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May 15, 2007

Office of Food Additive Safety (HFS-255)
Center for Food Safety and Applied Nutrition
Food and Drug Administration
5100 Paint Branch Parkway
College Park, MD 20740-3835

RE: Notification of GRAS Determination for *trans*-Resveratrol in Bottled Water

Dear Sir/Madame:

In accordance with proposed 21 CFR § 170.36 (Notice of a claim for exemption based on a GRAS determination) published in the Federal Register (62 FR 18939-18964), I am submitting in triplicate, as the agent to the notifier, ATLA Holdings, LLC, a GRAS Notification for *trans*-Resveratrol in bottled water at levels up to 10 ppm.

Please let me know if you have any questions.

Sincerely,

Edward A. Steele
President

Enclosures

000003

EAS Consulting Group, LLC
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1. GRAS Exemption Claim

A. Claim of Exemption from the Requirement for Premarket Approval Requirements Pursuant to Proposed 21 CFR § 170.36(c)(1)

trans-Resveratrol, for use as a nutrient, has been determined to be Generally Recognized As Safe, and therefore, exempt from the requirement of premarket approval under the conditions of its intended use as described below. The basis for this finding is described in the following sections.

Signed,

_____ Date 5/14/07

Edward A. Steele

Agent for:

ATLA Holdings, LLC
75 Fifth Street, NW
Suite 325
Atlanta, GA 30308

B. Name and Address of Notifier

Greg Lamps
Vice President, Research and Development
ATLA Holdings, LLC
75 Fifth Street, NW
Suite 325
Atlanta, GA 30308
Phone: 404-920-0785
Fax: 404-920-0786
glamps@atlaholdings.com

C. Common or Usual Name of the Notified Substance

trans-Resveratrol

D. Conditions of Use

The intended use of *trans*-resveratrol is as a nutrient in bottled water ("near waters[1]").

trans-Resveratrol is intended to be added to bottled water at levels up to 10 ppm. Such application and use therefore correspond to resveratrol doses of up to 5 mg/500 ml serving or 2.4 mg/8 ounce serving of water (bottled). The estimated consumption of added resveratrol from the intended use levels of resveratrol at the mean and 90th percentile is determined as 2.09 and 3.99 mg/person/day or 0.04 and 0.07 mg/kg/day. There is sufficient qualitative and quantitative scientific evidence, including animal and human data, to determine safety-in-use or acceptable daily intake (ADI) for resveratrol. No systematic or typical 90-day toxicity study of resveratrol is available; however there are several experimental studies, such as short-term toxicity, chronic, and carcinogenicity, which support the safety-in-use and the ADI determinations.

In two separate 28 day studies, a NOAEL for resveratrol of 300 mg/kg/day was established. Using this experimental data and an uncertainty factor of 1000 (10 for interspecies, 10 for intraspecies, and 10 for chronic extrapolation), an ADI of 0.3 mg/kg/day (18 mg/person/day) for resveratrol is determined. This ADI is supported by a chronic study in mouse, in which a NOAEL of 22.4 mg/kg/day (highest dose used) was determined. In this study, mice were fed a high fat diet with resveratrol. Applying an uncertainty factor of 100 to the NOAEL of 22.4 mg/kg/day from the chronic mouse study yields an ADI of 0.224 mg of resveratrol/kg *per* day. Another study that supports the ADI determination is a six month p53 knockout mouse

[1]"Near waters" is a category of water-based soft drinks that are not defined by FDA and do not meet the standard of identity for bottled water as stated in 21 CFR 165.110. "Near waters" is described as waters that are fortified with minerals, vitamins, herbs, electrolytes, or other such ingredients.

carcinogenicity study in which the NOAEL for resveratrol of 1000 mg/kg/day was established. Using an uncertainty factor of 1000 (10 for interspecies, 10 for intraspecies and 10 for database deficiency), an ADI of 1 mg/kg/day for resveratrol may be determined. These studies support an ADI determination of 0.3 mg/kg/day. The estimated 90th percentile intake of resveratrol (0.07 mg/kg/day) from its intended uses is approximately 4-fold lower than the ADI (0.3 mg/kg/day) determined on the basis of available safety data.

Because of lack of typical standardized toxicity study, in addition to the above described standard approach of ADI determination, a "weight of the evidence" approach was also used to assess safety in use of resveratrol at the estimated daily intake level. All available studies on resveratrol were critically reviewed for quality and suitability for use in this safety assessment. The available dataset on resveratrol show that adverse effects of resveratrol are not likely to occur at resveratrol exposure levels of 0.07 mg/kg/day. Based on this approach, it is concluded that, under the intended and expected conditions of use, resveratrol is safe for human consumption.

Moderate consumers of red wine with high levels of resveratrol are likely to ingest 4 mg resveratrol/day or 0.067 mg/kg/day. The resulting 90th percentile intake of resveratrol from the intended uses is similar to individuals consuming red wine (in moderation) with high levels of resveratrol.

The estimated daily intake (0.07 mg/kg/day), if ingested daily over a lifetime, is considered as safe.

E. Basis for the GRAS Determination

Pursuant to 21 CFR § 170.30, *trans*-resveratrol, has been determined to be GRAS by scientific procedures. A comprehensive search of the scientific literature was also utilized for this review.

F. Availability of Information

The data and information that serve as a basis for this GRAS determination are available for the Food and Drug Administration's review and copying during business hours at the offices of:

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President
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Or

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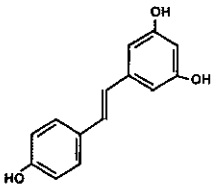
glamps@atlaholdings.com

II. A. Detailed Information About the Identity of the Substance

The substance that is the subject of this notification is *trans*-resveratrol chemically known as 3,4',5-stilbenetriol or 3,4',5-trihydroxystilbene. The chemical structure of resveratrol is as follows:



***trans*-Resveratrol**

trans-Resveratrol has a melting point of 260-264°C. It is prepared in the form of off-white to cream powder with ≥99% purity as determined by HPLC methodology. *trans*-Resveratrol has been shown to be stable over long periods of time (over 2 years) in well sealed containers kept away from light.

B. Method of Manufacturing

Resveratrol is extracted from roots of the plant *Polygonum cuspidatum*. Before the extraction process, the plant root is identified and confirmed as *P. cuspidatum* Sieb. Et Zucc[2]. The majority of the resveratrol in the root is in the form of piceid (glucosidic form of resveratrol). The concentration of piceid and resveratrol in *P. cuspidatum* root is generally between 1-2% and 0.1-0.3%, respectively. The root is dried and cut to small pieces. The extraction of resveratrol from the *P. cuspidatum* root also results in extraction of piceid. Piceid is extracted from the pieces of roots with ethanol. The piceid extract is evaporated to dryness under reduced pressure. The isolated piceid is hydrolyzed with sulfuric acid. Hydrolyzation of 1 g piceid yields approximately 0.58 g resveratrol. Normally hydrolysis produces a product containing

[2]A particular species of the 80 known species

approximately 50% resveratrol. Subsequent purification steps (column chromatography) results in a highly purified resveratrol (>99%) concentrate. The solvents and acids used in the isolation and purification process are food grade

C. Specifications for Resveratrol

The specifications for the final product are summarized below

Parameter	Typical specifications
Loss on drying	≤ 1%
Melting point	260-264°C
Total ash	≤ 1%
Purity (% by weight)	≥99%
Identification	HPLC and IR corresponding to standards
Impurities (area HPLC)	
Each unknown impurities	≤ 0 1%
Total impurities	≤ 1%
Solvent residues	Complies with ICH guidelines (CPMH/ICH/283/95 limits)
Ethanol	≤ 500 ppm (actual from 5 batches- < 55 ppm)
Ethyl acetate	≤ 100 ppm (actual from 5 batches- ND)
Heavy metals	
Cadmium	<2.5 ppm (actual from five batches- ND)
Lead	< 5 0 ppm (actual from five batches- ND)
Arsenic	< 5 0 ppm (actual from five batches- ND)
Mercury	<0.5 ppm (actual from five batches- ND)
Microbiological assays	
Total plate count	Not more than 1000 cfu/g (actual from 5 batches- < 200 cfu/g)
Yeast and Mold	Not more than 100 cfu/g (actual from 5 batches- < 25 cfu/g)
Escherichia coli	Negative
Salmonella	Negative
Pseudomonas aeruginosa	Negative

cfu = colony forming units; ND = not detectable; ppm= parts per million

III. Summary of the Basis for the Notifier's Determination that Resveratrol is GRAS

An independent panel of recognized experts, qualified by their scientific training and relevant national and international experience to evaluate the safety of food and food ingredients, was requested by ATLA Holdings, LLC to determine the Generally Recognized As Safe (GRAS) status of resveratrol intended for use as a dietary nutrient. A comprehensive search of the scientific literature was also utilized for this review.

Based on a critical evaluation of the pertinent data and information summarized here, the Expert Panel members have individually and collectively determined by scientific

procedures that addition of resveratrol to bottled water ("near waters"), meeting the specification cited above and manufactured according to current Good Manufacturing Practice, is Generally Recognized As Safe (GRAS) under the conditions of intended use in water (bottled), as specified herein.

In coming to its decision that resveratrol is GRAS, the Expert Panel relied upon, the conclusions that neither resveratrol nor any of its degradation products pose any toxicological hazards or safety concerns at the intended use levels, as well as published toxicology studies and other articles relating to the safety of the product. It is also their opinion that other qualified and competent scientists, reviewing the same publicly available toxicological and safety information, would reach the same conclusion.

IV. **Summary of the Basis for a Conclusion that Resveratrol is GRAS for its Intended Use.**

DETERMINATION OF THE GENERALLY RECOGNIZED AS SAFE (GRAS) STATUS OF RESVERATROL AS A NUTRIENT SUPPLEMENT

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DETERMINATION OF THE GENERALLY RECOGNIZED AS SAFE (GRAS) STATUS OF RESVERATROL AS A NUTRIENT SUPPLEMENT

1. INTRODUCTION

The undersigned, an independent panel of recognized experts (hereinafter referred to as the Expert Panel)[3], qualified by their scientific training and relevant national and international experience to evaluate the safety of food and food ingredients, was convened by EAS Consulting Group LLC at the request of ATLA Holdings, LLC, USA, to determine the Generally Recognized As Safe (GRAS) status of *trans*-Resveratrol as a nutrient supplement [21 CFR 170.3(o)(20)][4] at levels up to 10 ppm in bottled water (near waters). A comprehensive search of the scientific literature for safety and toxicity information on resveratrol was conducted through October 2006 and made available to the Expert Panel. The Expert Panel independently and critically evaluated materials submitted by ATLA Holdings, LLC and other materials deemed appropriate or necessary. Following an independent, critical evaluation, the Expert Panel conferred and unanimously agreed to the decision described herein.

1.1. Historical perspective

trans-Resveratrol, a polyphenol, occurs naturally in grapes, peanuts, and a number of other plants. It is commonly found in foods/drinks made from grapes and peanuts, and in a number of herbal remedies. As a constituent of red wine, resveratrol has been identified as one possible explanation for the 'French paradox,' *i.e* the finding that the incidence of coronary heart disease is relatively low in southern France despite the high intake of dietary saturated fats or similar risk factor profile (Hendler and Rorvik, 2001). Recent reports on the potential for resveratrol to inhibit the development of cancer and extend life expectancy in animal and cell culture models have continued to generate scientific interest. Available scientific evidence show that resveratrol has a wide range of desirable biological effects such as cardioprotection (Hung *et al.*, 2000), chemoprevention (Jang and Pezzuto, 1999), anticancer (Gusman *et al*, 200) and prolongation of life-span in several species (Howitz *et al.*, 2003; Valenzano *et al.*, 2006; Horn, *et al*, 2007).

1.2. Description, occurrence, manufacturing process and specifications

Resveratrol (CAS No. 501-36-0) is chemically known as 3,4',5-stilbenetriol or 3,4',5-trihydroxystilbene (Figure 1). It is produced by various plants to help defend against invading fungi, stress, injury, infection, and too much sunlight. Resveratrol may exist in both *cis*- and *trans*-stereoisomeric forms. Both *cis*- and *trans*-resveratrol also occur in their respective glucoside forms (bound to a glucose molecule). Resveratrol-3-*O*-

[3]Modeled after that described in section 201(s) of the Federal Food, Drug, and Cosmetic Act, As Amended. See also attachments (curriculum vitae) documenting the expertise of the Panel members.

[4]Nutrient supplements. Substances which are necessary for the body's nutritional and metabolic processes.

β-glucoside is also called piceid. Several methods including high-performance liquid chromatography (HPLC), gas chromatography (GC), gas chromatography-mass spectrometry (GC-MS), and capillary electrophoresis (CE) have been employed to extract resveratrol from wine and to isolate the *trans*- and *cis*- isomers of resveratrol (NIEHS, 2002). Resveratrol (>98% purity) can be isolated from *Polygonum cuspidatum* by employing high-speed counter-current chromatography (Yang *et al.*, 2001).

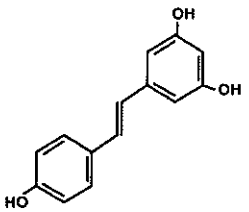

Figure 1. Chemical structure of resveratrol

The ATLA Holdings, LLC's *trans*-resveratrol is a purified, off-white to cream-colored solid product in powder form. It is extracted from the roots of *P. cuspidatum* Sieb Et Zucc (Knotweed herb; bushy knotweed, Japanese knotweed). *trans*-Resveratrol is isolated by solvent extraction and the purified product is reported to contain >99% *trans*-resveratrol. General descriptive parameters and properties of *trans*-resveratrol are summarized in Table 1.

Table 1. General descriptive characteristics of Resveratrol

Parameter	Description
Synonyms	3, 5, 4′-Trihydroxystilbene, 1,3-Benzenediol, 5-[(1*E*)-2-(4-hydroxyphenyl)ethenyl] (9CI); CA 1201; (*E*)-Resveratrol, 3,4′, 5-Stilbenetriol (7CI, 8CI); (*E*)-5-[2-(4-Hydroxyphenyl)ethenyl]-1,3-benzenediol; (*E*)-5-(*p*-Hydroxystyryl)resorcinol
CAS No.	501-36-0
Molecular formula	$C_{14}H_{12}O_3$
Molecular weight	228 2
Physical state	Solid powder
Appearance	Off-white to cream
Odor	Characteristic
Taste	Characteristic
Storage	Well-sealed in dry place away from light
Shelf life	24 months

1.2.1. Occurrence

Resveratrol is found in over 70 common plant species (NIEHS, 2002). The presence of *trans*-resveratrol in varying concentrations has been reported in grapes, peanuts, eucalyptus, spruce, lily, mulberries, groundnut, members of the knotweed and hellebore genera (*Polygonum* and *Helleborus*), and fescue grass. The highest concentration of resveratrol has been reported in *P cuspidatum*. Resveratrol is primarily found in lignified plant tissues, in leaves, and in berries of *Vaccinum* species, including

blueberries, bilberries, and cranberries. *trans*-Resveratrol was also detected in vines, and leaf tissues of *Vitis vinifera* infected with fungi or exposed to UV light. Infection of grapes with the fungus *Botrytis cinerea* (gray mold) has been reported to result in increased concentrations of resveratrol in nearby (unaffected) grapes. Stimulating a grape plant's production of resveratrol and other defense chemicals has been shown to increase its resistance to fungal infection.

