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National Toxicology Program
U.S. Department of Health and Human Services

NTP Technical Report on the Toxicity Studies of (+)-Usnic Acid (CASRN 7562-61-0) Administered in Feed to F344/N Nctr Rats and B6C3F1/Nctr Mice

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Foreword

The National Toxicology Program (NTP), established in 1978, is an interagency program within the Public Health Service of the U.S. Department of Health and Human Services. Its activities are executed through a partnership of the National Institute for Occupational Safety and Health (part of the Centers for Disease Control and Prevention), the Food and Drug Administration (primarily at the National Center for Toxicological Research), and the National Institute of Environmental Health Sciences (part of the National Institutes of Health), where the program is administratively located. NTP offers a unique venue for the testing, research, and analysis of agents of concern to identify toxic and biological effects, provide information that strengthens the science base, and inform decisions by health regulatory and research agencies to safeguard public health. NTP also works to develop and apply new and improved methods and approaches that advance toxicology and better assess health effects from environmental exposures.

The Toxicity Report series began in 1991. The studies described in the NTP Toxicity Report series are designed and conducted to characterize and evaluate the toxicological potential of selected substances in laboratory animals (usually two species, rats and mice). Substances (e.g., chemicals, physical agents, and mixtures) selected for NTP toxicity studies are chosen primarily on the basis of human exposure, level of commercial production, and chemical structure. The interpretive conclusions presented in the toxicity reports are derived solely from the results of these NTP studies, and extrapolation of these results to other species, including characterization of hazards and risks to humans, requires analyses beyond the intent of these reports. Selection for study per se is not an indicator of a substance's toxic potential.

NTP conducts its studies in compliance with its laboratory health and safety guidelines and Food and Drug Administration Good Laboratory Practice Regulations and meets or exceeds all applicable federal, state, and local health and safety regulations. Animal care and use are in accordance with the Public Health Service Policy on Humane Care and Use of Laboratory Animals. Studies are subjected to retrospective quality assurance audits before they are presented for public review. Draft reports undergo external peer review before they are finalized and published.

The NTP toxicity reports are available free of charge on the NTP website and cataloged in PubMed, a free resource developed and maintained by the National Library of Medicine (part of the National Institutes of Health). Data for these studies are included in NTP's Chemical Effects in Biological Systems database.

For questions about the reports and studies, please email NTP or call 984-287-3211.

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Peer Review

The National Toxicology Program (NTP) conducted a peer review of the draft *NTP Technical Report on the Toxicity Studies of (+)-Usnic Acid (CASRN 7562-61-0) Administered in Feed to F344/N Nctr Rats and B6C3F1/Nctr Mice* by letter in November 2021 by the experts listed below. Reviewer selection and document review followed established NTP practices. The reviewers were charged to:

(1) Peer review the draft *NTP Technical Report on the Toxicity Studies of (+)-Usnic Acid*.

(2) Comment on NTP's interpretations of the data.

NTP carefully considered reviewer comments in finalizing this report.

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Abstract

(+)-Usnic acid is a secondary metabolite of lichens belonging to the *Usnea* genus. *Usnea* lichens and purified usnic acids have been used historically in traditional herbal medicine as bactericidal and antimicrobial agents. (+)-Usnic acid exhibits membrane proton uncoupling activity, which not only forms the mechanistic basis of its bactericidal action, but also has provided a rationale for its use as a fat-burning, weight-loss agent. Purified (+)-usnic acid has been marketed in the United States for this purpose either alone or in combination with other chemical agents. Use of some of these fat-burning products that contain (+)-usnic acid has resulted in serious liver damage. This study investigated the potential toxicity of (+)-usnic acid in male and female F344/N Nctr rats and B6C3F1/Nctr mice that were exposed via feed for 3 months. F344/N Nctr rats were administered 0, 30, 60, 120, 360, or 720 ppm in feed, while B6C3F1/Nctr mice were administered 0, 15, 30, 60, 180, or 360 ppm in feed.

Exposure of F344/N Nctr rats and B6C3F1/Nctr mice to (+)-usnic acid in feed for 3 months resulted in hepatotoxicity in male rats at exposure levels above 120 ppm. Mild toxicity as demonstrated by increased serum enzyme activity was observed in female rats at exposure levels of 720 ppm. In male mice, moderate but significant increases in alanine aminotransferase and alkaline phosphatase were observed at exposure levels of 360 ppm, moderate significant increases in blood urea nitrogen were observed at exposure levels of 180 and 360 ppm, whereas moderate significant increases in serum creatinine were observed at exposure levels of 60, 180, and 360 ppm. There were significantly fewer female rats cycling in the 720 ppm group than in the control group, due to extended diestrus. Significant body weight decreases were achieved at exposure levels of 720 ppm in male and female rats. Exposure to 600 ppm (+)-usnic acid for 14 days significantly increased the incidence of micronuclei in erythrocytes or reticulocytes from both male and female B6C3F1/Nctr mice; exposure to 1,200 ppm significantly increased the incidence of micronuclei in reticulocytes in male B6C3F1/Nctr mice. No-observed-adverse-effect levels (NOAELs) of 120 ppm and 30 ppm of (+)-usnic acid administered in feed were established for F344/N Nctr rats and B6C3F1/Nctr mice, respectively, on the basis of the results of these subchronic studies.

Synonyms: 2,6-diacetyl-7,9-dihydroxy-8,9b(R)-dimethyldibenzofuran-1,3(2H,9bH)-dione; (d)-usnic acid; usneine; usninic acid; usniacin

Trade names: *usnea* extract, usnic acid

Summary of Subchronic Toxicology Studies of (+)-Usnic Acid in F344/N Nctr Rats and B6C3F1/Nctr Mice

	Male F344/N Nctr Rats	Female F344/N Nctr Rats	Male B6C3F1/Nctr Mice	Female B6C3F1/Nctr Mice
Exposure Concentrations of (+)-Usnic Acid in NIH-41 Feed	0, 30, 60, 120, 360, 720 ppm	0, 30, 60, 120, 360, 720 ppm	0, 15, 30, 60, 180, 360 ppm	0, 15, 30, 60, 180, 360 ppm
Body Weight Effects	60, 720 ppm groups < controls	360, 720 ppm groups < controls	No effect	No effect
Survival	No effect	No effect	No effect	No effect
Liver, Hepatocellular Degeneration	1/10, 0/10, 3/10, 4/10, 10/10, 10/10	No effect	No effect	No effect
Liver, Inflammation	0/10, 1/10, 3/10, 3/10, 8/10, 10/10	No effect	1/10, –[a], –, –, –, 0/10	No effect
Kidney, Hydronephrosis	0/10, –, –, 0/10, 2/10, 5/10	No effect	No effect	No effect
Clinical Pathology	↑ Creatinine	↑ Alanine aminotransferase ↑ Blood urea nitrogen ↑ Creatinine	↑ Alanine aminotransferase ↑ Blood urea nitrogen ↑ Creatinine	No effect
Estrous Cycle	N/A	↑ Diestrus stage length ↑ Estrous cycle length	N/A	No effect
Genetic Toxicology				
Micronucleated Erythrocytes (In Vivo)				
Mouse peripheral blood:		Positive in males and females		

[a]These groups were not histopathologically examined.
N/A = not applicable.

Introduction

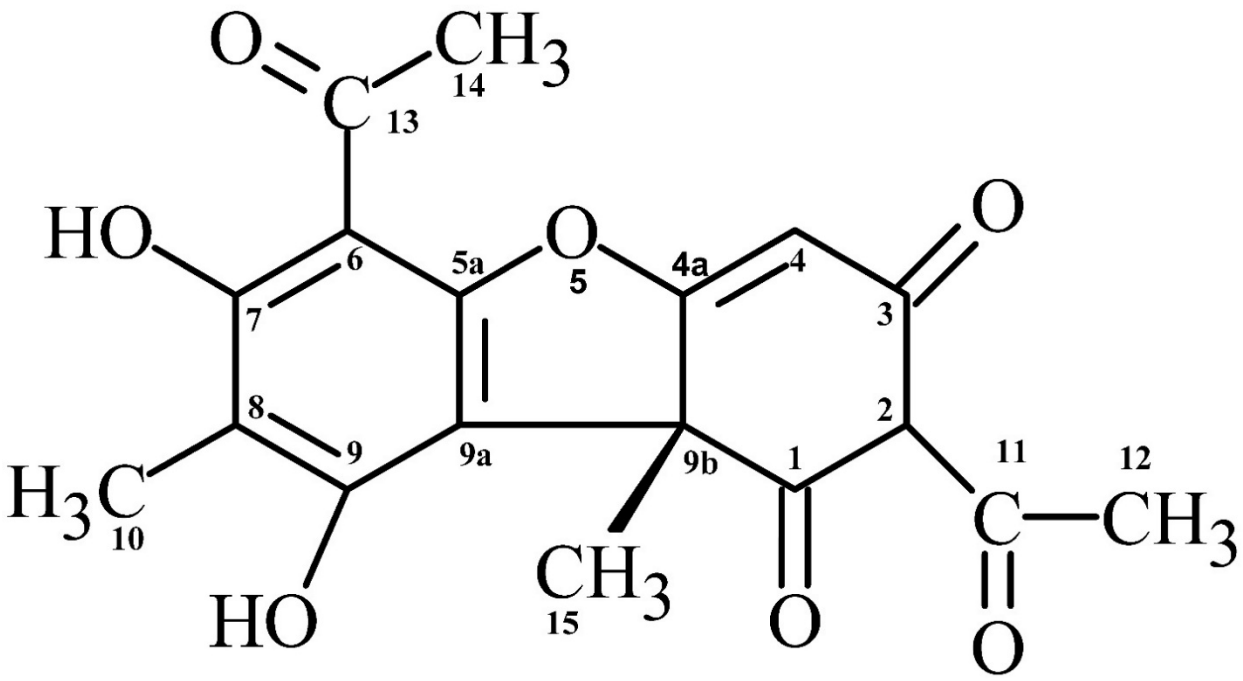

Figure 1. (+)-Usnic Acid (CASRN 7562-61-0; Chemical Formula: $C_{18}H_{16}O_7$; Molecular Weight: 344.32)

Synonyms: 2,6-diacetyl-7,9-dihydroxy-8,9b(R)-dimethyldibenzofuran-1,3(2H,9bH)-dione; (d)-usnic acid; usneine; usninic acid; usniacin. Trade names: *Usnea* extract, usnic acid.

Chemical and Physical Properties

2,6-diacetyl-7,9-dihydroxy-8,9b(R)-dimethyldibenzofuran-1, 3(2H,9bH)-dione ((+)-Usnic acid), CASRN 7562-61-0, is a bright-yellow dibenzofuran compound, which occurs naturally as a secondary metabolite of lichens of the *Usnea* genus and several other genera.[1] While both the (+)- and (−)-usnic acid enantiomers (also denoted as d and l enantiomers, respectively) have been isolated from lichens, the (+)-usnic acid enantiomer usually predominates in *Cladina, Evernia, Flavocetraria, Lecanora, Lobaria, Melanelia, Nephroma, Parmelia, Ramalina, Usnea,* and *Xanthoparmelia* species.[1-7] (−)-Usnic acid (CASRN 6159-66-6) predominates in most species of *Alectoria, Cladonia, Rhizoplaca,* and several other genera.[1] The two enantiomers differ in their R or S projection of the angular -CH_3 group at the chiral 9b position. Racemic mixtures of (+)- and (−)-usnic acid enantiomers are listed under the CASRN 125-46-2. There has been some controversy over the correct absolute enantiomeric structure of (+)- or (−)-usnic acid.[8] Huneck and collaborators assigned the R configuration to the chiral methyl group of (+)-usnic acid by using x-ray crystallography to determine the structure of the (−)-α-phenylalanine derivative[9] and this configuration has been confirmed by others.[10] However, the structure of (+)-usnic acid frequently appears in the literature drawn as the S configuration of (−)-usnic acid.[8] Authentic (+)-usnic acid isolated from *Usnea* lichens is dextrorotatory with a specific rotation of +478° [c 0.2%, $CHCl_3$, (deg mL) (g dm)-1].[8] It has a melting point of 204°C and is practically insoluble in water, moderately soluble in acetone and ethyl acetate, and more soluble in tetrahydrofuran

and furfural.[11; 12] The sodium dihydrate salt of usnic acid is slightly soluble in water.[11] (+)-Usnic acid can be chemically synthesized from methylphloroacetophenone by oxidative coupling followed by hydrolysis in sulfuric acid.[13] In 1969, Taguchi and coworkers[14] confirmed that methylphloroacetophenone, which is produced from acetyl CoA, was also an intermediate in the biosynthesis of both enantiomers of usnic acid in lichens. (+)-Usnic acid absorbs light predominantly in the UVB (280–315 nm) region with a peak extinction coefficient at 283 nm of 45,000 $M^{-1}cm^{-1}$, which has led investigators to suggest that one biological function of (+)-usnic acid is to protect the lichen from the high levels of ultraviolet radiation that is ubiquitous to many of the environments where lichens thrive.[15]

Of the three hydroxyl groups present in the usnic acid molecule, the enolic hydroxyl at the 1 position has the strongest acidic character (pKa 4.4) due to the inductive effect of the keto groups at positions 3 and 11, whereas the hydroxyl groups at positions 7 and 9 are less acidic with pKa values of 8.8 and 10.7, respectively.[4] (+)-Usnic acid is highly lipophilic in both neutral and anionic forms due to its β-triketone groups, which absorb the negative charge of the anion by resonance stabilization.[16] This lipophilicity of usnic acid and the usniate anion allows (+)-usnic acid to behave as a membrane uncoupler as is described in the Toxicity section of this report.

Production, Use, and Human Exposure

Because of usnic acid's bright-yellow color, usnic acid-containing lichens were extensively used as a fabric dye for many years in Europe prior to the advent of synthetic aniline dyes.[17] Racemic (+/−)-usnic acid was first isolated from *Usnea* lichens in 1843,[4] and was first chemically synthesized in 1956.[13] Currently, commercial preparations of (+)-usnic acid are purified from dried wild-collected lichen or from segments of thalli of *Usnea* and *Ramalina* species grown in tissue culture.[18] An alternative synthesis method has been reported for (+/−)-usnic acid.[19] This method involves a two-step procedure that uses commercially available phloracetophenone—methylated with iodomethane to form trihydroxyacetophenone which is oxidized to usnic acid with horseradish peroxidase. (+)-Usnic acid can be purified from racemic mixtures by chiral chromatography.[20; 21]

(+)-Usnic acid has been formulated into creams, toothpaste, mouthwash, deodorants, antibiotic ointments, sunscreen products, and antimicrobial preparations at concentrations of 0.1%–2%.[18; 22] (−)-Usnic acid has not been produced in commercial quantities. In Italy, usnic acid has been used in vaginal creams, foot creams, powders, and hair shampoo.[23; 24] There has recently been renewed interest in (+)-usnic acid's therapeutic potential. In the United States and Canada, (+)-usnic acid has been marketed as a "fat burner" due to its activity as a mitochondrial uncoupler.[18; 25] It has also been suggested that usnic acid could be used as a biomarker to assess pollution, because its concentration in lichens can increase with the increased exposure to toxicants.[4] It has also been proposed that (+)-usnic acid may be of use as a sunscreen agent[15] and as a marine antifouling agent for treating ship's hulls as a replacement for tributyltin.[26]

Pharmacology

Antimicrobial Activity

Prior to the discovery of penicillin, usnic acid was under active investigation for its broad-spectrum antibiotic activities. In fact, from the mid-1940s until the end of the 1950s, most of the 64 research publications on usnic acid were related to its antimicrobial activity. During the 1980s, interest in usnic acid was renewed because of increasing multi-drug resistance caused by overuse of synthetic antibiotics.[3] Both of the optical enantiomers of usnic acid are active against Gram-positive bacteria and mycobacteria.[4] In subsequent years, the antibacterial properties of (+)-usnic acid have been confirmed by several researchers. In preliminary clinical trials, a mouthwash containing 1% (+)-usnic acid was administered to volunteers, and at regular intervals the samples of oral bacterial flora were examined. It was reported that the growth of *Streptococcus mutans* involved in the etiology of dental caries was selectively suppressed.[27] A number of preparations containing (+)-usnic acid have been marketed.[3] Using standardized assays, the in vitro susceptibility of pathogenic Gram-positive and anaerobic bacteria toward usnic acid has been confirmed.[4] Usnic acid has been shown to suppress the growth of Gram-positive organisms mainly responsible for body odor.[18] (+)-Usnic acid has been shown recently to be effective, in vitro, against *Staphylococcus epidermidis*, *S. aureus*, and *S. haemolyticus* with minimum inhibitory concentrations of 3.12, 12.5, and 12.5 µg/mL, respectively.[28] In another in vitro study, (+)-usnic acid was reported to be active against many strains of *Helicobacter pylori* with minimum inhibitory concentrations of <2 µg/mL.[29] (+)-Usnic acid was also found to be effective against *Mycobacterium aurum*.[30] In in vitro assays, (+)-usnic acid and its salt were reported to inhibit the growth of Mycobacterium tuberculosis at relatively low concentrations.[18] Liposomes,[31; 32] cationic polyacrylamide nanoparticles,[33] or nanofluids[34] containing (+)-usnic acid have been investigated as potential therapeutic agents for treating antibiotic resistant bacterial infections because encapsulation increases the potency of (+)-usnic acid. Liposomal encapsulation reduced the in vitro minimal inhibitory concentration of (+)-usnic acid against multi-drug resistant Mycobacterium tuberculosis from 31.25 to 0.98 µg/mL.[31] However, liposomal encapsulation of (+)-usnic acid has also been shown to enhance its uptake by macrophages.[35] While encapsulation is expected to reduce the toxicity of (+)-usnic acid, it may also reduce its bactericidal activity by an equivalent factor. A recent study by Martinelli and coworkers[36] reported a minimum inhibitory concentration for (+)-usnic acid incorporated into carboxylated poly-L-lactide microparticles of 160 µg/mL against planktonic *S. epidermidis* compared to a minimum inhibitory concentration of 16 µg/mL for free (+)-usnic acid. The encapsulated preparation provided a slow release of (+)-usnic acid and was more efficient than solubilized (+)-usnic acid in inhibiting bacterial biofilm growth. Recently, (+)-usnic acid has been used as an antimicrobial additive in experimental collagen-based 3D matrices for use for tissue regeneration; these matrices allowed proliferation of osteoblasts but suppressed growth of *S. aureus*.[37] (+)-Usnic acid has been used experimentally as an additive to polymethylmethacrylate surgical bone cement to inhibit bacterial biofilm formation.[38] While relatively high concentrations of (+)-usnic acid (approximately 12% in the cement) did significantly inhibit biofilm formation by methicillin-resistant *S. aureus*, it also induced small changes in the material characteristic of the cement. Nevertheless, the investigators supported a potential role for (+)-usnic acid in controlling bacterial biofilm formation in bone cement.

Antimycotic Activity

During a short-term treatment with a usnic acid salt (copper usnate), 65 patients with tinea pedis (athlete's foot) exhibited a significant improvement in their clinical conditions.[3] (+)-Usnic acid (25–100 µg/mL) has been reported to inhibit biofilm formation in in vitro cultures of *Candida albicans*, but did not inhibit its proliferation.[39] Conversely, in another study (+)-usnic acid isolated from *Ramalina farinacea* and identified by infrared spectography and polarimetry was reported to inhibit growth of *C. albicans* and *C. glabrata* with minimal inhibitory concentrations of <2 µM.[40] Also, Pires and coworkers reported that (+)-usnic acid inhibited both the planktonic and biofilm growth of *C. orthopsilosis* and *C. parapsilosis*, with minimal fungicidal concentrations of 125 and 250 µg/mL, respectively, for the two species.[41]

Antiprotozoal Activity

(−)-Usnic acid exhibited significant inhibitory effects against the pathogenic protozoan *Trichomonas vaginalis* at comparatively lower concentrations than metronidazole,[42] whereas (+)-usnic acid isolated from Chilean lichens showed leishmanicidal properties in both in vitro and in vivo studies; intralesional administration produced a reduction in lesion weight as well as parasite body burden.[43] In a recent Chinese study,[44] (+)-usnic acid was shown to be active against the toxoplasmosis parasite, *Toxoplasma gondii* in an in vitro assay at a dose range of 0.25–4.0 µM and increased the survival time of mice infected with *T. gondii* when given at oral doses of 10 or 20 mg/kg body weight/day (mg/kg/day) in a 20-day exposure study. When encapsulated in liposomes and given at an oral dose of 10 mg/kg/day (+)-usnic acid improved survival to a greater extent than either dose given in unencapsulated form. (+)-Usnic acid was also found to be active against the malarial parasites, *Plasmodium berghei* and *Plasmodium falciparum*[45]; reported median inhibitory concentration (IC_{50}) values were 2.3 and 45 µM, respectively, for the liver stage of *P. berghei* and the blood stage of *P. falciparum*, and toxicity was associated with inhibition of fatty acid metabolism.

Antiviral Activity

In a cancer chemoprevention assay, (+)-usnic acid isolated from *Usnea longissima* was found to be significantly effective against teleocidin b4-induced Epstein-Barr virus with a median effective dose (ED_{50}) of 1.0 µg/mL.[46] (+)-Usnic acid also inhibited the cytopathic effects of herpes simplex type 1 and polio type 1 viruses in infected African green monkey kidney cells.[4] In a clinical trial, the effect of an intravaginal formulation containing (+)-usnic acid and zinc sulfate as an adjuvant therapy on radiosurgical treatment was evaluated in 100 women with genital infections of human papilloma virus. The treatment significantly improved the time of re-epithelization 1 month after the radio surgery.[47]

Insecticidal Activity

(+)-Usnic acid showed strong larvicidal activity and caused 100% mortality in the third to fourth larval stages of *Culex pipiens* (house mosquito) at 24 hours at the doses of 5 and 10 ppm.[48] (+)-Usnic acid was also reported to be larvicidal against the second and third instar larvae of the mosquito *Culiseta longiareolata* with median lethal dose (LD_{50}) and 90% lethal dose (LD_{90}) values of 0.48 and 1.54 ppm, respectively.[49]

Antiproliferative Activity

(+)-Usnic acid caused moderate inhibition in the murine P388 leukemia assay, and also exhibited cytotoxic activity against cultured L1210 cells; it was inferred that the p-tri-ketone moiety was essential for optimum activity.[50] On the other hand, (+)-usnic acid (50 µg/mL) reduced the cell counts of leukemic (K-562) and endometrial (Ishikawa and HEC-50) carcinoma cell cultures.[51; 52] (+)-Usnic acid exhibited cytotoxic activity against human keratinocyte cell cultures.[53] Recent studies reviewed by Kapoor[54] have demonstrated that (+)-usnic acid is cytotoxic to cultured cancer cells derived from many different types of tumors. For example, Bačkorová and coworkers reported IC_{50} values ranging from 48 to 178 µM for nine cell lines,[55] with the cytotoxicity being related to loss of mitochondrial membrane potential.[56] Sahu and coworkers reported a median lethal concentration (LC_{50}) value of 30 µM for a human hepatoma (HepG2) cell line,[57] and Brisdelli and coworkers reported IC_{50} values of 17.7, 23.7, and 75.7 µM for HCT116, HeLa, and MCF-7 cells, respectively.[58] Singh and coworkers reported concentrations of 10–100 µM (+)-usnic acid induced cell cycle arrest, mitochondrial membrane depolarization, and apoptosis in lung carcinoma, A549 cells.[59] Chen and coworkers demonstrated that (+)-usnic acid exposure induced both apoptosis and autophagy in HepG2 cells.[60] However, none of these studies compared the toxicity of (+)-usnic acid in cancer cells with that in normal cells in primary culture or in vivo. Recently, Bruno and coworkers reported that (+)-usnic acid gave an IC_{50} value of 24 µg/mL for spontaneously transformed human keratinocytes after 24 hours in culture.[61] (+)-Usnic acid has also been reported to inhibit migration and invasive activity of A549 lung carcinoma cells in vitro by a mechanism that involved downregulation of AP-1 mediated pathways and that was additive to the inhibitory action of the EGF-receptor antibody drug cetuximab.[62] In another study, Einarsdóttir and coworkers[63] demonstrated that both (+)- and (−)-usnic acid were equally potent in inhibiting the proliferation and cell size of two carcinoma cell lines, T-47D (mammary) and Capan-2 (pancreatic cancer), by mechanisms that involved disruption of the cell's inner mitochondrial membrane potential. (+)-Usnic acid has been shown to strongly inhibit angiogenesis in both chick embryo chorioallantoic membrane and mouse corneal angiogenesis assays.[64] In the same study, it also inhibited growth of implanted Bcap-37 breast tumors in a mouse xenograft model.

