin treatment
- Meek, K., *et al.*, 2005, “Changes in collagen orientation and distribution in keratoconus corneas”, *Invest Ophthalmol Vis Sci*, 46: 1948 – 1956.
 - Proof of concept; human disease pathology
- Raiskup-Wolf, F., *et al.*, 2008, “Collagen crosslinking with riboflavin and ultraviolet –A light in keratoconus: Long term results”, *J Cataract Refract Surg*, 34: 796 – 801
 - Human efficacy data
- Roizenblatt, R., 2010, “Comparison study of ultraviolet A irradiance of 3mW/cm² versus 9mW/cm² with riboflavin on corneal collagen cross- linking efficacy in rabbit eyes”
 - Abstract/Poster
- Schein, O., *et al.*, 2001, “Patient outcomes of refractive surgery. The refractive status and vision profile”, *J Cataract Refract Surg*, 27(5): 665 – 673
 - Human efficacy and pathology
- Seiler, T., *et al.*, 2000, “Manifest diabetes and keratoconus: A retrospective case-control study”, *Graefes Arch Clin Exp Ophthalmol*, 238(10): 822 – 825
 - Human efficacy and pathology
- Sliney, 2010, “Optical radiation safety of the Avedro KXL System and accelerated cross-linking procedure”,
 - Consult on device safety and human hazards; review
- Spoerl, E., *et al.*, 2004, “Increased resistance of crosslinked cornea against enzymatic degradation”, *Curr Eye Res*, 29(1): 35 – 40.
 - Proof of concept not relevant to current indication
- Spoerl, E., *et al.*, 2004, “Thermomechanical behavior of collagen cross linked porcine cornea”, *Ophthalmologica*, 218(2): 136 – 140
 - Proof of concept; thermomechanics of cross-linked collagen
- Sung, H., *et al.*, 2003, “Crosslinking of biological tissues using genipin and/or carbodiimide”, *J Biomed Mater Res A*, 64(3): 427 – 438

- Proof of concept; abstract only

Pharmacokinetics

- Araie, M., and D. Maurice, 1987, “The rate of diffusion of fluorophores through the corneal epithelium and stroma”, *Exp Eye Res*, 44: 73 – 87
 - Absorption study (no UVA or riboflavin treatment)
- Axelrod, A., *et al.*, 1941, “A study of urinary riboflavin excretion in man”, *J Clin Invest*, 20(2): 229 – 32
 - Excretion study in humans
- Bates, C., 1997, “Bioavailability of riboflavin”, *Eur J Clin Nutr*, 51(Suppl1): S38 – S42.
 - Review human data, dietary riboflavin
- Stripp, B., 1965, “Intestinal absorption of riboflavin by man”, *Acta Pharmacol et Toxicol*, 22: 353 – 362.
 - Human PK data
- Lee, S., and D. McCormick, 1983, “Effect of riboflavin status on hepatic activities of flavin-metabolizing enzymes rats”, *J Nutr*, 113: 2274 – 2279
 - Effect of dietary riboflavin content on riboflavin metabolizing enzyme activity
- Zempleni, J., *et al.*, 1996, “Pharmacokinetics of orally and intravenously administered riboflavin in healthy humans”, *Am J Clin Nutr*, 63: 54 – 66.
 - Human PK data
- Ohkawa, H., *et al.*, 1983, “New metabolites of riboflavin appear in human urine”, *J Biol Chem*, 258(9): 5623 – 5628.
 - Human PK data
- Innami, S. and O. Mickelsen, 1969, “Clinical nutrition: Riboflavin-transport and excretion”, *Nutr Rev*, 27: 285.
 - Editorial review of human data
- Ramakrishnan, P. and U. Sheth, 1977, “Serum flavin levels and urinary excretion of riboflavin and riboflavin tetrabutryate – a comparative evaluation”, *Indian J Med Res*, 66(4):618 – 626.
 - Human PK data
- Gastaldi, G., *et al.*, 1999, “Energy depletion differently affects membrane transport and intracellular metabolism of riboflavin taken up by isolated rat enterocytes”, *J Nutr*, 129(2):406 – 409.
 - Characterization of the transport mechanism of riboflavin across the intestinal epithelia, not considered relevant
- Christiansen, S., 1973, “The biological fate of riboflavin in mammals: A survey of literature and own investigations”, *Acta Pharma Toxicol*, 32(Suppl2): 3 – 72
 - Review article
- Criado, S., and N. Garcia, 2004, “Vitamin B2-sensitized photooxidation of the ophthalmic drugs Timolol and Pindolol: kinetics and mechanism”, *Redox Rep*, 9(5): 291 – 297
 - Drug-drug interaction study

- de La Rochette, A., *et al.*, 2003, “Advanced glycation endproducts as UVA photosensitizers of tryptophan and ascorbic acid: consequences from the lens”, *Biochim Biophys Acta*, 1621(3): 235 – 241.
 - Effect of UVA on lens constituents (riboflavin not studied)
- Levy, G., and W. Jusko, 1996, “Factors affecting absorption of riboflavin in man”, *J Pharm Sci*, 55(3): 285 – 289
 - Pharmacokinetics in man
- Nagataki, S., *et al.*, 1983, “The diffusion of fluorescein in the stroma of rabbit cornea”, *Exp Eye Res*, 36: 764 – 771
 - Rabbit physiology; no riboflavin/UVA treatment
- Ono, S., 1987, “Riboflavin metabolism in the lens”, *Vitamins*, 61(4): 135 – 146
 - Article in Japanese
- Ota, Y., *et al.*, 1974, “Endothelial permeability of the living cornea to fluorescein”, *Invest Ophthalmol*, 13(12): 945 – 949
 - Human and rabbit physiology; no riboflavin/UVA treatment
- Patel, R., *et al.*, 1977, “Pharmacokinetics and pharmacology of riboflavin tetrabutryate (Lipabol) in animals”, *The Medicine & Surgery*, April 1977
 - Animal systemic PK study with other molecular entity
- Ramakrishnan, P. and U. Sheth, 1977, “Serum flavin levels and urinary excretion of riboflavin and riboflavin tetrabutryate – a comparative evaluation”, *Indian J Med Res*, 66(4): 618 – 626
 - Human pharmacokinetic study
- Reddi, A., *et al.*, 1990, “Riboflavin excretion in normal and diabetic rats”, *Int J Vitam Nutr Res*, 60(3): 252 – 254.
 - Comparative PK study in rats, no ophthalmic endpoints
- Roth-Maier, D., *et al.*, 2000, “Investigations on the effect of maternal dietary riboflavin supplementation during gravidity and lactation on riboflavin concentrations in blood, liver, and total body of rat offspring”, *J Anim Physiol Anim Nutr*, 83: 158 – 167
 - Dietary riboflavin pharmacokinetic study in pregnant rats

Toxicology

- Sugiyama, M., *et al.*, 1992, “Potentiation of sodium chromate(VI)-induced chromosomal aberrations and mutation by vitamin B₂ in Chinese hamster V79 cells”, *Mutat Res*, 283: 211 – 214.
 - Effect of riboflavin on genotoxicity of chromate compounds

4 Pharmacology

4.1 Primary Pharmacology

Pharmacology

Under physiological conditions, riboflavin acts as the parent molecule of two coenzymes, flavin adenine dinucleotide and flavin mononucleotide, which catalyze specific oxidation/reduction reactions in the body including glucose oxidation, amino acid deamination, and fatty acid breakdown.

(b) (4)

Riboflavin displays a biphasic absorption spectrum with peak absorption occurring at 370 nm and 465 nm. Once excited by UVA irradiation, riboflavin can elicit either a Type I or Type II photochemical reaction. In a Type I reaction, there is a direct electron transfer between the substrate (e.g., lysine bond within collagen fibril) and the excited riboflavin followed by oxidation. In a Type II photochemical reaction, energy transfer occurs between the excited riboflavin and molecular oxygen which produces singlet oxygen that then interacts with a substrate (e.g. collagen fibrils) in an oxidation type reaction to form physical cross-links. It is believed that both Type I and Type II mechanisms play a role in the collagen cross-linking process (Wollensak, G., *et al.*, 2003, [Treatment of keratoconus with collagen crosslinking]., *Ophthalmologe*, 100(1): 44 – 49.; De La Rochette, A., *et al.*, 2003, “Advanced glycation endproducts as UVA photosensitizers of tryptophan and ascorbic acid: consequences for the lens”, *Biochim Biophys Acta*, 1621(3): 235 – 241].

The sponsor cites a study which concluded that the stress/strain relationship of corneal collagen was not significantly altered by exposure to riboflavin alone while exposure to UVA alone tended to increase the amount of stress required to induce a given amount of strain, although this difference did not reach statistical significance ($p > 0.05$). Exposure to 436 nm UVA/riboflavin caused a weakly significant increase in stress ($p < 0.05$) and exposure to 365 nm UVA/riboflavin ($p < 0.0001$) or simulated sunlight/riboflavin ($p < 0.001$) caused highly significant increases in stress at strains of 4, 6, and 8%. A similar relationship was observed with porcine and human corneal tissue samples treated with UVA (370 nm, 3mW/cm², 30 minutes) and riboflavin (0.1% in Dextran T-500 20%) (Wollensak, G., *et al.*, 2003, “Stress-strain measurements of human and porcine corneas after riboflavin-ultraviolet-A-induced cross-linking”, *J Cataract Refract Surg*, 29(9): 1780 – 1785).

Reviewer’s note: The applicant’s statement [REDACTED] (b) (4) is supported by extensive literature and known chemical properties of riboflavin. No alternate hypotheses regarding the mechanism of riboflavin/UVA treatment on corneal collagen were found.

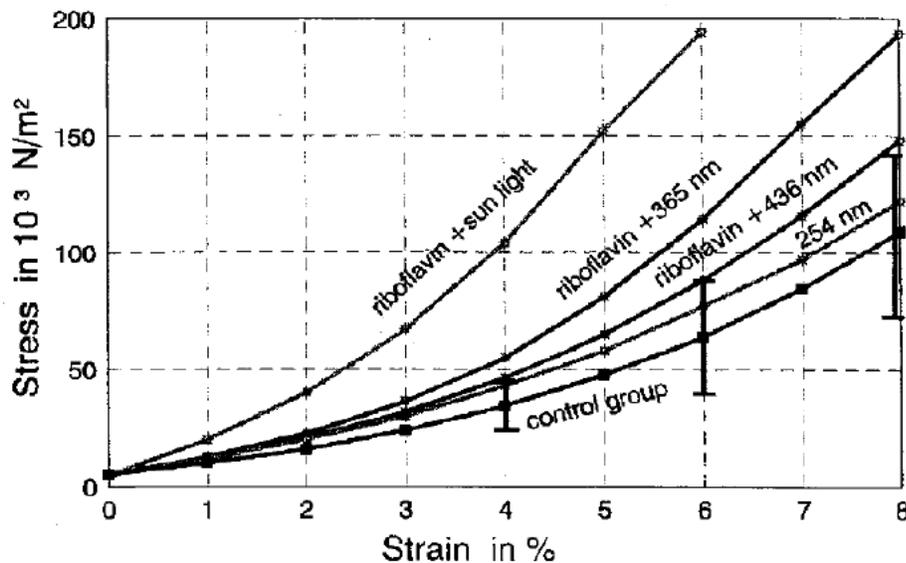
Spoerl, E., *et al.*, 1998, “Induction of cross-links in corneal tissue”, *Exp Eye Res*, 66(1): 97 – 103.