The concentration of resveratrol in food substances varies widely. Grape skin (fresh) contains approximately 50 to 100 μg/g (0.22 to 0.44 μmol/g) of *trans*-resveratrol (NIEHS, 2002). Stewart *et al* (2003) reported that resveratrol accounts for 5–10% of the grape skin biomass. Generally, non-muscadine red wine is reported to contain between 0.2 and 5.8 mg resveratrol/L (Gu *et al.,* 1999). The amount of reseveratrol in wine depends on the grape variety. White wine has much less resveratrol compared to red, the reason being that red wine is fermented with the skins, allowing the release of resveratrol from skin, whereas white wine is fermented after the skin has been removed. Wines produced from muscadine grapes, however, both red and white, contain higher levels of resveratrol, up to 13.4 mg/L (NIEHS, 2002).

1.2.2. Manufacturing process

Resveratrol is extracted from roots of the plant *P cuspidatum*. Before the extraction process, the plant root is identified as *P. cuspidatum* Sieb. Et Zucc. In plants, the majority of the resveratrol present occurs as glucoside called piceid. The concentration of piceid and resveratrol in *P. cuspidatum* root is generally between 1-2% and 0.1-0.3%, respectively. The root is dried and cut to small pieces. The extraction of resveratrol from the *P. cuspidatum* root also results in extraction of piceid. Piceid is extracted from the pieces of roots with ethanol. The piceid extract is evaporated to dryness under reduced pressure. The isolated piceid is hydrolyzed with sulfuric acid. Hydrolyzation of 1 g piceid yields approximately 0.58 g resveratrol. Normally hydrolysis produces a product containing approximately 50% resveratrol. Subsequent purification steps results in highly purified resveratrol (>99%) concentrate. The solvents and acids used in the isolation and purification process are food grade.

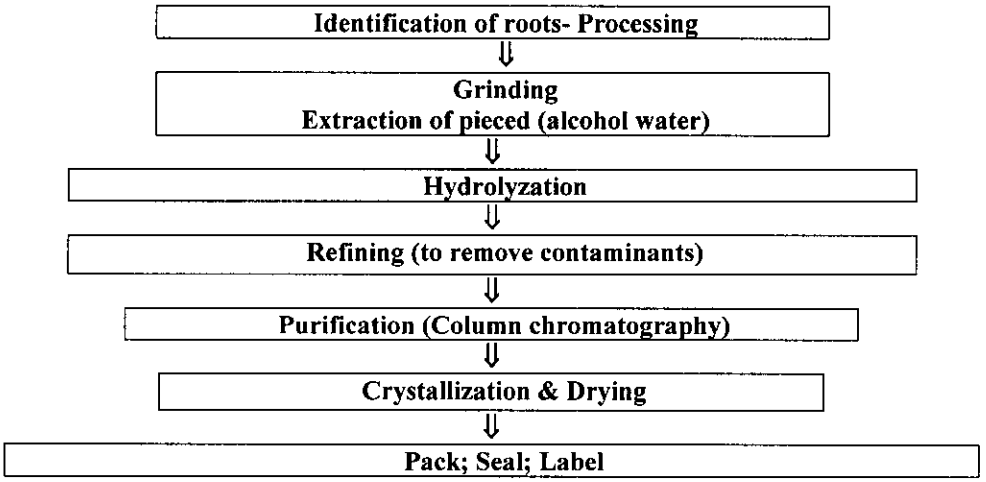

Figure 2. Schematic of manufacturing process of Resveratrol

1.2.3. Identity and specifications

Physical characteristics and specifications of resveratrol from ATLA Holdings, LLC, are presented in Table 2. Analytical results of five lots from non-consecutive batches indicate that the resveratrol product meets these specifications.

Table 2. Specifications of Resveratrol

Parameter	Specifications (ATLA Holding, LLC.)
Loss on drying	≤ 1%
Melting point	260-264°C
Total ash	≤ 1%
Purity (% by weight)	≥99%
Identification	HPLC and IR corresponding to standards
Impurities (area HPLC)	
Each unknown impurities	≤ 0.1%
Total impurities	≤ 1%
Solvent residues	Complies with ICH guidelines (CPMH/ICH/283/95 limits)
Ethanol	≤ 500 ppm (actual from 5 batches- < 55 ppm)
Ethyl acetate	≤ 100 ppm (actual from 5 batches- ND)
Heavy metals	
Cadmium	<2.5 ppm (actual from five batches- ND)
Lead	< 5.0 ppm (actual from five batches- ND)
Arsenic	< 5.0 ppm (actual from five batches- ND)
Mercury	<0.5 ppm (actual from five batches- ND)
Microbiological assays	
Total plate count	Not more than 1000 cfu/g (actual from 5 batches- < 200 cfu/g)
Yeast and Mold	Not more than 100 cfu/g (actual from 5 batches- < 25 cfu/g)
Escherichia coli	Negative
Salmonella	Negative
Pseudomonas aeruginosa	Negative

cfu = colony forming units; ND = not detectable; ppm= parts per million

1.2.4. Technical effects

Resveratrol is intended for uses in food as a nutrient supplement, for individuals who wish to increase their daily intake of resveratrol. The nutritive contribution of resveratrol is well recognized. It is naturally present in a variety of commonly consumed foods such as grapes, peanut, blueberries and mulberry. As indicated earlier, resveratrol has wide-ranging biological activities including inhibition of lipid peroxidation, free-radical scavenging activity, anti-inflammatory activity, modulation of lipid metabolism, and anticancer activity. Its antioxidant activities are believed to be due to its protective effects on the cellular membranes. Although resveratrol is present at certain amount in foods, its supplementation to food is aimed at gaining certain health benefits. Resveratrol's effects are reported to be due to its amphiphatic character (as the structure has both hydrophilic and hydrophobic sites), which allows the protection of cellular and subcellular components.

1.3. Historical and current uses

Humans have been exposed to resveratrol since ancient times, at least as long as grapes, wine-making and the consumption of other resveratrol-containing plants have been popular. In traditional Asian remedies, the root of *P. cuspidatum,* a source of resveratrol, has long been used as a circulatory tonic, among other uses (NIEHS, 2002). Approximately 4500 years ago, *Ayurveda,* the ancient medicinal book of Hindus described "Darakchasava" (fermented juice of red grapes) as a heart tonic (Hendler and Rorvik, 2001). At present, several dietary supplements marketed in the United States contain *trans*-resveratrol. The source of these supplements is primarily an extract of *P. cuspidatum* or ground dried red grape skins. The *P. cuspidatum* extracts are usually standardized to deliver approximately 8% resveratrol. Some supplements deliver 16 mg resveratrol *per* serving or higher.

1.4. Intended uses and estimated daily intake

ATLA Holdings, LLC, intends to use *trans*-resveratrol as a nutrient supplement [21CFR170.3(o)(20)][5] at levels up to 10 ppm in bottled water ("near water") [21CFR 170.3(n)(3)][6]. The daily estimated intake of *trans*-resveratrol was calculated using the intended maximum use level value and the consumption of water (bottled) containing resveratrol. To obtain the estimated daily intake of resveratrol from the intended uses, CanTox Health Sciences International was commissioned to undertake these determinations. The CanTox report is attached as Appendix II.

As resveratrol is intended to be marketed in bottled water, intake analysis based on consumption of bottled water was considered as the most appropriate and applicable method for exposure analysis. The representative data for bottled water consumption from individual dietary records in the CSFII 1994-1996, 1998 is lacking. Hence, the

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[5] Nutrient supplements· Substances which are necessary for the body's nutritional and metabolic processes.

[6] The category includes Beverages and beverage bases, nonalcoholic, including only special or spiced teas, soft drinks, coffee substitutes, and fruit and vegetable flavored gelatin drinks

methodology presented by the Environmental Protection Agency for the calculation of direct bottled water consumption from the CSFII 1994-1996 was replicated in the intake assessment performed by CanTox using data from the CSFII 1994-1996, 1998. Household and sample person data from additional record types (Record types 15 and 25, respectively) available within the CSFII dataset were merged with the individual dietary record file (Record type 30) to determine the amount of plain bottled water consumed by survey respondents. The method used differed only in the manner in which files were merged. A new food code was generated to represent bottled water intake (95000000, Water, bottled) in the current intake assessment. The intakes of bottled water represented by this new food code were then added to the individual dietary record for each bottled water consumer, in order to allow estimation of the intake of resveratrol from bottled water, and to derive the total intake of resveratrol from all currently identified food-uses. Based on this analysis, the estimated consumption of added resveratrol from the intended use levels of 10 ppm at mean and 90th percentile for all-users total population ("users only") is determined as 2.09 and 3.99 mg resveratrol/person/day or 0.02 and 0.07 mg/kg/day. The CanTox derived estimated daily intake of resveratrol by the US population from the intended food uses of resveratrol is summarized in Table 3.

Table 3. Summary of the Estimated Daily Intake of Resveratrol from All Investigated Food Categories in the US by Population Group (1994-1996, 1998 USDA CSFII Data)

Population Group	Age Group (Years)	% Users	Actual # of Total Users	All-Person Consumption (mg)		All-Users Consumption (mg)	
				Mean	90th Percentile	Mean	90th Percentile
Infant	0 to 2	19.5	698	0.26	0.35	1.35	1 42
Child	3 to 11	21.0	1,326	0.52	0 71	2.74	2 84
Female Teenager	12 to 19	20.1	141	0.36	1 06	1.78	3.19
Male Teenager	12 to 19	16.4	114	0 32	1 06	1.96	4 26
Female Adult	20 and Up	20.4	933	0.47	1.77	2.10	4 26
Male Adult	20 and Up	18.4	872	0.40	1.42	1.99	4.26
Total Population	All Ages	19.8	4,084	0 43	1.42	2 09	3.99

In a report prepared in 2000 for the Rockefeller University and conducted by Yankelovich Partners, it was determined that of the total daily estimated intake of 6.1 servings (each serving consisting of eight ounce fluid) of water consumed in the US, 2.3 servings were consumed daily as bottled water (Saint-Jean, 2001). Based on this report, the daily consumption of bottled water in the US is approximately 544 ml (2.3 servings X 8 ounce = 18.4 ounce; 18.4 ounces X 29.6 ml = 544 ml). Based on the results of this report, the intended use of resveratrol at use levels of 10 ppm in bottled drinking water will result in an estimated intake of 5.44 mg resveratrol/person/day or 0.09 mg/kg/day (for an individual weighing 60 kg). In this survey, adults were the only population group from which consumption data were obtained. Additional details of the report and methods of determination used were not available. However, the resulting estimated daily intake is lower than the tolerable daily intake.

1.4.1. Daily intake *via* natural occurrence in food

Humans have been exposed to resveratrol since ancient times primarily through the widespread consumption of grapes and red wine. The current and primary sources of human exposure to *trans*-resveratrol are primarily *via* ingestion of peanuts, grapes, and the products containing either these products or derived from them (particularly wine) and *via* consumer use of dietary supplements. The concentration of resveratrol in grapes, grape juice, and wine is highly variable. *Per capita* wine consumption in the US during 2001 was estimated as 8.55 liters (Wine Institute, 2006). The highest *per capita* consumption of wine is reported in Luxembourg (64.02 liters/year). Based on CSFII "eaters only" data (2002), *per capita* total wine consumption from all sources (including wine used in food preparation) in the US is reported as 16 ml/person/day. Details about the levels of *trans*-resveratrol in different wines are presented in Table 4. Compared to grape products, the concentration of resveratrol in peanuts and peanut products is relatively low. In roasted peanuts, boiled peanuts and peanut butter, resveratrol concentrations were reported as 0.05, 5.14, and 0.32 μg/g, respectively (NIEHS, 2002). In other reports, the levels of *trans*-resveratrol in peanuts ranged from 0.02 -1.79 μg/g (0.09-7.84 nmol/g).

Analysis of exposure from the reports described above indicate that the daily intake of resveratrol from existing food and beverage sources will be a small amount and difficult to determine accurately. As noted earlier, red wine is much higher than white wine in resveratrol as red wine is prepared by fermentation with the skins. If one considers for exposure estimate purposes that all the wine consumed is red wine with maximum levels of resveratrol (13.4 mg/L) and *per capita* consumption of wine (16 ml), the resulting intake of resveratrol in US will be 0.21 mg/person/day. As the reported intake of wine is high in Luxembourg, the likely intake of resveratrol is expected to be eight-fold greater than in the US.

An individual ingesting red wine (containing the highest levels of resveratrol) regularly and in moderation (2 drinks/day = ~300 ml) is likely to ingest 4 mg resveratrol/day or 0.067 mg/kg/day for an individual weighing 60 kg. These values are similar to the estimated daily intake of resveratrol (0.07 mg/kg/day at 90th percentile) resulting from its intended use in bottled water. Although, exact figures are not available, it is well known that there are significant numbers of individuals who consume red wine daily in moderation. As noted earlier, the amount of resveratrol in dietary supplements varies from product to product and depends on dosage recommendations. The dietary supplement recommended dosage of resveratrol covered a range from 3 to 1000 mg (NIEHS, 2002).

Above described information suggest that red wine is the major natural source of resveratrol intake. Very small amount of resveratrol is ingested by other sources such as peanut. Moderate consumers of red wine containing high levels of resveratrol are likely to ingest about 4 mg resveratrol/day or 0.067 mg/kg/day. The estimated 90th percentile daily intake of resveratrol (3.99 mg/person) is similar to that of consumers of red wine (in moderation) with high levels of resveratrol (4 mg/person).