Anti-inflammatory Activity

In an acute rat paw edema and a chronic rat cotton pellet assay at 100 mg/kg oral dose level, the anti-inflammatory action of (+)-usnic acid was comparable to ibuprofen at the same dose level.[65] Pretreatment (50 or 100 mg/kg oral gavage for 5 days) with (+)-usnic acid has also been shown to protect mice from lipopolysaccharide (LPS)-induced inflammatory lung injury[66] and to downregulate nuclear factor-κB-dependent tumor necrosis factor-α and inducible nitric oxide synthase expression in LPS-stimulated macrophages.[67] (+)-Usnic acid has also been reported to accelerate wound-healing in rat and mouse in vivo wound closure assays.[61]

Analgesic and Antipyretic Activity

The analgesic and antipyretic effects of (+/−)-usnic acid purified from *Usnea diffracta* were evaluated in two mouse studies.[68] At 100 mg/kg oral dose level, usnic acid exhibited a significant analgesic effect as indicated by an acetic acid-induced writhing test and a tail pressure test. At oral dose levels up to 300 mg/kg, usnic acid also expressed significant antipyretic activity determined through LPS-induced hyperthermia.

Absorption, Distribution, Metabolism, and Excretion

Experimental Animals

The pharmacokinetics of (+)-usnic acid were studied in rabbits following intravenous or oral administration of doses of 5 and 20 mg/kg body weight, respectively.[69] Plasma usnic acid levels following intravenous administration showed a tri-exponential elimination with a terminal half-life of 10.7 ± 4.6 hours. The volume of distribution of the central compartment and systemic clearance was 43.9 ± 21.3 mL/kg and 12.2 ± 3.0 mL/hr/kg, respectively. Peak plasma level (C_{max}) of 32.5 ± 6.8 µg/mL was achieved in 12.2 ± 3.8 hours (t_{max}). The mean absolute bioavailability of (+)-usnic acid following oral administration was 77.8%.[70] In rats treated intraperitoneally (i.p.) with 25 mg/kg (+)-usnic acid, the usnic acid accumulated in the liver and lungs at levels similar to plasma concentrations, but accumulated at lower concentrations in brain, fat, testis, and other organs.[70] A protein binding of (+)-usnic acid in rabbit plasma and bovine serum albumin revealed that (+)-usnic acid was extensively bound to protein with approximately 99.2% in bound form.[70] A tissue disposition study in rats following 25 mg/kg (i.p.) administration showed that (+)-usnic acid distributed into various tissues and tissue levels were high in lung, liver, and blood with mean tissue/plasma ratios of 1.777, 1.503, and 1.192, respectively.[70] (+)-Usnic acid was reported to penetrate porcine skin in vitro with significant amounts accumulating in the stratum corneum and dermis layers after 12 hours of exposure.[71]

In Vitro

The in vitro metabolism of (+)-usnic acid was investigated using human plasma, hepatocytes, and liver subcellular fractions.[72] To identify metabolites, (+)-usnic acid was incubated in human liver S9 fractions and samples were analyzed by LC/MS (liquid chromatograph/mass spectrometry) ion chromatograms. Various metabolites, including three oxidized metabolites and two glucuronide conjugates, were identified. Two of the oxidation products were regioisomeric hydroxy ketones and were not differentiated by LC/MS. A third monohydroxylated metabolite was also identified but not characterized. The two isomeric glucuronides were conjugates of parent (+)-usnic acid. In this in vitro study, the authors also reported that the half-life of (+)-usnic acid in human liver microsomes was 19.3 minutes with an intrinsic clearance of 45.24 mL/min/kg. This half-life predicted a human hepatic clearance of 13.86 mL/min/kg. Phase I metabolizing enzymes (cytochrome P450 [CYP] isoforms) involved in oxidative metabolism of (+)-usnic acid were also investigated using human liver microsomes pre-incubated with (+)-usnic acid and several CYP inhibitors.[72] The inhibitors, used to infer the CYP isoforms responsible for catalyzing the turnover of (+)-usnic acid, included furafylline (CYP1A2), thiotepa (CYP2B6), quercetin (CYP2C8), sulfaphenazole (CYP2C9), (s)-(+)–3-benzylnirvanol (CYP2C19), quinidine (CYP2D6), and ketoconazole (CYP3A4/5). Among the inhibitors investigated, only furafylline, the inhibitor of CYP1A2, showed the effect on the turnover rate of (+)-usnic acid by increasing its half-life tenfold. This observation suggested that the oxidative metabolism of usnic acid was probably mediated by CYP1A2. This study suggested that (+)-usnic acid was a weak inhibitor of CYP2D6, a potent inhibitor of CYP2C19 and CYP2C9, and a less potent inhibitor of CYP2C8 and CYP2C18. Assays using 12 recombinant human UDP-glucuronosyltransferase (UGT) isoforms suggested that UGT1A1 and UGT1A3 played a major role in glucuronidating (+)-usnic acid, with a minor contribution of UGT1A8.[72]

Toxicity

Experimental Animals

Irrespective of a long history of usnic acid-containing products, only a few animal studies had been conducted to evaluate the clinical safety of (+)-usnic acid when it was nominated for evaluation by NTP; no systemic subchronic and chronic general toxicity studies had been conducted; and the conduct and quality of some of the available studies were questionable.[18] LD_{50} values for usnic acid (enantiomer not specified) were reported as 25, 75, and 838 mg/kg for mice exposed via intravenous, subcutaneous, and oral routes, respectively, and 500 mg/kg for rabbits exposed orally.[18] More recent studies of mice have reported LD_{50} values for oral and i.p. exposure to (+)-usnic acid of 388 and 75 mg/kg, respectively.[73; 74]

Acute toxicity studies of (+)-usnic acid have been reported for both animals and plants. In several experimental animal or wild animal species—such as guinea pigs, mice, rats, domestic sheep, cow elk, and mosquitoes—either general toxicity or organ-specific toxicity, or both, have been reported. In female guinea pigs with tuberculosis, subcutaneous injection of usnic acid (20 mg per animal for 6 days, followed by 10 mg per animal for 24 days; enantiomer not specified) caused a slight weight loss in the first week and a significant inhibition of weight gain during the next 3 weeks.[75] Even after the discontinuation of usnic acid, weight gain was still reduced 44%–68% for at least 2 weeks. This report was the first to show that usnic acid could cause weight loss with the possibility of general toxicity, although this possibility was largely ignored in the ensuing decades. Of note, no apparent organ-specific toxicities in the liver, spleen, or lung were observed in this report, and no apparent therapeutic effects were observed.[75] In healthy male Swiss mice, treatment with (+)-usnic acid i.p. at 15 mg/kg for 15 days caused no apparent general toxicity, as evidenced by the negative observations in clinical signs or changes of body weight.[76; 77] However, strong hepatotoxicity, including elevated serum transaminase activity and extensive liver necrosis, was observed. No toxicity in other organs, such as kidney and spleen, was detected in the study. A similar pattern of toxicity was also revealed in the tumor-bearing mice in the study.[76; 77] In male Wistar albino rats, (+)-usnic acid (i.p., 50 or 200 mg/kg for 5 days) induced remarkable swelling of the liver mitochondria and endoplasmic reticulum assessed by electron microscopy, although no changes in serum transaminase activity were observed, suggesting that only mild hepatotoxicity occurred.[78] Conversely, a more recent study that administered (+)-usnic acid at 100, 200, and 240 mg/kg to male Wistar rats via oral gavage for 8 days observed significantly elevated alanine aminotransferase and total bilirubin after 3 and 6 days in the 100 and 200 mg/kg groups and hepatic degeneration in the 200 mg/kg group, but not in the 100 mg/kg group. Less than 50% of the 240 mg/kg dosed group survived the experiment.[79] Another study investigated potential cardiotoxicity in female rats receiving 30 or 100 mg (+)-usnic acid in methylcellulose/kg/day via oral gavage for 14 days.[80] Neither dose caused alterations in blood chemistry parameters but cytoplasmic rarefaction of myocardium in conjunction with mitochondrial swelling and increased prohibitin expression were observed in animals from the 100 mg/kg/day group.

Feeding domestic sheep with (+)-usnic acid of 323–776 mg/kg/day for a maximum of 9 days induced several clinical signs such as lethargy and anorexia, or even death, with the estimated median toxic dose between 485 and 647 mg/kg/day.[81] Other toxicity indices, such as serum lactate dehydrogenase, aspartate aminotransferase, and creatine kinase, were also increased. A complete postmortem examination revealed that pathological changes occurred exclusively in the

skeletal muscle.[82] This observation contrasts sharply with mice, rats, and humans, in which the liver is considered to be the organ most vulnerable with usnic acid insults. Usnic acid is also the assumed toxicant associated with some 400–500 cow elk deaths that occurred in Wyoming in 2004.[82; 83] Necropsy revealed extensive muscle damage, such as muscle pallor and streaking, particularly in the semitendinosus, semimembranosus, and pelvic limbs. Histological examination showed necrosis, rupture, inflammation, and degeneration of myofiber. However, a causative relation between muscle damage and usnic acid exposure was not established.

Usnic acid also acts as a strong toxicant toward certain insects, such as mosquitoes. It has been reported that both (+)- and (−)-usnic acids (5 and 10 ppm) killed all the larvae of certain species of mosquitoes during their third and fourth stages with similar potency, suggesting that they might be developed as a novel natural insecticide.[48] In addition to the toxicity toward animals, usnic acid displays phytotoxicity as well. It exerts toxic effects on the growth of onion and lettuce, possibly by inhibition of plant p-hydroxyphenylpyruvate dioxygenase, indicating the potential usage as an herbicide.[84] In another study, (+)-usnic acid added to cultures of *Nicotiana tabacum* protoplasts reduced protoplast viability at concentrations of 5–200 µg/mL.[51] (+)-Usnic acid has also been shown to inhibit growth of the free living alga, *Scenedesmus quadricauda*, concurrent with increased oxidative damage and decreased chlorophyll production.[85]

Subchronic Toxicity Studies

No subchronic animal studies were found in the literature.

Chronic Toxicity Studies

Extensive library searches (Frankos,[18] updated in 2017) did not provide any information about chronic toxicity studies.

In Vitro

(+)-Usnic acid is highly lipophilic in both neutral and anionic forms because it is able to absorb the negative charge of the usnate anion by resonance stabilization over its β-triketone groups (Figure 2),[16] which allows (+)-usnic acid to act as a membrane uncoupler in a manner similar to that of 2,4-dinitrophenol (Figure 2).[25; 51; 86] According to chemiosmotic theory, such molecules easily diffuse through biological membranes in both their charged and neutral forms, resulting in the breakdown or uncoupling of ion gradients.[87] For example, (+)-usnic acid can pass through the inner mitochondrial membranes by passive diffusion into the matrix where it is ionized, releasing a proton into the matrix. The resulting usnate anion can then diffuse back into the inter-membrane space where it binds to a proton on the acidic side of the inner membrane proton gradient to re-form (+)-usnic acid, which can then diffuse back into the matrix.

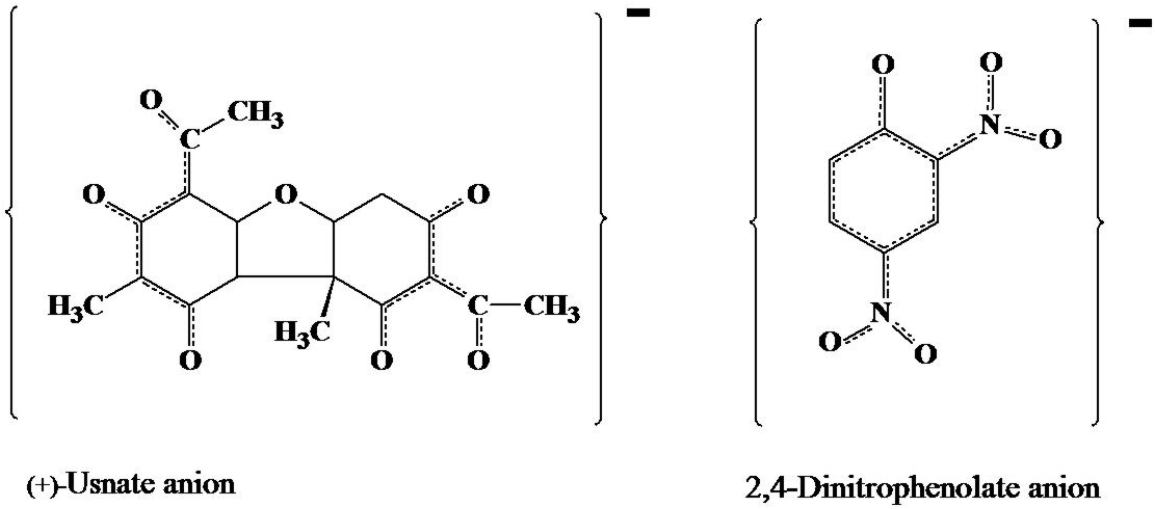

Figure 2. Structures of the Monoanionic Forms of (+)-Usnic Acid and 2,4-Dinitrophenol Showing the Resonance Stabilization of Their Negative Charges by Delocalization of Their π Orbital Electrons (Dashed Lines) as Described by Mitchell[87]

The resulting cycle (Figure 3) causes proton leakage that eventually can dissipate the proton gradient across the inner membrane, disrupting the tight coupling between electron transport and adenosine 5'-triphosphate (ATP) synthesis. This mitochondrial uncoupling activity of (+)-usnic acid has been demonstrated in vitro in several studies.[78; 88-90]

Uncoupling of oxidative phosphorylation by (+)-usnic acid was confirmed in mouse liver mitochondria by Abo-Khatwa et al.[88] Concentrations as low as 0.75 µM (+)-usnic acid decreased the phosphate/oxygen ratio dramatically, without inhibition of oxygen consumption. Stimulation of oxygen consumption by (+)-usnic acid was observed in the presence of the ATP synthase inhibitor oligomycin, confirming that usnic acid was acting to uncouple oxidative phosphorylation. Interestingly, approximately 10× the concentration of the classic uncoupler, 2,4-dinitrophenol, was required to reproduce the level of uncoupling produced by the usnic acid exposure. In observations similar to those of Johnson and Feldott,[91] Abo-Khatwa et al.[88] reported inhibition of mitochondrial oxygen consumption at (+)-usnic acid concentrations above 1 µM, again suggesting adverse effects on mitochondrial function not limited to uncoupling. They noted that usnic acid possessed physical properties like that of a "membrane disruptor," consistent with its uncoupling actions. Sahu and coworkers[57] recently reported that (+)-usnic acid was cytotoxic to cultured human hepatoblastoma, HepG2 cells, with a 24-hour LC_{50} value of 30 µM. These HepG2 cells exhibited a statistically significant reduction in mitochondrial membrane potential and increased oxidative stress when exposed to (+)-usnic acid concentrations of 20, 50, or 100 µM, but not at lower concentrations.

Pramyothin et al.[78] reported, however, that in isolated rat liver mitochondria, (+)-usnic acid concentrations as low as 0.3 µM could significantly increase ATPase activity and oxygen consumption. The same study showed that in isolated rat hepatocytes (+)-usnic acid also stimulated markers of lipid peroxidation, but that much higher concentrations were required (100 µM). These effects have been confirmed and extended by other studies,[89] which used mouse hepatocytes. Unlike classic mitochondrial membrane uncouplers such as 2,4-

dinitrophenol, usnic acid stimulates the production of reactive oxygen species while also depleting ATP levels.[89] The resulting lipid peroxidation and oxidative damage causes cytotoxicity and cell death. (+)-Usnic acid was recently shown to decrease ATP concentrations in perfused livers from fasted rats[92]; hepatic ATP/ADP ratios were reported to decrease after 90 minutes perfusion from 3.24 for control media, to 1.78 or 0.62 with perfusion media containing either 5 or 10 µM (+)-usnic acid, respectively.

In addition, (+)-usnic acid causes similar permeability effects on lysosomal membranes, reducing lysosomal acidity and disrupting autophagic processes.[93] This membrane uncoupling and disrupting action of (+)-usnic acid, which also occurs with synthetic liposomes,[32] is thought to play a major role in (+)-usnic acid-induced hepatotoxicity. However, usnic acid also produces the same uncoupling actions on bacterial cell membranes, and this forms the basis for its antimicrobial activity.

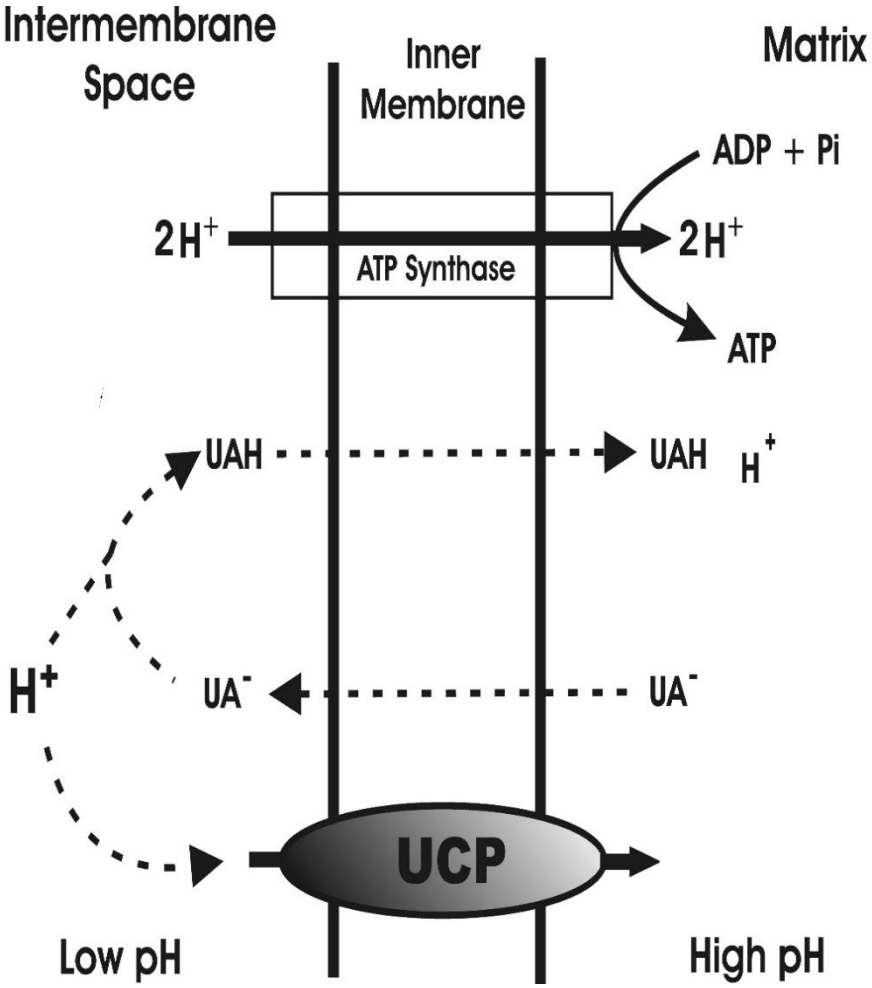

Figure 3. Mechanism of Mitochondrial Uncoupling as Originally Proposed by Mitchell[87]

Chemicals with membrane uncoupling activity, such as UA, are lipophilic and can diffuse through biological membranes in both their ionized and unionized forms; they can therefore transport protons across the inner mitochondrial membrane by passive diffusion, analogous to mitochondrial UCPs, resulting in a reduced proton gradient to drive ATP synthesis and the generation of heat.
H^+ = proton; ATP = adenosine triphosphate; ADP = adenosine diphosphate; Pi = inorganic phosphate; UAH = (+)-usnic acid; UA = usnate anion; UCP = uncoupling protein.

Humans

The idea of utilizing chemicals with mitochondrial uncoupling activity for weight loss originated in the early 1930s after it was noticed that munitions workers exposed to 2,4-dinitrophenol lost weight.[94] Subsequently, 2,4-dinitrophenol was formulated into an anti-obesity drug, which was prescribed by some physicians or directly marketed to the public with some claims of efficacy. However, many serious side effects were also recorded, including liver, heart, and muscle toxicity and cataract formation so that, in 1938, the U.S. Food and Drug Administration (FDA) finally declared 2,4-dinitrophenol too toxic for use under any circumstances.[94] Following this declaration, reports of 2,4-dinitrophenol misuse became less frequent. Interest in uncoupling chemicals has resurfaced primarily in the body-building community with the advent of the internet and the passage of the Dietary Supplement Health and Education Act of 1994, resulting in the clandestine trade of 2,4-dinitrophenol[95; 96] and the open marketing of usnic acid and other natural products in dietary supplements formulated for weight loss.[18] Such formulations generally contain relatively high usnic acid concentrations, either alone or in combination with other ingredients, and their use has been reported to be associated with hepatotoxicity.

In 2000, Favreau et al.[97] reported that seven previously healthy patients developed acute hepatitis after ingesting *LipoKinetix* (Syntrax, Cape Girardeau, MO) and recovered spontaneously after discontinuing its use. Subsequently, two more cases of acute hepatitis were reported after taking *LipoKinetix,* with one resulting in a liver transplant.[98] *LipoKinetix* was a multi-ingredient product; one capsule contained 25 mg of norephedrine hydrochloride, 100 mg of (+)-usnic acid, 100 µg of 3,5-diiodothyronine, 3 mg of yohimbine hydrochloride, and 100 mg of caffeine. It was sold as a dietary supplement to promote weight loss. The manufacturer claimed that *LipoKinetix* "affects oxidative phosphorylation in such a way that an incredible amount of fatty acids are burned," therefore promoting weight loss. The recommended dose of *LipoKinetix* was one or two capsules three times per day, which is 3–6× higher than usnic acid doses of 60–180 mg used in traditional Chinese medicine. Production and sale of *LipoKinetix* was terminated in 2001, although Syntrax continued to produce a product with similar ingredients, but without (+)-usnic acid, which was called *AdipoKinetix*.

UCP-1 (BDC Nutrition, Richmond, KY) was marketed as a weight-loss product containing 150 mg of (+)-usnic acid, 525 mg of L-carnitine, and 1,050 mg of calcium pyruvate per capsule. The recommended dose of *UCP-1* was three capsules three times per day. Sanchez et al.[99] reported the development of severe liver failure in two patients who were taking the recommended dose of *UCP-1*; one resulted in a liver transplant. Durazo et al.[100] also reported one case of a healthy woman who, after taking pure (+)-usnic acid (Industrial Strength AAA Services, Frazer Park, CA) for weight loss, presented with liver failure requiring a transplant. The recommended dose of pure (+)-usnic acid from this manufacturer was 500 mg/day.