This study investigated the induction of cross-links in corneal tissue of debrided, enucleated porcine eyes. Groups of eyes (n=10/group) were as follows:

| Group | Irradiation | Corneal treatment |
|-------|-----------------------|----------------------------|
| 1 | 254 nM, 20 minutes | None |
| 2 | 365 nm, 45 minutes | Riboflavin 0.5%, 45 min |
| 3 | 436 nM, 45 minutes | Riboflavin 0.5%, 45 min |
| 4 | Sunlight, 120 minutes | Riboflavin 0.5%, 45 min |
| 5 | None | Riboflavin 0.5%, 45 min |
| 6 | None | Glutaraldehyde 1%, 10 min |
| 7 | None | Glutaraldehyde 1%, 10 min |
| 8 | None | Karnovsky solution, 10 min |
| 9 | None | None |

Strips of cornea were tested for stress-strain behavior to assess the cross-linking process. Compared to untreated corneas, treatment with riboflavin and UV-irradiation resulted in an increased stress required to induce 4, 6, and 8% strain in the corneas (Figure 1).

Figure 1. Influence of UV-radiation and riboflavin on the stress-strain relation of the cornea



Wollensak, G., et al., 2004, "Collagen fiber diameter in the rabbit cornea after collagen crosslinking by riboflavin/UVA", *Cornea*, 23: 503 – 507.

In this study, the corneas of the right eyes of 10 New Zealand White albino rabbits were cross-linked by topical application of riboflavin and exposure to UVA light (370 nm, 3 mW/cm²) to the debrided cornea for 30 minutes. The riboflavin solution (0.1% riboflavin-

5-phosphate and 20% dextran T-500) was dropped onto the cornea 5 minutes before the irradiation and every 5 minutes during the irradiation.

The left fellow control eyes were either left untreated (rabbits 1-4), debrided (rabbits 5-7), or debrided and treated with riboflavin/dextran solution (rabbits 8-10) to exclude an influence of epithelial debridement or hydration changes on the fiber diameter.

In the anterior stroma, the collagen fiber diameter in the treated corneas was significantly increased by 12.2% (3.96 nm), and in the posterior stroma by 4.6% (1.63 nm), compared with the contralateral eyes. In UVA/riboflavin treated eyes, the collagen fiber diameter was also significantly increased by 9.3% (3.1 nm) in the anterior compared with the posterior stroma within the same eye.

Bottós, K., et al., 2008, “Immunofluorescence confocal microscopy of porcine corneas following cross-linking treatment with riboflavin and ultraviolet A”, *J Refract Surg*, 24: S715 – S719

This study assessed the ultrastructural stromal modifications in porcine corneas after riboflavin and UVA irradiation using immunofluorescence confocal imaging.

Freshly enucleated porcine eyes were studied as follows:

- Control (n=5)
- Epithelium removed and cross-linked with riboflavin 0.1% solution (20% dextran-T-500) and exposed to UVA (365 nm, 3 mW/cm²) for 30 minutes
- Intact epithelium and cross-linked with riboflavin 0.1% solution (20% dextran-T-500) and exposed to UVA (365 nm, 3 mW/cm²) for 30 minutes (n=5).
- Epithelium removed and soaked with riboflavin without irradiation (n=5)
- Epithelium removed and eyes irradiated without exposure to riboflavin

Sections of the corneas were stained with anti-collagen I. Only the cross-linked corneas (riboflavin and UVA, second group) showed a pronounced, highly organized anterior zone of organized collagen fibers of 182.5±22.5 μm. Using 4',6-diamidino-2-phenylindole (DAPI) staining, an anterior and concentrated displacement of cell nuclei due to collagen compaction was observed after crosslinking. Treatment of the cornea with riboflavin and UVA without previous de-epithelialization did not induce any cross-linking effect. No structural changes were observed in all other groups.

Hayes, S., et al., 2008, “Effect of complete epithelial debridement before riboflavin–ultraviolet-A corneal collagen crosslinking therapy”, *J Cataract Refract Surg*, 34: 657–661

This study evaluated removal of the corneal epithelium before riboflavin–UVA crosslinking therapy. Riboflavin (10 mg/mL in 20% dextran T-500) was applied at 5-minute intervals for 35 minutes to the corneal surface of porcine eyes (12 with no epithelial removal, 12 with superficial epithelial removal but with an intact basal epithelium, and 12 with a fully removed epithelium). The corneal surface was then exposed to UVA light for 30 minutes during riboflavin administration. The light

transmission spectra of the enucleated corneas were analyzed with a spectrophotometer and compared with those of 9 untreated porcine corneas.

Corneas with a fully removed epithelium and treated with riboflavin showed an abnormal dip in the transmission spectrum between 400 nm and 510 nm ($p < .01$). This was attributed to the presence of riboflavin in the corneal stroma. The spectra of riboflavin-treated corneas with intact epithelium and those with superficial epithelial removal did not differ from those of the non-riboflavin-treated controls. Exposure to UVA following riboflavin administration did not alter corneal light transmission. The authors conclude that complete removal of the corneal epithelium is necessary for penetration of riboflavin into the corneal stroma.

4.2 Safety Pharmacology

Riboflavin is considered a GRAS substance for dietary consumption. No safety pharmacology studies were submitted by the applicant.

5 Pharmacokinetics/ADME/Toxicokinetics

5.1 PK/ADME

Ocular Pharmacokinetics

Ono, S., and H. Hirano, 1983, "Riboflavin metabolism in the single lens of rat", *Ophthalmic Res*, 15: 140 – 145.

The uptake of ^{14}C -labeled riboflavin and synthesis of ester forms of riboflavin over 60 minutes were assayed in the isolated whole lens of male Wistar rats. Uptake was dose proportional and reached a plateau at 60 minutes. Approximately 3% of the ^{14}C -riboflavin was bound to lens protein. Within 15 minutes, approximately 35% of the transported riboflavin was converted to ester forms flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD). These results suggest that flavokinase and flavin adenine dinucleotide pyrophosphorylase which convert riboflavin to FMN and FAD, respectively, are active in the lens of rats. In the presence of metabolic inhibitors, uptake into the lens was not inhibited suggesting that this process was not energy dependent.

Hirano, H., et al., 1988, "Riboflavin metabolism in the optic nerve of albino rabbit", *Int J Vitam Nutr Res*, 58(4): 470.

The binding and metabolism of riboflavin in isolated optic nerve tissue of rabbits was examined. Results suggest the presence of riboflavin metabolizing enzymes flavokinase and flavin adenine dinucleotide pyrophosphorylase since FMN and FAD were synthesized. Results also suggest the riboflavin binds to albino rabbit optic nerve protein (Table 1).

Table 1 Riboflavin metabolism in the optic nerve of normal albino rabbits

| | | |
|---|-----|---------------|
| Ester forms of riboflavin (nmol/g wet weight/hr) | | 2.019 ± 0.189 |
| Ester fractions | FAD | 14.7% |
| | FMN | 85.3% |
| Riboflavin binding capacity (nmol/g wet weight) | | 0.028 ± 0.005 |

Spoerl, E., et al., “Safety of UVA-riboflavin cross-linking of the cornea”, *Cornea*, 26: 385 – 389.

This published article used available data to discuss the parameters needed for safe diffusion of riboflavin into the cornea following topical application. Riboflavin must reach the stroma of the cornea to facilitate cross-linking of the collagen fibrils present there. The authors note that the cornea must be debrided of the most anterior epithelial layer to facilitate absorption to the deeper layers of the cornea and aqueous humor. The authors describe the penetration of riboflavin into the cornea as a concentration- and time-dependent event that saturates at concentrations of greater than 0.04%. The authors described a rabbit study which showed that thirty minutes after riboflavin application onto the de-epithelialized cornea, an absorption coefficient of 0.7 cm^{-1} was measured at the corneal endothelium and lens resulting in a concentration of 0.002% riboflavin in the aqueous humor.

Systemic Pharmacokinetics

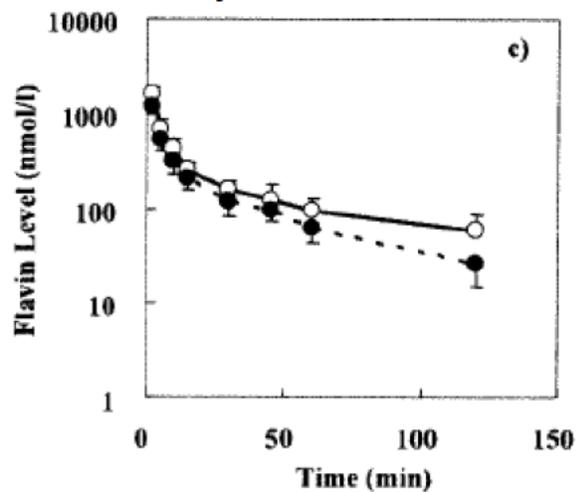
Absorption

Akimoto, M., et al., 2007, “Pharmacokinetic profile of flavin adenine dinucleotide, flavin mononucleotide and riboflavin following intravenous administration or its coenzymes in rats”, *J Health Sci*, 53(3): 332 – 338.

The pharmacokinetics of intravenously administered riboflavin was assessed in rats. Wister Hannover GALAS outbred rats were injected with 376 ng/kg (500 nmol/kg) riboflavin. Serial blood samples were taken into syringes containing EDTA to prevent hydrolysis of riboflavin. EDTA-treated plasma was analyzed for riboflavin, FMN and FAD content. Endogenous levels of each compound were subtracted from the concentration values obtained. Pharmacokinetic results are shown in Table 2. The authors express C_{max} as C_0 as apparently levels of riboflavin decrease immediately following intravenous injection. Elimination of riboflavin from plasma appeared to be biphasic with a rapid phase followed by a slower terminal phase (Figure 2).

Table 2. Pharmacokinetic parameters of riboflavin after intravenous administration in rats

| C_0 | C_{max} | AUC (nmol·min/L) | CL (L/min/kg) | MRT (min) | V_{dss} (L/kg) | $T_{1/2}$ (min) |
|-----------------|-----------|---------------------|------------------|--------------|---------------------|--------------------|
| 1906.3 ± 377 | - | 24469.6 ± 6121.9 | 0.022 ± 0.01 | 43.5 ± 5.7 | 0.94 ± 0.22 | 49.6 ± 9.8 |

Figure 2. Total flavins measured with two different techniques following intravenous injection of riboflavin in rats

Distribution

Yang, C., and D. McCormick, 1967, "Degradation and excretion of riboflavin in the rat", *J Nutrition*, 93: 445 – 453.

The distribution and excretion of ^{14}C -riboflavin following intraperitoneal injection was determined in male Sprague Dawley rats. After 24 hours, distribution and excretion in the urine and feces were determined (Table 3). Intact riboflavin was found predominantly in the liver >> kidney = small intestine>>>blood. A large percentage was also found outside of the blood indicating riboflavin is predominantly distributed to peripheral tissues. Excreted riboflavin accounted for ~13% of the administered dose at 24 hours and was predominantly found in urine as opposed to feces. Further experiments determined the half-life to be approximately 16 days following intraperitoneal injection.