Table 4 Concentrations of *trans*-resveratrol in wines

Wine type (grape species used)	Concentration range
White (*V vinifera*)	≤0.02 mg/L (0 09 μM)
White (*V. rotundifolia*)	0 29-1 18 mg/L (1.3-5.17 μM)
Spanish rose (grape species n.p.)	~1 2-2 2 μM (0 27-0.50 μg/ml)
Red (muscadine [where noted]; otherwise, species n.p.)	≤0.02-13 4 mg/L (0 09-58.7 μM) (upper concentration from muscadine grapes)

n p = not provided. Adapted from NIEHS (2002)

1.5. Consumption summary

The intended use of resveratrol at levels of 10 ppm in bottled water ("near waters") will result in estimated mean and 90th percentile exposures of 2.09 and 3.99 mg resveratrol/person/day or 0.04 and 0.07 mg/kg/day, respectively. The mean intake of resveratrol from consumption of wine in US is estimated as 0.21 mg/person/day (0.004 mg/kg/day). The intake of resveratrol from other natural dietary sources is very small. The total estimated intake of resveratrol from all sources including its natural consumption is approximately 4 mg/person/day. The estimated daily intake from intended uses of resveratrol is below acceptable daily intake determined on the basis of available safety information on resveratrol (see discussion below). In summary the 90th estimated intake of resveratrol from the intended uses is determined as 3.99 mg/person/day.

2. Toxicology

trans-Resveratrol is naturally present in a number of commonly consumed foods such as grapes (red-wine) and peanuts. Because of its potential health benefits, there has been a considerable effort to elucidate the potential biological effects of *trans*-resveratrol. As a result, literature is full of information on resveratrol. The relevant biological and toxicological studies on *trans*-resveratrol are included in the following section. The results of metabolism, subchronic, chronic toxicity, and carcinogenicity studies are also discussed from a mechanism of toxicity perspective.

2.1. Absorption, metabolism and excretion

Available animal studies and human data show that following oral ingestion, resveratrol is absorbed from the gastrointestinal tract. A summary of the metabolic and toxicokinetic studies of resveratrol is presented in Table 5. The human (*in vivo*) studies of resveratrol detailing absorption, metabolism and excretion are described in section 2.4.

2.1.1. *In vitro* studies

Andlauer *et al.* (2000) investigated the bioavailablity of *trans*-resveratrol in the perfused small intestine of the rat (Table 5). Following perfusion of the small intestine with a synthetic perfusate containing either 28, 34, or 57 μM resveratrol, the majority of the absorbed resveratrol was found in the luminal effluent (54%). Approximately 20% of the administered resveratrol appeared at the vascular site, with the major product being

the conjugated glucuronide form. In another study, Kuhnle *et al.* (2000) reported that small amounts of unmetabolized resveratrol were absorbed across the enterocytes of the jejunum and ileum and significant amounts of its glucuronide were found in the serosal fluid (Table 5). In this study, 100 μM of resveratrol was administered and the glucuronide noted in jejunum and ileum was reported as 1.19 and ~0.45 nmol/cm, respectively.

In human liver microsomes, the maximum rate of *trans*-resveratrol glucuronidation occurred at a neutral pH, and the resveratrol-glucuronide amount increased linearly with time up to 40 min (Table 5). The highest rate of glucuronidation was noted at concentrations of up to 1 mM resveratrol and 1 mM uridine 5′-diphosphoglucuronic acid in incubation mixture. The reaction of resveratrol sulphation at a concentration up to 2 μM resveratrol and 0.4 μM 3′-phosphoadenosine-5′-phosphosulphate was also linear for about 40 min. The rates of resveratrol sulphation were inhibited (mixed and noncompetitive) by flavonoids such as quercetin, fisetin, myricetin, kaempferol and apigenin. Flavonoids also inhibited resveratrol glucuronidation, but the extent of inhibition was less than that for sulphation (De Santi *et al.*, 2000a,b,c).

Table 5. Metabolism and toxicokinetic studies of resveratrol

Test system/Species	Protocol Details	Results/observations	Reference
***In vitro* studies**			
Rat- small intestine	Single pass perfusion for 60 min, 6 4, 7 8, and 13 μg/ml	In the luminal perfusate resveratrol degradation was ~16% after 2 h at 37°C, in the vascular perfusate no degradation occurred. The recovery of free resveratrol, glucuronide and sulfate in luminant effluent was ~40, 11 and 3%, respectively. In vascular side the recovery was 3, 7 and 0.3%, respectively	Andlauer *et al* (2000)
Rat- small intestine	Single pass perfusion for 90 min; 22.8 μg/ml	~96% of the absorbed amount was found as resveratrol glucuronide on the serosal side of the enterocytes of the jejunum versus the amount of unmetabolized resveratrol	Kuhnle *et al* (2000)
Human liver microsomes	Incubation for 30 min; 1 mM in 0 05 ml incubation mixture	The rate of resveratrol glucuronidation ranged from0.23 to 1 2 nmol/min/mg	De Santi *et al* (2000a)
Human liver microsomes	Incubation for 30 min; 0 0625, 0.125, 0.25, 0 5 and 1 mM in incubation mixture	Glucuronosyl transferase towards resveratrol followed Michaelis-Menten kinetics Km = 0.15 mM; Vmax = 1 3 nmol/min/mg; Intrinsic clearance = 11 ml/min/mg	De Santi *et al.* (2000a)

***In vivo* studies**

Species	Dose	Duration	Route	Results/observations	Reference
Rat Wistar	86 μg/kg in red wine	0, 0.5, 1, 2, 4, 8 and 12 h	Gavage	Maximum resveratrol in plasma at 1 hour. Maximum concentration in liver, heart, and kidney 21, 2, and 20 ng/g, respectively	Bertelli *et al* (1998a)

Rats Wistar	43 µg/kg in red wine	15 days	Gavage	Maximum concentration in plasma, liver, heart, and kidney ~8, 54, 3, and 44 ng/g, respectively Equilibrium reached between absorbed and excreted resveratrol	Bertelli *et al* (1998a)
Rats Wistar	28 µg/kg in red wine	0, 0.5, 1, 2, 4, 8 and 12 h	Gavage	Plasma resveratrol at 0 5, 1 and 2 h· one compartmental model- Clearance=739 ml/h, V1=533 ml, Ka=1.46/h In two compartmental model- Half life- absorption = 0 46 h, distribution α= 0 48, elimination (kidneys) = 0 5 h, elimination (plasma) = 0.5 h, terminal plasma β= 25 h	Bertelli *et al* (1998b)
Rats Sprague Dawley	2 mg/kg	One dose	IP*	Blood concentration measured for ~ 300 min. Resveratrol absorbed rapidly. Blood concentration declined as "two-exponential" pathway The elimination rate constant for phase 1 (Kel) = 0.185/min. Half life (t1/2) = 3.74 min. AUC = 9917 min-ng/ml.	Zhu *et al.* (2000)
Mice C57BL/6 (60 female)	4000 mg/kg	0.25, 0 5, 1, 2, 4, 8 and 24 h	Gavage	rapidly absorbed, completely metabolized; glucuronide and sulfate formation; Cmax free resveratrol = 20 to 40 µM (range)	Horn *et al*, 2007

IP = intraperitoneal

2.1.2. *In vivo* studies

Oral administration of resveratrol (86 µg/kg or 43 µg/kg) daily for 15 days in red wine to rats indicates that resveratrol was rapidly absorbed from the intestine in rats (Bertelli *et al.*, 1998a) (Table 5). Within one hour of administration, maximum levels of resveratrol were noted in the blood. Following single (86 µg/kg) or repeated (43 µg/kg/day for 15 days) administration of resveratrol to rats, the highest amount was noted in liver (20.7 and 53.5 ng/g). The investigators reported that the "main excretion pathways appear to be renal." Kinetic studies revealed equilibrium between the absorbed and the eliminated resveratrol. In another report by these investigators, significant cardiac bioavailability was noted, as well as a strong affinity for the liver and kidneys (Bertelli *et al.*, 1998b). Zhu *et al.* (2000) reported that following intraperitoneal administration of *trans*-resveratrol (2 mg/kg), it was rapidly absorbed and the concentration in rat blood declined in a "two-exponential" manner.

Abd El-Mohsen *et al.* (2006) investigated the distribution of [^{3}H]-*trans*-resveratrol and its metabolites, following gavage administration. Male Sprague Dawley rats were gavaged with 50 mg/kg [^{3}H]-radiolabeled resveratrol. At 2 hours after the injection, plasma concentrations of resveratrol reached 1.7% of the administered dose, while liver and kidney concentrations were 1.0 and 0.6%, respectively. At the end of 18 hours, plasma levels were 0.5% in plasma and a total of 0.35% in tissues. At 18 hours, kidney and liver concentrations fell to 10 and 25%, respectively, of concentrations at 2

hours, the brain retained 43% of that measured at 2 hours. The major metabolite was identified as resveratrol-glucuronide, reaching 7 μm in plasma at 2 hours. However, at 18 hours the main form identified in liver, heart, lung and brain was native resveratrol aglycone. Unlike flavonoids, resveratrol does not appear to be metabolized by colonic microflora as no phenolic degradation products were detected in urine or tissues. The results of this study indicate that resveratrol and not its metabolite might be responsible for its *in vivo* biological effects.

Hebbar *et al* (2005) investigated the effects of resveratrol on stress-related genes and drug detoxifying enzymes by using cDNA array analysis and Quantitative Real-Time PCR. Male and female CD-rats were treated (gavage) with resveratrol (300, 1000 and 3000 mg/kg/day) for 28 days. The gene expression profiles of Phase I drug metabolizing enzymes changed only slightly from control rats among the low and intermediate dose groups. The induction in these dose groups did not change more than 2-fold from control rats. Investigators used a cut-off of 1.5 X fold of control in at least one treatment dose level, to explain gene expression changes in the array data. The investigators concluded that resveratrol, especially at higher doses moderately induced Phase II enzyme activities and inhibited Phase I enzyme activities in the rat liver. As evidenced from the cDNA array data, resveratrol modulated the expression of certain Phase I drug metabolizing as well as antioxidant genes. The clinical significance of the changes noted in a variety of genes is difficult to interpret. In addition to gene array, the investigators also studied various safety related parameters. Evaluation of toxicity parameters revealed that administration of resveratrol at 300 mg/kg/day for 28 days did not cause adverse effects. The safety related results from this study are described below in short-term and subchronic studies (see section 2.3).

As cited in the NIEHS (2002) report, based on an abstract publication, oral administration of *cis*- and *trans*-resveratrol (1 mg/kg/day for five or ten days) to CD2F1-mice caused inhibition of 7-ethoxyresorufin-*o*-dealkylation (EROD) activity (CYP1A2). However, resveratrol did not affect ethoxycoumarin-*o*deethylation (ECOD) activity (CYP1A2/2E1) or benzo[a]pyrene metabolism. The details of this publication were not available. In an *in vitro* study, also cited in NIEHS (2002) report, incubation of resveratrol with human microsomes inhibited CYP1A2 (methoxyresorufin *O*-demethylation) and CYP3A4 (erythromycin demethylation) without affecting CYP2E1 activity.

By inhibiting the expression and activity of certain cytochrome P450 enzymes, resveratrol may prevent activation of some carcinogens. In contrast, increasing the activity of phase II biotransformation enzymes generally promotes the excretion of potentially toxic or carcinogenic chemicals. Although such interactions have not been reported in humans, high intakes of resveratrol could theoretically increase the bioavailability and the risk of toxicity of drugs that undergo extensive first-pass metabolism by cytochrome P450. Available *in vivo* and *in vitro* studies indicate that resveratrol-mediated changes in drug metabolizing enzymes occur at doses greater than ≥ 1 mg/kg/day. The intended use of resveratrol at the low doses in water (bottled) is unlikely to alter the drug metabolizing enzymes. The available studies show that resveratrol is rapidly absorbed from the intestine and eliminated *via* kidney. As discussed

in human, because of its rapid metabolism and elimination, bioavailability of resveratrol is low.

2.2. Acute studies

In a brief report, Juan *et al* (2002) investigated the acute toxic effects of *trans*-resveratrol in rats (apparently Sprague Dawley). The study was conducted according to the Organization for Economic Cooperation and Development (OECD) guidelines at a dose level of 2000 mg/kg. The investigators concluded that the absence of symptoms, the lack of any negative effects on growth and the normal appearance of vital organs in the gross necropsy suggest that *trans*-resveratrol was practically non-toxic even under these exaggerated exposure conditions.

In unpublished studies cited by Boocock *et al.* (2006), reported as part of Phase I human trials of *trans*-resveratrol, the toxicity of pure *trans*-resveratrol was studied in rats and mice. In rats, administration of a single intravenous dose of resveratrol (80 mg/kg; highest dose) failed to elicit any manifestation of toxicity, as reflected by lack of any effects on weight loss or histological alterations investigated at a range of time points post-dosing. Similarly, repeated oral dosing of resveratrol (250 mg/kg daily for 5 days) in mice was without adverse effect.

In summary, results from an acute toxicity study show that the LD_{50} (median lethal dose) of resveratrol is likely to be higher than 2 g/day. The results of the acute toxicity study indicate that *trans*-resveratrol is practically nontoxic. Single intravenous administration of resveratrol (80 mg/kg) to rats or oral repeat-dose administration (250 mg/kg/day for 5 days) to mice did not reveal any adverse effects.

2.3. Short term and subchronic studies

Juan *et al* (2002) investigated the effects of repeated oral administration of *trans*-resveratrol in male Sprague-Dawley rats. *trans*-Resveratrol dissolved in carboxymethylcellulose was administered orally to male Sprague-Dawley rats at a dose of 20 mg/kg/day for 28 days. Animals in the control group received carboxymethylcellulose. Compared to the control group, resveratrol administration did not affect body weight, food or water consumption. The results of the hematologic tests did not differ between control and treatment groups. *trans*-Resveratrol treatment did not affect serum lipids, enzymes, electrolytes or other metabolites except alanine aminotransferase (ALT), which was 30% higher compared to the control group. *trans*-Resveratrol treatment did not affect the final relative weights of lungs, spleen, heart, liver, kidney or adrenal gland. However, relative brain weight was greater in the treatment group compared to control rats. Histopathology examination of the organs did not reveal any alterations between the groups. The increase in ALT noted in this study was not associated with histological changes.

Crowell *et al.* (2004) investigated the potential adverse effects of resveratrol in rats. Male and female CD-rats (20/sex/group) were dosed once daily with resveratrol (0, 300, 1000, or 3000 mg/kg/day) for 28 days *via* gavage. The majority of the adverse

effects were noted in the rats receiving 3000 mg/kg/day of resveratrol. Adverse effects included decreased final body weights and food intake; increased clinical signs of toxicity; increases in plasma levels of BUN, creatinine, alkaline phosphatase, ALT, total bilirubin, and albumin; decreases in hemoglobin, hematocrit, and red cell counts; and increased white cell counts. Additionally, at this dose level (3000 mg/kg/day) increased kidney weights, gross renal pathology changes, and an increased incidence and severity of histopathological changes in the kidneys were noted. Although clinical chemistry changes suggest liver toxicity, this was not supported by histopathology. In rats treated with 1000 mg/kg/day of resveratrol, reduced body weight gain (females only) and elevated white blood cell count (males only) were noted. The investigators determined the no-observed-adverse-effect level (NOAEL) as 300 mg/kg/day in rats. In a review article of toxicological studies of resveratrol prepared for the NIEHS (2002), this article was considered to determine the NOAEL for resveratrol (300 mg/kg/day). The study referenced above was conducted in compliance with Good Laboratory Practices and meets FDA core standards for safety studies of food additives.