FDA has received at least 21 adverse event reports including one death attributed to weight-loss supplements containing (+)-usnic acid (*LipoKinetix* and *UCP-1*) or pure (+)-usnic acid. Twelve cases associated with hepatotoxicity appeared in the literature and are summarized in Guo et al.[25] These cases included eight females and four males; the median age of the patients was 31 years old. Two patients required liver transplantation and the others ultimately recovered. While the total number of people who have experimented with weight-loss supplements containing (+)-usnic acid is unknown, the manufacturer of *LipoKinetix* has claimed to have sold over 30,000 bottles of the supplement.[101]

Reproductive and Developmental Toxicity

In a 35-day oral study in 5- to 6-week-old male Swiss mice, no adverse effects of 200 mg/kg/day of (+)-usnic acid on the number, motility, and structure of epididymal spermatozoa were observed. Additionally, no quantitative differences in the content of testicular protein, RNA, and DNA were recorded.[102]

Carcinogenicity

There are no reports of carcinogenic activity for either usnic acid or *Usnea* lichen preparations. The primary focus of this study of (+)-usnic acid is acute and subchronic toxicity rather than carcinogenesis.

Genetic Toxicity

Many plants contain endogenous compounds that are genotoxic.[103] Using the Ames *Salmonella*/microsome assay, Shibamoto and Wei[90] tested the mutagenicity of pure (+)-usnic acid along with two other lichen constituents: physodic (5′-carboxy-3,4′-dihydroxy-5-methyl-caproyl-6′-pentyl-6-carboxy-diphenyl ether 2′,6 lactone) and physodalic acid (3′-acetoxyl-5′-carboxy-3,4′-dihydroxy-2-formyl-5,6′-dimethyl-3′-methylacetoxy-6-carboxy-diphenyl ether-2′,6-lactone) in two *Salmonella typhimurium* strains (TA98 and TA100) with or without S9 addition. Physodalic acid exhibited a clear dose-related mutagenicity in TA100, the tester strain; the addition of S9 mix increased mutagenicity fourfold at the high dose (400 µg/plate). In contrast, (+)-usnic and physodic acids showed no mutagenicity in tested strains, including TA98 and TA100 with or without S9 addition at the highest dose of 200 µg per plate for both chemicals.

NTP studies confirmed that (+)-usnic acid was negative in Ames tests with *S. typhimurium* strains TA98 and TA100 and *E. coli* strain WP2 *uvrA* (pkM101), with and without the addition of rat liver S9.[104] Koparal and coworkers[105] evaluated both (+)-usnic acid and (−)-usnic acid genotoxicity in human lymphocytes from two healthy male donors in vitro using the cytokinesis-blocked micronucleus (CBMN) assay. The results obtained from their study suggest that even though the number of micronuclei was higher in both usnic acid enantiomers-treated human lymphocytes in comparison to those in the control, the induction was not significant statistically. The authors concluded that both (+)- and (−)-usnic acid were nongenotoxic as shown by the absence of micronucleus induction in human lymphocytes. In another study, (+)-usnic acid was also found to be nongenotoxic in cultured human lymphocytes, inducing neither micronuclei nor chromosomal aberrations at concentrations up to 200 µg/mL in the absence of S9.[106]

Oral administration of a single dose of either 100 or 200 mg/kg usnic acid caused a slight increase in micronucleated erythrocytes in the mice 24 and 48 hours after treatment, which did not reach statistical significance and returned to control levels by 72 hours.[102] In another study, Leandro and coworkers[107] treated Swiss mice with 25, 50, 100, or 200 mg/kg (+)-usnic acid via oral gavage. No significant treatment-related increases in either bone marrow micronucleated erythrocyte frequency or hepatic DNA damage, evaluated using a comet assay, were noted after 24 hours. However, (+)-usnic acid did significantly increase DNA damage to V79 cells in vitro when added to the culture medium at 60 or 12 µg/mL.[107]

Study Rationale

Informed by the adverse events described above that were first reported by Medwatch in November 2001,[18] the Center for Food Safety and Applied Nutrition (CFSAN) of the FDA issued a warning letter[108] on November 19, 2001, entitled "FDA Warns Consumers Not to Use the Dietary Supplement *LipoKinetix*," because it had been implicated in a number of serious liver injuries. After receiving additional reports of persons who developed liver injury or liver failure while using *LipoKinetix*, FDA subsequently issued a strong recommendation to the manufacturer, Syntrax Innovation Inc., to withdraw the product from the market (Letter to Distributor on Hazardous Dietary Supplement *LipoKinetix*).[109] However, botanical extracts of *Usnea* lichen species are still marketed as herbal antimicrobials.

To further understand the risk to human health of usnic acid and *Usnea* preparations, the Office of Dietary Supplement Programs of CFSAN nominated usnic acid to NTP for the evaluation of acute and subchronic toxicity in January 2005 (see Frankos[18]). Long-term carcinogenesis studies were not required due to the focus on acute toxicity seen in humans. The study documented in this report is a 3-month subchronic study of (+)-usnic acid, administered in feed to B6C3F1/Nctr mice and F344/N Nctr rats. It was conducted in conjunction with a 14-day range-finding study (Appendix J), which was used to set the exposure levels for these subchronic studies and correlate them to daily oral dose levels derived from observed feed consumption. A companion study of *Usnea* lichens containing both (+)- and (−)-usnic acid is reported on in NTP TOX 105.[110]

Materials and Methods

Study Test Facility

The study was conducted between August 2008 and July 2009 under the U.S. Food and Drug Administration (FDA) 21 Code of Federal Regulations (CFR) Part 58, Good Laboratory Practices (GLP) conditions at the National Center for Toxicological Research (NCTR), 3900 NCTR Road, Jefferson, AR. The study followed NCTR policy relevant to this time period and utilized both F344/N Nctr rats and B6C3F1/Nctr mice, which were provided by the NCTR rodent breeding facility.

Chemical Procurement and Characterization

(+)-Usnic acid [(d)-usnic acid, 2,6-diacetyl-7,9-dihydroxy-8,9b(R)-dimethyldibenzofuran-1,3(2H,9bH)-dione], CASRN 7562-61-0, lot 02503HD, was purchased from Sigma-Aldrich Co., Milwaukee, WI. Chemical purity [98% per Sigma-Aldrich Certificate of Analysis (8/10/2006)] was re-evaluated by high-performance liquid chromatography-photodiode array (HPLC-PDA), nuclear magnetic resonance spectroscopy (NMR), and high-performance liquid chromatography with tandem mass spectrometry (HPLC-MS/MS) by the NCTR Division of Biochemical Toxicology/Chemical Group (NCTR Chemistry) prior to the initiation of the study (Appendix H). (+)-Usnic acid was stable under normal temperatures and pressures.

Dose Formulation

(+)-Usnic acid was incorporated in the NIH-41 irradiated meal chow by first grinding the usnic acid with the chow using a mortar and pestle, and then blending in a mixer. For rats, concentrations of 0, 30, 60, 120, 360, and 720 ppm (+)-usnic acid in feed were prepared to provide target doses of 0, 2.5, 5, 10, 30, and 60 mg (+)-usnic acid/kg body weight/day (mg/kg/day), respectively, based on historical body weight and feed consumption data for the F344/N Nctr rat colony. For mice, concentrations of 0, 15, 30, 60, 180, and 360 ppm (+)-usnic acid in feed were prepared to provide target doses of 0, 2.5, 5, 10, 30, and 60 mg/kg/day based on historical body weight and feed consumption data for the B6C3F1/Nctr mouse colony. These doses were selected based on the results of the preliminary 2-week toxicology study (Appendix J). A sample from each preparation of each dose was analyzed for dose certification. In addition, homogeneity samples were collected from each preparation of the lowest dose level (15 ppm) and analyzed. Dosed feed was within 10% of target with a coefficient of variation (CV) $< \pm$ 10% (Appendix H). New preparations were prepared at least 14 days prior to the expiration of the preparation in use, or more frequently when required by the rate of consumption. Preparations were stored at 2°C–8°C until delivery to the animal rooms. Test article formulations in feed were determined to be stable in feed for at least 14 days at room temperature and up to 17 weeks at 2°C–8°C (Appendix H).

Animal Breeding and Dosing

Animal exposure was conducted between August 11, 2008, and December 7, 2008. The study design followed guidelines as specified in the Specifications for the Conduct of Studies to Evaluate the Toxic and Carcinogenic Potential of Chemical, Biological and Physical Agents in Laboratory Animals for the National Toxicology Program (NTP).[111] The Multigeneration

Support System (MGSS) laboratory data system (designed and maintained by Z-Tech) was used to weight rank the animals according to NTP guidelines and to collect and maintain all in-life data on the study animals.

Male and female F344/N Nctr rats and male and female B6C3F1/Nctr mice were provided by the NCTR breeding colony and delivered at 3 weeks of age weighing approximately 35–50 grams for rats and 15–25 grams for mice. A total of 140 F344/N Nctr rats (70 males and 70 females) along with 140 B6C3F1/Nctr mice (70 males and 70 females) (Table 1) were delivered in five consecutive weekly shipments of 14 animals/sex/species for a total of 28 rats and 28 mice per shipment. Animals were acclimated in their designated animal room for a minimum of 10 days from date of receipt. At 7 weeks of age, the animals were weight ranked and randomized for the experiments by weight ranking. For each experiment, a total of 10 animals/sex/species were randomized to each of the six exposure groups; an additional 10 animals/sex were unassigned and considered extra. Due to the staggered exposures, two animals/sex/species were allocated to each of the six exposure groups/study from each of the five consecutive weekly shipments (12 rats and 12 mice were allocated to the study per sex each week). The animals were randomized across the exposure groups. At allocation, each animal received a three-digit tail tattoo (last three digits of the cage number). This tail tattoo was the physical link to the animal ID that was reflected when the cage was accessed by the MGSS system. The carcass identification number (CID)—composed of experiment/cage/test—was assigned to the animal and was used to track the animal through pathological evaluation.

One unallocated animal from the first shipment of each species and one unallocated animal from the fourth shipment of each species were assigned to the study as sentinels (total two rats and two mice). One sentinel from each species was removed on week 13 of the study and the other sentinel was removed on week 17 of the study. In-life data collection for sentinel animals included body weights and observations for mortality and morbidity daily. Daily observations for all animals, including sentinels, were conducted at morning and afternoon morbidity/mortality checks. After removal, the sentinels were sent for microbiological evaluation.

Table 1. Experimental Design for the Three-month Feed Studies of (+)-Usnic Acid in F344/N Nctr Rats and B6C3F1/Nctr Mice

Target Dose[a] (Estimated mg/kg/day)	Feed Concentration for Rats[b,c] (ppm)	Feed Concentration for Mice[b,c] (ppm)	Number of Animals/Sex
0	None	None	10
2.5	30	15	10
5	60	30	10
10	120	60	10
30	360	180	10
60	720	360	10
0	Sentinel[d]	Sentinel[d]	2[e]

[a]Target dose estimate was calculated from historical body weight and feed consumption data for the animal colonies.
[b]Feed concentrations are denoted by their (+)-usnic acid content as ppm added to feed.
[c]Doses were selected based on data obtained from 14-day feed studies (Appendix J) and historical data for the animal colonies.
[d]Sentinel animals received control feed.
[e]Sentinel animals were female only.

Animal Husbandry

Animal husbandry was performed per NTP guidelines.[111] Microbiological surveillance samples were collected by the animal care staff from the animal room(s) and analyzed. The environments of the animal rooms were continually monitored. Environmental controls were set to maintain the temperature at 22°C ± 4°C, with a relative humidity of 40%–70%. A 12-hour light/dark cycle was maintained. The animal rooms received 10–15 air changes per hour.

The test animals were fed irradiated NIH-41 ground feed ad libitum and filtered tap water was provided ad libitum. NIH-41 is an irradiated form of the NIH-31 diet and is the standard diet for rodent bioassays at NCTR that use F344/N Nctr rats and B6C3F1/Nctr mice. Its use was required so that animal data could be compared with the historical database. Animal body weights were recorded twice weekly, and feed consumption was recorded weekly. Cages were changed once a week. Random water bottles from the animal rooms were analyzed for microbiological contamination at start of dosing and during weeks 13 and 17.

Cage racks were washed every 4 weeks. Both rats and mice were singly housed. Hardwood chips (Northeastern Products Corp., Warrensburg, NY) were used as cage bedding and were autoclaved prior to use on the studies to preclude contamination at levels that would interfere with the studies. Random samples for this analysis of the autoclaved bedding were analyzed to monitor microbial load.

Necropsy and Histopathology

A gross examination was performed on all animals at the completion of each individual 3-month dosing schedule and on those that died during the experiment. These examinations were conducted under the supervision of a pathologist.

On the afternoon before a scheduled terminal sacrifice, the animals were weighed and delivered to the necropsy holding area. The animals were fasted overnight but had access to water. On the necropsy day, all animals were weighed and then anesthetized with carbon dioxide. A cardiac puncture was performed to collect blood for clinical pathology analyses of the following parameters: red and white blood cells, hemoglobin content, platelets, hematocrit, mean cell hemoglobin concentration, mean cell volume, glucose, total protein, albumin, creatine kinase, phosphorus inorganic, alanine aminotransferase, alkaline phosphatase, urea nitrogen, and creatinine. The animals were then euthanized by exposure to carbon dioxide (>99% in accordance with American Veterinary Medical Association guidelines). Further details of animal maintenance are summarized in Table 2.

Complete necropsies were performed on each animal in all exposure groups in all studies. Heart, right kidney, left kidney, liver, lung, right testis, left testis, right epididymis, left epididymis, and thymus weights were taken on terminal sacrifice animals. Liver and lung weights were taken on moribund animals. No weights were taken on early death animals. All gross lesions were recorded to include number, location, size, and color, as appropriate.

Organs and tissues were fixed in 10% neutral buffered formalin and then processed to paraffin blocks or slides for histopathological examination using a read-down approach. Histological sections of <6 µm in thickness were prepared, fixed, and stained with hematoxylin and eosin. Eyes and right testes and epididymides were fixed in modified Davidson's fixative. The left

testes were frozen in liquid nitrogen and stored at −80°C for spermatid evaluation. Liver tissues for histopathologic evaluation had a specified fixation time of 48 hours due to immunohistochemistry requirements.

A reproductive toxicity assessment on all animals receiving vehicle control feed and the three highest levels of exposure was conducted according to NTP Specifications,[111] with modifications. All male mice from the vehicle control, 60, 180, and 360 ppm (+)-usnic acid groups (40 mice total) were evaluated for sperm count and sperm motility. All male rats from the vehicle control, 120, 360, and 720 ppm groups (40 rats total) were evaluated for sperm count and sperm motility. All female mice from the vehicle control, 60, 180, and 360 ppm (+)-usnic acid groups (40 mice total) were evaluated for estrous cycling activity via cytological examination of vaginal lavage samples collected daily for 16 consecutive days preceding necropsy (i.e., vaginal cytology). All female rats from the vehicle control, 120, 360, and 720 ppm groups (40 rats total) were evaluated for estrous cycling activity via cytological examination of vaginal lavage samples collected daily for 16 consecutive days preceding necropsy.

Sperm count and sperm motility were conducted on the left epididymis (cauda). Vaginal lavage was conducted in the animal room. The fixed vaginal cytology slides and frozen testis were shipped to NTP's contract laboratory for evaluation. Samples of sperm suspension from the left epididymis (cauda) were shipped to the National Institute of Environmental Health Sciences/NTP Tissue Repository.

Table 2. Experimental Design and Materials and Methods in the Three-month Feed Studies of (+)-Usnic Acid

	Three-month Studies
Study Laboratory	
U.S. FDA National Center for Toxicological Research (NCTR, Jefferson, AR)	
Strain and Species	
Rats: F344/N Nctr	
Mice: B6C3F1/Nctr	
Animal Source	
NCTR breeding colony	
Time Held before Studies	
10 days	
Average Age When Studies Began	
8 weeks	
Date of First Exposure (Staggered Loading)	
Rats: August 11, 18, 25; September 01, 08, 2008	
Mice: August 11, 18, 25; September 01, 08, 2008	
Duration of Exposure	
3 months	
Date of Last Exposure (Staggered Loading)	
Rats: November 09, 16, 23, 30; December 7, 2008	
Mice: November 09, 16, 23, 30; December 7, 2008	
Necropsy Dates (Staggered Loading)	
Rats: November 10, 17, 24; December 1, 8, 2008	
Mice: November 10, 17, 24; December 1, 8, 2008	
Average Age at Necropsy	
21 weeks	
Size of Study Groups	
Rats: 60 males and 62 females	
Mice: 60 males and 62 females	

Method of Distribution
Animals were distributed randomly into groups of approximately equal initial body weights.
Animals per Cage
Rats: 1
Mice: 2 (divided)
Method of Animal Identification
Rats: tail tattoo
Mice: tail tattoo
Diet
Irradiated rodent chow (Harlan Teklad, Madison, WI) (also designated NIH-41 IR), available ad libitum
Water
Filtered tap water (Jefferson Laboratories potable water supply, monitored monthly for bacteriological quality and quarterly for state health criteria), available ad libitum
Cages
Polycarbonate cages (Lab Products, Inc., Seaford, DE and Allentown Caging Equipment Co., Inc., Allentown, NJ), changed once weekly
Bedding
Autoclaved hardwood chip bedding (Northeastern Products Corp., Warrensburg, NY), changed twice weekly (rats) or once weekly (mice)
Cage Filters
MicroVENT cage filtration with 0.2-micron HEPA filter (Allentown Caging Equipment Co., Inc., Allentown, NJ), changed weekly
Racks
Stainless steel (Allentown Caging Equipment Co., Inc., Allentown, NJ), changed every 4 weeks
Animal Room/Chamber Environment
Temperature: 22°C ± 4°C
Relative humidity: 40%–70%
Room fluorescent light: 12 hours/day
Room air changes: 10–15/hour
Exposure Concentrations
Rats: 0, 30, 60, 120, 360, and 720 ppm (+)-usnic acid
Mice: 0, 15, 30, 60, 180, and 360 ppm (+)-usnic acid
Type and Frequency of Observation
Observed twice daily; animals weighed twice weekly; feed and water consumption measured weekly
Method of Euthanasia
Carbon dioxide (>99%)
Necropsy
Necropsies were performed on all animals. Organs weighed were heart, right kidney, left kidney, liver, lung, right testis, left testis, right epididymis, left epididymis, and thymus; organs were not weighed for dead animals.
Clinical Pathology
Blood was collected via cardiac puncture during euthanasia
Hematology: erythrocyte cell count, hematocrit, hemoglobin, mean cell volume, mean cell hemoglobin, mean cell hemoglobin concentration, leukocyte cell count, and platelet count
Clinical Chemistry: glucose, total protein, albumin, creatine kinase, phosphorus inorganic, alanine aminotransferase, alkaline phosphatase, urea nitrogen, and creatinine
Histopathology
Histopathology was performed on all animals from the control and high exposure groups with a read-down approach and all gross lesions. The following tissues were examined: adrenal cortex, bone with marrow, brain, clitoral glands, epididymis, esophagus, eyes, femur, gallbladder (mouse), gross lesions, Harderian glands, heart and aorta, intestine, large intestine (cecum, colon, rectum), small intestine (duodenum, jejunum, ileum), kidneys, liver, lungs, lymph nodes (mandibular, lumbar, and mesenteric), mammary gland, muscle (thigh), nasal cavity and nasal turbinates, ovaries, pancreas, parathyroid glands, pharynx, pituitary gland, preputial glands, prostate, salivary glands, seminal vesicle, skin, spinal cord, spleen, stomach, testis, thymus, thyroid gland, trachea, urinary bladder, and uterus, vagina, and Zymbal's gland.

Sperm Motility and Vaginal Cytology
The left testis, left cauda, and left epididymis from males in the three highest exposed groups and the control group (rats: 0, 120, 360, and 720 ppm; mice: 0, 60, 180, and 360 ppm) were evaluated for percent motile sperm, number of sperm/mg cauda, total number of sperm/cauda, number of homogenization-resistant spermatids/mg testis, and total number of spermatids/testis. Vaginal lavage samples from females in the three highest exposed groups and the control group (rats: 0, 120, 360, and 720 ppm; mice: 0, 60, 180, and 360 ppm) were collected for 16 consecutive days prior to the end of the studies for estrous cycle evaluation.

Statistical Methods

Statistical analysis on body weights and mean daily feed consumption (calculated from the weekly [rats] or twice weekly [mice] feeder weights, for each week of the 13 weeks of dosing) were performed by the NCTR Statistics Group. Water consumption (calculated from the individual water bottle weights), for each week of the 13 weeks of dosing, was evaluated for rats but not for mice when excessive spillage occurred. Statistical analyses of organ weights, clinical chemistry, hematology, and survival were also performed by the NCTR Statistics Group. Within-group correlations were modeled using a heterogeneous first-order autoregressive [ARH(1)] correlation structure, which allows for correlated differences in variability across time points. Under the assumption of normally distributed data, trend tests used linear regression, and comparisons of exposed groups to control were performed with Dunnett's method for adjusted contrasts.[112] The probabilities of survival were estimated by the product-limit procedure of Kaplan and Meier.[113] Animals found dead of other than natural causes were censored from the survival analyses.[114-117]

Analysis of continuous variables for clinical chemistry and mutagenicity data were conducted using a linear regression trend test, with Dunnett's test[112] used to compare the exposed group means to the vehicle control means. The exact Cochran-Armitage trend test was used to test for a trend in nonneoplastic incidence with exposure.[118; 119] Fisher's exact test was used to compare incidences between exposed groups and the control group.[120] Tests for trend and comparisons of exposed groups to control were performed as one-sided tests.[121]

Sperm counts and estrous cycle lengths were analyzed using the nonparametric multiple comparison methods of Shirley (as modified by Williams) and Dunn. Necropsy body weights and organ weights that were evaluated as part of the reproductive tissue analysis were analyzed using the parametric multiple comparison methods of Williams and Dunnett. Jonckheere's test[122] was used to assess the significance of the exposure-related trends and to determine, at the 0.01 level of significance, whether a trend-sensitive test (Shirley's or Williams' test) was more appropriate for pairwise comparisons than a test that does not assume a monotonic exposure-related trend (Dunn's or Dunnett's test).[123] Estrous cyclicity data were also analyzed using a Markov transition matrix approach[124] in which exposure effects were investigated by testing for increased probabilities for deviations in cycling relative to the vehicle control group using the Chi-square Test.

Quality Assurance Methods

These 3-month studies were conducted in compliance with FDA GLP Regulations (21 CFR, Part 58). In addition, records from these studies, including protocol and any amendments, deviations, or related information; study-related standard operating procedures and documentation; test article accountability and characterization; raw data generated in operational areas as defined in

applicable standards of practice; computer records containing in-life and pathology raw data; daily animal room logs; and the NCTR final report are maintained in the NCTR Archives.

Results

Rats

Survival

Disposition data and survival probabilities for the male and female F344/N Nctr rats are shown in Table 3. All rats in all exposure groups survived to terminal sacrifice. No further statistical analysis was performed due to all animals surviving until the end of the study.

Body and Organ Weight Analysis

Body weight curves are shown in Figure 4. The effect of (+)-usnic acid exposure on body weight was of interest because human exposure is primarily due to its use as a weight-loss agent. There were statistically significant differences in mean body weights between the high exposed group (720 ppm) and the control group for both females and males. Mean body weights for females and males were consistently lower (4.2%–14.5% and 3.0%–7.9%, respectively) for the 720 ppm group than for the control group throughout the study. Mean body weights for females in the 360 ppm group were lower (6.4% at week 13) than in the control group. Mean body weights for males in the 60 ppm group were lower (4.7% at week 13) than in the control group (see Appendix D for details).

Absolute and relative organ weights are reported in Appendix E. Mean absolute and relative liver weights for females and males were higher for the 720 ppm group than for controls. Relative liver weight was higher at 360 ppm for females. Mean weights for male left and right (relative only) testis and left epididymis in the 720 ppm group were higher compared to the control group.

The body weight data and feed consumption data (Appendix D and Appendix F) were used to estimate the actual dose of (+)-usnic acid in each exposure group on days 28, 46, and 88 of the study. The data are shown in Table F-5 and summarized in Table 3. The observed dosage was similar to the target dose of (+)-usnic acid for all exposure groups except the females in the 720 ppm group in which the observed dose was 28% greater than the target dose.