Table 3 Distribution of radioactivity 24 hours after administration of ¹⁴C-riboflavin

| Tissue | % ¹⁴ C-riboflavin |
|---------------------------------------|------------------------------|
| Liver | 27.86 |
| Kidney | 4.32 |
| Small intestine (minus contents) | 4.57 |
| Contents of small intestine | 1.50 |
| Cecum and large intestine | 1.82 |
| Contents of cecum and large intestine | 0.49 |
| Blood | 0.02 |
| Rest of carcass | 40.3 |
| Urine | 10.17 |
| Feces | 2.89 |
| Total | 93.89 |

Metabolism/Excretion

Ohkawa, H., et al., 1983, "New metabolites of riboflavin appeared in rat urine", *Biochem Int*, 6(2): 239 – 247.

While other publications suggested that riboflavin is excreted intact into the urine, the authors of this study note that some riboflavin derivatives with modified isoalloxazine ring could be excreted in the urine and remain undetected by former techniques. In this study, male Wistar rats were orally administered ¹⁴C-riboflavin (37.2 µg/rat) and urine was collected for 24 hours following administration. Chromatographic separation of the flavin metabolites in urine revealed two compounds, 7-carboxy lumichrome and 8-carboxy lumichrome, as metabolites of riboflavin. The distribution of radioactivity of sum 7-carboxy lumichrome and 8-carboxy lumichrome, riboflavin, chloroform-soluble compounds and other metabolites was estimated to be 46, 30.5, 0.5, and 22.7 percent of total radioactivity found in the urine, respectively.

Photodegradation of riboflavin

The sensitivity of riboflavin to photodegradation has been extensively studied. The photolysis of riboflavin in aqueous solution is thought to occur (b) (4)

The potential genotoxicity (b) (4) are discussed below.

Pharmacokinetics/ADME Summary

It should be noted that full ocular tissue distribution studies of riboflavin were not submitted by the applicant, so it remains unknown whether topically applied riboflavin to the debrided cornea facilitates deeper penetration of riboflavin to more posterior portions of the eye.

6 General Toxicology

Systemic Toxicity

(b) (4)

Unna, K., and J. Greslin, 1942, “Studies on the toxicity and pharmacology of riboflavin”, *J Pharmacol Exp Ther*, 76(1): 75 – 80.

In this report, the authors determined the oral, subcutaneous and intraperitoneal toxicity of riboflavin in rats weighing 150 – 200 grams (strain not reported). An oral dose of 10 grams/kg (10,000 mg/kg) showed no signs of toxicity. Subcutaneous injection of riboflavin in doses of 5 grams/kg (5000 mg/kg) produced no toxic effects. An LD₅₀ of 560 mg/kg in the rat was reported following intraperitoneal injection.

Toxic doses resulted in a period of anuria for 1 or 2 days, with only scant amounts of deep yellow colored urine excreted after this period. Rats were listless, refused food, and lost weight. Tremors were frequently observed. Death occurred within 2 to 5 days. On autopsy, the kidneys appeared mottled brownish yellow in color and contained bright yellow crystals in the collecting tubules and the pelvis.

Appeldorn, M., et al., 1999, “WHO Food Additive Series 42: Safety evaluation of certain food additives”, Prepared by the Fifty-first meeting of the Joint FAO/WHO Expert Committee on Food Additives (JECFA).

The authors report LD₅₀ values of > 40,000 mg/kg for riboflavin in acute oral toxicity studies in the mouse and >20,000 mg/kg for riboflavin in acute toxicity studies in the rat. Following subcutaneous injection in the rat, a LD₅₀ of 5000 mg/kg was reported for riboflavin (mouse data not reported). Following intraperitoneal injection, an LD₅₀ of 890 mg/kg and 560 mg/kg (riboflavin phosphate) were reported for the mouse and rat, respectively. Following intravenous injection, LD₅₀ values for the mouse of 560 mg/kg (riboflavin) and 780 mg/kg (riboflavin phosphate) for the rat were reported, respectively.

The authors cite unpublished data as the source (Bächtold, H., 1980, “Acute toxicity of riboflavin, some intermediates and by-products of the synthesis, degradation products and metabolites. Unpublished report No. 9024 from F. Hoffmann La Roche dated 16 June 1980”. Submitted to WHO by F. Hoffmann La Roche Ltd, Basel, Switzerland.

Reviewer's note:

(b) (4)

[Redacted]

(b) (4)

[Redacted]

(b) (4)

[Redacted]

Apeldoorn, M., *et al.*, 1999, "WHO Food Additive Series 42: Safety evaluation of certain food additives", Prepared by the Fifty-first meeting of the Joint FAO/WHO Expert Committee on Food Additives (JECFA).

The authors describe a GLP 13-week repeat oral dietary dose study of riboflavin in detail:

| | |
|----------------------------|--------------------|
| GLP compliance: | Yes |
| QA statement: | Yes |
| Drug, lot #, and % purity: | 99.4 – 100% purity |

Key Study Findings

Methods

Doses: 0 (control), 20, 50 or 200 mg/kg
Frequency of dosing: Daily for 13 weeks (*ad libitum*)
Route of administration: Oral/dietary
Formulation: Three different synthesis methods were compared, complete details of results for each were not given. The most conservative result (i.e. lowest dose producing effect) is reported in this review.
Species/Strain: Wistar rat
Number/Sex/Group: 10/sex/group
Age: 6 weeks
Weight: Males: 160 – 170 g
Females: 130 – 140 g
Satellite groups: 6/sex/treatment (TK, urinalysis, recovery)

Observations and Results

- The study authors concluded that the NOEL was 200 mg/kg.
- The Committee concluded that the NOEL was 50 mg/kg based on significant decreases in mean hemoglobin concentration and erythrocyte count and a significant increase in mean reticulocyte count in females at 200 mg/kg. No other correlates related to this finding.
- Given the findings, the NOAEL should be considered 200 mg/kg.

Mortality (twice daily)

- No deaths reported

Clinical Signs (twice daily)

- No changes attributable to test article

Body Weights (weekly)

- No changes attributable to test article

Feed Consumption (weekly)

- No changes attributable to test article

Ophthalmoscopy (pre- and post- dosing phase)

- No changes attributable to test article

Hematology (Week 6 and Week 13)

The following represents the author's description of the results, animal data was not included to confirm or further characterize the results:

At the end of treatment (week 13), females in the 200 mg/kg dose group had a slightly but statistically significant lower mean hemoglobin value and erythrocyte count, whereas the mean reticulocyte count was significantly increased. The individual reticulocyte counts in these animals were markedly increased in two of 10 females and moderately increased in another two. At the end of the recovery period, all of the hematological parameters were normal, except for a statistically significant decrease in mean thrombocyte counts in females at 200 mg/kg.

Clinical Chemistry (Week 7 and Week 13)

- No changes attributable to test article

Urinalysis (Week 7 and Week 13)

- No changes attributable to test article

Gross Pathology (at sacrifice)

- No changes attributable to test article

Organ Weights (at sacrifice)

A slight but statistically significant increase in relative liver weight was seen in females at 200 mg/kg, and a slight but statistically significant increase in relative spleen weight was observed in males at 50 and 200 mg/kg, but with no dose-response relationship. No histological correlate was noted. The changes in organ weights were considered to be of questionable biological relevance because there was no dose-response relationship and they were not accompanied by histopathological changes or changes in clinical chemistry. At the end of the recovery period there were no notable differences in organ weights.

Histopathology

Battery: testes, epididymides, kidneys, liver, lungs, and all gross lesions

Peer Review: Not noted

Histological Findings: Microscopic examination did not reveal any treatment related abnormalities.

Toxicokinetics (Weeks 2, 6, and 13)

- Not reported by authors

Dosing Solution Analysis

The test substances were homogeneously distributed in the diets and showed storage stability under simulated experimental conditions. The actual measured concentrations in the diet were within $\pm 10\%$ of the nominal concentration.

(b) (4)

Unna, K., and J. Greslin, 1942, "Studies on the toxicity and pharmacology of riboflavin", *J Pharmacol Exp Ther*, 76(1): 75 – 80.

The authors briefly describe a study in which four dogs (~10 weeks of age), were fed 25 mg/kg of riboflavin daily over a period of 5 months, with 2 litter-mates serving as controls. Their growth was normal and no toxic symptoms were observed. All dogs were sacrificed at the end of the feeding period. Macroscopic examination of the organs failed to reveal any change. No other details were provided.

Reviewer's note:

(b) (4)

Riboflavin as an oral dietary vitamin is generally recognized as safe (GRAS) by FDA. The following constitutes the Database of Select Committee on GRAS Substances conclusion on dietary riboflavin:

Riboflavin, an essential nutrient, is a constituent of two coenzymes: riboflavin-5'-phosphate [flavin mononucleotide (FMN)] and flavin adenine dinucleotide (FAD), which are essential components of a number of oxidative enzyme systems. Various foods such as bakery, cereal and pasta products are commonly enriched by the addition of 2 to 5 mg per kg products. Also, many commonly used vitamin supplements contain riboflavin. The amount of riboflavin-5'-phosphate added to food is minuscule.

The Recommended Dietary Allowance of riboflavin is 0.6mg per 1000kcal for persons of all ages with an additional 0.3mg daily for pregnant and 0.5mg for lactating women. A recent U.S. survey of over 20,000 persons, 1 to 74 years of age, revealed a mean average intake of 1.92 mg/day and a median of 1.69 mg per day.

The acute toxicity in animals of riboflavin or FMN given orally is extremely low, with LD₅₀ values several orders of magnitude greater than the dietary requirements or the estimated addition to food. The relative insolubility of riboflavin limits the absorption when large amounts are ingested. No reports have come to the attention of the Select Committee suggesting carcinogenic, mutagenic, or teratogenic effects of riboflavin. Normal reproductive performance was observed in three generations of rats fed several hundred times their daily requirements. Toxic effects in man have not been reported apart from rare instances of sensitivity.

In light of these considerations, the Select Committee concludes that: There is no evidence in the available information on riboflavin or riboflavin-5'-phosphate that demonstrates or suggest reasonable grounds to suspect, a hazard to the public when they are used at levels that are now current or that might reasonably be expected in the future.

Reviewer's note: Assuming 100% absorption of the ocular dose (1.12 mg), this amount is less than the daily intake consumed by the average United States citizen. Furthermore, as the conclusion notes, toxicity has not been noted except at levels which far exceed the indicated dose. No further review of systemic toxicity of riboflavin is deemed necessary for this review.

Ocular toxicity

Riboflavin and flavins are efficient photosensitizers inducing increased oxidative damage to light-exposed tissues. Riboflavin-mediated photooxidation of DNA, purines and pyrimidines, proteins and lipids have been shown in animal and human cells.