As mentioned earlier, Hebbar *et al.* (2005) studied the dose-response effects of resveratrol in rats. Male and female CD-rats were gavaged with resveratrol (300, 1000 and 3000 mg/kg/day) for 28 days. At termination, clinical chemistry parameters such as ALT, alkaline phosphatates, total bilirubin, blood urea nitrogen, creatinine were not affected at low (300 mg/kg/day) and intermediate (1000 mg/kg/day) doses of resveratrol. At the high dose level (3000 mg/kg/day), nephrotoxicity was observed in the male and female rats and dehydration and labored breathing were seen among the female rats at the intermediate dose level (1000 mg/kg/day). At the highest dose level, slight but significant increases in ALT and alkaline phosphatatse in male and female rats were noted. Increases in total bilirubin, blood urea nitrogen and creatinine were noted only in female rats at the highest dose of resveratrol. Based on the results of this study, the NOAEL of resveratrol is determined as 300 mg/kg/day in rats. The results from these investigations support the observation from the Crowell *et al* (2004) study.

In a four week study, C57BL/6-mice (10/sex/group) were treated daily *via* gavage with resveratrol at doses of 0 (vehicle control), 1000, 2000, 3000, 4000, or 5000 mg/kg/day for 28 consecutive days (Horn *et al.,* 2007). Animals were observed twice daily for morbidity and mortality, and weighed weekly. On study day 29, blood was drawn for clinical pathology evaluations and a limited gross necropsy was performed with collection of gross lesions data. In male mice receiving the highest dose of resveratrol (5000 mg/kg /day), a 40% mortality was noted. Necropsy in all early deaths observed in the high-dose group revealed a large amount of unabsorbed resveratrol in the stomach and/or intestine. Except for the mortality associated with the impaction of resveratrol in the gastrointestinal tract, no clear evidence of resveratrol toxicity was identified in any dose group. The study was designed as part of an oncogenicity study (Horn *et al.,* 2007). In other NCI-sponsored preclinical toxicity studies of resveratrol (cited in Boocock *et al*, 2006), doses of up to 1000 mg/kg/day in rats treated for 28 days, or doses up to 2000 mg/kg/day in dogs treated for 14 days, did not reveal any toxic effects.

In a subchronic study, male Sprague-Dawley rats were administered 20 mg/kg/day of resveratrol *via* gavage for 90-days (Juan *et al.,* 2005). The investigators reported that the selected dosage is not harmful and corresponds to 1000 times the amount of resveratrol consumed by a 70-kg person who drinks 250 mL of red wine a day containing 1.4 mg of *trans*-resveratrol. The objective of this study was to investigate the effect of *trans*-resveratrol on the testis and on spermatogenesis (See Section 2.5. Reproductive and Developmental Studies). Although the study was designed to investigate reproductive toxicity of resveratrol, it does provide additional information on the safety of the resveratrol. During the course of study, rats were examined daily for any changes in skin, eyes, mucous membranes, respiratory system, autonomic and central nervous system conditions, somatomotor pattern, and behavior. Body weight and food and water consumption were recorded daily. The growth rate was calculated as the difference between the final weight and the initial weight divided by 90 day. The food and water intake and body weight gain of the resveratrol-treated group did not differ from that of the controls. Resveratrol administration did not adversely affect any of the parameters studied. As discussed later in Section 2.5, resveratrol did not affect testicular gross anatomy, wet weight or relative weight.

In summary, available short-term studies of resveratrol indicate that daily administration of resveratrol at doses up to 300 mg/kg/day for 28 days to rats did not reveal any adverse effects. In one study (Juan *et al.* 2002), resveratrol administration at a dose of 20 mg/kg/day for 28 days revealed increased serum ALT levels without any histopathological changes. Contrary to the increased serum ALT levels noted by Juan *et al* (2002), results from two separate subsequent well-designed studies by Crowell *et al.* (2004) and Hebbar *et al.* (2005) did not observe any increase in ALT levels at dose levels of 300 mg/kg/day (15-fold higher). In both these subsequent studies, elevation of ALT levels was noted at a very high dose of 3000 mg/kg/day, but without any histopathological alteration. Additionally, in an NCI sponsored study, resveratrol did not reveal any adverse effects at dose level of 1000 mg/kg/day. In a study in mice, resveratrol at doses up to 4000 mg/kg/day did not reveal any adverse effects. Based on the results of two separate studies in rats, the NOAEL of resveratrol is determined as 300 mg/kg/day.

2.4. Chronic toxicity and carcinogenicity studies

In an extensive and well designed study, Baur *et al.* (2006) investigated the long-term effects of *trans*-resveratrol on the health and survival of mice. Cohorts of one year old male C57BL/6NIA-mice were maintained on a standard diet or an otherwise equivalent high-calorie diet alone (60% calories from fat) or high caloric diet with resveratrol that provided an average 5.2 or 22.4 mg resveratrol/kg body weight/day for the remainder of their life. At the end of six months, a clear trend towards increased survival and insulin sensitivity was noted and hence the investigators published an interim report of the study at the end of one year. Several parameters, including safety-related evaluations such as clinical pathology and histopathology were investigated. The published report contained results from three groups of mice that received one of the following regimens: standard diet (SD); high calorie diet (HCD) and; high calorie diet plus resveratrol (HCDR). The HCDR diet provided an average of 22.4 mg resveratrol/kg body weight/day. No significant effects of resveratrol on body weight, body temperature,

food consumption, total fecal output or lipid content, or post-mortem body fat distribution were noted compared to animals receiving HCD. At 60 weeks of age, the survival in HCDR mice was 3-4 months longer than SD or HCD mice. At the end of 114 weeks, 58% of the HCD-control mice died as compared to 42% HCDR and 42% of the SD-controls. Mice in HCDR-group steadily improved their motor skills compared to HCD group.

Administration of resveratrol for one year at a dose of 22.4 mg/kg/day did not affect serum clinical chemistry parameters including safety related indices such as ALT, AST, creatinine phosphokinase, alkaline dehydrogenase, bilirubin, albumin, and creatinine. The HCDR mice group had significantly lower levels of insulin, glucose, and IGF-1 compared to mice in HCD-group. The levels of these markers in the HCDR-group paralleled the SD-group. Mice receiving resveratrol had higher levels of insulin sensitivity and low insulin resistance. Plasma amylase was elevated in the HCD-group and was significantly reduced by resveratrol feeding. At 18 months of age, resveratrol prevented an increase in size and weight of livers of mice in HCDR-group compared to HCD-group. Histological examinations of the liver revealed a loss of cellular integrity and the accumulation of large lipid droplets in the liver of the HCD but not the HCDR-group. The livers of mice receiving resveratrol had more mitochondria compared to mice receiving HCD alone.

Recently, Horn *et al.* (2007) investigated the carcinogenic potential of resveratrol in p53 knockout mice. This model is accepted by the FDA as an alternative model for oncogenicity bioassay. TSG-p53(±) (heterozygous p53 knockout) mice (25/sex/group) received daily *via* gavage either vehicle only (negative control), resveratrol doses of 1000, 2000, or 4000 mg/kg/day, or *p*-cresidine (400 mg/kg/day; positive-control) for six months (180 days). Because of high mortality in the high-dose group, the 4000 mg/kg/day dose was reduced to 3000 mg/kg/day at the end of study week 4. Animals were observed twice daily for mortality or evidence of toxicity. Body weights, food consumption, and detailed clinical observations were performed once *per* week. Blood samples were collected immediately prior to the terminal necropsy for clinical evaluations. At termination, weights of the adrenals, brain, heart, kidneys, liver, spleen, testes/ovaries, and thymus were collected. Histopathology evaluations on approximately 45 tissues *per* animal were performed in control, low and mid-dose groups (Horn *et al.*, 2007).

No mortality was noted at 1000 mg/kg/day. Administration of resveratrol at 2000 and 4000/3000 mg/kg/day induced mortality in both sexes of p53(±) mice. At the termination of the study (26 weeks), survival in male mice was 64 and 28% in the middle and high dose groups, respectively. In females the survival at mid- and high-dose was 60 and 24%, respectively. The early mortality in groups receiving the mid- and high-doses of resveratrol appears to be related to the mass of resveratrol administered, rather than a result of any agent-specific toxicity. Dose- and time-related mortality patterns were noted in both male and female mice. Clinical observations, body weight measurements, and quantitation of food intake failed to identify any evidence of toxicity in animals exposed to resveratrol. Blood analysis for clinical pathology did not reveal any adverse effects at the lowest dose (1000 mg/kg/day). Modest (<40%) increases in serum cholesterol were seen in male mice at the highest dose and in female mice at the mid- and

high dose. At the low dose, resveratrol administration did not affect serum cholesterol levels. The investigators did not consider these changes of any toxicological significance. Small (<15%) but significant reductions in red blood cell count, hematocrit, and hemoglobin were seen only in male mice receiving the high dose (4000/3000 mg/kg/day) of resveratrol (Horn *et al.,* 2007). These changes were not noted in male mice receiving lower doses or in female mice in any dose group. Occasional significant differences from control were noted in several other clinical pathology parameters in mice exposed to resveratrol. However, these changes were small (<10%), not dose related, observed in one sex only, and involved values that were within normal range limits of the assay. These changes were not considered to be biologically significant.

With the exception of a dose-related increase in absolute and relative liver weights (without any microscopic changes) in both sexes of mice, resveratrol administration did not affect organ weight. At the lowest dose of resveratrol, the effects on liver weight were not significant. Histopathology evaluation of 45 tissues did not reveal any evidence of oncogenicity of resveratrol. In the positive-control group, *p*-cresidine induced urinary bladder neoplasms in both sexes of mice. Resveratrol treatment resulted in a dose-related hydronephrosis and epithelial cell hyperplasia of the urinary bladder. Neither hydronephrosis nor urothelial hyperplasia were linked to any functional alteration. Hydronephrosis was noted at 1000 and 2000 mg/kg/day dose, while hyperplasia was noted only at 2000 mg/kg/day. Hydronephrosis was noted in 20% low-dose male, 12% low-dose female, 18% mid-dose male and 60% mid-dose female. Epithelial hyperplasia of the urinary bladder was noted in 44% surviving males and 33% surviving females in the group exposed to mid-dose of resveratrol. The results of this study demonstrate that chronic oral administration of resveratrol at its maximum tolerated dose (3000 mg/kg/day) does not increase the incidence of any malignant or benign neoplasm in p53 knockout mice (Horn *et al.,* 2007). The results of this study also demonstrate that except for a hydronephrosis in few animals without any functional alterations, resveratrol administration at a dose of 1000 mg/kg/day for 180 days did not cause any toxicity.

In summary, the results from a well-designed chronic toxicity study in mice demonstrate that administration of resveratrol (22.4 mg/kg/day) had no adverse effects on overall health and lifespan as determined by several indicators including survival, motor function, insulin sensitivity, organ pathology, and mitochondrial number. Resveratrol administration for one year did not affect serum clinical chemistry parameters such as ALT, AST, creatinine phosphokinase, alkaline dehydrogenase, bilirubin, albumin, and creatinine. Based on the results of the chronic study, a NOAEL for resveratrol of 22.4 mg/kg/day can be determined. The carcinogenicity study in p53 knockout mice shows that resveratrol does not possess carcinogenic potential. Hydronephrosis (at 1000 and 2000 mg/kg/day) and epithelial cell hyperplasia (at 2000 mg/kg/day) of the urinary bladder noted in the carcinogenicity study appear to be related to the elimination of high doses of resveratrol. Based on the results of this study, a NOAEL for resveratrol of 1000 mg/kg/day can be determined. FDA has accepted the p53 mouse model as an alternative model for an oncogenicity bioassay. Based on the information provided in chronic and carcinogenecity studies, the data quality and study design for both these studies is very good and both studies appear to have followed Good Laboratory Practices.

2.5. Reproduction and developmental studies

Nikaido *et al* (2005) investigated the effect of prepubertal exposure to resveratrol on development of the reproductive tract and mammary glands in female mice. Beginning at 15 days of age, female CD-1 mice were administered four daily subcutaneous injections of 10 mg/kg/day of resveratrol. Vaginal opening was checked and estrous cyclicity was monitored from 5, 9 or 21 weeks of age for 21 consecutive days. At 4, 8 and 24 weeks of age, animals were necropsied. Prepubertal exposure to resveratrol did not affect body weight gain, puberty onset (vaginal opening) or the estrous cycle length. Necropsy at 4, 8 and 24 weeks of age did not reveal any significant adverse effects on corpora lutea. Resveratrol did not alter the uterine or vaginal morphology or mammary gland growth. The results of this study indicate that resveratrol does not exhibit estrogenic biological activity. In a similar previously reported study by this group, resveratrol at same dose (10 mg/kg/day) caused transient effects (increased length of estrus cycle by prolonging diestrus during week 9-11 on the reproductive tract (Nikaido *et al.,* 2004). However, as described above, these effects were not confirmed in the more recent study by the same group.

In a multigenerational mouse study, Kyselova *et al* (2003) investigated the effects of resveratrol on the body weight, organ weight, histology of testes and ovaries, acrosomal integrity of the spermatozoa, and litter size. Adult CD-1 outbred mice two-months old (parental or P-generation) were exposed to resveratrol (3 mg/L) in drinking water for four weeks, and then mated. Based on an average daily intake of water and an average body weight of mice, resveratrol intake was determined as 0.75 mg/kg/day. The F_1 generation was exposed to resveratrol during gestation, lactation, the prepubertal period and the pubertal period, up to adulthood. Exposure to resveratrol did not affect body weight in either males or females in the P or F_1 generation. Resveratrol decreased the absolute (20%) and relative seminal vesicle (12%) weight and spleen weight (18%) as compared to controls in the P generation and the absolute vesicle (18%) and kidney (10%) weight in the F_1 generation compared to control. Although, resveratrol administration resulted in decreased absolute and relative seminal vesicle weight and spleen weight in the P generation, similar decreases, in absolute and relative seminal vesicle weight, and spleen weight were not noted in F_1 generation. The absolute and relative ovary weight was decreased in the P-generation females treated with resveratrol. However, such a decrease was not noted in F_1 generation. The differential changes noted in the P and F_1 generations indicate that the decreases noted in the P generation may not be related to the treatment. It is anticipated that changes noted in the P generation should also be present in F_1 generation. Resveratrol did not affect litter size or sex ratio (female/male). Resveratrol exposure had no affect on sperm number or sperm quality. Histological examinations did not reveal any alteration of testes or ovarian morphology in either generation. The investigators concluded that resveratrol had no effects on *in vivo* fertility.