Table 3. Survival, Disposition, and Body Weights of Rats in the Three-month Feed Study of (+)-Usnic Acid

Parameter[a]	0 ppm	30 ppm (2.5)[b]	60 ppm (5)	120 ppm (10)	360 ppm (30)	720 ppm (60)
Male						
Rats Initially in Study	10	10	10	10	10	10
Natural Deaths	0	0	0	0	0	0
Moribund	0	0	0	0	0	0
Rats Surviving to Study Termination	10	10	10	10	10	10
Probability of Survival to End of Study	100%	100%	100%	100%	100%	100%
Mean Survival (Days)[c]	94	94	94	94	94	94
Initial Body Weight (g)[d]	194.4 ± 5.1	196.2 ± 3.9	195.4 ± 5.1	194.5 ± 3.8	197.1 ± 5.5	195.3 ± 4.3

Final Body Weight (g)[d]	359.4 ± 5.5***	357.1 ± 5.1	342.6 ± 6.1*	353.0 ± 4.2	350.5 ± 6.0	334.2 ± 3.5***
Change in Body Weight (g)	165.0	160.9	147.2	158.5	153.4	138.9
Final Weight as % of Controls	–[e]	99.4	95.3	98.2	97.5	93.0
Observed Dose (mg/kg/day)[f]	–	2.26 ± 0.08	4.45 ± 0.13	9.09 ± 0.31	27.78 ± 0.80	59.93 ± 1.80
Female						
Rats Initially in Study	10	10	10	10	10	10
Natural Deaths	0	0	0	0	0	0
Moribund	0	0	0	0	0	0
Rats Surviving to Study Termination	10	10	10	10	10	10
Probability of Survival to End of Study	100%	100%	100%	100%	100%	100%
Mean Survival (Days)	94	94	94	94	94	94
Initial Body Weight (g)	140.4 ± 1.8	141.5 ± 1.6	144.5 ± 2.0	139.3 ± 2.0	139.6 ± 2.2	141.5 ± 2.4
Final Body Weight (g)	209.1 ± 4.4***	212.6 ± 3.2	210.2 ± 3.6	205.5 ± 4.0	195.8 ± 3.2**	178.7 ± 2.1***
Change in Body Weight (g)	68.7	71.1	65.7	66.2	56.2	37.2
Final Weight % of Controls	–	101.7	100.5	98.3	93.6	85.5
Observed Dose (mg/kg/day)	–	2.91 ± 0.07	5.51 ± 0.14	11.64 ± 0.28	35.64 ± 0.56	76.98 ± 0.96

[a]Complete details of the dosing schedule are given in the methods section.
[b]Denotes target dose as mg (+)-usnic acid per kg/day, calculated from historical body weight and feed consumption data.
[c]Animals were assigned to the study for 94 days but were exposed to dosed feed for 90 days.
[d]Body weight (g) as mean ± standard error. Asterisks denote significant trend (control column) or significant pairwise comparison to control group (Dunnett's test, other columns): $p \leq 0.05$ (*); $p \leq 0.01$ (**); $p \leq 0.001$ (***).
[e]Not applicable.
[f]Observed values calculated from the observed weekly mean feed consumption and observed weekly mean body weights for surviving rats in each exposed group. Observed feed consumption values do not correct for spillage. Data presented as mean ± standard error for the 13 weekly values.

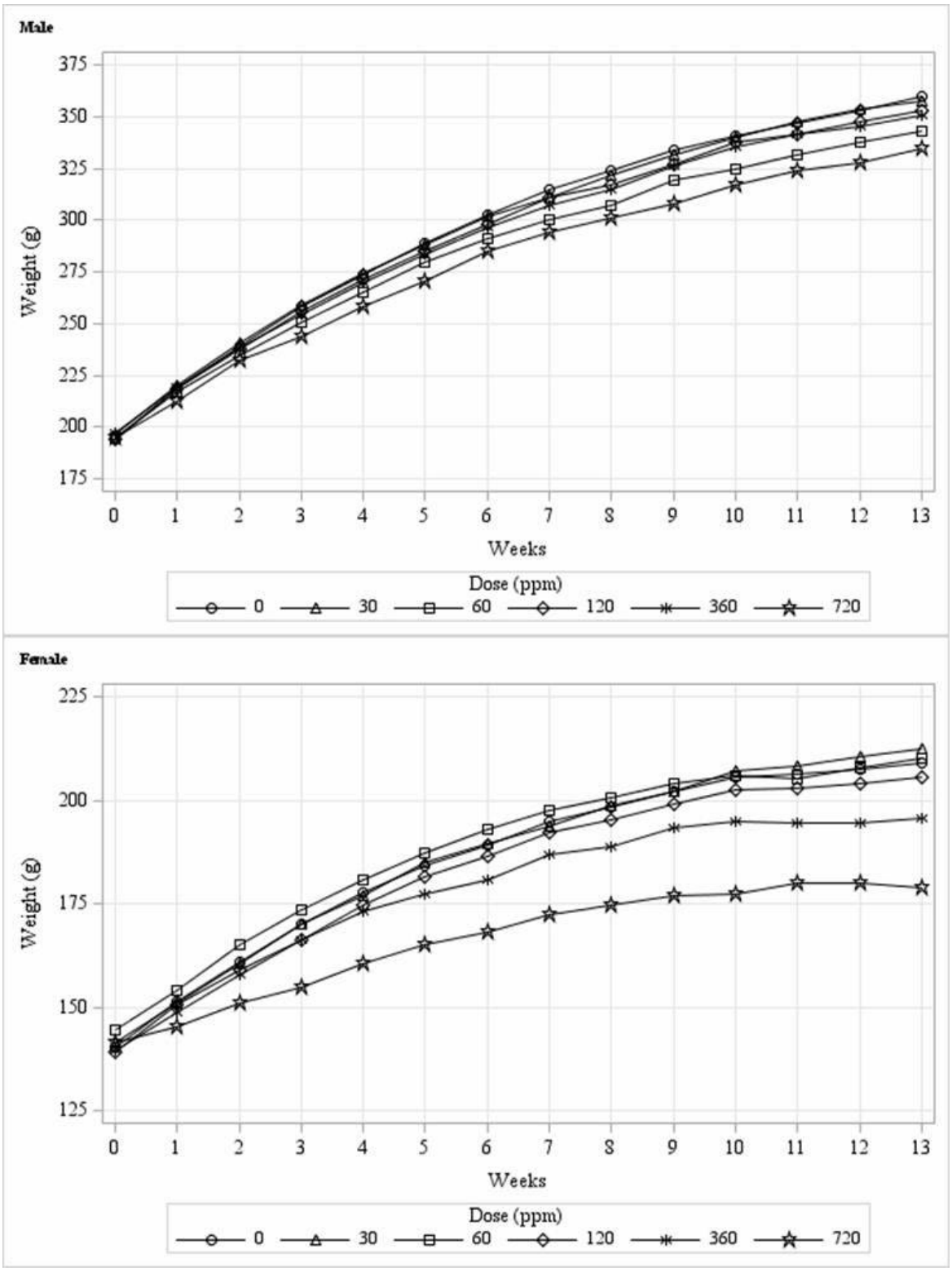

Figure 4. Growth Curves for Male and Female Rats Exposed to (+)-Usnic Acid in Feed for Three Months

Plotted as mean body weights of each exposure group.

Pathology and Statistical Analyses

The only gross observation in rats that may have had an exposure relationship was renal pelvis dilatation (hydronephrosis) present in males. The remainder of the gross observations were considered to be common background changes.

While no exposure-related histopathological changes were noted in females, the incidence of renal nephropathy was significantly increased (10/10, average score 1.2 versus 2/10, average score 1.0 for the control group $p \leq 0.001$; see Appendix A) in the 720 ppm group relative to the control group. Renal nephropathy is a common lesion in F344/N Nctr rats and was observed in 10/10 of the male controls. Serum alanine aminotransferase (ALT), blood urea nitrogen (BUN), and creatinine concentrations were moderately increased in the 720 ppm groups relative to the control group in female rats, and creatinine was moderately increased in the 720 ppm group in males (Appendix C).

The histopathological changes observed in male rats that were considered exposure-related are summarized in Table 4 and Figure 5, Figure 6, Figure 7, and Figure 8. Hepatocellular degeneration (Figure 8.) was observed in male rats from the 60 and 120 ppm exposure groups and had increased incidence and severity in the 360 and 720 ppm exposure groups relative to the control group. The affected animals displayed one or more of the following changes: cell swelling as well as cell contraction, cytoplasmic vacuolization or clearing, clumping (increased densities) of organelles, and in many animals an increased cytoplasmic eosinophilia. These lesions were primarily noted in the centrilobular zone with midzonal involvement in many of the same animals (Figure 9). Nuclear chromatin clumping with early karyorrhexis was occasionally observed and less frequently noted were single necrotic cells characterized by their dark appearance and by being dislodged from their normal position. Vacuolar degeneration was the most prominent change and was characteristic of either water accumulation with distortion of endoplasmic reticulum following cellular membrane damage, markedly dilated mitochondria due to a primary injury to mitochondria, or lipidosis, a result of an overload of metabolic pathways.

Hepatic inflammation was significantly increased in the males exposed to 360 and 720 ppm relative to the control group (Table 4). Also, hydronephrosis (dilatation of the renal pelvis and loss of medullary tissue) had a higher incidence only in males exposed to 720 ppm. These tissues were examined microscopically in progressively lower exposure levels until a no-observed-adverse-effect level (NOAEL) was reached at 120 ppm.

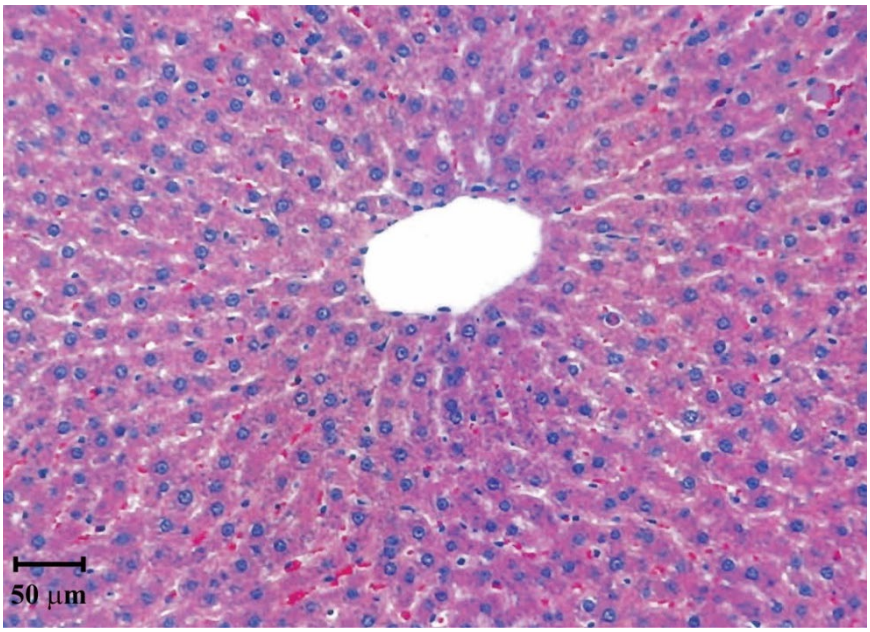

Figure 5. Section of the Liver from 0 ppm F344/N Nctr Rats from the Three-month Feed Study of (+)-Usnic Acid (H&E)

H&E = hematoxylin and eosin stain.

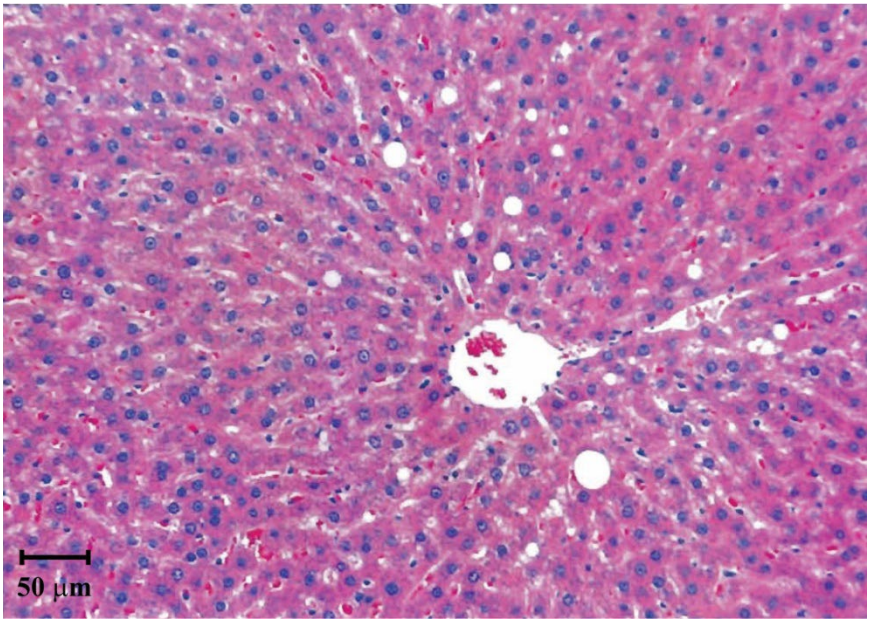

Figure 6. Section of the Liver from 120 ppm F344/N Nctr Rats from the Three-month Feed Study of (+)-Usnic Acid (H&E)

H&E = hematoxylin and eosin stain.

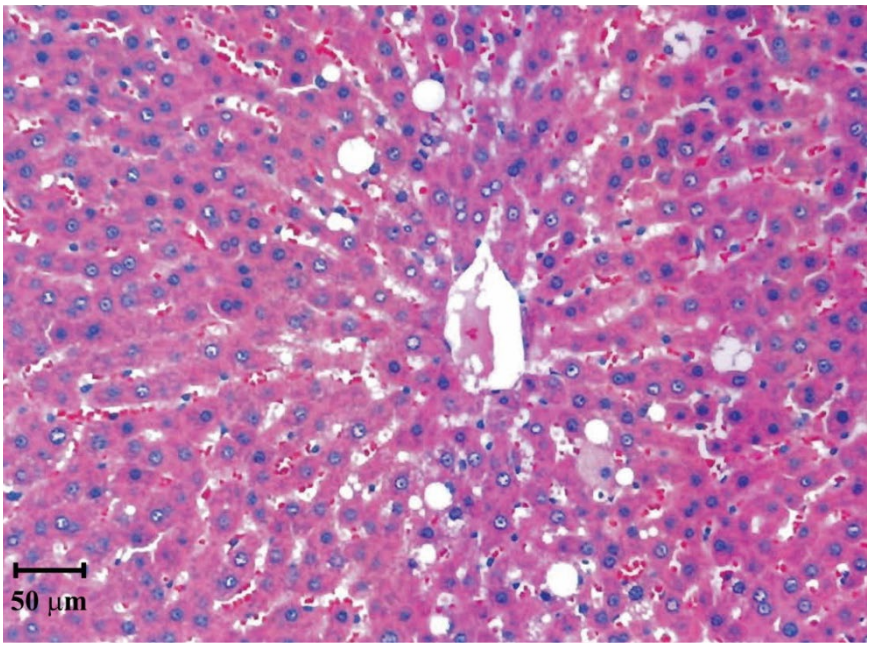

Figure 7. Section of the Liver from 360 ppm F344/N Nctr Rats from the Three-month Feed Study of (+)-Usnic Acid (H&E)

H&E = hematoxylin and eosin stain.

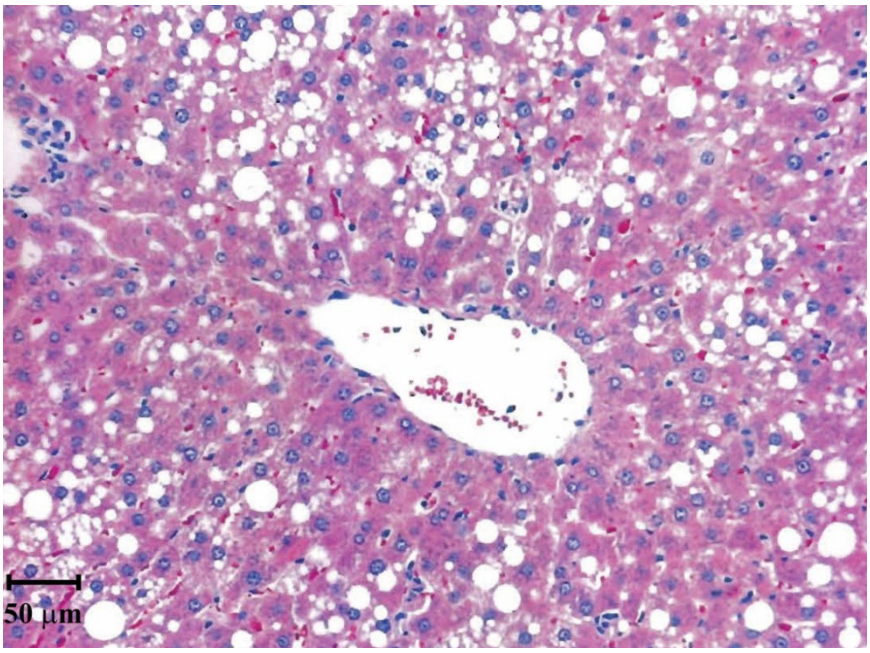

Figure 8. Section of the Liver from 720 ppm F344/N Nctr Rats from the Three-month Feed Study of (+)-Usnic Acid (H&E)

H&E = hematoxylin and eosin stain.

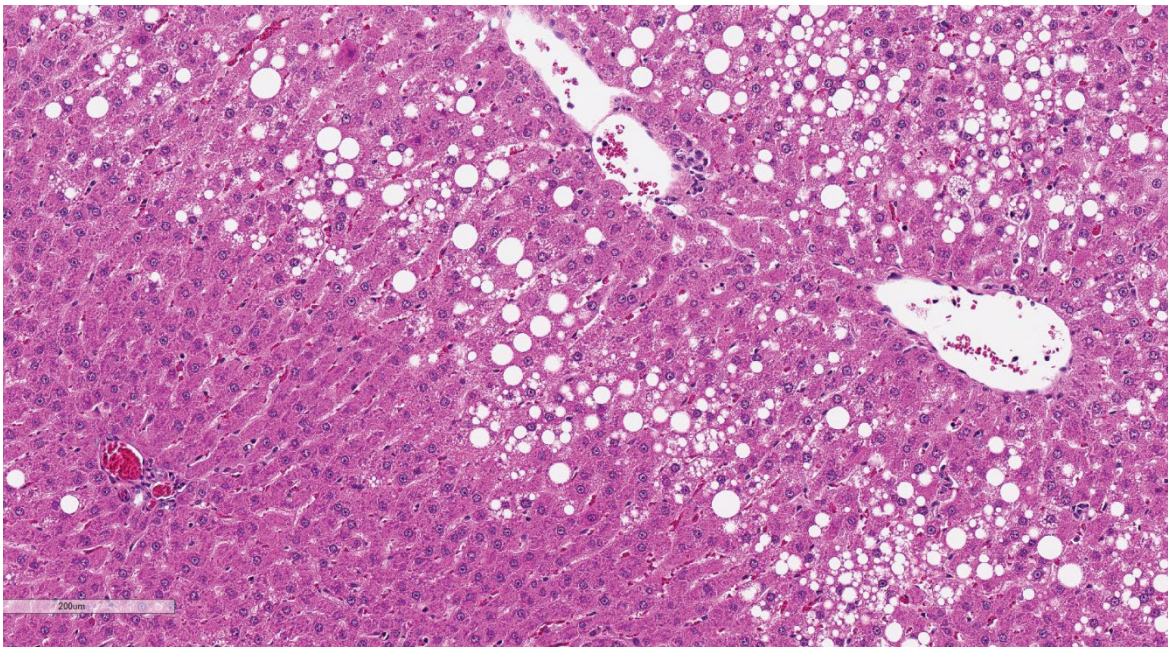

Figure 9. Section of the Liver from a 720 ppm F344/N Nctr Rat from the Three-month Feed Study of (+)-Usnic Acid (H&E)

Extended view to show centrilobular location of degeneration (upper right) with minimal damage to the periportal region (lower left).
H&E = hematoxylin and eosin stain.

There was a test article-related statistically significant decrease in the proportion of females cycling at 720 ppm (7/10 versus 10/10 in other groups, including control groups) that was due to extended diestrus (Table G-2). Five females in the 720 ppm group, including four females that were not cycling, displayed extended diestrus (5 or more consecutive days in diestrus) compared to none in the other groups, including controls, which translated to a significant increase in the percentage of days in diestrus at 720 ppm compared to controls (69.4% versus 57.5%). As a result of the extended diestrus, the percentage of days in proestrus was decreased at 720 ppm compared to the control female rats although it did not reach statistical significance (means of 13.1% and 19.4%, respectively). The percentage of days in estrus was similarly decreased without statistical significance at 720 ppm compared to control female rats (means of 13.8% and 19.4%, respectively). The percentage of days in metestrus was similar across groups. The mean cycle length in cycling females was significantly increased in the 720 ppm group compared to the control group.

In the male rats, exposure to 720 ppm (+)-usnic acid resulted in significantly increased absolute and/or relative weights of the testes and epididymides, but sperm parameters were not significantly altered (see Table E-1 and Table G-1).

Table 4. Statistical Analysis of Select Nonneoplastic Lesions in Male Rats in the Three-month Feed Study of (+)-Usnic Acid[a]

	0 ppm	30 ppm (2.5)[b]	60 ppm (5)	120 ppm (10)	360 ppm (30)	720 ppm (60)
Liver						
Hepatocellular Degeneration						
Overall rate[c]	1/10 (10.0%)	0/10 (0.0%)	3/10 (30.0%)	4/10 (40.0%)	10/10 (100.0%)	10/10 (100.0%)
Terminal rate[d]	1/10 (10.0%)	0/10 (0.0%)	3/10 (30.0%)	4/10 (40.0%)	10/10 (100.0%)	10/10 (100.0%)
CAFÉ p value[e]	**p ≤ 0.001**	p = 0.500N	p = 0.291	p = 0.152	**p ≤ 0.001**	**p ≤ 0.001**
Average severity[f]	1.0	–[g]	1.0	1.3	1.9	3.6
Inflammation						
Overall rate	0/10 (0.0%)	1/10 (10.0%)	3/10 (30.0%)	3/10 (30.0%)	8/10 (80.0%)	10/10 (100.0%)
Terminal rate	0/10 (0.0%)	1/10 (10.0%)	3/10 (30.0%)	3/10 (30.0%)	8/10 (80.0%)	10/10 (100.0%)
CAFÉ p value	**p ≤ 0.001**	p = 0.500	p = 0.105	p = 0.105	**p ≤ 0.001**	**p ≤ 0.001**
Average severity	–	1.0	1.0	1.0	1.3	1.3
Kidney						
Hydronephrosis						
Overall rate	0/10 (0%)	–	–	0/10 (0.0%)	2/10 (20%)	10/10 (100%)
Terminal rate	0/10 (0%)	–	–	0/10 (0.0%)	2/10 (20%)	10/10 (100%)
CAFÉ p value	**p = 0.001**	–	–	–	p = 0.237	**p = 0.016**
Average severity	–	–	–	–	1.5	1.4

[a]Complete details of the dosing schedule are given in the methods section.
[b]Denotes target dose as mg (+)-usnic acid per kg/day, calculated from historical body weight and feed consumption data.
[c]Number of nonneoplastic lesion-bearing animals over number of animals examined.
[d]Observed incidence at terminal sacrifice.
[e]The exact Cochran-Armitage trend test was used to test for a trend in nonneoplastic incidence with exposure. Fisher's exact test was used to compare incidences between exposed groups and the control group. Tests for trend and comparisons of exposed groups to control were performed as one-sided tests. A negative trend or a lower incidence in an exposure group is indicated by N. Significant p values are bolded.
[f]Severity was scored as: 1 = minimal, 2 = mild, 3 = moderate, and 4 = marked.
[g]Indicates no data were collected.