Of particular note is that nonclinical studies submitted to support this application did not include studies to assess the potential toxicity of photosensitized riboflavin on non-corneal ocular tissues or periocular skin. Given the intended riboflavin dosing regimen, it is expected that ocular tissues other than cornea will contain increased amounts of riboflavin, as will the periocular skin, due to tear spillover. An assessment of available clinical data will be necessary to ensure safety using the intended clinical dosing/irradiance protocol.

The following studies were submitted to support safety of the proposed clinical dosing regimen, as it relates to corneal tissues.

Wollensak, G. et al., 2003, "Endothelial cell damage after riboflavin-ultraviolet-A treatment in the rabbit", *J Cataract Refract Surg*, 29: 1786 – 1790.

The applicant cited a study which determined the cytotoxic effect of riboflavin treatment and UVA irradiance on the rabbit cornea, particularly the deepest, posteriorly positioned endothelial layer.

In the experiments, 36 right eyes of 36 female New Zealand White rabbits were divided into two groups and studied. The first group of rabbits was to be sacrificed 4 hours following treatment of the debrided cornea with UVA only and the second group was to be sacrificed at 24 hours after treatment of the debrided cornea with a riboflavin/dextran T-500 solution combined with UVA irradiation. Rabbits weighed 2.0 to 2.5 kg.

In the first groups (UVA-only; 4 hour sacrifice), following induction of general anesthesia, the central 5.0 mm portion of the epithelium of the cornea was removed and rabbits were treated as follows:

| Group (n) | Surface epithelial dose | Calculated endothelial dose |
|-----------|--|---|
| 1 (n=1) | Corneal abrasion only | NA |
| 2 (n=1) | 1.3 J/cm ² (0.75 mW/cm ²) | 0.16 J/cm ² (0.09 mW/cm ²) |
| 3 (n=2)* | 2.7 J/cm ² (1.5 mW/cm ²) | 0.33 J/cm ² (0.18 mW/cm ²) |
| 4 (n=2) | 5.4 J/cm ² (3 mW/cm ²) | 0.65 J/cm ² (0.36 mW/cm ²) |
| 5 (n=1) | 7.2 J/cm ² (4.0 mW/cm ²) | 0.9 J/cm ² (0.50 mW/cm ²) |

*approximates UVA dose proposed for clinical indication

In the second group of animals (24 hour sacrifice), following induction of general anesthesia, the central 5.0 mm portion of the epithelium of the cornea was removed. Five minutes before irradiation, a riboflavin solution (0.1% riboflavin-5-phosphate and 20% dextranT-500) was administered to the cornea and repeated every 5 minutes during the 30 minute irradiation. The eyes were exposed to a surface UVA (370 nm) irradiance ranging from 0.75 to 4.0 mW/cm² for 30 minutes using a double UVA diode 1.0 cm from the cornea.

Reviewer's note: This regimen differs from the proposed clinical regimen. In the regimen reported in this study, the 0.1% riboflavin solution is applied up to 8 times [5 minutes prior to irradiation, time 0 (i.e. start of irradiation), and then at 5, 10, 15, 20, 25, and 30 minutes]. Assuming a 0.03 mL drop, the total dose of riboflavin in this nonclinical study would result in 0.24 mg riboflavin applied to the debrided cornea over ~35 minutes whereas the proposed clinical regimen applies a total of 1.12 mg riboflavin over a period of approximately 60 minutes.

The authors do not specify whether saturation of the corneal stroma was confirmed, but state that the solution "was placed on the cornea to achieve good corneal penetration of the solution". Several literature reports suggest that saturation of the cornea (i.e. appearance of riboflavin the aqueous humor) is required for maximal activity.

The irradiance at the epithelium was used to estimate irradiance at the endothelial layer based on the equation:

$$I_{\text{depth}} = I_{\text{surface}} \times e^{(-d\mu)}, \text{ (Beer Equation)}$$

where depth (d) was 400 μ m and the UVA-absorption coefficient (μ) was 53 cm⁻¹. The rabbits were treated as follows:

| Group (n) | Surface epithelial dose | Calculated endothelial dose |
|-----------|--|---|
| 1 (n=1) | Corneal abrasion only | NA |
| 2 (n=3) | 1.3 J/cm ² (0.75 mW/cm ²) | 0.16 J/cm ² (0.09 mW/cm ²) |
| 3 (n=6)* | 2.7 J/cm ² (1.5 mW/cm ²) | 0.33 J/cm ² (0.18 mW/cm ²) |
| 4 (n=5) | 3.4 J/cm ² (1.9 mW/cm ²) | 0.42 J/cm ² (0.23 mW/cm ²) |
| 5 (n=3) | 4.0 J/cm ² (2.3 mW/cm ²) | 0.49 J/cm ² (0.27 mW/cm ²) |
| 6 (n=3) | 4.7 J/cm ² (2.6 mW/cm ²) | 0.58 J/cm ² (0.32 mW/cm ²) |
| 7 (n=5) | 5.4 J/cm ² (3.0mW/cm ²) | 0.65 J/cm ² (0.36 mW/cm ²) |
| 8 (n=3) | 7.2 J/cm ² (4.0 mW/cm ²) | 0.9 J/cm ² (0.50 mW/cm ²) |

*approximates endothelial UVA dose proposed for clinical indication

The central corneal thickness of the rabbit corneas was determined preoperatively using an ultrasound pachymeter. At sacrifice, right eyes were enucleated, bisected, fixed in neutral buffered 4% formalin for light microscopy or fixed in 2% glutaraldehyde for transmission electron microscopy (TEM). Small central corneal sections were examined by TEM. Detection of apoptosis at the corneal endothelium was accomplished with the TUNEL assay on sections fixed for light microscopy.

Mean central corneal thickness was 400 μm. In all animals sacrificed at 4 hours and those that underwent corneal epithelial debridement alone, neither TUNEL-positive endothelial cells nor loss of endothelium was observed. In the animals killed at 24 hours, significant endothelial cell necrosis with complete loss of endothelial cells and a few remaining apoptotic endothelial cells were observed in the treatment area in the eyes treated with surface irradiances of 3 mW/cm² and 4 mW/cm². The few remaining endothelial cells in these 2 groups were TUNEL-positive. On TEM, apoptotic endothelial cells showed typical features of apoptosis-like chromatin condensation, formation of apoptotic bodies, and cell shrinkage. The authors calculation for the toxic endothelial UVA doses resulting from these surface irradiances were 0.5 mW/cm² and 0.36 mW/cm² for the 4 mW/cm² and 3 mW/cm² surface doses, respectively.

In rabbits irradiated with surface doses of 0.75 – 2.6 mW/cm² [calculated endothelial UVA doses of 0.09 to 0.32 mW/cm², the endothelial cells were intact. Wollensak *et al* conclude that that the LOAEL toxic endothelial dose in rabbit, 0.36 mW/cm², was approximately twice as high as the therapeutic endothelial dose in humans at 0.18mW/cm². The authors also conclude that riboflavin–UVA treatment should be safe for the endothelium, as long as the endothelial UVA dose is less than the cytotoxic dose of 0.32mW/cm². The sponsors additionally stated that in human corneas, the endothelial cytotoxic UVA dose would only be reached in corneas thinner than 400μm. The rabbit NOAEL UVA endothelial dose was established at 0.32mW/cm², which provides a 1.75-fold safety margin over the estimated UVA clinical endothelial dose (0.18 mW/cm²) provided by the applicant.

Reviewer's note: It is very important to note that the surface UVA irradiance (or dose) the applicant proposes for human use (3mW/cm²) is the same as that which resulted in

endothelial toxicity in the rabbit. The authors state that the surface dose used in the rabbit study will produce a lower endothelial dose in humans of 0.18 mW/cm^2 (as opposed to 0.36 mW/cm^2 in rabbits). Therefore, it was concluded by the applicant that the surface dose resulting in toxicity in rabbits is safe in humans (3 mW/cm^2). The difference in calculated endothelial doses between rabbits and humans following a 3 mW/cm^2 surface dose appears to be based on different corneal absorption coefficients between rabbits and humans. The source of the absorption coefficients used could not be confirmed. The authors cite a published article as the source of these absorption coefficients (Spoerl E, et al. Untersuchungen zur Verfestigung der Hornhaut am Kaninchen. *Ophthalmologe*, 2000; 97:203–206). This article appears to be in German and an English version was not retrievable by this reviewer.

The information request sent on 2-19-2014 asked the applicant to explain the differences between species and show how these calculations were made. The applicant's response (eCTD24; dated 2-25-2014) states that the differences in calculated endothelial doses between species are based upon different assumed corneal depths between the rabbit ($270 \mu\text{M}$) and human ($400 \mu\text{M}$) and a constant absorption coefficient of 53 cm^{-1} used for both humans and rabbits. Using these assumptions, endothelial irradiances of 0.36 mW/cm^2 and 0.72 mW/cm^2 were found for humans and rabbits, respectively. This calculation maintains the 2-fold margin claimed by Wollensak but does not replicate Wollensak's calculations of the endothelial doses (0.18 and 0.36 mW/cm^2 for the human and rabbit, respectively). The applicant states the difference for this discrepancy is unknown. This explanation by the applicant does not appear valid since Wollensak specifically states in the article that the depth of the rabbit cornea was $400 \mu\text{M}$.

Further investigation by this reviewer yielded a potential explanation. A review of the literature revealed that the absorption coefficient of the cornea varies across species. In a review by Spoerl *et al* (2007, *Cornea*, 26: 385 – 389), the authors state the riboflavin saturated human cornea has an absorption coefficient of 70 cm^{-1} . In humans, using a corneal depth of $400 \mu\text{M}$ and an absorption coefficient of 70 cm^{-1} , the result of the Beer equation yields an endothelial irradiance of 0.18 mW/cm^2 . This exactly matches Wollensak's calculation for the human endothelial dose resulting from a 3 mW/cm^2 surface dose. For the rabbit calculation, if the corneal depth is $400 \mu\text{M}$ and an absorption coefficient of 53 cm^{-1} is used, the result of the Beer equation yields an endothelial irradiance of 0.36 mW/cm^2 for a 3 mW/cm^2 surface dose. This number also exactly replicates Wollensak's calculations and represents a 2-fold difference between the toxic dose in rabbits and the calculated human endothelial dose at the same surface irradiance.

However, there appear to be discrepancies in the absorption coefficients for a given species among different literature reports. In the article published by Spoerl *et al*, the authors list the absorption coefficients of debrided, riboflavin treated corneas across species (2007, "Safety of UVA-riboflavin cross-linking of the cornea", *Cornea*, 26: 385 - 389). The article cites the source of these coefficients as unpublished data. Spoerl states a absorption coefficient of 63 cm^{-1} in the rabbit whereas Wollensak *et al* use a

coefficient of 53 cm^{-1} . Using the Beer equation, the calculated endothelial irradiance in the rabbit using an absorption coefficient of 63 cm^{-1} and a corneal depth of $400 \text{ }\mu\text{m}$ would be 0.24 mW/cm^2 following a surface irradiance of 3 mW/cm^2 . This only represents a 1.5-fold difference between the toxic dose in rabbits and the calculated human endothelial dose at the same surface irradiance. If the endothelial dose of the NOAEL surface irradiance in the Wollensak study (2.6 mW/cm^2) is recalculated based on the absorption coefficient of 63 cm^{-1} , the Beer Equation yields 0.21 mW/cm^2 which approximates the human endothelial dose of 0.18 mW/cm^2 and thus established no safety margin.