In a comparative study, Kubo *et al* (2003) investigated the effects of resveratrol, bisophenol-A, diethylstilbestrol or vehicle (control) on the sexual differentiation of open-field behavior and the sexually dimorphic nuclei in the brain in the offspring of rats exposed to these compounds during the fetal and suckling periods. After copulation,

female Wistar rats received water containing *trans*-resveratrol (5 mg/L) until their pups were weaned on postnatal day 21. The dose of resveratrol for dams was determined as 1.5 mg/kg/day. Resveratrol exposure did not affect male sexual development, whereas resveratrol delayed the day of vaginal opening in females (their body weights on the day of vaginal opening increased in comparison to the control females). However, in a mouse study described above, Nikaido *et al.* (2005) reported that resveratrol did not affect vaginal opening. In the open-field test, compared to control group, resveratrol exposure did not show any effects. In male rats, resveratrol did not affect the number of mounts or intromission. The intromission rate, however, was decreased. The investigators concluded that resveratrol did not change sexual behavior in male rats. In females, resveratrol did not affect the number of ear wiggles, but decreased the lordosis[7] quotient and increased the rejection score suggesting reduced receptivity.

Resveratrol did not affect the weight of epidydymis, ventral prostate, seminal vesicle or quality/quantity of sperms in males as determined by the total spermatid present in the left testis, the caudal sperm, or the percentage of motile sperms. In either male or female offspring, resveratrol exposure had no significant effects on serum hormone levels of luteinizing hormone, follicle-stimulating hormone, prolactin, testosterone or 17β-estradiol. In female offspring, resveratrol did not affect weight of the uterus or bilateral ovaries. Resveratrol exposure resulted in reduced number of estrus cycles. Resveratrol treatment had no effect on the sex differences in the brain weight. The volume of female locus coeruleus[8] was significantly larger than that of males in the respective control groups, and resveratrol administration abolished this difference. Comparatively the estrogenicity of resveratrol was less than bisophenol-A or diethylstilbestrol. Interestingly, the delayed vaginal opening in female offspring noted in this study appears to contradict the claimed estrogenic activity, as estrogens are known to accelerate the vaginal opening (Kubo *et al.*, 2003).

Juan *et al.* (2005) investigated the effect of *trans*-resveratrol on testis and spermatogenesis. Male Sprague-Dawley rats were administered 20 mg/kg/day of resveratrol *via* gavage for 90-days. A period of 90-days was chosen to cover the complete spermatogenesis cycle of rats. The food intake and body weight gain of the resveratrol-treated group did not differ from that of the controls. Resveratrol did not affect testicular gross anatomy, wet weight or relative weight. Histological examinations of testis did not reveal microscopic lesions such as cytoarchitectural alterations or disorganization of the tubular elements. Resveratrol treatment reduced the seminiferous tubules diameter and increased the tubular density. Moreover, sperm counts were significantly greater in the resveratrol-treated group compared to control, but sperm quality did not differ. Serum levels of gonadotrophins and testosterone were higher in the resveratrol-treated group. The investigators concluded that resveratrol enhanced sperm production by stimulating the hypothalamic-pituitary-gonadal axis, without inducing adverse effects. These investigators also suggested that resveratrol might be useful in the treatment of male infertility.

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[7] abnormal forward curvature of the spine in the lumbar region

[8] a bluish area of the brain stem with many norepinephrine-containing neuron

Henry and Witt (2006) investigated the effects of maternal resveratrol exposure to male and female offspring. In this study, reproductive physiology, behavior and brain morphology were examined in adult offspring of dams exposed to resveratrol throughout the lactation period. Time-mated female Sprague Dawley rats following both delivery and during the entire lactation period received water (control), 10% ethanol (vehicle), or resveratrol (5, 50 or 100 μM in 10% ethanol) as a daily drinking water solution. At weaning, rats were housed in same sex groups and were provided water and food *ad libitum.* The mean daily consumption of drinking solution was similar (~70 ml) in all groups and ranged from 61-79 ml. The resulting daily resveratrol intake was approximately 0.08, 0.8 and 1.6 mg/day, respectively. During adulthood, female offspring exposed to resveratrol throughout nursing exhibited reduced body weight and increased ovarian weight. However, these offspring exhibited normal estrous cyclicity and sociosexual behavior, without changes in the volume of the sexually dimorphic nucleus of the preoptic area or the anteroventral periventricular nucleus of the hypothalamus. Adult males exposed to resveratrol during nursing exhibited decreased body weight and plasma testosterone concentration, increased testicular weight, and reduced sociosexual behavior. These males also had significantly smaller sexually dimorphic nucleus of the preoptic area volumes and larger anteroventral periventricular nucleus volumes compared to male controls. The investigators suggested that postnatal exposure to resveratrol may affect estrogenic activity in specific peripheral tissues (e.g., the gonads), while inducing antiestrogenic effects in the brain.

In a previous study, Henry and Witt (2002) reported that resveratrol acts as a possible agonist/antagonist depending on the availability of specific estrogen receptor isoforms localized in the reproductive tract and brain of the female rats. In this previous study, exposure of resveratrol (100 μM in 10% ethanol) in drinking water to female Sprague-Dawley rats for seven days decreased body weight, disrupted estrous cyclicity, and induced ovarian hypertrophy. In ovariectomized females, subcutaneous resveratrol (0.01-1 mg) injection did not appear to mimic 17β-estradiol benzoate induced behavioral responses and had no subsequent estrogen sensitivity or sociosexual behavior

The results of the Henry and Witt (2002; 2006) studies are difficult to interpret because of confounding factors. Although the investigators used an ethanol vehicle group, it is not clear how high levels of ethanol (10%; 10,000 mg/kg/day) in combination with resveratrol may interact. Studies have shown that heavy alcohol consumption results in reduced testosterone levels in the blood. Alcohol is also known to impair the function of the testicular Sertoli cells that play an important role in sperm maturation (Emanuele and Emanuele, 1998). Ethanol used in Henry and Witt (2002; 2006) study is likely to cause several unknown effects that were not investigated in the study. To put this into perspective, the ethanol used in this study is equivalent to a human weighing 70 kg drinking 70 ml of ethanol daily. A typical alcoholic beverage contains 12 g of alcohol, corresponds to a dose of about ~12 ml/day for a 70-kg adult, and produces a peak blood ethanol concentration of approximately 25 mg/dl.

In summary, prepubertal exposure to resveratrol did not affect development of the reproductive tract and mammary glands in female mice. In a multigenerational mouse study, resveratrol treatment resulted in decreased absolute and relative seminal vesicle

weight and spleen weight in the P generation and the absolute vesicle and kidney weight in the F_1 generation. Some of the changes noted in the P generation were not found in the F_1 generation. For example, resveratrol administration resulted in decreased absolute and relative seminal vesicle weight and spleen weight in the P generation, similar decreases, in absolute and relative seminal vesicle weight, and spleen weight were not noted in F_1 generation. The results of this multigeneration study show that resveratrol has no effect on *in vivo* fertility. In a comparative study of different endocrine disruptors, resveratrol exposure did not affect male sexual development, but delayed the day of vaginal opening in females with an increase observed in body weight on the day of vaginal opening. However, in another study, resveratrol did not affect vaginal opening. As estrogens are known to accelerate vaginal opening, the delayed vaginal opening noted in one of the study contradicts the claimed estrogenic activity. In a 90-day study, gavage administration of resveratrol at a dose of 20 mg/kg/day enhanced sperm production by stimulating the hypothalamic-pituitary-gonadal axis, without inducing adverse effects. Maternal resveratrol exposure in drinking water with 10% ethanol to male and female offspring, indicate that postnatal exposure to resveratrol along with ethanol may affect estrogenic activity in specific peripheral tissues (*e.g.*, the gonads), while inducing antiestrogenic effects in the brain. The results of this study are difficult to interpret as very high levels of ethanol used in this study could compound any specific effects observed.

2.6. Estrogenic activity

The structural similarity of resveratrol to that of the synthetic estrogen agonist, diethylstilbestrol, suggest that resveratrol might also function as an estrogen agonist. *In vitro* experiments in cell culture indicate that resveratrol acts as an estrogen agonist under some conditions, and an estrogen antagonist under other conditions. Initial concerns regarding resveratrol's effect on breast cancer arose from a paper by Gehm *et al* (1997) describing resveratrol's estrogenic actions. These investigators found that resveratrol bound specifically to estrogen receptors and stimulated proliferation of a breast cancer cell line (T47D). In swift succession, several papers found the exact opposite for resveratrol: inhibition of several breast cancer cell lines with and without estrogen receptors, including the T47D breast cancer cell line (Mgbonyebi *et al.*, 1998; Hsieh *et al*, 1999; Lu and Serrero, 1999; Damianaki *et al.*, 2000).

Additional *in vitro* studies with mammary cancer cell lines also indicated that resveratrol acts as a mixed estrogen agonist/antagonist. However in the presence of 17β-estradiol, it was an antiestrogen (Bhat *et al.*, 2001; Gehm *et al.*, 1997). For example, in MCF-7 and S30 cells, resveratrol alone showed weak estrogenic response, but when combined with estradiol (1 nM), a dose-dependent antagonism occurred. In addition, progesterone receptor (PR) protein expression was induced with resveratrol alone, but when combined with estradiol, the expression was suppressed. In T47-D and LY2 cells, resveratrol was a pure estrogen antagonist and it significantly down regulated steady state and estradiol-induced PR protein levels. With LY2 and S30 cells, presnelin 2-protein expression was down-regulated (Bhat *et al.*, 2001).

Nakagawa *et al.* (2001) reported that exposure to resveratrol at pharmacological doses (52-74 μM [12-17 μg/mL]) suppressed the growth of ER-positive breast cancer cells (KPL-1 and MCF-7) and ER-negative breast cancer cells (MKL-F) stimulated by linoleic acid, a potent stimulator of these cells. High fat diet, particularly linoleic acid has been shown to play a key role in breast cancer stimulation. Resveratrol (1 pM-1 μM [2.28 x 10^{-7}-0.2 μg/mL]) was also an agonist of steroid receptors. In the MCF-7 cells, resveratrol at the nanomolar range interacted with estradiol simultaneously with PRs (at the picomolar range). In T47-D hormone-sensitive breast cancer cell line, the same interactions were seen but to a lesser extent. In MDA-MB-231 hormone-independent breast cancer cell line, no steroid binding was noted observed (Damianaki *et al.*, 2000).

Both *trans*- and *cis*-resveratrol (10 and 25 μM [2.3 and 5.7 μg/mL]) significantly increased the growth of MCF-7 cells. Cell growth was decreased at a high dose of 50 μM (11 μg/mL), and this concentration was found to be cytotoxic. In the presence of estradiol, and at 25 and 50 μM *trans*-resveratrol, and 50 μM *cis*-resveratrol, significant reductions in cell proliferation were observed. In MVLN cells, *trans*-resveratrol (10 and 25 μM) and *cis*-resveratrol (25 μM) significantly increased luciferase activity compared to estradiol. In the presence of estradiol, both isomers at the same doses acted as superagonists of estradiol. In both cell lines, *cis*-resveratrol was less effective than *trans*-resveratrol (Basly *et al.*, 2000). Resveratrol was observed to exhibit estradiol antagonist activity for ER-α with select estrogen response elements and no such activity was noted with ER-β (Bowers *et al.*, 2000). In human endometrial adenocarcinoma (Ishikawa) cells at concentrations of 10 μM (2.3 μg/mL), resveratrol mediated antiestrogenic effects by selective down-regulation of ER-α but not ER-β (Bhat and Pezzuto, 2001).

Contrary to some of the above described *in vitro* studies, an *in vivo* study with oral administration of resveratrol (1, 4, 10, 40, and 100 μg/day for six days) to weanling rats had no effect on estrogen target tissues (bone formation and mineralization rates) *versus* the estrogen 17β-estradiol. Resveratrol administration had no significant effect on body weight, serum cholesterol, radial bone growth, epithelial cell height, or messenger RNA levels for insulin-like growth factor I. Resveratrol treatment resulted in slight increases in uterine wet weight, but significance was only achieved at the 10 μg/day dose. These observations suggest that resveratrol was not an agonist at the estrogen receptor. Simultaneous administration of resveratrol (1000 μg) and 17β-estradiol (100 μg) showed a synergistic effect (*i.e.,* a significant decrease in cholesterol levels was seen in the animals). The inability of low doses (1 and 10 μg, respectively) of resveratrol to lower serum cholesterol levels suggested antagonism by resveratrol at the estrogen receptor (Turner *et al.*, 1999). In other studies in rats, oral or subcutaneous administration of *trans*-resveratrol (0.03-575 mg/kg) had no estrogenic response in uterine tissue (Ashby *et al.*, 1999; Freyberger *et al.*, 2000). Observations from *in vivo* studies raise questions regarding the extent to which estrogenicity data from *in vitro* assays using MCF-7 or other cell lines can be used to predict the hormonal effects likely to occur in animals or humans.

In summary, available *in vitro* data indicate that resveratrol has complex estrogenic and antiestrogenic effects depending on the biological environment. Resveratrol appears to act consistently as an antiestrogen to breast tumors under

physiological conditions. These data suggest that resveratrol may have beneficial effects if used as a chemopreventive agent for breast cancer. Results from an *in vivo* study demonstrate that resveratrol does not stimulate indices of uterine growth and differentiation in immature rats. Even very high doses of resveratrol generally had insignificant effects on uterine wet weight, epithelial cell height, and IGF-I gene expression. Additionally, resveratrol had no effect on other estrogen target tissues. Resveratrol treatment did not alter cortical bone growth, serum cholesterol concentration , or body weight. The available evidence shows that resveratrol is not an estrogen agonist.

2.7. Cytotoxicity

In an *in vitro* study, Brakenhielm *et al.* (2001) investigated dose-related effects of resveratrol in bovine capillary endothelial (BCE) and porcine aortic cell lines. Resveratrol dose-dependently (1-10,000 nm) inhibited the cell growth of capillary endothelial cells stimulated with fibroblast growth factor-2 (FGF-2). Resveratrol also inhibited the phosphorylation of mitogen-activated protein kinases (MAPKs) (10 and 20 μM), and FGF-2 and vascular endothelial growth factor (VEGF)-induced proliferation of porcine aortic cell lines expressing PAE/FGFR-1 and PAE/VEGFR-2, respectively, in a dose-dependent manner (0.5-10 μM).