Mice

Survival

Disposition data and survival probabilities for the male and female B6C3F1/Nctr mice are shown in Table 5. Except for one female mouse, which died prematurely due to a cage incident, all mice in all exposure groups survived to terminal sacrifice. No further statistical analysis was performed because there were no premature removals due to exposure.

Body and Organ Weight Analysis

Summary statistics of body weight by week are given in Appendix D and body weight growth curves are shown in Figure 10. The effect of (+)-usnic acid exposure on body weight was of interest because human exposure is primarily due to its use as a weight-loss agent. For males, there was a significant exposure effect ($p = 0.001$). For both females and males, there were significant effects for study week ($p \leq 0.001$) and covariate baseline body weight ($p \leq 0.001$). Weekly pairwise comparisons of each exposure group to the control group are also presented in detail in Appendix D. For female B6C3F1/Nctr mice, there was a negative overall dose trend and a negative dose trend at weeks 5 through 8. There was a significant overall difference from the control group for the 360 ppm group with the treated group showing lower body weights (97.8%, $p = 0.044$) compared to the control group. The 360 ppm group also had a lower mean weight compared to controls at week 6 ($p \leq 0.05$). The 30 ppm group had a lower mean weight compared to controls at weeks 5 and 6 ($p \leq 0.05$). For male B6C3F1/Nctr mice, there was a negative overall dose trend and a negative dose trend at weeks 3 through 9 and at week 11.

A full statistical analysis of the effects of 3-month exposure of B6C3F1/Nctr mice exposed to (+)-usnic acid on organ weights is presented in Appendix E. Relative weights, calculated as organ weight (mg) to receiving weight (g), were also evaluated. In females, there were significant positive overall trends for absolute and relative liver weights ($p \leq 0.001$), and there was a significant difference between the control group and the 360 ppm exposure group with the treated group showing higher mean liver weights (21.0%, $p \leq 0.01$ and 19.3% greater, $p \leq 0.001$ for absolute and relative, respectively) compared to the control group. In males, there was a significant positive overall trend for absolute and relative liver weights ($p \leq 0.05$ and $p \leq 0.001$, respectively) and compared to the control group, there were significant differences for the 180 ppm and 360 ppm exposure groups in relative liver weights with the treated groups showing higher mean weights (9.8% greater, $p \leq 0.05$ and 17.7% greater, $p \leq 0.001$, respectively).

Table 5. Survival, Disposition, and Body Weights of Mice in the Three-month Feed Study of (+)-Usnic Acid

Parameter[a]	0 ppm	15 ppm (2.5)[b]	30 ppm (5)	60 ppm (10)	180 ppm (30)	360 ppm (60)
Male						
Mice Initially in Study	10	10	10	10	10	10
Natural Deaths	0	0	0	0	0	0
Moribund	0	0	0	0	0	0
Mice Surviving to Study Termination	10	10	10	10	10	10
Probability of Survival to End of Study	100%	100%	100%	100%	100%	100%
Mean Survival (Days)[c]	94	94	94	94	94	94
Initial Body Weight (g)[d]	22.4 ± 0.5	22.0 ± 0.6	22.1 ± 0.7	22.2 ± 0.6	22.2 ± 0.5	21.5 ± 0.4
Final Body Weight (g)[d]	30.7 ± 0.7	30.9 ± 0.5	30.6 ± 0.5	31.0 ± 0.8	29.8 ± 0.7	29.5 ± 0.4
Change in Body Weight (g)	8.3	8.9	8.5	8.8	7.6	8.0
Final Weight as % of Controls	–[e]	100.7	99.7	101.0	97.1	96.1
Observed Dose (mg/kg/day)[f]	–	4.04 ± 0.15	8.25 ± 0.46	15.58 ± 0.36	46.82 ± 0.96	98.88 ± 3.19
Female						
Mice Initially in Study	10	10	10	10	10	10
Accidental Deaths	0	0	1	0	0	0
Natural Deaths	0	0	0	0	0	0
Moribund	0	0	0	0	0	0
Mice Surviving to Study Termination	10	10	9	10	10	10
Probability of Survival to End of Study	100%	100%	90%	100%	100%	100%
Mean Survival (Days)	94	94	86.4	94	94	94
Initial Body Weight (g)	17.8 ± 0.2	18.1 ± 0.3	18.3 ± 0.3	17.3 ± 0.3	18.0 ± 0.2	18.5 ± 0.3
Final Body Weight (g)	24.6 ± 0.4	24.3 ± 0.4	24.1 ± 0.6	23.8 ± 0.4	24.6 ± 0.3	24.4 ± 0.4
Change in Body Weight (g)	6.8	6.2	5.8	6.5	6.6	5.9
Final Weight as % of Controls	–	98.8	98.0	96.7	100.0	99.2
Observed Dose (mg/kg/day)	–	5.38 ± 0.20	9.84 ± 0.28	20.92 ± 0.80	58.04 ± 1.59	124.37 ± 2.04

[a]Complete details of the dosing schedule are given in the methods section.
[b]Denotes target dose as mg (+)-usnic acid per kg/day, calculated from historical body weight and feed consumption data.
[c]Animals were assigned to the study for 94 days but were exposed to dosed feed for 90 days.
[d]Body weight (g) as mean ± standard error.
[e]Not applicable.
[f]Observed values calculated from the observed weekly mean feed consumption and observed weekly mean body weights for surviving mice in each exposure group. Observed feed consumption values do not correct for spillage. Data presented as mean ± standard error for the 13 weekly values.

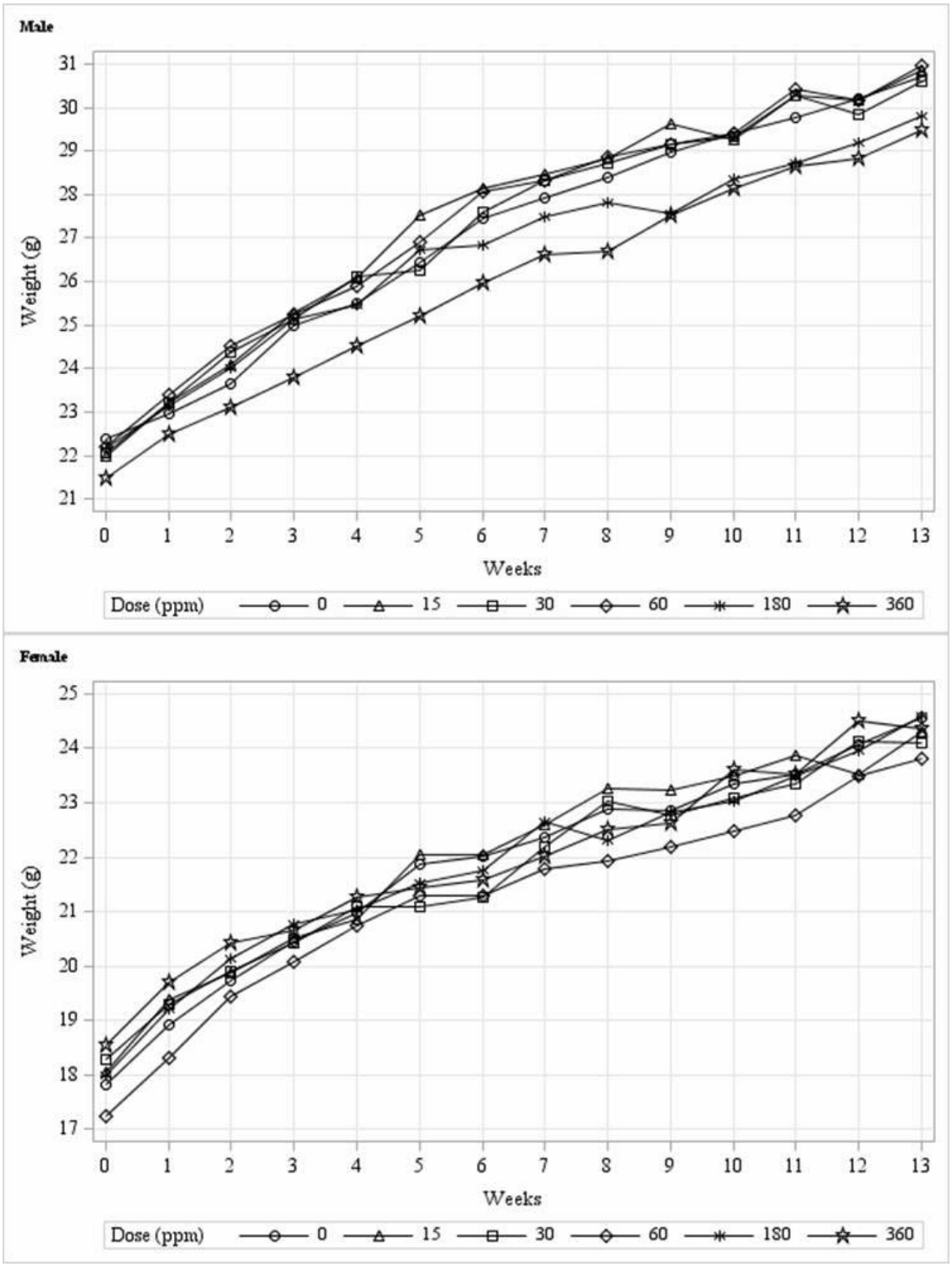

Figure 10. Growth Curves for Male and Female Mice Exposed to (+)-Usnic Acid in Feed for Three Months

Plotted as mean body weights of each exposure group.

The body weight data and feed consumption data (Appendix D and Appendix F) were used to estimate the actual dose of (+)-usnic acid in each exposure group on days 28, 46, and 88 of the study. The data are shown in Table F-6 and summarized in Table 5. In contrast to the rats, the mice appeared to consume more feed than expected so that the observed (+)-usnic acid exposure was considerably greater than the target dose for all exposure groups. However, the observed feed consumption, which varied considerably between individual mice, included feed lost to spillage so that the actual feed consumption, and hence ingested dose, would be expected to be less. Because of this, exposure levels for these studies are based on the ppm concentration of (+)-usnic acid in feed and target mg/kg/day values are provided as an approximate comparison to human exposure levels.

Pathology and Statistical Analyses

There were no exposure-related histopathological findings noted in either male or female B6C3F1/Nctr mice. All lesions that were coded were considered spontaneous background changes (Appendix A). However, as shown in Appendix C, serum creatinine, alanine aminotransferase, alkaline phosphatase, and BUN were moderately elevated in the 180 and/or 360 ppm exposure groups from male, but not female, B6C3F1/Nctr mice. There were no significant differences in reproductive toxicity parameters observed in either male or female mice (see Appendix G).

Genetic Toxicology

Exposure to (+)-usnic acid for 14 days significantly increased the incidence of micronuclei in erythrocytes or reticulocytes from both male and female B6C3F1/Nctr mice (Appendix B).

Discussion

The selection of exposure levels of (+)-usnic acid for F344/N Nctr rats and B6C3F1/Nctr mice was based on a 2-week range-finding study conducted prior to the 3-month study (Appendix J) in which feed concentrations of 1,250 and 2,500 ppm in rats and 1,200 ppm in mice caused rapid weight loss and some morbidity. The selected exposure levels corresponded to target doses of 5, 10, 30, 100, and 200 mg/kg body weight/day (mg/kg/day) for both rats and mice, calculated from historical feed consumption and body weight data from the relevant strains. Human exposure to formulations associated with hepatotoxicity was approximately 1–12 mg/kg/day.[18] The observed daily doses calculated from the study data correlated closely for the rats but were higher than the target dose in mice (Appendix F).

(+)-Usnic acid was relatively nontoxic to female F344/N Nctr rats and male and female B6C3F1/Nctr mice at the doses used in this 3-month feed study. In contrast, exposure of male F344/N Nctr rats to (+)-usnic acid concentrations of 360 and 720 ppm in feed resulted in increased incidence and severity of hepatocellular degeneration and hepatic inflammation. Males from the 720 ppm exposure group also had higher incidence of hydronephrosis. Although serum alanine aminotransferase levels were moderately increased (e.g., by 17–41 U/l, Table C-1) in the highest exposure groups of both male mice and female rats relative to controls, the increases were much less than those generally observed in rats treated with hepatotoxic doses of acetaminophen or carbon tetrachloride (e.g., 390–460 U/l, Sun et al.[125]) or those observed in patients with (+)-usnic acid-associated severe hepatotoxicity (e.g., 1,000–14,000 U/l[97]). These observations suggest that (+)-usnic acid-induced hepatotoxicity in this study did not produce severe necrosis. Exposure to higher concentrations of (+)-usnic acid equivalent to 100 or 200 mg/kg/day for 2-weeks (Appendix J) was significantly more toxic, producing clear hepatocellular degeneration in both male and female (200 mg/kg/day only) mice and in male and female rats. Greater toxicity was observed in rats for the 2-week study, with some mortality occurring in the first week of exposure.

Hepatotoxicity has occurred in humans when (+)-usnic acid is used as a weight-loss supplement and "fat burner." In this study rats exposed to 720 ppm had significantly lower body weights compared to the control group, but not in mice exposed to 360 ppm. In the 2-week range-finding study significant weight loss was observed at exposure levels of 1,250 and 2,500 ppm in rats and 1,200 ppm in mice.

(+)-Usnic acid is known to be an uncoupler of mitochondrial oxidative phosphorylation[25] and this uncoupling activity is thought to be the basis of (+)-usnic acid-induced hepatotoxicity due to its resultant depletion of intracellular ATP concentrations.[25; 88; 89] Significant ATP depletion was observed in livers of male rats exposed for 2 weeks to (+)-usnic acid at exposure concentrations of 120, 360, 1,250, and 2,500 ppm (Table J-5), whereas liver damage was noted in only the 1,250 and 2,500 ppm exposure groups (Table J-3). While this supports the hypothesis that mitochondrial uncoupling provides a mechanistic basis for (+)-usnic acid-induced hepatotoxicity, it implies that severe prolonged depletion of ATP is required for hepatotoxicity to develop.

When isolated mouse or rat hepatocytes were exposed to (+)-usnic acid concentrations in the range of 1–10 µM, intracellular ATP concentrations are rapidly depleted to <5% of initial values and the cells die.[25; 89] In contrast, when female F344/N Nctr rats or male B6C3F1/Nctr mice were

exposed to 360 or 180 ppm (+)-usnic acid, respectively, for 14 days, which did not result in overt hepatotoxicity, hepatic (+)-usnic acid levels increased to steady-state concentrations of 75–80 or 38–58 µM, respectively (Appendix K). In female F344/N Nctr rats fed control feed for 14 days, hepatic ATP concentrations were measured as 1.09 ± 0.13 µmol/g, whereas hepatic ATP levels in rats exposed to 360 ppm (+)-usnic acid were measured as 0.60 ± 0.14 µmol/g (55% of control, p = 0.16, Table J-5). In male B6C3F1/Nctr mice fed control feed for 14 days, hepatic ATP concentrations were measured as 1.64 ± 0.21 µmol/g, whereas hepatic ATP levels in male mice exposed to 180 ppm (+)-usnic acid were measured as 0.85 ± 0.22 µmol/g (52% of control, p = 0.016, Table J-6). This suggests that hepatocytes in vitro are considerably more susceptible to (+)-usnic acid-induced ATP depletion than hepatocytes in vivo and that rats are more susceptible than mice. In both rats and mice, hepatic (+)-usnic acid had accumulated after 14 days exposure in feed to levels that were more than fourfold greater than concentrations that are lethal to cultured hepatocytes, but hepatic ATP concentrations had decreased by <50%. Interestingly, hepatic tyrosine aminotransferase activity was shown to be induced by (+)-usnic acid exposure in the male rats used in the 14-day study (Appendix J). Tyrosine aminotransferase is a rate-limiting enzyme that facilitates catabolism of tyrosine.[126] Mammalian mitochondria generally express membrane uncoupling proteins, which generate heat at the expense of ATP synthesis.[127; 128] Mice express higher levels of uncoupling protein *UCP-1* than rats because they require a greater capacity for heat generation due to their smaller body size. *UCP-1* is upregulated by leptin and downregulated by corticosterone in rodents.[127] It is probable that rodents compensate for the uncoupling action of (+)-usnic acid by downregulating *UCP-1* expression. Serum leptin concentrations were found to be depleted to <10% of control values in female F344/N Nctr rats exposed to 1,250 ppm (+)-usnic acid for 14 days (Appendix J), which may be a mechanism through which the animals reduce *UCP-1* expression. Because mice express higher *UCP-1* concentrations than rats, it is probable that they have a greater capacity to compensate for (+)-usnic acid toxicity.

Exposure of F344/N Nctr rats and B6C3F1/Nctr mice to (+)-usnic acid in feed for 3 months resulted in significant hepatotoxicity in male rats at exposure levels of 360 and 720 ppm. Moderate increases in serum creatinine, blood urea nitrogen (BUN), and alanine aminotransferase activity were observed in female rats at exposure levels of 720 ppm. In male mice, moderate but significant increases in alanine aminotransferase and alkaline phosphatase were observed at exposure levels of 360 ppm, moderate significant increases in BUN were observed at exposure levels of 180 and 360 ppm, whereas moderate significant increases in serum creatinine were observed at exposure levels of 60, 180, and 360 ppm. There were significantly fewer female rats cycling in the 720 ppm group than in the control group, due to extended diestrus. Significant body weight decreases were achieved at exposure levels of 720 ppm in male and female rats. Exposure to 600 ppm (+)-usnic acid for 14 days significantly increased the incidence of micronuclei in erythrocytes or reticulocytes from both male and female B6C3F1/Nctr mice; exposure to 1,200 ppm significantly increased the incidence of micronuclei in reticulocytes in male B6C3F1/Nctr mice. No-observed-adverse-effect levels (NOAELs) of 120 ppm and 30 ppm of (+)-usnic acid administered in feed were established for F344/N Nctr rats and B6C3F1/Nctr mice, respectively, on the basis of the results of these subchronic studies.

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Appendix A. Summary of Nonneoplastic Lesions in Rats and Mice

Tables

Table A-1. Summary of the Incidence of Nonneoplastic Lesions in Male Rats in the Three-month Feed Study of (+)-Usnic Acid

	0 ppm	30 ppm	60 ppm	120 ppm	360 ppm	720 ppm
Disposition Summary						
Animals Initially in Study	10	10	10	10	10	10
Early Deaths	0	0	0	0	0	0
Survivors						
Terminal sacrifice	10	10	10	10	10	10
Alimentary System						
Liver	(10)[a]	(10)	(10)	(10)	(10)	(10)
Angiectasis	0	0	0	1 (10%)	0	0
Eosinophilic focus	0	0	0	0	1 (10%)	0
Inflammation, chronic	0	1 (10%)	3 (30%)	3 (30%)	8 (80%)	10 (100%)
Hepatocellular degeneration	1 (10%)	0	3 (30%)	4 (40%)	10 (100%)	10 (100%)
Pancreas	(10)	(0)	(0)	(0)	(0)	(10)
Acinus, degeneration	0	–[b]	–	–	–	1 (10%)
Cardiovascular System						
Heart	(10)	(0)	(0)	(0)	(0)	(10)
Cardiomyopathy	9 (90%)	–	–	–	–	7 (70%)
Endocrine System						
Pituitary Gland	(10)	(0)	(0)	(0)	(0)	(10)
Pars distalis, cyst	2 (20%)	–	–	–	–	0
Pars intermedia, cyst	0	–	–	–	–	1 (10%)
Thyroid Gland	(10)	(0)	(0)	(0)	(0)	(10)
Ectopic thymus	4 (40%)	–	–	–	–	1 (10%)
General Body System						
None						
Genital System						
Preputial Gland	(10)	(0)	(0)	(0)	(0)	(10)
Inflammation, suppurative	2 (20%)	–	–	–	–	6 (60%)
Inflammation, chronic active	1 (10%)	–	–	–	–	1 (10%)
Duct, dilatation	0	–	–	–	–	4 (40%)
Prostate	(10)	(0)	(0)	(0)	(0)	(10)
Inflammation, suppurative	1 (10%)	–	–	–	–	0
Hematopoietic System						
Lymph Node, Mandibular	(10)	(0)	(0)	(0)	(0)	(10)
Infiltration cellular, plasma cell	0	–	–	–	–	1 (10%)
Lymph Node, Mesenteric	(10)	(0)	(0)	(0)	(0)	(10)
Hyperplasia, lymphoid	1 (10%)	–	–	–	–	2 (20%)
Infiltration cellular, mast cell	8 (80%)	–	–	–	–	7 (70%)
Integumentary System						
None						
Musculoskeletal System						
None						
Nervous System						
None						
Respiratory System						
Lung	(10)	(0)	(0)	(0)	(0)	(10)

Infiltration cellular, histiocyte	0	–	–	–	–	1 (10%)
Special Senses System						
None						
Urinary System						
Kidney	(10)	(0)	(0)	(10)	(10)	(10)
Hydronephrosis	0	–	–	–	2 (20%)	5 (50%)
Nephropathy	10 (100%)	–	–	10 (100%)	10 (100%)	9 (90%)

[a]Number of animals examined microscopically at the site and the number of animals with lesion.
[b]Indicates no data were collected.