Corneal absorption coefficients published by Spoerl *et al*

| Cornea type | Absorption coefficient (cm^{-1}) | |
|-------------|---|----------------------|
| | Without riboflavin | With riboflavin 0.1% |
| Porcine | 13.6 | 59 |
| Rabbit | 13.8 | 63 |
| Human | 14 | 70 |

Another published article questions the absorption coefficients used by Wollensak and Spoerl (Koppen, C, *et al*, 2010, "The absorption characteristics of the human cornea in ultraviolet crosslinking", *Eye Contact Lens*, 36(2): 77 – 80). In human cadaver corneas, the absorption coefficient of the riboflavin saturated cornea (0.1% riboflavin) was calculated to be 32 cm^{-1} for debrided corneal depths of $\sim 650 \text{ }\mu\text{m}$. Using this coefficient and corneal depth, the Beer Law yields an endothelial dose of 0.37 mW/cm^2 for a surface irradiance of 3 mW/cm^2 . Wollensak found that an endothelial dose of 0.36 mW/cm^2 was toxic in the rabbit. The authors conclude that based on these data, the cornea should have a theoretical thickness of $660 \text{ }\mu\text{m}$ to adequately protect the endothelium. The authors note however that in clinical experience and in confocal microscopy examinations until now there have been no reports on endothelial damage. The authors theorize that perhaps human endothelial cells are more resistant to UVA irradiation than their animal counterparts or some other effect such as riboflavin in the tear film following the clinical protocol increases the human absorbance coefficient (and thus safety).

Based upon review of all the data presented by the applicant and found through independent literature review, no clear safety margin can be established for the human endothelial dose over the endothelial dose concluded as safe in the animal studies. It is clear that toxicity to the corneal endothelium occurs at some threshold of surface irradiance. This threshold appears to be based on a constant absorption coefficient that differs between species and the depth of the cornea. In the rabbit, the same surface UVA dose proposed for approval in this NDA (3 mW/cm^2) caused endothelial toxicity at corneal depths of $400 \text{ }\mu\text{m}$. To date, the literature suggests that the same effect does not occur in humans at the same surface dose and corneal depth. Whether this implied safety is based upon differences in absorption coefficients between species or some other factor remains unproven. Given these uncertainties and a steep dose response to UVA irradiation, higher UVA doses are expected to result in endothelial toxicity and should be strictly avoided particularly at corneal depths at or near $400 \text{ }\mu\text{m}$.

Reviewer note: It should be noted that the total amount of riboflavin administered in this rabbit model was ~5 times less than that intended in the human, and was administered over 35-minute period, as compared to a 60-minute period intended in the human. The differences in dosing regimen may limit extrapolation of these rabbit data to the human.

Wollensak, G., et al., 2004, “Keratocyte cytotoxicity of riboflavin/UVA-treatment *in vitro*”, *Eye*, 18: 718 – 722.

This study was undertaken to investigate the cytotoxic effect of combined riboflavin/UVA-treatment on corneal keratocytes *in vitro* and to compare it to UVA-irradiation alone.

Cell cultures established from primary porcine keratocytes were treated with 0.025% riboflavin solution and various UVA (370 nm)-irradiances ranging from 0.4 to 1.0 mW/cm² and with UVA alone between 2 and 9 mW/cm² for 30 min. The concentration of riboflavin was chosen in an attempt to mimic the clinical situation wherein the stromal riboflavin concentration is thought to be 0.024% based on diffusion. The cell cultures were evaluated for cell death 24 h after irradiation using trypan-blue and Yopro-fluorescence staining.

When keratocytes were treated with UVA in combination with riboflavin, a cytotoxic irradiance level was found at 0.5 mW/cm² while UVA alone caused cytotoxicity at 5 mW/cm². This data suggests that the riboflavin sensitizes keratocytes to cytotoxic irradiance making them susceptible to cytotoxicity at UVA irradiances of 10-fold lower intensity.

| Irradiance level (mW/cm ²) | Riboflavin + UVA | UVA alone |
|--|------------------|-----------|
| 9 | | + |
| 8 | | + |
| 7 | | + |
| 6 | | + |
| 5 | | + |
| 4.5 | | - |
| 4 | | - |
| 3 | | - |
| 2 | | - |
| 0.8 | + | |
| 0.7 | + | |
| 0.6 | + | |
| 0.55 | + | |
| 0.5 | + | |
| 0.45 | - | |
| 0.4 | - | |

(+) = necrosis; (-) = no cell damage

The authors suggest that a cytotoxic effect of combined riboflavin/UVA-treatment on keratocytes is to be expected at 0.5 mW/cm². A study by Spöerl (cited above) suggests this level of irradiance is achieved following surface UVA-irradiance of 3 mW/cm² in human corneas to a depth of 300 micrometers in the clinical setting. This conclusion supports the minimum corneal depth of 400 μM to ensure corneal endothelial cell protection.

7 Genotoxicity

The applicant makes the following statement in the labeling:



This statement is supported by:

Appeldorn, M., et al., 1999, "WHO Food Additive Series 42: Safety evaluation of certain food additives", Prepared by the Fifty-first meeting of the Joint FAO/WHO Expert Committee on Food Additives (JECFA).

The authors cite unpublished data as the source (Albertini, S., 1989, Mutagenicity evaluation of Ro 01-3131/055 (riboflavin 96% ex fermentation) in the Ames test (Study No. 139M95). Unpublished report from F. Hoffmann L Roche Ltd. Dated 8 September 1995. Submitted to WHO by F. Hoffmann La Roche Ltd, Basel, Switzerland). The results are summarized as follows:

| Test Object | Endpoint | Strains | Concentrations | Results |
|---|------------------|--|----------------------------|---|
| Riboflavin 96% ex fermentation (purity 99.4%) | Reverse mutation | S. <i>typhimurium</i> : TA97, TA98, TA100, TA102, TA1535 | 50 – 5000 µg/plate in DMSO | Negative; no toxicity; increasingly milky suspensions at ≥ 50 µg/plate. At 1666 and 5000 µg/plate, precipitation prevented colony counting in preincubation assay |
| Riboflavin 98% ex fermentation (purity: 100.2%) | Reverse mutation | S. <i>typhimurium</i> : TA97, TA98, TA100, TA102, TA1535 | 50 – 5000 µg/plate in DMSO | Negative; no toxicity; increasingly milky suspensions at ≥ 50 µg/plate. At 1666 and 5000 µg/plate, precipitation prevented colony counting in preincubation assay |

Ishidate, M., et al., 1988, “A comparative analysis of data on the clastogenicity of 951 chemical substances tested in mammalian cell cultures”, *Mutat Res*, 195: 151 – 213.

A comparative analysis of metadata on the clastogenicity of various chemical substances tested in mammalian cell cultures was conducted. The authors report that riboflavin (300 µg/mL in the absence of S9) resulted in an increase in polyploidy in human lung cells after 48 hours of incubation but results were negative in the Ames assay and a rodent carcinogenicity assay. No other discussion of riboflavin was found. The reference containing the original results was not identifiable.

Hayashi, M., et al., 1988, “Micronucleus tests in mice on 39 food additives and eight miscellaneous chemicals”, *Food Chem Toxicol*, 26(6): 487 – 500.

The tests were carried out at Department of Food and Drug Science, Kanagawa Prefectural Public Health Laboratory in Japan. Eight-week-old male ddY mice were used. The sampling time after the administration was 24 hr. The compounds were administered by one ip injection. One thousand polychromatic erythrocytes per mouse were scored and the number of micronucleated polychromatic erythrocytes (MNPCEs)

was recorded. The proportion of polychromatic erythrocytes (PCEs) among the total erythrocytes was also evaluated by observing 1000 erythrocytes on the same slide. Results showed that riboflavin no increase in micronucleated erythrocytes compared to controls.

Photochemical genotoxicity

The applicant did not include any data regarding the potential photochemical genotoxicity of UVA or riboflavin in the presence of UVA light. A literature search revealed several published articles detailing the ability of riboflavin to induce gene mutations in the presence of UVA light. The genotoxicity of UVA has been established from *in vitro* experiments. Since DNA absorbs light poorly between 315nm and 400 nm, it is thought that the damage induced may be due to the absorption of photons by endogenous non-DNA chromophores. These UVA activated chromophores may then transfer energy to DNA via reactive oxygen intermediates or radicals and can cause direct modification of DNA via a type I reaction (Griffiths, H., *et al.*, 1998, "Molecular and cellular effects of ultraviolet light-induced genotoxicity", *Crit Rev Clin Lab Sci*, 35(3): 189 – 237. (b) (4)

(b) (4)

(b) (4)

In this study, the mutagenic potentials of riboflavin (b) (4) (b) (4) were assessed in the Ames assay, *umu* test and the SOS chromotest.

In Vitro Reverse Mutation Assay in Bacterial Cells (Ames)

GLP compliance: Not stated

QA statement: No

Key Study Findings

- Riboflavin alone or following metabolic activation was not considered mutagenic in the assays performed

- (b) (4) in the absence of metabolic activation, was not considered mutagenic though an increase in revertants over the control value was observed for strain TA97a only
- Upon metabolic activation with rat S9 fractions or rat cecal cell-free extract (CCE), (b) (4) was considered mutagenic in the Ames assay, *umu* test and the SOS chromotest

Methods

The Ames assay

| | |
|-------------------------------------|--|
| Strains: | <i>Salmonella typhimurium</i> : TA100, TA98, TA97a |
| Concentrations in definitive study: | 25, 50 or 100 µg/mL |
| Basis of concentration selection: | Not specified |
| Negative control: | Phosphate buffered saline (PBS) |
| Positive control: | S9 activated benzo[<i>a</i>]pyrene |
| Formulation/Vehicle: | Not specified, but presumably PBS |
| Incubation & sampling time: | 30 minute incubation in liquid culture in presence of compound prior to plating. Incubation time of plates prior to colony counting not specified. |

The *umu* assay

Derived from technique of Oda (Oda, Y., *et al.*, 1985, "Evaluation of a new system (*umu* test) for the detection of environmental mutagens and carcinogens", *Mutat Res*, 147: 219 – 229. Briefly, the *umu* operon in *E. coli* is considered part of the SOS response and is inducible by DNA-damaging agents. A plasmid carrying a fused gene *umuC::lacZ* (pSK1002) was introduced into *S. typhimurium* strain TA1535. The inducible *umu* operon expression is then used to monitor the levels of DNA damage by measuring the β-galactosidase activity in the cells produced by the fusion gene. Riboflavin and (b) (4) concentrations of 0 – 100 µg/mL (with and without S9 or CCE activation) were incubated for 180 minutes with exponentially growing *Salmonella typhimurium* TA1535/psk 1002 strain. A substrate of *B*-galactosidase (*O*-nitrophenyl-α-D-galactopyranoside) was added and *B*-galactosidase activity determined by absorbance characteristics of the reaction.