Babich *et al* (2000) investigated sensitivity of human gingival epithelial S-G cells to resveratrol using the neutral red dye uptake assay. The following sequence of sensitivity to resveratrol (doses up to 500 μM) was determined: tongue squamous carcinoma SCC-25 cells > Smulow-Glickman (S-G) human gingival epithelial cells > RHEK-1 keratinocytes >> fibroblasts. In a 3-day continuous exposure to resveratrol assay (5-150 μM) with S-G cells, toxicity was found to level off between day 2 and 3. At concentrations >75 μM, irreversible damage to cell proliferation was noted, and the presence of hepatic S9 microsomal fraction did not affect the cytotoxicity.

2.8. Genotoxicity

Matsuoka *et al.* (2001) investigated the genotoxic potential of *trans*-resveratrol in bacterial reverse mutation assay, in the *in vitro* chromosome aberration test, the *in vitro* micronucleus test, and in the sister chromatid exchange test (Table 6). Resveratrol was negative in the bacterial reverse mutation assay (*Salmonella typhimurium* strains TA98 and TA100 and *E coli* WP2*uvr*A) in the absence and presence of a microsomal metabolizing system. Resveratrol induced structural chromosomal aberrations (mainly chromatid breaks and exchanges) and showed weak aneuploidy induction in a Chinese hamster lung cell line. In the *in vitro* micronucleus test, resveratrol induced mononuclear, polynuclear and karyorrhectic cells at the end of 48 h treatments. In the sister chromatid exchange test, resveratrol induced sister chromatid exchanges dose-dependently at dose levels up to 10 μg/ml. Cell cycle analysis indicated that resveratrol caused S-phase arrest, and 48 h treatment induced apoptosis.

In another study, Fukuhara and Miyata (1998) reported that *trans*-resveratrol cleaved plasmid DNA in the presence of Cu^{2+} at neutral pH and under aerobic conditions. Under anaerobic conditions, however, increasing the concentration of resveratrol failed to

enhance the efficiency of DNA cleavage, suggesting the cleavage to be dependent on the presence of both Cu^{2+} and oxygen. Ahmad *et al.* (2005) also found resveratrol induced DNA changes in plasmid DNA in the presence of elevated levels of copper ions.

Table 6. Genotoxicity studies of resveratrol

Test system	Type of assay	Concentration	Results	Reference
Salmonella typhimurium Strains TA98 and TA 100, and *Escherichia coli* WP2*uvr*A	Bacterial reverse mutation	0.2 to 5000 µg/plate for 20 min	Negative[a]	Matsuoka *et al.* (2001)
Chinese hamster lung cells	Cytotoxicity	Cells seeded at 1.5 x 10^5/plate incubated for 17 h and then treated with 2 5, 5, 10, 20 µg/ml for 24, 29, 36, 48, 54 or 72 h	Dose dependent decrease in survival	Matsuoka *et al* (2001)
	Chromosomal aberrations	As above	Dose dependent increase in structural chromosomal aberrations	
	Micronucleus	As above	Dose dependent increase in micronucleus, polynuclear, and karyorrhectic cells with 48 h treatment up to 10 µg/ml dose	
	Sister chromatid exchanges	As above	Dose dependent increase in Sister chromatid exchanges	
	S phase arrest	As above	The number of cells decreased in G1, increased in S Increase in apoptosis with the 48 h treatment	

a = With and without metabolic activation

The positive genotoxicity findings are understood to be an artifact of the test systems as physiological concentrations of ascorbic acid or glutathione, normally present, are lacking in the test system. In these test systems, resveratrol was found to promote hydroxyl-radical formation by DNA-bound Cu^{2+} ions, thereby acting as a reducing agent. Burkitt and Duncan (2000) have shown that in the presence of either ascorbic acid or glutathione (i.e., under more physiological conditions), resveratrol lost this property and behaved as an antioxidant. In the ascorbate system, resveratrol had no effect on the rate of hydroxyl radical formation, but protected DNA from damage by acting as a radical scavenging antioxidant. Further, in the glutathione system, resveratrol inhibited hydroxyl radical formation *via* a novel mechanism involving the inhibition of glutathione disulfide formation. These investigators concluded that the DNA-damaging properties of resveratrol will be of no significance under physiological conditions. Burkitt and Duncan (2000) demonstrated that resveratrol behaves as a powerful antioxidant, both *via* classical, hydroxyl-radical scavenging and *via* a novel, glutathione-sparing mechanism.

The mechanistic data of Burkitt and Duncan (2000) are consistent with majority of the published data which demonstrate an antimutagenic effect of resveratrol in combination with known mutagens and carcinogens, usually thought to be associated with its antioxidant properties. For example, two studies found that resveratrol inhibited the mutagenic effects of the carcinogens Trp-P-1 and MNNG on *Salmonella typhimurium* bacteria (Uenobe *et al*, 1997; Kim *et al.*, 2002).

Studies in animal cell lines have also largely corroborated these findings. Sgambato *et al.* (2001) found that resveratrol reduced DNA fragmentation in a variety of cell lines (including rat fibroblast, mouse mammary cells, and human cell lines) that were exposed to mutagenic agents such as tobacco-smoke and hydrogen peroxide. Topical administration of resveratrol to mouse epidermis inhibited binding of a carcinogen, DMBA, to DNA (Szaefer *et al.*, 2004). Resveratrol also inhibited binding of the carcinogen benzo[a]pyrene to DNA of human oral epithelial cells (Walle *et al.*, 2006).

In summary, the genotoxicity studies of resveratrol clearly indicate that it is generally antimutagenic and anticarcinogenic. Although, in *in vitro* assays, DNA damaging effects of resveratrol have been reported, these effects are not physiologically comparable to *in vivo* biochemistry and allow for Cu^{2+} -induced hydroxyl radical formation by resveratrol. Under physiological conditions that have ascorbate and glutathione present, resveratrol does not induce DNA damage by Cu^{2+} but acts as a potent antioxidant. Therefore, resveratrol is not considered to pose any genotoxic risk *in vivo*.

2.9. Angiogenesis

Some studies indicate that resveratrol inhibits angiogenesis. Because angiogenesis plays an important role in physiologic process of wound healing and pathophysiologic cancer growth processes, it is important to determine under what conditions resveratrol inhibits angiogenesis. It is important to note that resveratrol potently inhibits angiogenesis related to tumor growth. Garvin *et al* (2006) demonstrated significantly less angiogenesis in human breast cancer grafts in mice. Similarly, other investigators found an inhibitory effect of resveratrol on angiogenesis in Kaposi's Sarcoma (Tseng *et al.*, 2004), brain glioma (Baliestrieri *et al.*, 2006), ovarian cancer (Cao *et al.* 2004), and lung cancer (Kimura *et al* ,2001).

Brakenhielm *et al* (2001) reported that oral administration of resveratrol significantly inhibits the growth of a murine fibrosarcoma in mice, and delays angiogenesis-dependent wound healing in mice. Resveratrol (5.7 μg/ml) was added to the drinking water of mice with a 5 mm surgical wound 2 days before the operation and throughout the experiment. Resveratrol significantly delayed wound healing in mice, as determined by measuring the sizes of wounds and the percentage of animals with healed wounds. In another experiment with corneal micropockets of the mice, oral administration of resveratrol (0.4 μg/mL) given three days before growth factor implantation and for 15 days after surgery significantly inhibited VEGF- and FGF-2-induced corneal neovascularization compared to the control (Brakenhielm *et al*, 2001).

Experimental studies generally do not indicate an adverse effect of resveratrol. Although resveratrol was found to inhibit corneal neorevascularization in mice (induced by the vascular endothelial growth factor [VEGF] (Oak *et al*, 2005), other studies clarify this by showing that resveratrol appears to act as a "brake" to unregulated angiogenesis (in tumors), but does not inhibit normal angiogenesis. Wound healing, in particular, requires neorevascularization and resveratrol appears to accelerate dermal wound healing (Khanna *et al.*, 2002) by actually upregulating VEGF in skin cells (Khanna *et al*, 2001; Sen *et al*, 2002). These observations contradict the above described findings from Brakenhielm *et al.* (2001). Similarly, resveratrol upregulates VEGF in rat models of myocardial infarction, leading to improved collateral formation (Fukuda *et al.*, 2006; Kaga *et al.*, 2005). Thus, based on the above, resveratrol is not considered to pose any risk of altering normal angiogenesis.

2.10. Platelet aggregation

The effects of resveratrol on platelet aggregation and cholesterol metabolism are controversial. Some *in vitro* studies indicate that resveratrol may block platelet aggregation (Kirk *et al*, 2002; Pace-Asciak *et al*, 1995). Kirk *et al.* (2002) reported that resveratrol, at 10-50 μM, blocked aggregation of washed platelets induced by collagen (5 μg/ml), thrombin (0.2 units/ml), and ADP (10 μM). Compared to washed platelets, in whole blood, resveratrol has poor antiplatelet activity and platelet aggregation was not affected by 50-100 μM resveratrol. Concentrations of 200 μM resveratrol were required to cause a decrease in platelet aggregation in whole blood.

Freedman *et al* (2002) suggested that quercetin or resveratrol are not the main substances of wine responsible for the platelet inhibition or nitric oxide–releasing effects (Violi *et al*, 2002). Although, *in vitro* studies have shown that flavonoids including quercetin, resveratrol, and catechin inhibit platelet aggregation (Pace-Asciak *et al.*, 1995), the physiological relevance of these findings has been questioned in humans because oral supplementation with quercetin causes markedly increased plasma levels but does not alter total, LDL, or HDL cholesterol levels or change thrombogenic markers including platelet aggregation and platelet thromboxane B2 production (Conquer *et al.*, 1998). The inhibitory effects on platelet aggregation were observed in cells cultured in the presence of resveratrol.

2.11. Observations in humans

In human studies, orally administered *trans*-resveratrol appears to be well-absorbed. Walle *et al.* (2004) studied absorption, metabolism, and excretion of ^{14}C-resveratrol in six human volunteers following both oral and intravenous administration. Following an oral dose of 25 mg of *trans*-resveratrol, the absorption was at least 70%, with peak plasma levels of resveratrol and metabolites of 491 ng/ml (peaked at 30-60 min) and a plasma half-life of 9.2 h. Only trace amounts of unchanged resveratrol (<5 ng/ml) were detected in plasma. The majority of the oral dose was recovered in urine as sulfate and glucuronic acid conjugates of the phenolic groups of resveratrol.

Recently, the National Cancer Institute (NCI) initiated clinical toxicity studies on *trans*-resveratrol (Abrams *et al.* 2004; Boocock *et al.* 2006). In a single dose pilot trial, four human participants were treated with 1 g of resveratrol and the samples assayed using a high performance liquid chromatography assay with metabolite identification by liquid chromatography-mass spectrometry. Data from this study indicate that at a C_{max} (between 60 -120 min), the mean concentration of resveratrol was 103.2 ng/mL with minimum and maximum concentrations of 58.8 and 208.0 ng/mL, respectively (Abrams *et al.* 2004). In a subsequent clinical study protocol, Boocock *et al.* (2006) reported that administration of a single oral dose of resveratrol at levels of 0.5, 1.0, 2.5, 5.0 g demonstrated bioavailability, albeit not greatly, with a C_{max} between 30 and 90 minutes and peak plasma levels for the 1.0 g dose of ~1 μM. No serious adverse events have been recorded during this study.

Zamora-Ros *et al.* (2006) conducted two randomized, cross-over trials and a cohort study to determine whether urinary resveratrol metabolites could serve as a biomarker of moderate wine consumption. In this study investigators used liquid chromatography-tandem mass spectrometry to analyze urinary total resveratrol metabolites In the first study, 10 healthy men consumed 30 g of ethanol/day as sparkling wine or gin for 28 days. In the second trial, 10 healthy women consumed 20 g of ethanol/day as white or red wine for 28 days. Urinary total resveratrol metabolites were analyzed using liquid chromatography-tandem mass spectrometry. A significant increase in total resveratrol metabolite (72.4, 211.5, and 560.5 nmol/g of creatinine) was noted after consumption of sparkling, white, or red wine, respectively, but no changes after the washout or gin periods. In the cohort study, the reported daily dose of wine consumption correlated directly with total resveratrol metabolites. These investigators concluded that resveratrol metabolites in urine may be useful biomarkers of wine intake.

In summary, available pharmacokinetic and safety studies in human subjects indicate that orally administered *trans*-resveratrol is well absorbed by humans. Due to its rapid metabolism and elimination, bioavailability of resveratrol is low. Resveratrol determination in urine samples may serve as a marker of wine consumption. In a pilot study, a single oral dose of resveratrol (1 g) was without any serious adverse effects. As the majority of the basic research on resveratrol has been conducted in cultured cells exposed to unmetabolized resveratrol at concentrations that are often 10-100 times greater than peak concentrations observed in human plasma after oral consumption, Gescher and Steward (2003) reported that bioavailability is important for its action. In a review article, Wenzel and Somoza (2005) reported that the oral bioavailability of resveratrol is almost zero due to rapid and extensive metabolism and the consequent formation of various metabolites as resveratrol glucuronides and resveratrol sulfates.

3. SUMMARY AND DISCUSSION

Resveratrol, a polyphenol, is commonly found in grapes, red wine, purple grape juice, peanuts and some berries. Various plants produce resveratrol to help defend against invading fungi, stress, injury, infection, and too much sunlight. Resveratrol may exist in both *cis*- and *trans*-stereoisomeric forms. In nature, both the forms also occur as the glucoside (bound to a glucose molecule). The richest source of resveratrol is the roots of

the plant *Polygonum cuspidatum* (Ko-jo-kon), mainly cultivated in China and Japan. *trans*-Resveratrol (>98% pure) can be isolated from this plant by employing high-speed counter-current chromatography. In recent years, resveratrol has been extensively studied for its wide range of desirable biological effects such as cardioprotection, chemoprevention, anti-cancer, and prolongation of life span in several species.

ATLA Holdings, LLC, intends to use standardized *trans*-resveratrol (>99% pure), extracted from *P cuspidatum,* as a dietary nutrient at levels up to 10 ppm in bottled water ("near waters"). *trans*-Resveratrol is isolated by solvent extraction and the purified product contains >99% *trans*-resveratrol. The intended use levels in bottled water will result in an estimated daily mean and 90th percentile exposure of 2.09 and 3.99 mg/person/day or 0.04 and 0.07 mg/kg/day. Moderate consumers of red wine containing high levels of resveratrol are likely to ingest about 4 mg resveratrol/day or 0.067 mg/kg/day. The estimated 90th percentile daily intake of resveratrol (3.99 mg/person) is similar to that of consumers of red wine (in moderation) with high levels of resveratrol (4 mg/person). The dietary supplement recommended dosages of resveratrol have been reported to range from 3 to 1000 mg/person/day.