Table A-2. Statistical Analysis of Nonneoplastic Lesions in Male Rats in the Three-month Feed Study of (+)-Usnic Acid

	0 ppm	30 ppm	60 ppm	120 ppm	360 ppm	720 ppm
Liver						
Hepatocellular Degeneration						
Overall rate[a]	1/10 (10.0%)	0/10 (0.0%)	3/10 (30.0%)	4/10 (40.0%)	10/10 (100.0%)	10/10 (100.0%)
Terminal rate[b]	1/10 (10.0%)	0/10 (0.0%)	3/10 (30.0%)	4/10 (40.0%)	10/10 (100.0%)	10/10 (100.0%)
First incidence (days)[c]	90 (T)	–[d]	90 (T)	90 (T)	90 (T)	90 (T)
CAFÉ p value[e]	p ≤ 0.001	p = 0.500N	p = 0.291	p = 0.152	p ≤ 0.001	p ≤ 0.001
Average severity[f]	1.0	–	1.0	1.3	1.9	3.6
Inflammation						
Overall rate	0/10 (0.0%)	1/10 (10.0%)	3/10 (30.0%)	3/10 (30.0%)	8/10 (80.0%)	10/10 (100.0%)
Terminal rate	0/10 (0.0%)	1/10 (10.0%)	3/10 (30.0%)	3/10 (30.0%)	8/10 (80.0%)	10/10 (100.0%)
First incidence (days)	–	90 (T)	90 (T)	90 (T)	90 (T)	90 (T)
CAFÉ p value	p ≤ 0.001	p = 0.500	p = 0.105	p = 0.105	p ≤ 0.001	p ≤ 0.001
Average severity	–	1.0	1.0	1.0	1.3	1.3
Heart						
Cardiomyopathy						
Overall rate	9/10 (90.0%)	–	–	–	–	7/10 (70.0%)
Terminal rate	9/10 (90.0%)	–	–	–	–	7/10 (70.0%)
First incidence (days)	90 (T)	–	–	–	–	90 (T)
CAFÉ p value	p = 0.291N	–	–	–	–	p = 0.291N
Average severity	1.3	–	–	–	–	1.3
Pituitary Gland						
Pars Distalis Cyst						
Overall rate	2/10 (20.0%)	–	–	–	–	0/10 (0.0%)
Terminal rate	2/10 (20.0%)	–	–	–	–	0/10 (0.0%)
First incidence (days)	90 (T)	–	–	–	–	–
CAFÉ p value	p = 0.237N	–	–	–	–	p = 0.237N
Average severity	1.5	–	–	–	–	–
Thyroid Gland						
Ectopic Thymus						
Overall rate	4/10 (40.0%)	–	–	–	–	1/10 (10.0%)
Terminal rate	4/10 (40.0%)	–	–	–	–	1/10 (10.0%)
First incidence (days)	90 (T)	–	–	–	–	90 (T)
CAFÉ p value	p = 0.152N	–	–	–	–	p = 0.152N
Average severity	5.0	–	–	–	–	5.0
Preputial Gland						
Duct Dilatation						
Overall rate	0/10 (0.0%)	–	–	–	–	4/10 (40.0%)
Terminal rate	0/10 (0.0%)	–	–	–	–	4/10 (40.0%)
First incidence (days)	–	–	–	–	–	90 (T)
CAFÉ p value	p = 0.043	–	–	–	–	p = 0.043
Average severity	–	–	–	–	–	3.8

Suppurative Inflammation						
Overall rate	2/10 (20.0%)	–	–	–	–	6/10 (60.0%)
Terminal rate	2/10 (20.0%)	–	–	–	–	6/10 (60.0%)
First incidence (days)	90 (T)	–	–	–	–	90 (T)
CAFÉ p value	p = 0.085	–	–	–	–	p = 0.085
Average severity	3.0	–	–	–	–	2.5
Mesenteric Lymph Node						
Hyperplasia						
Overall rate	1/10 (10.0%)	–	–	–	–	2/10 (20.0%)
Terminal rate	1/10 (10.0%)	–	–	–	–	2/10 (20.0%)
First incidence (days)	90 (T)	–	–	–	–	90 (T)
CAFÉ p value	p = 0.500	–	–	–	–	p = 0.500
Average severity	2.0	–	–	–	–	2.0
Infiltration Cellular						
Overall rate	8/10 (80.0%)	–	–	–	–	7/10 (70.0%)
Terminal rate	80/10 (80.0%)	–	–	–	–	7/10 (70.0%)
First incidence (days)	90 (T)	–	–	–	–	90 (T)
CAFÉ p value	p = 0.500N	–	–	–	–	p = 0.500N
Average severity	1.8	–	–	–	–	2.3
Kidney						
Hydronephrosis						
Overall rate	0/10 (0.0%)	–	–	0/10 (0.0%)	2/10 (20.0%)	5/10 (50.0%)
Terminal rate	0/10 (0.0%)	–	–	0/10 (0.0%)	2/10 (20.0%)	5/10 (50.0%)
First incidence (days)	–	–	–	–	90 (T)	90 (T)
CAFÉ p value	p = 0.001	–	–	–	p = 0.237	p = 0.016
Average severity	–	–	–	–	1.5	1.4
Nephropathy						
Overall rate	10/10 (100.0%)	–	–	10/10 (100.0%)	10/10 (100.0%)	9/10 (90.0%)
Terminal rate	10/10 (100.0%)	–	–	10/10 (100.0%)	10/10 (100.0%)	9/10 (90.0%)
First incidence (days)	90 (T)	–	–	90 (T)	90 (T)	90 (T)
CAFÉ p value	p = 0.250N	–	–	–	–	p = 0.500N
Average severity	1.3	–	–	1.0	1.0	1.2

[a]Number of nonneoplastic lesion-bearing animals over number of animals examined.
[b]Observed incidence at terminal sacrifice.
[c]Time to first lesion in days. T indicates terminal sacrifice.
[d]Indicates no data were collected.
[e]The exact Cochran-Armitage trend test was used to test for a trend in nonneoplastic incidence with dose. Fisher's exact test was used to compare incidences between exposed groups and the control group. Tests for trend and comparisons of exposed groups to control were performed as one-sided tests. A negative trend or a lower incidence in an exposure group is indicated by N.
[f]Severity was scored as: 1 = minimal, 2 = mild, 3 = moderate, and 4 = marked.

Table A-3. Summary of the Incidence of Nonneoplastic Lesions in Female Rats in the Three-month Feed Study of (+)-Usnic Acid

	0 ppm	30 ppm	60 ppm	120 ppm	360 ppm	720 ppm
Disposition Summary						
Animals Initially in Study	10	10	10	10	10	10
Early Deaths	0	0	0	0	0	0
Survivors						
Terminal sacrifice	10	10	10	10	10	10
Alimentary System						
Liver	(10)[a]	(0)	(0)	(0)	(0)	(10)
Angiectasis	0	–[b]	–	–	–	1 (10%)
Bile duct, hyperplasia	0	–	–	–	–	1 (10%)
Cardiovascular System						
Heart	(10)	(0)	(0)	(0)	(0)	(10)
Cardiomyopathy	7 (70%)	–	–	–	–	7 (70%)
Endocrine System						
Thyroid Gland	(10)	(0)	(0)	(0)	(0)	(10)
Cyst	0	–	–	–	–	1 (10%)
Ectopic thymus	1 (10%)	–	–	–	–	3 (30%)
Ultimobranchial cyst	0	–	–	–	–	1 (10%)
General Body System						
None						
Genital System						
Clitoral Gland	(10)	(0)	(0)	(0)	(0)	(10)
Infiltration cellular, lymphocyte	3 (30%)	–	–	–	–	3 (30%)
Inflammation, suppurative	1 (10%)	–	–	–	–	1 (10%)
Inflammation, chronic active	2 (20%)	–	–	–	–	2 (20%)
Duct, dilatation	0	–	–	–	–	2 (20%)
Uterus	(10)	(0)	(0)	(0)	(0)	(10)
Lumen, dilatation	3 (30%)	–	–	–	–	1 (10%)
Hematopoietic System						
Lymph Node, Mesenteric	(10)	(0)	(0)	(0)	(0)	(10)
Hyperplasia, lymphoid	3 (30%)	–	–	–	–	1 (10%)
Infiltration cellular, mast cell	9 (90%)	–	–	–	–	10 (100%)
Integumentary System						
None						
Musculoskeletal System						
None						
Nervous System						
None						
Respiratory System						
Lung	(10)	(0)	(0)	(0)	(0)	(10)
Infiltration cellular, histiocyte	1 (10%)	–	–	–	–	1 (10%)
Special Senses System						
Harderian Gland	(10)	(0)	(0)	(0)	(0)	(10)
Infiltration cellular, lymphocyte	3 (30%)	–	–	–	–	0
Urinary System						
Kidney	(10)	(0)	(0)	(0)	(0)	(10)
Cyst	2 (20%)	–	–	–	–	1 (10%)
Mineralization	10 (100%)	–	–	–	–	10 (100%)
Nephropathy	2 (20%)	–	–	–	–	10 (100%)

[a]Number of animals examined microscopically at the site and the number of animals with lesion.
[b]Indicates no data were collected.

Table A-4. Statistical Analysis of Nonneoplastic Lesions in Female Rats in the Three-month Feed Study of (+)-Usnic Acid

	0 ppm	30 ppm	60 ppm	120 ppm	360 ppm	720 ppm
Heart						
Cardiomyopathy						
Overall rate[a]	7/10 (70.0%)	–[b]	–	–	–	7/10 (70.0%)
Terminal rate[c]	7/10 (70.0%)	–	–	–	–	7/10 (70.0%)
First incidence (days)[d]	90 (T)	–	–	–	–	90 (T)
CAFÉ p value[e]	p = 0.686	–	–	–	–	p = 0.686
Average severity[f]	1.1	–	–	–	–	1.4
Thyroid Gland						
Ectopic Thymus						
Overall rate	1/10 (10.0%)	–	–	–	–	3/10 (30.0%)
Terminal rate	1/10 (10.0%)	–	–	–	–	3/10 (30.0%)
First incidence (days)	90 (T)	–	–	–	–	90 (T)
CAFÉ p value	p = 0.291	–	–	–	–	p = 0.291
Average severity	5.0	–	–	–	–	5.0
Clitoral Gland						
Duct Dilatation						
Overall rate	0/10 (0.0%)	–	–	–	–	2/10 (20.0%)
Terminal rate	0/10 (0.0%)	–	–	–	–	2/10 (20.0%)
First incidence (days)	–	–	–	–	–	90 (T)
CAFÉ p value	p = 0.237	–	–	–	–	p = 0.237
Average severity	–	–	–	–	–	4.0
Chronic Active Inflammation						
Overall rate	2/10 (20.0%)	–	–	–	–	2/10 (20.0%)
Terminal rate	2/10 (20.0%)	–	–	–	–	2/10 (20.0%)
First incidence (days)	90 (T)	–	–	–	–	90 (T)
CAFÉ p value	p = 0.709N	–	–	–	–	p = 0.709N
Average severity	2.5	–	–	–	–	2.0
Infiltration Cellular						
Overall rate	3/10 (30.0%)	–	–	–	–	3/10 (30.0%)
Terminal rate	3/10 (30.0%)	–	–	–	–	3/10 (30.0%)
First incidence (days)	90 (T)	–	–	–	–	90 (T)
CAFÉ p value	p = 0.686N	–	–	–	–	p = 0.686N
Average severity	2.0	–	–	–	–	1.3
Uterus						
Lumen Dilatation						
Overall rate	3/10 (30.0%)	–	–	–	–	1/10 (10.0%)
Terminal rate	3/10 (30.0%)	–	–	–	–	1/10 (10.0%)
First incidence (days)	90 (T)	–	–	–	–	90 (T)
CAFÉ p value	p = 0.291N	–	–	–	–	p = 0.291N
Average severity	3.0	–	–	–	–	4.0
Mesenteric Lymph Node						
Hyperplasia						
Overall rate	3/10 (30.0%)	–	–	–	–	1/10 (10.0%)
Terminal rate	3/10 (30.0%)	–	–	–	–	1/10 (10.0%)
First incidence (days)	90 (T)	–	–	–	–	90 (T)
CAFÉ p value	p = 0.291N	–	–	–	–	p = 0.291N
Average severity	1.7	–	–	–	–	2.0
Infiltration Cellular						
Overall rate	9/10 (90.0%)	–	–	–	–	10/10 (100.0%)
Terminal rate	9/10 (90.0%)	–	–	–	–	10/10 (100.0%)
First incidence (days)	90 (T)	–	–	–	–	90 (T)
CAFÉ p value	p = 0.500	–	–	–	–	p = 0.500
Average severity	1.8	–	–	–	–	3.0

Harderian Gland						
Infiltration Cellular						
Overall rate	3/10 (30.0%)	–	–	–	–	0/10 (0.0%)
Terminal rate	3/10 (30.0%)	–	–	–	–	0/10 (0.0%)
First incidence (days)	90 (T)	–	–	–	–	–
CAFÉ p value	p = 0.105N	–	–	–	–	p = 0.105N
Average severity	1.3	–	–	–	–	–
Kidney						
Mineralization						
Overall rate	10/10 (100.0%)	–	–	–	–	10/10 (100.0%)
Terminal rate	10/10 (100.0%)	–	–	–	–	10/10 (100.0%)
First incidence (days)	90 (T)	–	–	–	–	90 (T)
CAFÉ p value	–	–	–	–	–	–
Average severity	3.0	–	–	–	–	3.4
Cyst						
Overall rate	2/10 (20.0%)	–	–	–	–	1/10 (10.0%)
Terminal rate	2/10 (20.0%)	–	–	–	–	1/10 (10.0%)
First incidence (days)	90 (T)	–	–	–	–	90 (T)
CAFÉ p value	p = 0.500N	–	–	–	–	p = 0.500N
Average severity	3.5	–	–	–	–	4.0
Nephropathy						
Overall rate	2/10 (20.0%)	–	–	–	–	10/10 (100.0%)
Terminal rate	2/10 (20.0%)	–	–	–	–	10/10 (100.0%)
First incidence (days)	90 (T)	–	–	–	–	90 (T)
CAFÉ p value	p ≤ 0.001	–	–	–	–	p ≤ 0.001
Average severity	1.0	–	–	–	–	1.2

[a]Number of nonneoplastic lesion-bearing animals over number of animals examined.
[b]Indicates no data were collected.
[c]Observed incidence at terminal sacrifice.
[d]Time to first lesion in days. T indicates terminal sacrifice.
[e]The exact Cochran-Armitage trend test was used to test for a trend in nonneoplastic incidence with dose. Fisher's exact test was used to compare incidences between exposed groups and the control group. Tests for trend and comparisons of exposed groups to control were performed as one-sided tests. A negative trend or a lower incidence in an exposure group is indicated by N.
[f]Severity was scored as: 1 = minimal, 2 = mild, 3 = moderate, and 4 = marked.

Table A-5. Summary of the Incidence of Nonneoplastic Lesions in Male Mice in the Three-month Feed Study of (+)-Usnic Acid

	0 ppm	15 ppm	30 ppm	60 ppm	180 ppm	360 ppm
Disposition Summary						
Animals Initially in Study	10	10	10	10	10	10
Early Deaths	0	0	0	0	0	0
Survivors						
Terminal sacrifice	10	10	10	10	10	10
Alimentary System						
Liver	(10)[a]	(0)	(0)	(0)	(0)	(10)
Inflammation, chronic active	1 (10%)	–[b]	–	–	–	0
Tension lipidosis	1 (10%)	–	–	–	–	2 (20%)
Vacuolization cytoplasmic	6 (60%)	–	–	–	–	2 (20%)
Stomach, Forestomach	(10)	(0)	(0)	(0)	(0)	(10)
Epithelium, hyperplasia	1 (10%)	–	–	–	–	0
Cardiovascular System						
None						
Endocrine System						
Adrenal Cortex	(10)	(0)	(0)	(0)	(0)	(10)
Subcapsular, hyperplasia	1 (10%)	–	–	–	–	1 (10%)
General Body System						
None						
Genital System						
Preputial Gland	(10)	(0)	(0)	(0)	(0)	(10)
Cyst	0	–	–	–	–	1 (10%)
Hematopoietic System						
Lymph Node, Mesenteric	(10)	(0)	(0)	(0)	(0)	(10)
Hyperplasia, lymphoid	3 (30%)	–	–	–	–	2 (20%)
Spleen	(10)	(0)	(0)	(0)	(0)	(10)
Hematopoietic cell proliferation	0	–	–	–	–	2 (20%)
Hyperplasia, lymphoid	0	–	–	–	–	2 (20%)
Integumentary System						
None						
Musculoskeletal System						
None						
Nervous System						
None						
Respiratory System						
None						
Special Senses System						
None						
Urinary System						
Urinary Bladder	(10)	(0)	(0)	(0)	(0)	(10)
Lumen, dilatation	0	–	–	–	–	1 (10%)

[a]Number of animals examined microscopically at the site and the number of animals with lesion.
[b]Indicates no data were collected.

Table A-6. Statistical Analysis of Nonneoplastic Lesions in Male Mice in the Three-month Feed Study of (+)-Usnic Acid

	0 ppm	15 ppm	30 ppm	60 ppm	180 ppm	360 ppm
Liver						
Vacuolization Cytoplasmic						
Overall rate[a]	6/10 (60.0%)	–[b]	–	–	–	2/10 (20.0%)
Terminal rate[c]	6/10 (60.0%)	–	–	–	–	2/10 (20.0%)

First incidence (days)[d]	90 (T)	–	–	–	–	90 (T)
CAFÉ p value[e]	p = 0.085N	–	–	–	–	p = 0.085N
Average severity[f]	1.2	–	–	–	–	1.0
Tension Lipidosis						
Overall rate	1/10 (10.0%)	–	–	–	–	2/10 (20.0%)
Terminal rate	1/10 (10.0%)	–	–	–	–	2/10 (20.0%)
First incidence (days)	90 (T)	–	–	–	–	90 (T)
CAFÉ p value	p = 0.500	–	–	–	–	p = 0.500
Average severity	2.0	–	–	–	–	1.0
Spleen						
Hematopoietic Cell Proliferation						
Overall rate	0/10 (0.0%)	–	–	–	–	2/10 (20.0%)
Terminal rate	0/10 (0.0%)	–	–	–	–	2/10 (20.0%)
First incidence (days)	–	–	–	–	–	90 (T)
CAFÉ p value	p = 0.237	–	–	–	–	p = 0.237
Average severity	–	–	–	–	–	2.0
Lymphoid Hyperplasia						
Overall rate	0/10 (0.0%)	–	–	–	–	2/10 (20.0%)
Terminal rate	0/10 (0.0%)	–	–	–	–	2/10 (20.0%)
First incidence (days)	–	–	–	–	–	90 (T)
CAFÉ p value	p = 0.237	–	–	–	–	p = 0.237
Average severity	–	–	–	–	–	2.0
Mesenteric Lymph Node						
Lymphoid Hyperplasia						
Overall rate	3/10 (30.0%)	–	–	–	–	2/10 (20.0%)
Terminal rate	3/10 (30.0%)	–	–	–	–	2/10 (20.0%)
First incidence (days)	90 (T)	–	–	–	–	90 (T)
CAFÉ p value	p = 0.500N	–	–	–	–	p = 0.500N
Average severity	1.7	–	–	–	–	1.5

[a]Number of nonneoplastic lesion-bearing animals over number of animals examined.
[b]Indicates no data were collected.
[c]Observed incidence at terminal sacrifice.
[d]Time to first lesion in days. T indicates terminal sacrifice.
[e]The exact Cochran-Armitage trend test was used to test for a trend in nonneoplastic incidence with dose. Fisher's exact test was used to compare incidences between exposed groups and the control group. Tests for trend and comparisons of exposed groups to control were performed as one-sided tests. A negative trend or a lower incidence in an exposure group is indicated by N.
[f]Severity was scored as: 1 = minimal, 2 = mild, 3 = moderate, and 4 = marked.

Table A-7. Summary of the Incidence of Nonneoplastic Lesions in Female Mice in the Three-month Feed Study of (+)-Usnic Acid

	0 ppm	15 ppm	30 ppm	60 ppm	180 ppm	360 ppm
Disposition Summary						
Animals Initially in Study	10	10	10	10	10	10
Early Deaths						
Accidentally killed	0	0	1	0	0	0
Survivors						
Terminal sacrifice	10	10	9	10	10	10
Alimentary System						
Liver	(10)[a]	(0)	(1)	(0)	(0)	(10)
Inflammation, chronic active	0	–[b]	0	–	–	2 (20%)
Tension lipidosis	1 (10%)	–	0	–	–	0
Vacuolization cytoplasmic	4 (40%)	–	1 (100%)	–	–	0
Cardiovascular System						
None						
Endocrine System						
Adrenal Cortex	(10)	(0)	(1)	(0)	(0)	(10)
Subcapsular, hyperplasia	10 (100%)	–	1 (100%)	–	–	10 (100%)
Thyroid Gland	(10)	(0)	(1)	(0)	(0)	(10)
Ectopic thymus	1 (10%)	–	0	–	–	0
General Body System						
None						
Genital System						
Uterus	(10)	(0)	(1)	(0)	(0)	(10)
Endometrium, hyperplasia	0	–	0	–	–	1 (10%)
Hematopoietic System						
Bone Marrow	(10)	(0)	(1)	(0)	(0)	(10)
Hyperplasia	1 (10%)	–	0	–	–	0
Lymph Node, Mesenteric	(10)	(0)	(1)	(0)	(0)	(10)
Hyperplasia, lymphoid	0	–	0	–	–	1 (10%)
Spleen	(10)	(0)	(1)	(0)	(0)	(10)
Hematopoietic cell proliferation	2 (20%)	–	0	–	–	2 (20%)
Hyperplasia, lymphoid	4 (40%)	–	0	–	–	0
Thymus	(10)	(0)	(1)	(0)	(0)	(10)
Hyperplasia, lymphoid	0	–	0	–	–	2 (20%)
Necrosis	1 (10%)	–	0	–	–	0
Integumentary System						
None						
Musculoskeletal System						
Bone	(0)	(0)	(1)	(0)	(0)	(0)
Cranium, fracture	–	–	1 (100%)	–	–	–
Cranium, hemorrhage	–	–	1 (100%)	–	–	–
Nervous System						
Brain, Brain Stem	(10)	(0)	(1)	(0)	(0)	(10)
Hemorrhage	0	–	1 (100%)	–	–	0
Brain, Cerebellum	(10)	(0)	(1)	(0)	(0)	(10)
Hemorrhage	0	–	1 (100%)	–	–	0
Brain, Cerebrum	(10)	(0)	(1)	(0)	(0)	(10)
Hemorrhage	0	–	1 (100%)	–	–	0
Respiratory System						
Lung	(10)	(0)	(1)	(0)	(0)	(10)
Hemorrhage	0	–	1 (100%)	–	–	0

Nose	(10)	(0)	(1)	(0)	(0)	(10)
Hemorrhage	0	–	1 (100%)	–	–	0
Special Senses System						
None						
Urinary System						
None						

[a]Number of animals examined microscopically at the site and the number of animals with lesion.
[b]Indicates no data were collected.

Table A-8. Statistical Analysis of Nonneoplastic Lesions in Female Mice in the Three-month Feed Study of (+)-Usnic Acid

	0 ppm	15 ppm	30 ppm	60 ppm	180 ppm	360 ppm
Liver						
Vacuolization Cytoplasmic						
Overall rate[a]	4/10 (40.0%)	–[b]	1/1 (100.0%)	–	–	0/10 (0.0%)
Terminal rate[c]	4/10 (40.0%)	–	–	–	–	0/10 (0.0%)
First incidence (days)[d]	90 (T)	–	17	–	–	–
CAFÉ p value[e]	p = 0.023N	–	p = 0.206	–	–	p = 0.043N
Average severity[f]	1.3	–	2	–	–	–
Inflammation						
Overall rate	0/10 (0.0%)	–	0/1 (0.0%)	–	–	2/10 (20.0%)
Terminal rate	0/10 (0.0%)	–	–	–	–	2/10 (20.0%)
First incidence (days)	–	–	–	–	–	90 (T)
CAFÉ p value	p = 0.214	–	–	–	–	p = 0.237
Average severity	–	–	–	–	–	1.0
Adrenal Cortex						
Subcapsular Hyperplasia						
Overall rate	10/10 (100.0%)	–	1/1 (100.0%)	–	–	10/10 (100.0%)
Terminal rate	10/10 (100.0%)	–	–	–	–	10/10 (100.0%)
First incidence (days)	90 (T)	–	17	–	–	90 (T)
CAFÉ p value	–	–	–	–	–	–
Average severity	1.0	–	1	–	–	1.1
Spleen						
Hematopoietic Cell Proliferation						
Overall rate	2/10 (20.0%)	–	0/1 (0.0%)	–	–	2/10 (20.0%)
Terminal rate	2/10 (20.0%)	–	–	–	–	2/10 (20.0%)
First incidence (days)	90 (T)	–	–	–	–	90 (T)
CAFÉ p value	p = 0.669	–	–	–	–	p = 0.709N
Average severity	2.0	–	–	–	–	2.0
Lymphoid Hyperplasia						
Overall rate	4/10 (40.0%)	–	0/1 (0.0%)	–	–	0/10 (0.0%)
Terminal rate	4/10 (40.0%)	–	–	–	–	0/10 (0.0%)
First incidence (days)	90 (T)	–	–	–	–	–
CAFÉ p value	p = 0.035N	–	–	–	–	p = 0.043N
Average severity	2.0	–	–	–	–	–
Thymus						
Lymphoid Hyperplasia						
Overall rate	0/10 (0.0%)	–	0/1 (0.0%)	–	–	2/10 (20.0%)
Terminal rate	0/10 (0.0%)	–	–	–	–	2/10 (20.0%)
First incidence (days)	90 (T)	–	–	–	–	90 (T)
CAFÉ p value	p = 0.224	–	–	–	–	p = 0.237
Average severity	–	–	–	–	–	2.0

[a]Number of nonneoplastic lesion-bearing animals over number of animals examined.
[b]Indicates no data were collected.