The SOS assay

The assay was performed using the procedure formerly described (Quillardet, P. and M. Hofnung, 1985, "The SOS Chromotest, a colorimetric bacterial assay for genotoxins: procedures", *Mutat Res*, 147: 65 – 78) with minor modifications. Briefly, in *E. coli* the *sfiA* gene is inducible by DNA-damaging agents as part of the SOS response. In this assay the *lacZ* gene (β-galactosidase) is engineered as a *sfiA::lacZ* fusion under

control of *sfiA* in the *E. coli* PQ37 strain. The strain also carries a deletion for the normal *lac* region so that β -galactosidase activity is strictly dependent on *sfiA* expression. Additionally, *uvrA* mutation renders the strain deficient in excision repair and accordingly increases the response to certain DNA-damaging agents.

A PQ37 cell suspension was incubated with 0-100 $\mu\text{g/ml}$ of riboflavin or (b) (4) (with or without S9 or CCE activation) for 10 min. The activity of β -galactosidase was determined and normalized to background alkaline phosphatase activity.

Study Validity: For the Ames assay, treatment of *Salmonella* with the positive control, S9 activated benzo[*a*]pyrene, resulted in 900 additional revertants. The strains of *Salmonella* used to validate the positive control were not identified. The negative control, PBS, generated low numbers of revertants consistent with expected results. A positive control for CCE and “neat” preparations of riboflavin and (b) (4) were not included in the results. Overall, given other literature reports regarding the lack of genotoxicity for riboflavin, the results for riboflavin appear accurate. The negative results for “neat” (b) (4) are unconfirmed without a positive control.

The *umu* assay and SOS assay

The authors did not report the use of positive controls (with or without metabolic activation). The assays are not typical aspects of the genotoxicity testing battery per ICHS2(R1). It is not the scope of this review to validate these assays and therefore results will be considered supportive only.

Results

Riboflavin did not demonstrate any mutagenic response, “neat” or after exposure to metabolic enzymes. These results are in agreement with other reports. (b) (4) “neat” showed no mutagenic potential; however (b) (4) was considered mutagenic after activation by S9 and CCE in the *umu* test, SOS chromotest and Ames/*Salmonella* assay.

In the Ames/*Salmonella* assay, S9-activated (b) (4) induced 1.9, 2.9 and 4.8 times the number of revertants compared to controls in *Salmonella typhimurium* strains TA100, TA98 and TA97, respectively. (b) (4) after exposure to CCE showed a mutagenic response in strain TA97a by increasing revertants to 3 times the control values while the other two strains remained unaffected. The results for strains TA98 and TA97a indicate the potential for metabolically activated (b) (4) to induce frameshift mutations while the results in strain TA100 indicate potential for base-pair substitution. In the presence of S9 or CCE, (b) (4) elicited up to a 2-fold increase in β -galactosidase activity in the *umu* test as well as in the SOS chromotest.

| Compound ($\mu\text{g/mL}$) | Number of revertants | | | | | | | | |
|----------------------------------|----------------------|-----------------------|----------------------|---------------------|----------------------|---------------------|-----------------------|-----------------------|-----------------------|
| | TA100 | | | TA98 | | | TA97a | | |
| | Neat | +S9 | +CCE | Neat | +S9 | +CCE | Neat | +S9 | +CCE |
| 0 | 104.4 \pm 15.60 | 85.66 \pm 21.30 | 68.30 \pm 13.50 | 26.22 \pm 4.10 | 21.80 \pm 5.30 | 23.00 \pm 5.70 | 105.30 \pm 12.41 | 120.10 \pm 31.19 | 119.20 \pm 20.80 |
| Riboflavin | | | | | | | | | |
| 25 | 73.37 \pm 12.80 | 81.00 \pm 22.50 | 78.16 \pm 32.50 | 18.25 \pm 6.60 | 25.40 \pm 3.90 | 19.50 \pm 5.50 | 101.00 \pm 10.80 | 99.12 \pm 12.50 | 105.12 \pm 19.70 |
| 50 | 79.70 \pm 17.10 | 77.12 \pm 19.85 | 72.00 \pm 23.50 | 17.25 \pm 4.10 | 21.66 \pm 7.90 | 23.20 \pm 3.80 | 111.50 \pm 28.40 | 143.60 \pm 27.40 | 126.80 \pm 24.70 |
| 100 | 85.50 \pm 16.80 | 67.40 \pm 23.19 | 87.80 \pm 14.30 | 23.00 \pm 7.30 | 22.50 \pm 5.0 | 24.10 \pm 3.90 | 88.00 \pm 5.70 | 127.25 \pm 15.40 | 106.80 \pm 10.22 |
| (b) (4) | | | | | | | | | |
| 25 | 95.25 \pm 32.20 | 139.10 \pm 33.20 | 71.50 \pm 0.70 | 28.60 \pm 3.70 | 35.10 \pm 4.50 | 25.40 \pm 3.80 | 186.00 \pm 20.21 | 197.80 \pm 29.73 | 280.40 \pm 46.70 |
| 50 | 92.33 \pm 12.61 | 146.33 \pm 24.33 | 97.60 \pm 19.90 | 20.85 \pm 4.37 | 48.33 \pm 7.80 | 24.00 \pm 3.39 | 177.10 \pm 28.41 | 488.18 \pm 108.7 | 295.40 \pm 61.80 |
| 100 | 60.28 \pm 12.20 | 163.37 \pm 26.40 | 66.60 \pm 15.20 | 25.50 \pm 5.80 | 63.40 \pm 12.80 | 22.40 \pm 2.30 | 172.90 \pm 16.20 | 583.12 \pm 69.90 | 360.00 \pm 49.50 |

The *umu* assay

| Compound ($\mu\text{g/mL}$) | β -galactosidase activity (Units / OD_{600}) | | |
|-------------------------------|--|-------------------|-------------------|
| | Neat | + S9 | + CCE |
| Negative control | 97.00 \pm 4.00 | 136.50 \pm 4.10 | 137.66 \pm 1.88 |
| Riboflavin | | | |
| 25 | 98.00 \pm 1.40 | 144.13 \pm 3.05 | 136.13 \pm 4.07 |
| 50 | 101.10 \pm 1.82 | 144.06 \pm 3.61 | 142.80 \pm 4.38 |
| 100 | 94.28 \pm 3.30 | 134.23 \pm 3.65 | 138.66 \pm 5.92 |
| (b) (4) | | | |
| 25 | 142.66 \pm 4.26 | 153.31 \pm 2.25 | 183.06 \pm 5.92 |
| 50 | 130.20 \pm 3.93 | 230.10 \pm 6.68 | 236.18 \pm 2.19 |
| 100 | 129.33 \pm 7.05 | 267.43 \pm 8.09 | 247.80 \pm 2.76 |

SOS assay

| Compound | Ratio β -galactosidase units / Alkaline phosphatase units | | | Induction factor (ratio at test concentration / ratio at concentration zero) | | |
|------------------|---|------|-------|--|------|-------|
| | Neat | + S9 | + CCE | Neat | + S9 | + CCE |
| Negative control | 0.14 | 0.19 | 0.16 | 1.00 | 1.00 | 1.00 |
| Riboflavin | | | | | | |
| 25 | 0.15 | 0.19 | 0.18 | 1.00 | 1.00 | 1.10 |
| 50 | 0.19 | 0.17 | 0.16 | 1.30 | 0.89 | 1.00 |
| 100 | 0.20 | 0.16 | 0.14 | 1.40 | 0.84 | 0.87 |
| (b) (4) | | | | | | |
| 25 | 0.14 | 0.33 | 0.28 | 1.00 | 1.73 | 1.75 |
| 50 | 0.15 | 0.38 | 0.31 | 1.00 | 2.00 | 1.93 |
| 100 | 0.15 | 0.40 | 0.32 | 1.00 | 2.10 | 2.00 |

(b) (4)

In this study, the mutagenic potential of riboflavin and (b) (4) following exposure to UV light were assessed in the Ames assay, *umu* test and the SOS chromotest.

In Vitro Reverse Mutation Assay in Bacterial Cells (Ames)

GLP compliance: Not stated
 QA statement: No
 Drug, lot #, and % purity: Not specified

Key Study Findings

- Following photo-activation, riboflavin and (b) (4) generated singlet oxygen and superoxide radicals and induced mutagenesis in the assays performed

Methods

The Ames assay

| | |
|-------------------------------------|--|
| Strains: | <i>Salmonella typhimurium</i> : TA102 |
| Concentrations in definitive study: | 25, 50 or 100 µg/mL |
| Basis of concentration selection: | Not specified |
| Negative control: | Phosphate buffered saline (PBS) |
| Positive control: | None specified |
| Formulation/Vehicle: | Not specified, but presumably PBS |
| Incubation & sampling time: | 30 minute incubation in liquid culture in presence of compound prior to plating. Incubation time of plates prior to colony counting not specified. |

The *umu* assay

(see above for general technique)

For both assays, to induce potential photodegradation of the flavin compounds, the incubation mixtures were treated with Philips 'Ultraphill' MLU, 300 W, 220-240 V, F.28 sunlamp with a glass filter (0.5 mm thickness). The filter was used to isolate specific effects of the radiation regime at 360-400 nm and to avoid possible effects of stray UV light.

Study Validity:

For the Ames assay, a positive control was not identified. The negative control, PBS, generated low numbers of revertants consistent with expected results.

The *umu* assay

The authors did not report the use of positive controls. The assay is not a typical aspect of the genotoxicity testing battery per ICHS2(R1). It is not the scope of this review to validate this assay and therefore results will be considered supportive only.

Results

The data suggest that both riboflavin and (b) (4) possess mutagenic potential in the presence of light due to production of reactive oxygen species. The authors presented data which shows that riboflavin and (b) (4) produce singlet oxygen and superoxide radicals in the presence of light (data not shown in this review). Incorporation of sodium azide was found to quench singlet oxygen formation by riboflavin and (b) (4) by 86% and 81%, respectively. Superoxide dismutase was found to quench superoxide radical formation by 88% and 90% for riboflavin and (b) (4), respectively. (b) (4) was found to be more efficient in generating singlet

oxygen and superoxide anion than riboflavin. The data presented confirm the genotoxic potential of riboflavin on exposure to light.

Following exposure to light, the histidine revertants of TA102 increased by 2.2 and 3 times, respectively. This photoactivation was inhibited by superoxide dismutase while sodium azide did not exert any influence.

In the presence of illuminated riboflavin and (b) (4) riboflavin and (b) (4) caused an increase in β -galactosidase activity in the umu assay by a factor of 2 and 2.2 times, respectively. In the presence of sodium azide, the mutagenic response was unaltered while in the presence of superoxide dismutase it was reduced to 1.4 times for both riboflavin and (b) (4).