Following oral ingestion in animals and humans, resveratrol is rapidly absorbed from the gastrointestinal tract. Although resveratrol appears to be well-absorbed by humans, its bioavailability is low because it is rapidly metabolized and eliminated. The plasma half-life of *trans*-resveratrol following oral administration (25 mg) has been determined as 9.2 hours. The pharmacokinetic studies indicate that resveratrol is rapidly eliminated from the body and is unlikely to accumulate.

In an acute toxicity study, the LD_{50} of resveratrol was found to be greater than 2 g/day indicating that *trans*-resveratrol is practically nontoxic. Single intravenous administration of resveratrol (80 mg/kg) to rats or oral repeat dose administration (250 mg/kg/day for 5 days) to mice did not reveal any adverse effects. In two separate studies, daily administration of resveratrol at doses up to 300 mg/kg/day for 28 days did not reveal any adverse effects. In one study, oral administration of resveratrol (20 mg/kg/day for 28 days) revealed some adverse effects such as increased serum ALT levels without liver histological changes. Contrary to these results, other studies did not reveal increases in ALT levels at a dose level of 300 mg/kg/day (a 15-fold higher dose). Additionally, in NCI sponsored study, resveratrol did not reveal any adverse effects at dose level of 1000 mg/kg/day. Oral administration of resveratrol to mice at a dose of 4000 mg/kg/day for 28 days did not reveal any adverse effects. In a review article prepared for the NIEHS, NOAEL for resveratrol was determined as 300 mg/kg/day on the basis of 28 day toxicity study in rats.

In a chronic toxicity study in mice, feeding of resveratrol (22.4 mg/kg/day) in the diet for one year had no adverse effects as evaluated by survival, motor function, insulin sensitivity, organ pathology, and mitochondrial number. Based on the results of this study, a NOAEL for resveratrol of 22.4 mg/kg/day can be determined. Results of a carcinogenicity study in p53 knockout mice show that resveratrol is not a carcinogen. In this extensive and well designed study, over 45 organs were studied for histopathology. Hydronephrosis (at dose levels of 1000 and 2000 mg/kg/day for 6 months) and epithelial

cell hyperplasia (at dose level of 2000 mg/kg/day) of the urinary bladder noted in the carcinogenicity study appear to be related to the elimination of high doses of resveratrol. Blood analysis for clinical pathology at resveratrol dose of 1000 mg/kg/day for six months did not reveal any adverse effects. Based on the results of this study a NOAEL for resveratrol of 1000 mg/kg/day can be determined.

Results of reproductive and developmental toxicity studies indicate that resveratrol is unlikely to cause toxicity. Prepubertal exposure to resveratrol did not affect development of the reproductive tract as well as the mammary glands in female mice. In a multigeneration mouse study, although resveratrol decreased absolute and relative seminal vesicle weight and spleen weight in the P generation and the absolute vesicle and kidney weight in the F_1 generation, some of the changes noted in P generation were not found in the F_1 generation. Although, resveratrol administration resulted in decreased absolute and relative seminal vesicle weight and spleen weight in the P generation, similar decreases, in absolute and relative seminal vesicle weight, and spleen weight were not noted in F_1 generation. In another multigeneration study, resveratrol did not affect fertility. Resveratrol exposure did not affect male sexual development. The available studies contradict the claimed estrogenic activity of resveratrol. In a 90-day study, oral administration of resveratrol at a dose of 20 mg/kg/day enhanced sperm production by stimulating the hypothalamic-pituitary-gonadal axis, without inducing adverse effects.

Available *in vitro* data suggest that depending on the biological environment, resveratrol can have both complex estrogenic and antiestrogenic effects. Under physiological conditions, resveratrol appears to act consistently as an antiestrogen relative to inducing breast tumors. Resveratrol does not stimulate indices of uterine growth and differentiation in immature rats. Resveratrol administration to rats even at high doses did not significantly affect uterine wet weight, epithelial cell height, IGF-I gene expression, or other estrogen target tissues. The available evidence shows that resveratrol is not an estrogen substance.

The structural similarity between resveratrol and some estrogenic substances such as diethylstilbestrol have raised questions about their pharmacological and toxicological similarity. However, available studies show that resveratrol does not possess estrogenic properties. In contrast to *trans*-resveratrol, diethylstilbestrol is known to have deleterious effects on the male reproductive tract. The distinct activity of *trans*-resveratrol and diethylstilbestrol can be explained by subtle differences in their molecules. Compared with *trans*-resveratrol, diethylstilbestrol lacks the 3-OH and 5-OH groups, but possesses a 4-OH group and two additional ethyl groups. These features provide differential binding characteristics to estrogen receptors. Diethylstilbestrol has an affinity to estrogen receptors similar to estradiol and acts as a potent agonist.

In an *in vitro* bacterial reverse mutation assay in the presence or absence microsomal activation system, resveratrol was not mutagenic. However, it induced structural chromosomal aberrations in a Chinese hamster lung cell line and mononuclear, polynuclear and karyorrhectic cells in micronucleus assay. In the sister chromatid exchange test, resveratrol induced sister chromatid exchanges. Contrary to these positive results, resveratrol is known for its antimutagenic and anticarcinogenic properties. The *in*

vitro DNA damaging effects of resveratrol are not physiologically comparable to *in vivo* conditions where resveratrol acts as a potent antioxidant. The available evidence shows that under *in vivo* conditions, resveratrol is unlikely to be genotoxic.

Resveratrol has been reported to inhibit corneal neorevascularization in mice. However, other studies have shown that resveratrol appears to act as a "brake" to unregulated angiogenesis (in tumors), but does not inhibit normal angiogenesis. Based on the available evidence, resveratrol is not considered to pose any risk of altering normal angiogenesis. Some *in vitro* studies indicate that resveratrol may inhibit platelet aggregation. However, available evidence from other similar antioxidants indicates that under *in vivo* conditions, resveratrol is unlikely to inhibit platelet aggregation. In *in vivo* conditions, resveratrol is rapidly metabolized and eliminated and it is unlikely to reach the concentrations required to inhibit platelet aggregation.

There is sufficient qualitative and quantitative scientific evidence, including animal and human data, to determine safety-in-use or acceptable daily intake (ADI) for resveratrol. Using the experimental data and an uncertainty factor (to bridge the lack of safety information in humans) an ADI of resveratrol for human use can be determined Although, no systematic or typical 90-day toxicity study of resveratrol is available to determine an ADI, there are several studies, such as short-term toxicity, subchronic toxicity, chronic toxicity and carcinogenicity, which support the safety-in-use and the ADI determinations. In two separate 28 day studies, a NOAEL for resveratrol of 300 mg/kg/day was established. Using this experimental data and an uncertainty factor of 1000 (10 for interspecies, 10 for intraspecies, and 10 for chronic extrapolation), an ADI of 0.3 mg/kg/day (18 mg/person/day) for resveratrol is determined. This ADI determination is supported by an extensive and well designed chronic study, in which mice were fed a high calorie diet and resveratrol for one year. In this study, 22.4 mg/kg/day was the highest dose of resveratrol used and the results support a NOAEL of 22.4 mg/kg/day. Application of an uncertainty factor of 100 to the NOAEL of 22.4 mg/kg/day from the chronic mouse study yields an ADI of 0.224 mg of resveratrol/kg *per* day. An additional investigation that supports the above ADI is a well designed six month carcinogenicity study in p53 knockout mouse. In the carcinogenicity study, the NOAEL for resveratrol of 1000 mg/kg/day was established. In this extensive study, clinical pathology and histology of 45 organs were evaluated. Using an uncertainty factor of 1000 (10 for interspecies, 10 for intraspecies and 10 for database deficiency), an ADI of 1 mg/kg/day for resveratrol is determined.

From the above discussion and supporting evidence, an ADI of 0.3 mg/kg/day or 18 mg/person/day (for an individual weighing 60 kg) is established. This daily level of intake, if ingested daily over a lifetime, is projected to be safe. The estimated 90th percentile intake of resveratrol (3.99 mg/day or 0.07 mg/kg/day) from the intended uses is approximately 4-fold lower than the ADI (0.3 mg/kg/day) determined on the basis of available safety data.

Because of lack of typical toxicity studies, in addition to the above described standard approach of ADI determination, a "weight of the evidence" approach was also used to determine safety in use of resveratrol at the estimated daily intake level. In this

context, any body of literature on biological effects and experience of historical ingestion of resveratrol from food is taken in to account to provide adequate assurance that any potential to produce adverse effects in humans at the estimated daily intake levels is understood and predictable.

All available studies on resveratrol were critically reviewed for quality and suitability for use in this safety assessment. Particular emphasis was placed on studies that focused on safety related parameters. The review yielded ten metabolism/ pharmacokinetic (*in vitro* and *in vivo*) studies, four short-term toxicity studies, one chronic study, one carcinogenicity, six reproduction/developmental studies, three mutagenicity studies, and several *in vitro* studies on estrogen-like activity, anti-angiogenesis, and platelet aggregation effects. Regarding these end points the review revealed that:

1. Following oral administration, resveratrol is rapidly absorbed, metabolized and eliminated. The bioavailability of resveratrol is low. The plasma half life of resveratrol in human was reported as 9.2 hours.
2. Short-term (28-day) toxicity studies did not reveal adverse effects at doses up to 300 mg/kg/day.
3. In a chronic one-year study in mice, resveratrol at a dose level of 22.4 mg/kg/day did not cause any adverse effects.
4. Resveratrol was not carcinogenic in a mouse study. In this study, safety related parameters such as clinical pathology and histology of 45 organs did not reveal toxicity at doses up to 1000 mg/kg/day.
5. The available evidence supports the conclusion that at low dose levels, resveratrol is unlikely to cause reproductive and developmental toxicity.
6. Depending on the biological environment, resveratrol has complex estrogenic and antiestrogenic effects. The available evidence shows that resveratrol is not estrogenic.
7. Based on the available evidence, resveratrol is not considered to pose any risk of altering normal angiogenesis or platelet aggregation.
8. There is a long history of resveratrol consumption by humans with written records dating back to ancient times.

Across the dataset, adverse effects of resveratrol on relevant endpoints were noted generally at doses greater than 300 mg/kg/day. The intended use levels of resveratrol, resulting in 90th percentile intake of 0.07 mg/kg/day is over 4000-fold lower than the lowest dose at which no significant adverse effects were noted. The available dataset show that adverse effects of resveratrol are not likely to occur at resveratrol exposure levels of 0.07 mg/kg/day. It is concluded that, under the intended and expected conditions of use, resveratrol does not pose a health risk to humans.

Moderate consumers of red wine (such as muscadine) with high levels of resveratrol are likely to ingest 4 mg resveratrol/day or 0.067 mg/kg/day. Although, exact figures are not available, it is well known that there are significant numbers of individuals who consume red wine daily in moderation for health benefits. The resulting 90th

percentile intake of resveratrol (3.99 mg/person/day) from the intended uses is similar to individuals consuming red wine (in moderation) with high levels of resveratrol.

In summary, on the basis of scientific procedures[9], and history of exposure from natural sources, the consumption of resveratrol as an added food ingredient is considered safe at levels up to 10 ppm in bottled water ("near waters"). The intended uses are compatible with current regulations, *i e*, resveratrol is used in bottled drinking water under the broad category non-alcoholic beverages [21 CFR §170.3(n)(3)], and is produced according to current good manufacturing practices (cGMP).

[9] 21 CFR §170.3 Definitions (h) Scientific procedures include those human, animal, analytical, and other scientific studies, whether published or unpublished, appropriate to establish the safety of a substance.

4. CONCLUSION

Based on a critical evaluation of the publicly available data summarized above, the Expert Panel members whose signatures appear below, have individually and collectively concluded that resveratrol, meeting the specifications cited above, and when used as a nutrient supplement [21 CFR 170.3(o)(20)] at maximum use levels of up to 10 ppm in bottled water ("near waters") [21 CFR170.3(n)(3)] is safe.

It is also our opinion that other qualified and competent scientists reviewing the same publicly available toxicological and safety information would reach the same conclusion. Therefore, we have also concluded that resveratrol, when used as described, is GRAS based on scientific procedures.

Signatures

Stanley M. Tarka, Jr. Ph.D.	7 May, 2007 Date
Sidney Green, Ph.D , FATS	8 May 2007 Date
Madhusudan G. Soni, Ph.D., FACN	10 May 2007 Date

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Appendix: I

Analytical data from five manufacturing lots

Typical Specifications of Resveratrol from five different lots

Parameter	Typical	Lot number				
		061109	**061114**	**061116**	**070208**	**061226**
Loss on drying (%)	≤ 1	0.40	0 30	0.45	0.35	0 30
Melting point °C	260-264	262-263	261-263	261-263	262-264	262-264
Total ash (%)	≤ 1	0.02	0.04	0 03	0.02	0.03
Purity (% by weight)	≥99	99.50	99.72	99 69	99.20	99.18
Identification	HPLC and IR	HPLC	HPLC	HPLC	HPLC	HPLC
Impurities (area HPLC)						
Each unknown impurities (%)	≤ 0.1	0.019	0.018	0.017	0 017	0.018
Total impurities (%)	≤ 1	0.075	0 083	0 072	0.12	0.10
Solvent residues	Complies with ICH guidelines	Complies	Complies	Complies	Complies	Complies
Ethanol (ppm)	≤ 500	50	35	45	40	50
Ethyl acetate (ppm)	≤ 100	Absent	Absent	Absent	Absent	Absent
Microbiological assays						
Total plate count (cfu/g)	≤1000	180	160	170	180	150
Yeast and Mold (cfu/g)	≤ 100	20	15	20	18	16
Escherichia coli	Negative	Absent	Absent	ntAbse	Absent	Absent
Pseudomonas aeruginosa	Negative	Absent	Absent	Absent	Absent	Absent
Heavy metals		**061101**	**061102**	**061103**	**061203**	**070201**
Cadmium (ppm)	<2 5	ND*	ND	ND	ND	ND
Lead (ppm)	< 5.0	ND	ND	ND	ND	ND
Arsenic (ppm)	< 5.0	ND	ND	ND	ND	ND
Mercury (ppm)	<0.5	ND	ND	ND	ND	ND

*ND = Not detectable at practical quantification limits of 2.5 ppm for cadmium, 5 ppm lead, 5 ppm arsenic and 0 5 ppm mercury.