[c]Observed incidence at terminal sacrifice.
[d]Time to first lesion in days. T indicates terminal sacrifice.
[e]The exact Cochran-Armitage trend test was used to test for a trend in nonneoplastic incidence with dose. Fisher's exact test was used to compare incidences between exposed groups and the control group. Tests for trend and comparisons of exposed groups to control were performed as one-sided tests. A negative trend or a lower incidence in an exposure group is indicated by N.
[f]Severity was scored as: 1 = minimal, 2 = mild, 3 = moderate, and 4 = marked.

Appendix B. Genetic Toxicology Studies

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Tables

B.1. Background

(+)-Usnic acid has been tested for genotoxicity in several in vitro systems. It showed no mutagenicity in tested strains including TA98 and TA100 without or with S9 addition at a highest dose of 200 µg per plate.[90] NTP studies confirmed that (+)-usnic acid was negative in Ames tests with *S. typhimurium* strains TA98 and TA100 and *E. coli* strain WP2 *uvrA* (pkM101), with and without the addition of rat liver S9.[104] (+)-Usnic acid was evaluated for genotoxicity in human lymphocytes in vitro using the cytokinesis-blocked micronucleus (CBMN) assay.[105] Although the number of micronuclei was higher in the lymphocytes treated with (+)-usnic acid in comparison to control lymphocytes, the induction was not significant statistically. The authors concluded that (+)-usnic acid was nongenotoxic as shown by the absence of significant micronucleus induction in human lymphocytes. Oral administration of a single dose of either 100 or 200 mg/kg usnic acid caused a slight increase in micronucleated erythrocytes in the mice 24 and 48 hours after treatment, which did not reach statistical significance and returned to control levels by 72 hours.[102]

The objective of this genetic toxicology evaluation was to determine whether in vivo exposure to (+)-usnic acid would significantly increase micronuclei formation in peripheral blood from mice that were exposed to (+)-usnic acid for the 2-week acute toxicity studies that were run in conjunction with this 3-month study.

B.2. Methods

Peripheral blood was collected at sacrifice from B6C3F1/Nctr mice evaluated for the 2-week acute toxicity studies (Appendix J), and aliquots were diluted with anticoagulant, fixed in cold (−80°C) methanol, and stored at –85°C. The fixed blood samples were shipped to Litron Laboratories (Rochester, NY) on dry ice for analysis. Micronucleated cells were identified and quantified using a *MicroFlow PLUS* mouse kit from Litron Laboratories.[129; 130] Briefly, reticulocytes were identified by fluorescein isothiocyanate-labeled antibodies against the CD71 mouse surface antigen, platelets were identified by phycoerythrin-labeled antibodies against CD61 antigen, and DNA, including micronuclei, was stained with propidium iodide. Data provided by Litron was compiled in the form of sorted spreadsheets of differences in reticulocyte micronucleus frequency between dose groups, and as audited study reports, which have been added to the Study Archive. The spreadsheet data were then analyzed at NCTR in SAS (version 9.1, TS level 1M3) to produce means, standard error values and significant differences between dose groups via a Dunnett test evaluation and a linear trend test run under the SAS General Linear Models program.

B.3. Results

The micronucleus frequencies for male and female B6C3F1/Nctr mice exposed to either 600 or 1,200 ppm (+)-usnic acid in feed for 14 days are shown in Table B-1. In males, (+)-usnic acid exposure did not significantly change the percentage of total reticulocytes in the samples (% RET), but significantly increased the percentage of micronucleated normochromatic erythrocytes (% NCE) at the 600 ppm dose level and the percentage of micronucleated reticulocytes (% micronucleated RET) at both the 600 and 1,200 ppm levels with a significant exposure trend. In female B6C3F1/Nctr mice, (+)-usnic acid exposure caused a large statistically

significant decrease in % RET at the 1,200 ppm exposure level and significantly increased both the % NCE and % micronucleated RET at the 600 ppm dose level but not at the 1,200 ppm level. The decrease in reticulocyte numbers at the high dose could reflect bone marrow toxicity. In both sexes, the increases in % NCE and % micronucleated RET were relatively small compared to certain other genotoxins. For example, perinatal exposure to 3'-azido-3'deoxythymidine (zidovudine) was reported to increase % NCE and % micronucleated RET 60- and 30-fold respectively in 10-day old mice B6C3F1/NTac mice.[131]

Table B-1. Frequency of Micronuclei in Peripheral Blood Erythrocytes and Reticulocytes in Mice in the Two-week Feed Study of (+)-Usnic Acid

	% RET	% Micronucleated NCE	% Micronucleated RET
Male			
Vehicle Control	5[a]	5	5
Mean ± standard error	1.7 ± 0.07	0.15 ± 0.005	0.28 ± 0.025
Trend test p value	p = 0.18[b]	p = 0.26	p ≤ 0.001
600 ppm	5	5	5
Mean ± standard error	2.2 ± 0.13	0.22 ± 0.008	0.61 ± 0.045
Dunnett's test p value	p = 0.92	p ≤ 0.001	p ≤ 0.001
1,200 ppm	4	4	3
Mean ± standard error	4.3 ± 2.2	0.15 ± 0.005	0.88 ± 0.061
Dunnett's test p value	p = 0.21	p = 0.41	p ≤ 0.001
Female			
Vehicle Control	5	5	5
Mean ± standard error	1.8 ± 0.12	0.12 ± 0.003	0.21 ± 0.015
Trend test p value	p ≤ 0.001	p = 0.010	–
600 ppm	5	5	5
Mean ± standard error	2.3 ± 0.19	0.16 ± 0.002	0.40 ± 0.023
Dunnett's test p value	p = 0.11	p = 0.002	p ≤ 0.001
1,200 ppm	3	3	
Mean ± standard error	0.04 ± 0.03	0.09 ± 0.015	ND[c]
Dunnett's test p value	p ≤ 0.001	p = 0.019	–

RET = reticulocytes expressed as percentages of total red blood cells; NCE = normochromatic erythrocytes
[a]Number examined.
[b]p values listed under the control group values denote trend test significance, and those beneath the dosed group values denote significance of Dunnett test pairwise comparisons between the feed controls and that dosed group. Two-tailed Dunnett tests were used.
[c]ND = not detected due to low reticulocyte concentration.

Appendix C. Hematology and Clinical Chemistry Data

Tables

Table C-1. Hematology and Clinical Chemistry Data for Rats in the Three-month Feed Study of (+)-Usnic Acid[a]

	0 ppm	30 ppm	60 ppm	120 ppm	360 ppm	720 ppm
Male						
Number of Animals	10	10	10	10	10	10
Leukocyte Cell Count (10^3/µl)	6.04 ± 0.45	6.39 ± 0.42	5.84 ± 0.45	5.81 ± 0.70	5.95 ± 0.44	6.68 ± 0.37
Erythrocyte Cell Count (10^3/µl)	9.55 ± 0.10	9.71 ± 0.05	9.55 ± 0.09	9.51 ± 0.10	9.50 ± 0.12	9.64 ± 0.09
Hemoglobin (g/dl)	17.08 ± 0.18*[b]	17.31 ± 0.08	17.28 ± 0.14	17.11 ± 0.14	17.12 ± 0.25	17.72 ± 0.20**
Hematocrit (%)	48.39 ± 0.52	49.27 ± 0.30	48.94 ± 0.38	48.38 ± 0.47	48.19 ± 0.64	49.67 ± 0.50
Mean Cell Volume (µm^3)	50.7 ± 0.26*	50.60 ± 0.16	51.30 ± 0.21	50.70 ± 0.15	50.70 ± 0.15	51.5 ± 0.27*
Mean Cell Hemoglobin (pg)	17.89 ± 0.07***	17.84 ± 0.06	18.10 ± 0.07	17.97 ± 0.09	18.03 ± 0.06	18.4 ± 0.10***
Mean Cell Hemoglobin Concentration (g/dl)	35.31 ± 0.08***	35.16 ± 0.10	35.33 ± 0.09	35.37 ± 0.10	35.52 ± 0.10	35.69 ± 0.10*
Platelet Count (10^3/µl)	518.4 ± 33.62	535.00 ± 14.00[b]	550.70 ± 15.62	540.44 ± 30.95[b]	565.50 ± 22.09	514.70 ± 36.52
Glucose (mg/dl)	169.70 ± 8.67	192.90 ± 19.32	196.50 ± 9.56	169.10 ± 10.41	156.60 ± 7.43	167.80 ± 13.11
Creatinine (mg/dl)	0.51 ± 0.02***	0.54 ± 0.02	0.55 ± 0.02	0.53 ± 0.02	0.57 ± 0.02	0.63 ± 0.02***
Blood Urea Nitrogen (mg/dl)	15.4 ± 0.54***	14.30 ± 0.33	14.80 ± 0.79	14.30 ± 0.33	15.40 ± 0.58	17.30 ± 0.67
Alanine Aminotransferase (U/l)	122.7 ± 75.2[c]	46.20 ± 3.79	46.00 ± 2.08	41.70 ± 1.32	49.20 ± 2.69	68.50 ± 14.84
Protein Concentration (g/dl)	7.79 ± 0.20	7.59 ± 0.07	8.04 ± 0.24	7.53 ± 0.14	8.03 ± 0.14	8.09 ± 0.17
Albumin (g/dl)	4.10 ± 0.10	4.04 ± 0.04	4.36 ± 0.13	4.03 ± 0.08	4.16 ± 0.08	4.38 ± 0.12
Serum Phosphate Concentration (mg/dl)	8.99 ± 1.20	5.93 ± 0.19	6.63 ± 0.30	5.87 ± 0.28	6.91 ± 0.31	6.52 ± 0.24
Alkaline Phosphatase (U/l)	107.00 ± 4.85	102.60 ± 2.85	118.60 ± 5.87	101.40 ± 2.47	102.90 ± 5.22	113.70 ± 3.96
Creatine Kinase (U/l)	372.80 ± 140.33	299.40 ± 34.13	197.60 ± 21.50	245.00 ± 29.52	444.80 ± 107.70	241.00 ± 30.91
Female						
Number of Animals	10	10	10	10	10	10
Leukocyte Cell Count (10^3/µL)	5.90 ± 0.42	5.09 ± 0.26	5.01 ± 0.21	5.23 ± 0.41	5.10 ± 0.46	5.68 ± 0.32
Erythrocyte Cell Count (10^3/µL)	8.98 ± 0.07***	8.97 ± 0.06	8.75 ± 0.06	8.82 ± 0.07	8.55 ± 0.10	8.46 ± 0.11
Hemoglobin (g/dl)	17.01 ± 0.13	17.03 ± 0.11	16.70 ± 0.11	16.98 ± 0.15	16.75 ± 0.23	16.89 ± 0.20
Hematocrit (%)	47.43 ± 0.38**	47.69 ± 0.34	46.41 ± 0.26	47.03 ± 0.45	45.92 ± 0.58	45.99 ± 0.57
Mean Cell Volume (µm^3)	52.9 ± 0.10***	53.10 ± 0.10	53.00 ± 0.15	53.20 ± 0.13	53.7 ± 0.15***	54.3 ± 0.21***
Mean Cell Hemoglobin (pg)	18.96 ± 0.03***	18.97 ± 0.04	19.08 ± 0.05	19.23 ± 0.07**	19.58 ± 0.07***	19.96 ± 0.04***
Mean Cell Hemoglobin Concentration (g/dl)	35.91 ± 0.08***	35.71 ± 0.10	35.97 ± 0.06	36.09 ± 0.12	36.46 ± 0.15**	36.71 ± 0.10***
Platelet Count (10^3/µL)	563.50 ± 16.26	537.80 ± 30.19	569.88 ± 13.68[d]	512.90 ± 37.63	580.11 ± 17.89[b]	473.75 ± 28.58
Glucose (mg/dl)	137.20 ± 6.52	124.40 ± 7.34	129.33 ± 1.97[b]	133.80 ± 15.66	125.22 ± 7.18[b]	137.30 ± 8.45
Creatinine (mg/dl)	0.46 ± 0.02***	0.46 ± 0.02	0.44 ± 0.02[b]	0.47 ± 0.02	0.53 ± 0.02*[b]	0.61 ± 0.02***
Blood Urea Nitrogen (mg/dl)	16.1 ± 0.64***	17.50 ± 0.97	17.22 ± 1.22[b]	17.00 ± 0.58	17.56 ± 0.69[b]	22.5 ± 1.01***
Alanine Aminotransferase (U/l)	40.2 ± 3.23*	46.60 ± 2.47	53.00 ± 14.12[b]	43.40 ± 3.21	41.56 ± 1.82[b]	80.8 ± 26.25*
Protein Concentration (g/dl)	7.56 ± 0.21	7.40 ± 0.08	7.29 ± 0.09[b]	7.61 ± 0.10	7.64 ± 0.14[b]	7.56 ± 0.24
Albumin (g/dl)	4.07 ± 0.14	3.98 ± 0.04	4.07 ± 0.11[b]	4.13 ± 0.06	4.12 ± 0.07[b]	4.07 ± 0.15

Serum Phosphate Concentration (mg/dl)	7.63 ± 0.41	6.83 ± 0.50	6.72 ± 0.37[b]	7.83 ± 0.46	7.11 ± 0.34[b]	7.07 ± 0.21
Alkaline Phosphatase (U/l)	94.00 ± 8.30**	75.50 ± 3.04	78.56 ± 2.99[b]	82.30 ± 4.35	75.67 ± 4.73[b]	113.60 ± 11.80
Creatine Kinase (U/l)	332.70 ± 78.56	358.20 ± 48.81	259.67 ± 31.69[b]	555.60 ± 108.09	264.78 ± 45.51[b]	355.90 ± 61.56

[a]Values are given as means ± standard error of the mean. For the control (0 ppm) group, asterisks represent significance for linear trend and for the exposed groups, asterisks represent significance in comparison to the control group using two-tailed Dunnett tests: $p \leq 0.05$ (*); $p \leq 0.01$ (**); $p \leq 0.001$ (***).

[b]Number of animals (n) = 9.

[c]One control animal, which had a pituitary cyst and hepatocellular degeneration gave a value of 799 (U/l). Removing this animal from the statistical analysis gave a mean alanine aminotransferase value of 47.67 ± 5.69 U/l. While this value was not significantly different from the 720 ppm value there was a significant positive dose trend ($p \leq 0.01$) with increasing dose.

[d]n = 8.

Table C-2. Hematology and Clinical Chemistry Data for Mice in the Three-month Feed Study of (+)-Usnic Acid[a]

	0 ppm	15 ppm	30 ppm	60 ppm	180 ppm	360 ppm
Male						
Number of Animals	10	10	10	10	10	10
Leukocyte Cell Count ($10^3/\mu L$)	1.84 ± 0.43	1.72 ± 0.56	1.26 ± 0.17	1.07 ± 0.17	1.51 ± 0.26	1.44 ± 0.19
Erythrocyte Cell Count ($10^3/\mu L$)	10.31 ± 0.12	10.14 ± 0.12	9.45 ± 0.49	10.02 ± 0.19	10.04 ± 0.23	10.27 ± 0.17
Hemoglobin (g/dl)	16.49 ± 0.18	16.38 ± 0.21	15.21 ± 0.75	16.11 ± 0.31	16.16 ± 0.40	16.50 ± 0.25
Hematocrit (%)	51.12 ± 0.59	50.54 ± 0.63	46.68 ± 2.47	49.45 ± 0.89	49.85 ± 1.26	50.31 ± 0.82
Mean Cell Volume (μm^3)	49.7 ± 0.21*	49.80 ± 0.25	49.30 ± 0.26	49.30 ± 0.15	49.80 ± 0.33	48.80 ± 0.33
Mean Cell Hemoglobin (pg)	15.99 ± 0.06	16.16 ± 0.06	16.11 ± 0.11	16.07 ± 0.05	16.10 ± 0.09	16.08 ± 0.10
Mean Cell Hemoglobin Concentration (g/dl)	32.26 ± 0.08*	32.44 ± 0.10	32.67 ± 0.24*	32.53 ± 0.11	32.41 ± 0.07	32.82 ± 0.08**
Platelet Count ($10^3/\mu L$)	749.50 ± 60.99	773.90 ± 27.79	777.50 ± 23.57	831.60 ± 29.84	814.90 ± 22.79	801.80 ± 21.61
Glucose (mg/dl)	145.00 ± 7.15	151.30 ± 9.50	137.70 ± 8.22	144.90 ± 6.06	156.20 ± 20.38	138.30 ± 8.84
Creatinine (mg/dl)	0.23 ± 0.02*	0.27 ± 0.02	0.27 ± 0.02	0.31 ± 0.01**	0.30 ± 0.02*	0.31 ± 0.02**
Blood Urea Nitrogen (mg/dl)	22.2 ± 0.59***	25.30 ± 2.05	26.70 ± 1.36	24.40 ± 0.69	34.3 ± 2.55***	29.9 ± 2.73*
Alanine Aminotransferase (U/l)	30.3 ± 1.66**	35.00 ± 4.36	37.50 ± 3.53	34.20 ± 1.33	38.20 ± 2.31	47.4 ± 6.46**
Protein Concentration (g/dl)	6.61 ± 0.14	6.40 ± 0.10	6.81 ± 0.27	6.32 ± 0.07	6.65 ± 0.25	6.39 ± 0.08
Serum Albumin Concentration (g/dl)	3.51 ± 0.05	3.47 ± 0.05	3.62 ± 0.09	3.43 ± 0.06	3.56 ± 0.10	3.42 ± 0.05
Serum Phosphate Concentration (mg/dl)	7.66 ± 0.26	8.70 ± 0.38	9.32 ± 0.70	7.94 ± 0.29	9.02 ± 0.50	9.14 ± 0.64
Alkaline Phosphatase (U/l)	78.4 ± 3.60**	73.30 ± 4.25	84.60 ± 5.01	78.00 ± 1.68	82.00 ± 5.57	94.5 ± 3.91*
Creatine Kinase (U/l)	293.20 ± 58.86	331.60 ± 77.97	477.50 ± 124.83	469.60 ± 113.06	374.80 ± 95.73	434.00 ± 137.07
Female						
Number of Animals	10	9	9	10	10	10
Leukocyte Cell Count ($10^3/\mu L$)	1.82 ± 0.41	2.13 ± 0.43	2.80 ± 0.47	2.37 ± 0.52	1.53 ± 0.44[b]	1.44 ± 0.12
Erythrocyte Cell Count ($10^3/\mu L$)	10.12 ± 0.13	10.21 ± 0.19	10.12 ± 0.22	9.83 ± 0.30	10.25 ± 0.17[b]	10.20 ± 0.25
Hemoglobin (g/dl)	16.17 ± 0.25	16.52 ± 0.27	16.21 ± 0.38	15.64 ± 0.53	16.56 ± 0.27[b]	16.59 ± 0.41
Hematocrit (%)	49.30 ± 0.78	50.64 ± 0.89	49.17 ± 1.28	47.71 ± 1.72	50.13 ± 0.94[b]	50.21 ± 1.24
Mean Cell Volume (μm^3)	48.60 ± 0.22	49.67 ± 0.24	48.56 ± 0.47	48.40 ± 0.37	48.89 ± 0.26[b]	49.10 ± 0.23
Mean Cell Hemoglobin (pg)	15.98 ± 0.07*	16.20 ± 0.07	16.03 ± 0.13	15.88 ± 0.09	16.13 ± 0.04[b]	16.27 ± 0.07*
Mean Cell Hemoglobin Concentration (g/dl)	32.82 ± 0.07*	32.64 ± 0.08	33.00 ± 0.16	32.80 ± 0.13	33.02 ± 0.13[b]	33.03 ± 0.08
Platelet Count ($10^3/\mu L$)	649.1 ± 57.17*	627.44 ± 45.42	715.89 ± 11.70	728.25 ± 32.18[c]	744.56 ± 36.04[b]	767.33 ± 22.81[b]
Glucose (mg/dl)	120.56 ± 9.97[b]	145.44 ± 12.73	126.56 ± 13.45	135.22 ± 15.11[b]	150.90 ± 19.81	150.89 ± 21.99[b]
Creatinine (mg/dl)	0.26 ± 0.02[c]	0.27 ± 0.02	0.26 ± 0.02	0.23 ± 0.02	0.28 ± 0.04[b]	0.30 ± 0.04
Blood Urea Nitrogen (mg/dl)	25.67 ± 1.93[b]	27.89 ± 1.86	31.33 ± 2.60	27.30 ± 3.45	28.10 ± 1.49	26.10 ± 2.43
Alanine Aminotransferase (U/l)	37.67 ± 5.33[b]	33.11 ± 1.54	44.57 ± 4.82[d]	31.44 ± 2.49[b]	33.00 ± 3.41	33.44 ± 3.40[b]
Protein Concentration (g/dl)	6.74 ± 0.28*[b]	6.56 ± 0.18	6.39 ± 0.28	6.38 ± 0.21	6.79 ± 0.26	7.06 ± 0.19
Albumin (g/dl)	3.64 ± 0.08[b]	3.72 ± 0.09	3.61 ± 0.10[c]	3.56 ± 0.10[b]	3.68 ± 0.07	3.89 ± 0.09[b]
Serum Phosphate Concentration (mg/dl)	8.62 ± 0.90[b]	8.18 ± 0.40	8.70 ± 0.56[c]	7.93 ± 0.37[b]	8.62 ± 0.90	9.08 ± 0.87[b]

Alkaline Phosphatase (U/l)	143.25 ± 14.42[c]	122.33 ± 6.96	125.29 ± 12.85[d]	114.00 ± 5.29[b]	137.10 ± 13.14	128.00 ± 9.15[b]
Creatine Kinase (U/l)	749.67 ± 280.88[b]	434.00 ± 69.88	439.88 ± 135.73[c]	328.33 ± 61.34[b]	331.80 ± 71.78	448.10 ± 113.15

[a]Values are given as means ± standard error of the mean. For the control (0 ppm) group, asterisks represent significance for linear trend and for the dosed groups, asterisks represent significance in comparison to the control group using two-tailed Dunnett tests: $p \leq 0.05$ (*); $p \leq 0.01$ (**); $p \leq 0.001$ (***).
[b]Number of animals (n) = 9.
[c]n = 8.
[d]n = 7.