Photomutagenicity of riboflavin and (b) (4) with the Ames assay

| Compound ($\mu\text{g/mL}$) | Histidine revertants / plate | | | |
|-------------------------------|------------------------------|------------------------------------|---|---|
| | Ambient light | Illuminated at 20 J / m^2 | Illuminated in presence of sodium azide | Illuminated in presence of superoxide dismutase |
| Negative control | 220.80 \pm 37.80 | 219.10 \pm 26.20 | 220.70 \pm 45.80 | 240.22 \pm 31.68 |
| Riboflavin | | | | |
| 25 | 229.00 \pm 26.50 | 301.00 \pm 37.10 | 301.20 \pm 33.50 | 231.20 \pm 13.07 |
| 50 | 218.04 \pm 23.35 | 410.70 \pm 33.20 | 410.60 \pm 37.30 | 260.80 \pm 28.80 |
| 100 | 217.50 \pm 28.20 | 503.90 \pm 41.40 | 504.00 \pm 23.80 | 334.20 \pm 43.00 |
| (b) (4) | | | | |
| 25 | 224.50 \pm 62.60 | 320.60 \pm 29.80 | 328.10 \pm 25.80 | 241.20 \pm 43.30 |
| 50 | 320.10 \pm 23.30 | 496.80 \pm 56.20 | 509.20 \pm 46.30 | 365.30 \pm 28.10 |
| 100 | 221.60 \pm 7.36 | 657.30 \pm 41.17 | 637.10 \pm 32.40 | 390.20 \pm 18.50 |

Photomutagenicity of riboflavin and (b) (4) with the umu test

| Compound ($\mu\text{g/mL}$) | β -galactoside activity (Units/ OD_{600}) | | | |
|-------------------------------|---|------------------------------------|---|---|
| | Ambient light | Illuminated at 20 J / m^2 | Illuminated in presence of sodium azide | Illuminated in presence of superoxide dismutase |
| Negative control | 112.00 \pm 15.87 | 110.10 \pm 17.23 | 115.60 \pm 17.23 | 110.90 \pm 5.43 |
| Riboflavin | | | | |
| 25 | 110.23 \pm 7.88 | 120.90 \pm 9.10 | 120.60 \pm 10.50 | 110.70 \pm 35.19 |
| 50 | 116.28 \pm 28.70 | 126.80 \pm 2.30 | 162.50 \pm 52.60 | 140.20 \pm 52.80 |
| 100 | 118.50 \pm 7.85 | 221.20 \pm 14.80 | 223.90 \pm 20.36 | 156.40 \pm 39.60 |
| (b) (4) | | | | |
| 25 | 110.50 \pm 4.76 | 179.30 \pm 19.90 | 180.90 \pm 19.00 | 123.80 \pm 20.00 |
| 50 | 117.30 \pm 6.39 | 200.10 \pm 20.10 | 201.00 \pm 37.30 | 142.60 \pm 40.20 |
| 100 | 128.20 \pm 39.00 | 246.00 \pm 24.20 | 248.10 \pm 43.39 | 159.00 \pm 29.50 |

(b) (4)

In Vitro Reverse Mutation Assay in Bacterial Cells (Ames)

GLP compliance: Not stated
 QA statement: No
 Drug, lot #, and % purity: Not specified

Key Study Findings

- Results suggested that (b) (4) is not genotoxic by Ames assay
- Further light activation or metabolic activation of (b) (4) (e.g. incubation with S9 liver fraction) was not performed (as was shown to induce genotoxic potential (b) (4)) so the genotoxic potential (b) (4) cannot be excluded under these other conditions

Methods

Strains: *Salmonella typhimurium* strains: TA98, TA100, TA1535, and TA1537 and *Escherichia coli* strain WP2 uvrA

Concentrations in definitive study: Not specified
 Basis of concentration selection: Maximum concentration tested was at the limit of solubility (b) (4)

Negative control: Not specified
 Positive control: Not specified
 Formulation/Vehicle: Not specified
 Incubation & sampling time: Not specified

Study Validity

The publication used (b) (4) as just one of many treatments in the assay; the others are not relevant to this review. Details on the concentrations (b) (4) used and the incubation time were not given. No other publications on the genotoxic potential (b) (4) were found by this reviewer.

Results

The results presented in the publication state that (b) (4) was found to not be genotoxic in the Ames assay. No other details were provided though given the close structural similarity (b) (4) the potential for genotoxicity following metabolic activation or further exposure to light remain.

8 Carcinogenicity

Study title: Pacernick, L., et al., 1975, “The inefficacy of riboflavin against ultraviolet-induced carcinogenesis”

Study report location: **J Invest Dermatol, 65: 547 – 548.**
Conducting laboratory and location: Section of Dermatology, Department of
Medicine
The University of Chicago Pritzker School
of Medicine
Chicago, IL
GLP compliance: Not stated
QA statement: No
Drug, lot #, and % purity: Not stated
CAC concurrence: No

Key Study Findings

- Previous *in vitro* data suggested that that riboflavin may protect against UV light induced DNA damage. This study sought to determine the protective effects of riboflavin against UV-light induced carcinogenesis *in vivo*.
- Results of this study suggest that riboflavin, administered as a daily “painting” or as a supplement to daily water consumption, neither alleviated nor exacerbated UV-light induced skin neoplasia in hairless mice

Reviewer’s note:

(b) (4)

Given that the tumors developed on the dorsal surface of the mice and the authors conclude that riboflavin neither promotes or prevents tumorigenesis in this model, it may be assumed that the dorsal surface of the mouse was painted and not the auricle. The authors did use to auricle to determine the

dermal concentrations of riboflavin achieved in the mouse following oral administration of riboflavin.

Adequacy of Carcinogenicity Study

- Endpoints to determine systemic neoplasia were not included. The number of mice per treatment group was small (9 males/group). Given its status as GRAS for dietary consumption, systemic exposure to riboflavin following topical ocular administration is not expected to present risk. In addition to the short period of use on a single day, these results support the conclusion that riboflavin treatment will not contribute to the development of topical (epidermal/dermal) neoplasia though relevance to ocular neoplasia remains undetermined.

Appropriateness of Test Models

- Results should only be used to predict possibility of skin carcinogenesis and not considered a determination of systemic or ocular carcinogenesis

Methods

| | |
|--------------------------|--|
| Doses: | Group II: 15 mg/mL Group III: 15 mg/mL (15mg/day) |
| Frequency of dosing: | Group II: daily "painting" Group III: daily (<i>ad libitum</i>) |
| Dose volume: | Group II: not specified Group III: ~1 mL consumed/mouse/day |
| Route of administration: | Group II: topical Group III: incorporation into drinking water |
| Formulation/Vehicle: | Group II: Propylene glycol Group III: Water |
| Basis of dose selection: | Group III animals consumed ~10,000 times the daily recommended intake for humans |
| Species/Strain: | Mouse: HR-hairless |
| Number/Sex/Group: | 9 males / group |
| Age: | 6 – 8 weeks, weight: ~14.8 g |

Special Study Design:

All groups, including Group I controls, were simultaneously irradiated in a light box with reflecting walls containing two Westinghouse FS20 sunlamps from a distance of 30 cm for a period of 5 minutes daily, 6 days a week throughout the experiment (11 months).

Histopathology

Peer Review: Not reported

Neoplastic lesions: By the 11th month, all surviving mice (6 – 7 / group) had started to develop several tumors on the dorsal skin. Some tumors were crusted and ulcerated. Biopsy revealed squamous cell carcinomas of various grades of differentiation. The total number (2 to 3 tumors measuring ≥ 5 mm in diameter and multiple incipient tumors) and the time of onset of tumors did not vary in the treated groups compared to controls. Dissections did not reveal metastatic progress.

| | Group I (controls) | Group II (dermal "painting") | Group III (daily supplementation in water) |
|------------------------|--------------------|------------------------------|--|
| Number of survivors | 6 | 7 | 6 |
| 9 th month | 3 | 4 | 2 |
| 10 th month | 5 | 7 | 6 |
| 11 th month | 6 | 7 | 6 |

Special Toxicokinetics:

In group III animals, 3 additional mice were sacrificed at 1 month to determine the degree of cellular penetration achieved by riboflavin in the auricular skin. At 1 month, the skin and urine had turned yellow and biopsy specimens were obtained and immediately frozen. Sections were compared by fluorescence microscopy and riboflavin concentration in the epidermis was estimated as 25 μ g/gm existing mainly in the cytoplasm.

Dosing Solution Analysis: Not performed

(b) (4)

Study title: Effect of massive doses of riboflavin, and other vitamins of the B group, on skin carcinogenesis in mice

Study report location: *Br J Cancer*, 16(2): 252 – 257
Conducting laboratory and location: F. Roe
Department of Cancer Research
London Hospital Medical College
Turner Street, London, England
GLP compliance: Not stated
QA statement: No
Drug, lot #, and % purity: Not stated
CAC concurrence: No

Key Study Findings

- Riboflavin supplementation into the diet of mice did not promote skin carcinogenesis in response to topical application of carcinogens
- Riboflavin may have slightly reduced or delayed onset of tumorigenesis but these differences did not reach statistical significance and incidence seemed to equilibrate between treated and non-treated groups over time

Adequacy of Carcinogenicity Study

- Given the short period of use on a single day for the proposed indication and the GRAS status of riboflavin for dietary consumption, these results should be considered as evidence that riboflavin does not contribute to the development of skin neoplasia. Relevance to ocular neoplasia remains undetermined.

Appropriateness of Test Models

- Results should only be used to support exclusion of the possibility of dermal carcinogenesis and not considered a determination of risk for systemic or ocular carcinogenesis

Methods:**Experiment I**

Strain “101” mice of 8 – 10 weeks of age (6/sex/group) began treatment with the diets and vitamins shown below. Four weeks later, all groups were given a single application of 9,10,-dimethyl-1,2-benzanthracene (DMBA; 225 µg / 0.15mL in acetone). After an additional 3 weeks, once weekly treatment with croton oil (0.1%, 0.25 mL applied) began and repeated for 15 total weeks.

| Group | Diet | In drinking water | Applications to skin (begun 4 weeks after starting on diet + vitamins in drinking water) | Incidence of papillomas at end of croton oil treatment | | | Incidence of papillomas per survivor one month after end of treatment | Average weight gain during experiment (g.) |
|-------|--------|-----------------------------------|--|--|----------------------|---------------------------------|---|--|
| | | | | Survivors | Mice with papillomas | Average papillomas per survivor | | |
| I | R.I.F. | — | 225 µg. DMBA in 0.15 ml. acetone once | 19 | 17 | 12.5 | 10.0 | 5.7 |
| II | | Riboflavine—2 mg. per mouse daily | | 19 | 19 | 12.5 | 10.7 | 5.4 |
| III | | Thiamine 0.2% | Then after 3-week interval | 20 | 18 | 9.3 | 7.3 | 6.4 |
| IV | | Pyridoxine 0.04% | | 18 | 17 | 14.1 | 9.1 | 5.8 |
| V | | Nicotinamide 0.2% | 0.25 ml. 0.1% croton oil in acetone once weekly for 15 weeks | 19 | 17 | 14.6 | 12.1 | 6.1 |
| VI | P.R.M. | — | | 19 | 18 | 8.4 | 7.5 | 7.4 |

¹ Formula of P.R.M. diet :

| | Per cent |
|---|----------|
| Wheat meal (millable grain) | 20 |
| English fine wheatfeed | 20 |
| Finely ground oats (thin husk) | 20 |
| Maize meal (yellow variety) | 9.4 |
| Barley meal (plump grains) | 5 |
| English white fish meal (66 per cent albuminoids) | 5 |
| Meat and bone meal (50 per cent protein) | 8.7 |
| Dried skim milk | 7.5 |
| Dried unextracted diastase yeast | 2.5 |
| Vitamin premix | 1.25 |
| Mineral supplement | 0.65 |

Vitamin Premix :

| | Per pound |
|---|--------------|
| Vitamin A (stabilized) | 140,000 I.U. |
| Vitamin D ₃ (stabilized) | 36,000 I.U. |
| Vitamin B ₂ (riboflavin, nicotinic acid, folic acid) | 300 mg. |
| Vitamin E | 900 mg. |

After 6 weeks of croton oil treatment, papillomas were initiated in all the groups. Their incidence rose steadily, though slightly less quickly, in Group III (given R.I.F. diet plus thiamine in the drinking water) and Group VI (given P.R.M. diet) than in the other 4 groups. Additionally, Groups III and VI had gained more weight than other treatment groups. These two groups also had a modest reduction in incidence of tumors though the difference did not reach statistical significance. It was concluded that P.R.M. diet, or thiamine in the drinking water of mice on R.I.F. diet, may have protected mice slightly against developing papillomas in response to treatment with DMBA and croton oil.