Appendix II

ESTIMATED DAILY INTAKE OF RESVERATROL FROM BOTTLED WATER BY THE U.S. POPULATION FROM PROPOSED FOOD-USES

(Attached separately)

CANTOX
HEALTH SCIENCES INTERNATIONAL

ESTIMATED DAILY INTAKE OF RESVERATROL FROM BOTTLED WATER BY THE U.S. POPULATION FROM PROPOSED FOOD-USES

- Draft for Discussion -

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ESTIMATED DAILY INTAKE OF RESVERATROL FROM BOTTLED WATER BY THE U.S. POPULATION FROM PROPOSED FOOD-USES

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ESTIMATED DAILY INTAKE OF RESVERATROL FROM BOTTLED WATER BY THE U.S. POPULATION FROM PROPOSED FOOD-USES

1.0 INTRODUCTION

Cantox Health Sciences International (Cantox) has completed an assessment of the consumption of resveratrol by the United States (U.S.) population in bottled water

Estimates for the intake of resveratrol were based on total intake of resveratrol from bottled water included in the United States Department of Agriculture's (USDA) 1994-1996 Continuing Survey of Food Intakes by Individuals (CSFII 1994-1996) and the 1998 Supplemental Children's Survey (CSFII 1998) (USDA, 2000) Calculations for the mean and 90th percentile all-person and all-user intakes were included The per person and per kilogram body weight intakes were reported for the following population groups:

infants, ages 0 to 2;
children, ages 3 to 11;
female teenagers, ages 12 to 19,
male teenagers, ages 12 to 19;
female adults, ages 20 and up;
male adults, ages 20 and up; and
total population (all population and gender groups combined)

2.0 FOOD CONSUMPTION SURVEY DATA

2.1 Survey Description

Nationwide dietary intake data for the years 2001-2002 are now available for public use; however, only Day 1 interview data are included in the present release. It is well established that the length of a dietary survey affects the estimated consumption of individual users and that short-term surveys, such as the typical 1-day dietary survey, overestimate consumption over longer time periods (Anderson, 1988) Because two 24-hour dietary recalls administered on 2 non-consecutive days (Day 1 and Day 2) are available from the CSFII 1994-1996, 1998 surveys, these data were used to generate estimates for the current intake analysis.

USDA CSFII 1994-1996 provides food consumption data on persons of all ages, whereas, CSFII 1998 is limited to children from birth through 9 years of age. Combined, these surveys provide the most appropriate data for evaluating food-use and food-consumption patterns in the

U S., containing 4 years of data on individuals selected *via* stratified, multistage area probability sampling of American households within all 50 states.

CSFII 1994-1996, 1998 survey data were collected from individuals and households *via* 24-hour dietary recalls administered on 2 non-consecutive days (Day 1 and Day 2) throughout all 4 seasons of the year. Data were collected in-person, a minimum of 3 days apart, on different days of the week, to achieve the desired degree of statistical independence. CSFII 1994-1996 contains 2-day dietary food consumption data for more than 15,000 individuals of all ages, and 1-day data for 16,103 individuals CSFII 1998 contributes data from an additional 5,559 children, birth through 9 years of age, to data reported for 4,253 children of the same ages within CSFII 1994-1996. The overall CSFII 1994-1996, 1998 response rate for individuals selected for participation in the survey was 81.5 and 77.5% for Day 1 and Day 2, respectively.

In addition to collecting information on the types and quantities of foods being consumed, CSFII 1994-1996, 1998 collected physiological and demographic information from individual participants in the survey, such as sex, age, self-reported height and weight, and other variables useful in characterizing consumption. The inclusion of this information allows for further assessment of food intake based on consumption by specific population groups of interest within the total population. USDA sample weights were developed and incorporated with CSFII 1994-1996, 1998 to compensate for the potential under-representation of intakes from specific population groups as a result of sample variability due to survey design, differential non-response rates, or other factors, such as deficiencies in the sampling frame (USDA, 2000)

2.2 Statistical Methods

Consumption data from individual dietary records, detailing food items ingested by each survey participant on each of the 2 survey days, were collated by computer and used to generate estimates for the intake of resveratrol from bottled water by the U.S. population. Estimates for the daily intake of resveratrol from bottled water represent projected 2-day averages for each individual from Day 1 and Day 2 of CSFII 1994-96, 1998 data These average amounts comprised the distribution from which mean and percentile intake estimates were produced. Mean and percentile estimates were generated using ratio estimation and non-parametric techniques, respectively, incorporating USDA survey weights in order to provide representative intakes for the entire U.S population All-person intake refers to the estimated intake of resveratrol averaged over all individuals surveyed, regardless of whether they consumed bottled water, and therefore includes "zero" consumers (those who reported no intake of bottled water during the 2 survey days). All-user intake refers to the estimated intake of bottled water by those individuals consuming bottled water, hence the 'all-user' designation Individuals were considered users if they consumed bottled water on either Day 1 or Day 2 of the survey

2.3 Statistical Reliability

Mean or percentile intake estimates based on small sample sizes or with high variability relative to the mean [assessed using the coefficient of variation (CV)] may be less statistically reliable than estimates based on adequate sample sizes or low variability relative to the mean (LSRO, 1995) Data presented herein for the estimated daily intake of resveratrol follow the guidelines proposed by the Human Nutrition Information Service/National Center for Health Statistics Analytic Working Group for evaluating the reliability of statistical estimates adopted in the "Third Report on Nutrition Monitoring in the United States", whereby an estimated mean may be unreliable if the CV is equal to or greater than 30% (LSRO, 1995). The CV is the ratio of the estimated standard error of the mean to the estimated mean, expressed as a percentage (LSRO, 1995). Therefore, for the estimated intakes of resveratrol presented herein, values were considered statistically unreliable if the CV was equal to or greater than 30% These values were not considered when assessing the relative contribution of specific food-uses to total resveratrol consumption and are marked with an asterisk

3.0 FOOD USAGE DATA

3.1 Individual Food-Uses and Use-Levels

Bottled water intakes were adjusted to account for the global and North American market share for table/distilled water *vs.* mineral/spring water (Canadean, 2002) The individual proposed food-uses and use-levels for resveratrol employed in the current intake analysis are summarized in Table 3.1-1. All food codes included in the current intake assessment are listed in Appendix A.

Table 3.1-1 Summary of the Individual Proposed Food-Uses and Use-Levels for Resveratrol in the United States

Food Category	Proposed Food-Use	Use-Level (%)
Beverages and Beverage Bases	Bottled Water	0 001

3.2 Methodology for Estimating Bottled Water Intake

The CSFII 1994-1996, 1998 contains approximately 7,400 food codes within 9 food groups, encompassing a broad range of commonly consumed U.S. foods and food mixtures for which consumption data were obtained from the surveyed sample. Unfortunately, there is no food code for bottled water, and therefore, the consumption of bottled water by participants in the CSFII 1994-1996, 1998 cannot be estimated directly from food consumption data contained in the individual dietary records

In order to obtain estimates for the intake of resveratrol from the consumption of bottled water, a variety of alternative data sources were examined. No current estimates of bottled water ingestion in the U.S. based on individual food intake assessments were identified from the available published literature. Dietary intake data have been used to estimate the intake of plain drinking water by the U.S. population in several instances (Ershow and Cantor, 1989; NCHS, 1994); however, water consumption in these examples refers to "plain water consumed as a beverage (drinking water)", including tap or spring water. The intake of water from bottled sources was not assessed A recent consumer usage survey conducted *via* telephone for The Rockefeller University and the International Bottled Water Association examined beverage consumption, particularly of bottled water (Yankelovich Partners, 2000), but adults were the only population group from which consumption data were obtained.

Alternatively, representative population estimates for bottled water intake are available from a report produced by the Office of Water of the U S Environmental Protection Agency (U.S. EPA) entitled "Estimated *Per Capita* Water Ingestion in the United States" (U S EPA, 2000) The U S EPA report provides estimates for the intake of water from both direct (plain drinking water) and indirect (water used in the preparation of foods and beverages) sources, including bottled water, community water, or other sources, for the total population (*per capita* estimates) and for consumers-only, based on data from the USDA, CSFII 1994-1996 (without the 1998 Supplemental Children's Survey) The U S EPA report provides representative data with which to estimate the intake of resveratrol from bottled water by the total U.S population and for specific population groups In the absence of representative data for bottled water consumption from individual dietary records in the CSFII 1994-1996, 1998, the methodology presented by the U S EPA (U.S. EPA, 2000) for the calculation of direct bottled water consumption from the CSFII 1994-1996 was replicated in the current intake assessment using data from the CSFII 1994-1996, 1998 Household and sample person data from additional record types (Record types 15 and 25, respectively) available within the CSFII dataset were merged with the individual dietary record file (Record type 30) to determine the amount of plain bottled water consumed by survey respondents. The current method differed only in the manner in which files were merged A new food code was generated to represent bottled water intake (95000000, Water, bottled, Appendix A) in the current intake assessment The intakes of bottled water represented by this new food code were then added to the individual dietary record for each bottled water consumer, in order to allow estimation of the intake of resveratrol from bottled water.

4.0 FOOD SURVEY RESULTS

Estimates for the total daily intakes of resveratrol from bottled water are provided in Tables 4 1-1 and 4 1-2 on a per person and per kilogram body weight basis, respectively

4.1 Estimated Daily Intake of Resveratrol from Bottled Water

Approximately 19.8% of the total U.S. population was identified as consumers of resveratrol from bottled water (4,084 actual users identified) Consumption of bottled water by the total U S. population resulted in estimated mean all-person and all-user intakes of resveratrol of 0 43 mg/person/day (0 01 mg/kg body weight/day) and 2 09 mg/person/day (0.04 mg/kg body weight/day), respectively (Tables 4 1-1 and 4.1-2). The 90th percentile all-person and all-user intakes of resveratrol from bottled water by the total population were 1 42 mg/person/day (0 02 mg/kg body weight/day) and 3.99 mg/person/day (0.07 mg/kg body weight/day), respectively

Table 4.1-1 Summary of the Estimated Daily Intake of Resveratrol from All Investigated Food Categories in the U.S. by Population Group (1994-1996, 1998 USDA CSFII Data)

Population Group	Age Group (Years)	% Users	Actual # of Total Users	All-Person Consumption (mg)		All-Users Consumption (mg)	
				Mean	90th Percentile	Mean	90th Percentile
Infant	0 to 2	19 5	698	0 26	0 35	1 35	1 42
Child	3 to 11	21 0	1,326	0 52	0.71	2 74	2 84
Female Teenager	12 to 19	20 1	141	0 36	1 06	1 78	3 19
Male Teenager	12 to 19	16 4	114	0 32	1 06	1 96	4 26
Female Adult	20 and Up	20 4	933	0 47	1 77	2 10	4 26
Male Adult	20 and Up	18 4	872	0 40	1 42	1 99	4 26
Total Population	All Ages	19 8	4,084	0 43	1 42	2 09	3 99

On an individual population basis, the greatest mean all-person and all-user intakes of resveratrol on an absolute basis were determined to occur in children, at 0.52 mg/person/day (0 02 mg/kg body weight/day) and 2 74 mg/person/day (0.10 mg/kg body weight/day), respectively Infants had the lowest intakes of resveratrol on an absolute basis, with mean all-person and all-users intakes of 0 26 and 1 35 mg/person/day, respectively. On a body weight basis, mean all-person and all-user intakes of resveratrol were highest in infants and children at 0.02 and 0.1 mg/kg body weight/day, respectively The lowest mean all-person and all-user intakes on a per kilogram body weight basis were observed in male adults at <0.01 and 0 02 mg/kg body weight/day, respectively (Table 4 1-2)

Table 4.1-2 Summary of the Estimated Daily Per Kilogram Body Weight Intake of Resveratrol from All Investigated Food Categories in the U.S. by Population Group (1994-1996, 1998 USDA CSFII Data)

Population Group	Age Group (Years)	% Users	Actual # of Total Users	All-Person Consumption (mg/kg)		All-Users Consumption (mg/kg)	
				Mean	90th Percentile	Mean	90th Percentile
Infant	0 to 2	19 5	698	0 02	0 03	0 10	0 12
Child	3 to 11	21 0	1,326	0 02	0 03	0 10	0 11
Female Teenager	12 to 19	20 1	141	0 01	0 02	0 03	0 06
Male Teenager	12 to 19	16 4	114	< 0 01	0 02	0 03	0 07
Female Adult	20 and Up	20 4	933	0 01	0 03	0 03	0 06
Male Adult	20 and Up	18 4	872	< 0 01	0.02	0 02	0 05
Total Population	All Ages	19 8	4,084	0 01	0 02	0 04	0 07

When heavy consumers (90th percentile) were assessed, all-person intake of resveratrol from bottled water was determined to be greatest in female adults at 1.77 and the greatest all-user intakes of 4 26 mg/person/day were determined to occur in male teenagers and male and female adults The lowest 90th percentile all-person and all-user intakes of resveratrol occurred in infants at 0.35 and 1 42 mg/person/day, respectively, on an absolute basis (Table 4.1-1) On a body weight basis, infants, children and female adults were determined to have the greatest 90th percentile all-person intakes, 0 03 mg/kg body weight/day, and infants had the greatest 90th percentile all-user intake, 0.12 mg/kg body weight/day, of resveratrol (Table 4.1-2). The lowest all-person 90th percentile intakes of resveratrol on a body weight basis were observed in female and male teenagers, and male adults at 0 02 mg/kg body weight/day, and male adults had the lowest 90th percentile all-user intake, 0 05 mg/kg body weight/day

5.0 CONCLUSIONS

Consumption data and information pertaining to bottled water was used to estimate the all-person and all-user intakes of resveratrol for specific demographic groups and for the total U S population This type of intake methodology is generally considered to be 'worst case' as a result of several conservative assumptions made in the consumption estimates For example, it is often assumed that all food products within a food category contain the ingredient at the maximum specified level of use In addition, it is well established that the length of a dietary survey affects the estimated consumption of individual users Short-term surveys, such as the typical 2 or 3-day dietary surveys, overestimate consumption of food products that are consumed relatively infrequently

In summary, on an all-user basis, the mean and 90th percentile intakes of resveratrol by the total U S population from bottled water were estimated to be 2.09 mg/person/day (0 04 mg/kg body weight/day) and 3.99 mg/person/day (0.07 mg/kg body weight/day), respectively.

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APPENDIX A

Representative CSFII 1994-1996, 1998 Food Codes for All Proposed Food-Uses of Resveratrol in the United States

Representative CSFII 1994-1996, 1998 Food Codes for All Proposed Food-Uses of Resveratrol in the United States

Beverage and Beverage Bases

Bottled Water

(Magnesium Sulfate concentration of 10 ppm, assuming market share for table/distilled water of 30%)
[Resveratrol] = 0 0003%

95000000	Water, bottled (Not an original CSFII food code, added for the purpose of estimating bottled water intake)

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SUBMISSION END