Experiment II

Six groups (10/sex/group) were treated as described below starting 4 weeks before applications of carcinogens to the skin [DMBA or 3,4-benzopyrene (BP)]. Carcinogens were applied for 20 weeks. Group XII received R.I.F. diet in addition to riboflavin as a subcutaneous injection (0.25 mg) twice weekly.

| Group | Diet | In drinking water | By subcutaneous injection | By application to the skin | Papillomas at 20th week | | | Malignant tumours arising before 33rd week | |
|-------|-----------------|-------------------|-----------------------------------|-------------------------------------|-------------------------|----------------------|------------------|--|---------------|
| | | | | | Survivors | Mice with papillomas | Total papillomas | Mice with tumours | Total tumours |
| VII | R.I.F. | — | — | 0.2 ml. 0.01% DMBA × 2 weekly | 19 | 18 | 124 | 17 | 35 |
| VIII | P.R.M. + Yeast* | — | — | | | | | | |
| IX | R.I.F. | — | — | 0.2 ml. 0.025% BP × 2 weekly | 18 | 13 | 37 | 12 | 29 |
| X | P.R.M. + Yeast* | — | — | | | | | | |
| XI | R.I.F. | Thiamine 0.2% | Thiamine 2.5 mg. × 2 weekly† | | | | | | |
| XII | R.I.F. | — | Riboflavin 0.25 mg. × 2 weekly | 19 | 8 | 13 | 8 | 21 | |

* 2 oz. per cage of 10 mice twice weekly.
† Stopped after 20 weeks.

Papillomas appeared in groups treated with DMBA (Groups VII and VIII) at the 11th week while those groups treated with BP not receiving thiamine or riboflavin supplementation initiated papillomas during the 13th week (Groups IX and X). Papillomas appeared in mice receiving thiamine or riboflavin supplementation and treated with BP during the 17th week (Groups XI and XII). Injections of thiamine for Group XI were stopped at 20 weeks because 12 of the mice had died. All other treatments continued.

Of the four BP-treated groups, Group XII (riboflavin) had a lower incidence of papillomas than the others. In addition, Groups VIII, IX and X had 1, 2 and 4 malignant skin tumors, respectively. Over the following 13 weeks, more malignant skin tumors arose in all the groups. Among the BP-treated groups the incidence was slightly, but not significantly, lower in riboflavin treated mice compared to Groups IX and X. It was concluded that neither the differences in diet nor the administration of thiamine or riboflavin affected skin carcinogenesis by repeated applications of DMBA; but that injected riboflavin may have slightly reduced the incidence of papillomas at 20 weeks, and of malignant tumors arising before the 33rd week, in mice treated repeatedly with BP.

Experiment III

Three groups of mice (20/sex/group) began treatment with the diets shown below. Four weeks later all groups began twice weekly dermal applications of BP (0.2 ml; 0.025% in acetone). Papillomas began to appear in mice which received no riboflavin supplement or those receiving a diet supplemented with 0.2% riboflavin (Groups XII and XIV) during the 11th week of treatment. Mice receiving a diet supplemented with 0.6% riboflavin (Group XV) showed papillomas at Week 13. Through Week 18, these mice developed less tumors, but from the 18th week onwards this difference progressively disappeared. By the 23rd week there was very little difference between the three groups.

| Group | Diet | Applications to skin (began 4 weeks after start of special diets) | Mice in group | Tumour incidence after 23 weeks | | | Average weight gain during experiment (g.) | |
|-------|-----------------------|---|---------------|---------------------------------|-------------------|----------------------|--|-------------------------|
| | | | | Survivors | Mice with tumours | Total benign tumours | | Total malignant tumours |
| XII | 41B | 0.2 ml. 0.025% BP in acetone twice weekly for 23 weeks | 40 | 30 | 21 | 76 | 6 | 8.0 |
| XIV | 41B + 0.2% Riboflavin | | 40 | 28 | 25 | 74 | 6 | 7.2 |
| XV | 41B + 0.6% Riboflavin | | 38 | 37 | 32 | 71 | 6 | 8.2 |

Reviewer's note: Collectively, these data suggest that riboflavin does not promote skin carcinogenesis in response to topical chemical carcinogen application and may slightly reduce or delay development of skin tumors though statistical significance in reduction or delay was not demonstrated.

9 Reproductive and Developmental Toxicology

(b) (4)

Unna, K., and J. Greslin, 1942, "Studies on the toxicity and pharmacology of riboflavin", *J Pharmacol Exp Ther*, 76(1): 75 – 80.

In this study, weanling male and female rats were fed oral dietary riboflavin (10 mg/day; calculated dose ~50 mg/kg) for about 140 days from weaning for each of 3 consecutive generations. The animals were mated, and normal litters averaging 10 young rats were obtained from both the riboflavin fed group and the control group. Offspring of the first generation were again fed daily with 10 mg/day riboflavin after they reached the age of 3 weeks. Daily feeding of riboflavin over periods of 140 days were continued through 3 generations. No effects on growth, development or gross necropsy findings were

reported. Body weights were comparable to controls through all generations. No difference in the development, growth, maturation or reproduction was observed between the rats fed riboflavin and those of the control group.

| Treatment group | | Average weight (g) after: | | | | | | | |
|------------------------------|----------------------------|---------------------------|---------|---------|---------|----------|---------|----------|---------|
| | | 20 days | | 50 days | | 100 days | | 140 days | |
| | | Males | Females | Males | Females | Males | Females | Males | Females |
| Control | 1 st generation | 96 | 87 | 222 | 156 | 294 | 207 | 317 | 237 |
| | 2 nd generation | 83 | 78 | 123 | 156 | 349 | 225 | 405 | 242 |
| | 3 rd generation | 91 | 80 | 241 | 169 | 339 | 214 | 381 | 239 |
| Riboflavin (10 mg/day) | 1 st generation | 98 | 83 | 230 | 154 | 317 | 206 | 347 | 243 |
| | 2 nd generation | 95 | 88 | 235 | 167 | 344 | 236 | 412 | 259 |
| | 3 rd generation | 91 | 84 | 239 | 171 | 351 | 219 | 416 | 246 |

Reviewer's note: The reproductive and developmental toxicology study submitted by the sponsor gives no information of the potential toxicity of riboflavin when administered during gestation. (b) (4)

Though no teratogenicity has been reported, it is prudent to keep the applicant's statement in the labeling regarding contraindicated use in pregnant patients (b) (4)

10 Integrated Summary and Safety Evaluation

Riboflavin is considered GRAS as a dietary supplement and the amount proposed to be administered approximates the recommended daily intake for human adults. Toxicity was not evident at levels within several orders of magnitude following oral administration.

Debridement of the corneal epithelium is required for riboflavin to reach and saturate the stroma of the cornea and subsequently the aqueous humor. Penetration of riboflavin to deeper ocular tissues was not determined. The proposed labeling states that pachymetry should be used to determine corneal thickness prior to undergoing the procedure. A minimum corneal depth of 400 μM is required, and this depth is supported by the nonclinical rabbit data.

The applicant cited a nonclinical study which examined the effect of different UVA doses on riboflavin-potentiated cytotoxic damage to the corneal endothelium. For debrided corneas with a thickness of 400 μM , significant endothelial cell necrosis with near complete loss of endothelial cells was produced at surface UVA (370 nm) irradiances of $\geq 3 \text{ mW/cm}^2$ (endothelial dose of $\geq 0.36 \text{ mW/cm}^2$) in the rabbit. In humans the same surface irradiance, 3 mW/cm^2 , appears to result in a nontoxic endothelial dose. The reason for this difference between species has not been definitely established, but may be due to differences in the absorption coefficient of riboflavin in treated human and rabbit corneas. Accordingly, others (Spoerl *et al*, 2007) calculate a human endothelial

dose of 0.18 mW/cm² at a surface irradiance of 3 mW/cm² and a corneal depth of 400 μM, which is 2-fold lower than the LOAEL endothelial dose in rabbits (0.36 mW/cm²). A NOAEL was established in this study at a surface irradiance of 2.6 mW/cm² (endothelial dose of 0.32 mW/cm²), which provides a ~1.75-fold margin of exposure over the sponsor-stated therapeutic endothelial irradiance in humans. It should be noted, however, that the accuracy of the absorption coefficients used by Wollensak in this paper is controversial, since other citations have presented different absorption coefficients in rabbits and humans. As such, the reliability of the above-listed exposure margins is uncertain.

Of particular note in the above rabbit study is that severe corneal endothelial toxicity was produced at a surface irradiance (3 mW/cm²), which represented only a 15% increase over that at NOAEL (2.6 mW/cm²). These data suggest that a narrow therapeutic window may exist given the steep UVA dose-response curve observed in this model.

The nonclinical studies submitted to support this application did not include studies to assess the potential toxicity of photoexcited riboflavin on non-corneal ocular tissues. Given the intended riboflavin dosing regimen, it is expected that ocular tissues other than cornea will contain increased amounts of riboflavin, and a theoretical concern exists regarding potential adverse effects due to photoexcitation of riboflavin contained within these tissues.

The genetic toxicity data suggest that riboflavin, itself, is not genotoxic. However, following UVA irradiation, photoexcited riboflavin ^{(b) (4)} have been shown to be genotoxic in the Ames test and in the SOS/*umu* test system. Under the conditions of use for the current indication, it is unknown whether the potential genotoxic effects of photoactivated riboflavin ^{(b) (4)} would promote ocular tumorigenesis. Nonclinical studies in which riboflavin was painted onto the skin or supplemented into the diet of hairless mice exposed daily to UV irradiation, however, did not show an increase in skin tumor incidence. These data provide some safety support regarding the potential for periocular photocarcinogenicity.

APPENDIX A: Original sponsor-proposed label (P/T recommended changes in red)

8 Page(s) of Draft Labeling have been Withheld in Full as b4 (CCI/TS) immediately following this page

This is a representation of an electronic record that was signed electronically and this page is the manifestation of the electronic signature.

/s/

AARON M RUHLAND
02/26/2014

LORI E KOTCH
02/26/2014