Appendix D. Body Weights

Tables

Table D-1. Body Weights of Male Rats in the Three-month Feed Study of (+)-Usnic Acid

Week [a]	0 ppm		30 ppm			60 ppm			120 ppm			360 ppm			720 ppm		
	N[b]	Mean ± SE[c]	N	Mean ± SE	Pct[d]	N	Mean ± SE	Pct	N	Mean ± SE	Pct	N	Mean ± SE	Pct	N	Mean ± SE	Pct
0	10	194.4 ± 5.1	10	196.2 ± 3.9		10	195.4 ± 5.1		10	194.5 ± 3.8		10	197.1 ± 5.5		10	195.3 ± 4.3	
1	10	218.9 ± 4.8**	10	220.0 ± 4.1	100.5	10	216.9 ± 5.2	99.1	10	218.8 ± 3.8	100.0	10	219.2 ± 5.1	100.1	10	212.4 ± 3.5*	97.0
2	10	239.5 ± 5.2*	10	240.5 ± 4.3	100.4	10	234.9 ± 5.2	98.1	10	237.8 ± 4.8	99.3	10	238.3 ± 4.8	99.5	10	232.1 ± 3.8*	96.9
3	10	258.0 ± 5.4**	10	259.3 ± 4.6	100.5	10	251.0 ± 5.1	97.3	10	255.6 ± 4.2	99.1	10	254.8 ± 4.1	98.8	10	243.7 ± 3.4**	94.5
4	10	273.4 ± 5.0**	10	274.0 ± 4.9	100.2	10	265.3 ± 5.2	97.0	10	270.9 ± 4.4	99.1	10	270.1 ± 4.9	98.8	10	258.2 ± 3.3**	94.4
5	10	289.1 ± 5.3**	10	287.8 ± 5.2	99.6	10	279.6 ± 5.5	96.7	10	284.9 ± 4.4	98.5	10	283.3 ± 5.3	98.0	10	270.2 ± 3.0**	93.5
6	10	302.3 ± 5.3**	10	301.8 ± 5.3	99.8	10	291.0 ± 6.0*	96.3	10	297.7 ± 3.8	98.5	10	296.1 ± 5.1	97.9	10	285.0 ± 3.2**	94.3
7	10	314.9 ± 4.7**	10	310.7 ± 4.3	98.7	10	300.3 ± 6.4**	95.4	10	310.7 ± 4.8	98.7	10	306.8 ± 5.1	97.4	10	294.2 ± 3.5**	93.4
8	10	323.9 ± 4.9**	10	321.2 ± 4.1	99.2	10	307.3 ± 6.2**	94.9	10	317.0 ± 4.6	97.9	10	314.5 ± 4.8	97.1	10	301.0 ± 3.1**	92.9
9	10	334.0 ± 5.5**	10	331.5 ± 4.7	99.3	10	319.0 ± 6.4*	95.5	10	327.0 ± 5.4	97.9	10	325.8 ± 5.0	97.5	10	307.7 ± 3.1**	92.1
10	10	341.0 ± 5.4**	10	340.0 ± 4.5	99.7	10	324.9 ± 6.6*	95.3	10	337.4 ± 4.7	98.9	10	335.0 ± 5.5	98.2	10	317.1 ± 2.9**	93.0
11	10	347.1 ± 5.0**	10	347.3 ± 4.3	100.1	10	331.2 ± 6.4**	95.4	10	341.5 ± 4.6	98.4	10	341.6 ± 5.5	98.4	10	324.2 ± 3.3**	93.4
12	10	352.7 ± 5.6**	10	353.8 ± 4.4	100.3	10	337.8 ± 6.6	95.8	10	347.5 ± 4.5	98.5	10	345.2 ± 6.3	97.9	10	327.6 ± 4.3**	92.9
13	10	359.4 ± 5.5**	10	357.1 ± 5.1	99.4	10	342.6 ± 6.1*	95.3	10	353.0 ± 4.2	98.2	10	350.5 ± 6.0	97.5	10	334.2 ± 3.5**	93.0
Mean for Weeks																	
1–13		305.2 ± 3.2**		302.8 ± 3.2	99.2		292.5 ± 3.2*	95.8		300.9 ± 3.2	98.6		297.0 ± 3.2	97.3		285.4 ± 3.2**	93.5

[a]Measured after each week of exposure.
[b]N = number of animals.
[c]Body weight (g) as mean ± standard error. Asterisks denote significant dose trend (control column) or significant pairwise comparison to control group (Dunnett's test, other columns): $p \leq 0.05$ (*); $p \leq 0.01$ (**); $p \leq 0.001$ (***).
[d]Mean weight as percentage of control.

Table D-2. Body Weights of Female Rats in the Three-month Feed Study of (+)-Usnic Acid

Week	0 ppm		30 ppm			60 ppm			120 ppm			360 ppm			720 ppm		
[a]	N[b]	Mean ± SE[c]	N	Mean ± SE	Pct[d]	N	Mean ± SE	Pct	N	Mean ± SE	Pct	N	Mean ± SE	Pct	N	Mean ± SE	Pct
0	10	140.4 ± 1.8	10	141.5 ± 1.6		10	144.5 ± 2.0		10	139.3 ± 2.0		10	139.6 ± 2.2		10	141.5 ± 2.4	
1	10	151.5 ± 2.4***	10	151.2 ± 2.2	99.8	10	154.3 ± 2.0	101.8	10	150.7 ± 2.3	99.5	10	148.9 ± 1.9	98.3	10	145.2 ± 1.6**	95.8
2	10	161.0 ± 2.6***	10	160.4 ± 2.8	99.6	10	165.1 ± 2.0	102.5	10	159.0 ± 2.7	98.8	10	157.8 ± 2.4	98.0	10	151.0 ± 1.7**	93.8
3	10	170.2 ± 3.0***	10	170.2 ± 2.6	100.0	10	173.5 ± 2.3	101.9	10	166.3 ± 2.9	97.7	10	166.3 ± 3.0	97.7	10	154.8 ± 1.8**	91.0
4	10	177.6 ± 2.7***	10	177.0 ± 2.6	99.7	10	180.7 ± 2.2	101.7	10	174.7 ± 2.4	98.4	10	173.3 ± 2.9	97.6	10	160.4 ± 2.0**	90.3
5	10	184.3 ± 3.5***	10	184.8 ± 2.8	100.3	10	187.3 ± 2.8	101.6	10	181.5 ± 3.3	98.5	10	177.4 ± 3.2	96.3	10	165.2 ± 2.1**	89.6
6	10	189.0 ± 3.9***	10	189.5 ± 3.1	100.3	10	193.1 ± 2.8	102.2	10	186.4 ± 3.4	98.6	10	180.6 ± 3.1*	95.6	10	168.3 ± 2.4**	89.0
7	10	195.0 ± 4.3***	10	193.7 ± 3.4	99.3	10	197.5 ± 3.0	101.3	10	192.2 ± 3.6	98.6	10	187.0 ± 3.5	95.9	10	172.3 ± 1.9**	88.4
8	10	198.1 ± 4.3***	10	198.7 ± 3.5	100.3	10	200.7 ± 3.4	101.3	10	195.2 ± 4.0	98.5	10	188.8 ± 3.0*	95.3	10	174.7 ± 2.3**	88.2
9	10	202.0 ± 4.4***	10	202.1 ± 3.6	100.0	10	204.1 ± 2.6	101.0	10	198.9 ± 4.0	98.5	10	193.5 ± 3.3*	95.8	10	176.8 ± 2.3**	87.5
10	10	205.5 ± 4.6***	10	206.9 ± 4.1	100.7	10	205.8 ± 3.2	100.1	10	202.5 ± 4.3	98.5	10	194.8 ± 3.7*	94.8	10	177.2 ± 2.5**	86.2
11	10	206.2 ± 4.5***	10	208.2 ± 4.1	101.0	10	205.2 ± 2.7	99.5	10	203.0 ± 4.0	98.4	10	194.5 ± 3.0**	94.3	10	180.0 ± 3.3**	87.3
12	10	207.3 ± 4.4***	10	210.4 ± 3.2	101.5	10	207.9 ± 3.1	100.3	10	204.1 ± 4.0	98.5	10	194.6 ± 3.4**	93.9	10	180.2 ± 2.3**	86.9
13	10	209.1 ± 4.4***	10	212.6 ± 3.2	101.7	10	210.2 ± 3.6	100.5	10	205.5 ± 4.0	98.3	10	195.8 ± 3.2**	93.6	10	178.7 ± 2.1**	85.5
Mean for Weeks																	
1–13		189.7 ± 1.7***		189.3 ± 1.7	99.8		188.2 ± 1.7	99.2		187.8 ± 1.7	99.0		182.4 ± 1.7*	96.2		167.7 ± 1.7**	88.4

[a]Measured after each week of exposure.
[b]N = number of animals.
[c]Body weight (g) as mean ± standard error. Asterisks denote significant dose trend (control column) or significant pairwise comparison to control group (Dunnett's test, other columns): $p \leq 0.05$ (*); $p \leq 0.01$ (**); $p \leq 0.001$ (***).
[d]Mean weight as percentage of control.

Table D-3. Body Weights of Male Mice in the Three-month Feed Study of (+)-Usnic Acid

Week [a]	0 ppm		15 ppm			30 ppm			60 ppm			180 ppm			360 ppm		
	N[b]	Mean ± SE[c]	N	Mean ± SE	Pct[d]	N	Mean ± SE	Pct	N	Mean ± SE	Pct	N	Mean ± SE	Pct	N	Mean ± SE	Pct
0	10	22.4 ± 0.5	10	22.0 ± 0.6		10	22.1 ± 0.7		10	22.2 ± 0.6		10	22.2 ± 0.5		10	21.5 ± 0.4	
1	10	23.0 ± 0.4	10	23.2 ± 0.4	100.9	10	23.2 ± 0.5	100.9	10	23.4 ± 0.6	101.7	10	23.1 ± 0.4	100.4	10	22.5 ± 0.3	97.8
2	10	23.7 ± 0.4	10	24.1 ± 0.4	101.7	10	24.4 ± 0.4	103.0	10	24.5 ± 0.5	103.4	10	24.0 ± 0.7	101.3	10	23.1 ± 0.3	97.5
3	10	25.0 ± 0.5*	10	25.3 ± 0.4	101.2	10	25.2 ± 0.5	100.8	10	25.3 ± 0.6	101.2	10	25.1 ± 0.5	100.4	10	23.8 ± 0.5	95.2
4	10	25.5 ± 0.4*	10	26.1 ± 0.4	102.4	10	26.1 ± 0.4	102.4	10	25.9 ± 0.6	101.6	10	25.5 ± 0.6	100.0	10	24.5 ± 0.5	96.1
5	10	26.4 ± 0.3**	10	27.5 ± 0.6*	104.2	10	26.3 ± 0.5	99.6	10	26.9 ± 0.6	101.9	10	26.7 ± 0.5	101.1	10	25.2 ± 0.5	95.5
6	10	27.5 ± 0.4**	10	28.2 ± 0.4	102.5	10	27.6 ± 0.5	100.4	10	28.1 ± 0.8	102.2	10	26.8 ± 0.5	97.5	10	26.0 ± 0.4	94.5
7	10	27.9 ± 0.5**	10	28.5 ± 0.4	102.2	10	28.3 ± 0.5	101.4	10	28.3 ± 0.6	101.4	10	27.5 ± 0.3	98.6	10	26.6 ± 0.5	95.3
8	10	28.4 ± 0.5**	10	28.8 ± 0.4	101.4	10	28.7 ± 0.5	101.1	10	28.9 ± 0.6	101.8	10	27.8 ± 0.5	97.9	10	26.7 ± 0.3	94.0
9	10	29.0 ± 0.4**	10	29.6 ± 0.4	102.1	10	29.2 ± 0.6	100.7	10	29.2 ± 0.5	100.7	10	27.6 ± 0.7	95.2	10	27.5 ± 0.5	94.8
10	10	29.4 ± 0.5	10	29.3 ± 0.7	99.7	10	29.3 ± 0.6	99.7	10	29.4 ± 0.6	100.0	10	28.4 ± 0.5	96.6	10	28.1 ± 0.4	95.6
11	10	29.8 ± 0.4**	10	30.3 ± 0.7	101.7	10	30.3 ± 0.5	101.7	10	30.4 ± 0.5	102.0	10	28.7 ± 0.4	96.3	10	28.7 ± 0.4	96.3
12	10	30.2 ± 0.6	10	30.2 ± 0.5	100.0	10	29.8 ± 0.5	98.7	10	30.2 ± 0.5	100.0	10	29.2 ± 0.6	96.7	10	28.8 ± 0.7	95.4
13	10	30.7 ± 0.7	10	30.9 ± 0.5	100.7	10	30.6 ± 0.5	99.7	10	31.0 ± 0.8	101.0	10	29.8 ± 0.7	97.1	10	29.5 ± 0.4	96.1
Mean for Weeks																	
1–13		27.2 ± 0.2***		27.9 ± 0.2	102.5		27.6 ± 0.2	101.5		27.7 ± 0.2	101.8		26.9 ± 0.2	98.8		26.6 ± 0.2	98.0

[a]Measured after each week of exposure.
[b]N = number of animals.
[c]Body weight (g) as mean ± standard error. Asterisks denote significant dose trend (control column) or significant pairwise comparison to control group (Dunnett's test, other columns): $p \leq 0.05$ (*); $p \leq 0.01$ (**); $p \leq 0.001$ (***).
[d]Mean weight as percentage of control.

Table D-4. Body Weights of Female Mice in the Three-month Feed Study of (+)-Usnic Acid

Week[a]	0 ppm		15 ppm			30 ppm			60 ppm			180 ppm			360 ppm		
	N[b]	Mean ± SE[c]	N	Mean ± SE	Pct[d]	N	Mean ± SE	Pct	N	Mean ± SE	Pct	N	Mean ± SE	Pct	N	Mean ± SE	Pct
0	10	17.8 ± 0.2	10	18.1 ± 0.3		10	18.3 ± 0.3		10	17.3 ± 0.3		10	18.0 ± 0.2		10	18.5 ± 0.3	
1	10	18.9 ± 0.2	10	19.4 ± 0.3	102.6	10	19.3 ± 0.3	102.1	10	18.3 ± 0.3	96.8	10	19.2 ± 0.2	101.6	10	19.7 ± 0.3	104.2
2	10	19.7 ± 0.2	10	19.9 ± 0.2	101.0	10	19.9 ± 0.3	101.0	10	19.4 ± 0.2	98.5	10	20.1 ± 0.3	102.0	10	20.4 ± 0.3	103.6
3	10	20.4 ± 0.2	10	20.5 ± 0.2	100.5	10	20.4 ± 0.3	100.0	10	20.1 ± 0.3	98.5	10	20.8 ± 0.3	102.0	10	20.7 ± 0.3	101.5
4	10	21.0 ± 0.1	10	20.9 ± 0.4	99.5	9	21.1 ± 0.4	100.5	10	20.7 ± 0.3	98.6	10	21.0 ± 0.3	100.0	10	21.3 ± 0.4	101.4
5	10	21.9 ± 0.3*	10	22.0 ± 0.3	100.5	9	21.1 ± 0.4*	96.3	10	21.3 ± 0.3	97.3	10	21.5 ± 0.3	98.2	10	21.4 ± 0.3	97.7
6	10	22.0 ± 0.1*	10	22.0 ± 0.3	100.0	9	21.3 ± 0.4*	96.8	10	21.3 ± 0.3	96.8	10	21.8 ± 0.4	99.1	10	21.6 ± 0.3*	98.2
7	10	22.4 ± 0.2*	10	22.6 ± 0.3	100.9	9	22.2 ± 0.4	99.1	10	21.8 ± 0.3	97.3	10	22.7 ± 0.4	101.3	10	22.0 ± 0.5	98.2
8	10	22.9 ± 0.3**	10	23.3 ± 0.4	101.7	9	23.0 ± 0.5	100.4	10	21.9 ± 0.3	95.6	10	22.3 ± 0.4	97.4	10	22.5 ± 0.4	98.3
9	10	22.9 ± 0.3	10	23.2 ± 0.4	101.3	9	22.8 ± 0.7	99.6	10	22.2 ± 0.2	96.9	10	22.8 ± 0.3	99.6	10	22.6 ± 0.5	98.7
10	10	23.3 ± 0.3	10	23.5 ± 0.4	100.9	9	23.1 ± 0.4	99.1	10	22.5 ± 0.3	96.6	10	23.0 ± 0.3	98.7	10	23.6 ± 0.6	101.3
11	10	23.5 ± 0.2	10	23.9 ± 0.4	101.7	9	23.3 ± 0.3	99.1	10	22.8 ± 0.3	97.0	10	23.5 ± 0.3	100.0	10	23.5 ± 0.5	100.0
12	10	24.1 ± 0.4	10	23.5 ± 0.3	97.5	9	24.1 ± 0.5	100.0	10	23.5 ± 0.3	97.5	10	24.0 ± 0.5	99.6	10	24.5 ± 0.5	101.7
13	10	24.6 ± 0.4	10	24.3 ± 0.4	98.8	9	24.1 ± 0.6	98.0	10	23.8 ± 0.4	96.7	10	24.6 ± 0.3	100.0	10	24.4 ± 0.4	99.2
Mean for Weeks																	
1–13		22.2 ± 0.1*		22.2 ± 0.1	99.6		21.8 ± 0.1	98.0		22.1 ± 0.1	99.2		22.1 ± 0.1	99.3		21.8 ± 0.1*	97.8

[a]Measured after each week of exposure.
[b]N = number of animals.
[c]Body weight (g) as mean ± standard error. Asterisks denote significant dose trend (control column) or significant pairwise comparison to control group (Dunnett's test, other columns): $p \leq 0.05$ (*); $p \leq 0.01$ (**).
[d]Mean weight as percentage of control.

Appendix E. Organ Weights and Organ-Weight-to-Body-Weight Ratios

Tables

Table E-1. Organ Weights and Organ-Weight-to-Body-Weight Ratios for Rats in the Three-month Feed Study of (+)-Usnic Acid

	0 ppm	30 ppm	60 ppm	120 ppm	360 ppm	720 ppm
n	10	10	10	10	10	10
Male						
Necropsy Body Weight[a]	345 ± 5	345 ± 5	330 ± 6	339 ± 4	336 ± 6	320 ± 3**
Heart[b]						
Absolute	1.05 ± 0.02	1.05 ± 0.02	1.01 ± 0.02	1.05 ± 0.02	1.03 ± 0.02	1.02 ± 0.02
Relative	3.05 ± 0.06	3.04 ± 0.06	3.08 ± 0.06	3.10 ± 0.06	3.08 ± 0.06	3.19 ± 0.06
R. Kidney						
Absolute	1.16 ± 0.02*	1.13 ± 0.02	1.09 ± 0.02	1.13 ± 0.02	1.07 ± 0.02*	1.09 ± 0.02
Relative	3.37 ± 0.05	3.29 ± 0.05	3.31 ± 0.05	3.34 ± 0.05	3.19 ± 0.05	3.41 ± 0.05
Liver						
Absolute	10.40 ± 0.27***[c]	10.14 ± 0.26	9.83 ± 0.26	10.35 ± 0.26	10.93 ± 0.26	11.94 ± 0.26**
Relative	30.23 ± 0.65***[c]	29.42 ± 0.61	29.78 ± 0.61	30.57 ± 0.61	32.48 ± 0.61	37.28 ± 0.61***
Lung						
Absolute	1.28 ± 0.03	1.23 ± 0.03	1.22 ± 0.03	1.25 ± 0.03	1.21 ± 0.03	1.24 ± 0.03
Relative	3.71 ± 0.09	3.57 ± 0.09	3.69 ± 0.09	3.68 ± 0.09	3.60 ± 0.09	3.88 ± 0.09
Thymus						
Absolute	0.22 ± 0.01*[c]	0.21 ± 0.01	0.22 ± 0.01	0.19 ± 0.01	0.19 ± 0.01	0.19 ± 0.01
Relative	0.63 ± 0.03[c]	0.62 ± 0.03	0.65 ± 0.03	0.57 ± 0.03	0.56 ± 0.03	0.60 ± 0.03
L. Testis						
Absolute	1.57 ± 0.02***	1.57 ± 0.02	1.54 ± 0.02	1.58 ± 0.02	1.60 ± 0.03	1.65 ± 0.02*
Relative	4.56 ± 0.07***	4.55 ± 0.07	4.67 ± 0.07	4.66 ± 0.07	4.78 ± 0.07	5.16 ± 0.07***
R. Testis						
Absolute	1.54 ± 0.02**	1.53 ± 0.02	1.52 ± 0.02	1.49 ± 0.02	1.57 ± 0.02	1.59 ± 0.02
Relative	4.47 ± 0.06***	4.45 ± 0.06	4.62 ± 0.06	4.41 ± 0.06	4.69 ± 0.06	4.98 ± 0.06***
L. Epididymis						
Absolute	0.48 ± 0.01**	0.48 ± 0.01	0.49 ± 0.01	0.46 ± 0.01	0.49 ± 0.01	0.52 ± 0.01*
Relative	1.37 ± 0.04***	1.40 ± 0.04	1.47 ± 0.04	1.36 ± 0.04	1.46 ± 0.04	1.61 ± 0.04***
R. Epididymis						
Absolute	0.47 ± 0.01	0.47 ± 0.01	0.47 ± 0.01	0.44 ± 0.01	0.48 ± 0.01	0.48 ± 0.01
Relative	1.37 ± 0.04**	1.35 ± 0.04	1.44 ± 0.04	1.31 ± 0.04	1.44 ± 0.04	1.51 ± 0.04*
Female						
Necropsy Body Weight	198 ± 5	202 ± 3	200 ± 3	196 ± 4	185 ± 3	169 ± 2
Heart						
Absolute	0.69 ± 0.02**	0.72 ± 0.02	0.70 ± 0.02	0.70 ± 0.02	0.66 ± 0.02	0.65 ± 0.02
Relative	3.49 ± 0.09**	3.59 ± 0.09	3.50 ± 0.09	3.60 ± 0.09	3.60 ± 0.09	3.85 ± 0.09*
R. Kidney						
Absolute	0.73 ± 0.02***[c]	0.72 ± 0.02	0.72 ± 0.02	0.73 ± 0.02	0.69 ± 0.02	0.66 ± 0.02**
Relative	3.68 ± 0.07**[c]	3.55 ± 0.06	3.60 ± 0.06	3.71 ± 0.06	3.72 ± 0.06	3.90 ± 0.06
Liver						
Absolute	5.15 ± 0.15***[c]	5.15 ± 0.14	5.21 ± 0.14	5.31 ± 0.14	5.68 ± 0.14	5.81 ± 0.14*
Relative	25.87 ± 0.59***[c]	25.57 ± 0.56	26.06 ± 0.56	27.17 ± 0.56	30.66 ± 0.56***	34.48 ± 0.56***

Lung						
Absolute	0.93 ± 0.02**	0.94 ± 0.02	0.92 ± 0.02	0.92 ± 0.02	0.89 ± 0.02	0.86 ± 0.02
Relative	4.69 ± 0.12**	4.68 ± 0.12	4.63 ± 0.12	4.72 ± 0.12	4.84 ± 0.12	5.08 ± 0.12
Thymus						
Absolute	0.17 ± 0.01	0.19 ± 0.01	0.18 ± 0.01	0.20 ± 0.01	0.18 ± 0.01	0.17 ± 0.01
Relative	0.86 ± 0.05	0.97 ± 0.05	0.89 ± 0.05	1.00 ± 0.05	0.98 ± 0.05	0.98 ± 0.05

[a]Body weights, which are given in grams, were obtained just prior to euthanasia after an overnight fast and were generally lower than the animal removal weights for week 13 of the study reported in Appendix D, which were obtained prior to the fast.
[b]Organ weights (absolute weights) are given in milligrams; organ-weight-to-body-weight ratios (relative weights) are given as mg organ weight/g body weight; necropsy body weights are given in grams. Values given as mean ± standard error. Asterisks denote significant dose trend (control column) or significant pairwise comparison to control group (Dunnett's test, other columns): $p \leq 0.05$ (*); $p \leq 0.01$ (**); $p \leq 0.001$ (***).
[c]n = 9.

Table E-2. Organ Weights and Organ-Weight-to-Body-Weight Ratios for Mice in the Three-month Feed Study of (+)-Usnic Acid

	0 ppm	15 ppm	30 ppm	60 ppm	180 ppm	360 ppm
n	10	10	10	10	10	10
Male						
Necropsy Body Weight[a]	28.3 ± 0.6	28.2 ± 0.7	27.9 ± 0.7	28.0 ± 0.7	27.2 ± 0.7	26.4 ± 0.4
Heart[b]						
Absolute	195.83 ± 9.73*	189.21 ± 9.73	199.50 ± 9.73	195.51 ± 9.73	181.64 ± 9.73	171.62 ± 9.73
Relative	6.97 ± 0.30	6.66 ± 0.30	7.17 ± 0.30	6.99 ± 0.30	6.62 ± 0.30	6.49 ± 0.30
R. Kidney						
Absolute	234.72 ± 7.69	231.69 ± 7.69	229.73 ± 7.69	234.92 ± 7.69	242.53 ± 7.69	221.55 ± 7.69
Relative	8.32 ± 0.17	8.18 ± 0.17	8.23 ± 0.17	8.40 ± 0.17	8.87 ± 0.17	8.39 ± 0.17
Liver						
Absolute	1,012.80 ± 35.13*	1,033.46 ± 35.13	1,029.29 ± 35.13	1,042.96 ± 35.13	1,075.33 ± 35.13	1,115.0 ± 33.13
Relative	35.86 ± 0.79***	36.52 ± 0.79	36.79 ± 0.79	37.29 ± 0.79	39.39 ± 0.79*	42.20 ± 0.79***
Lung						
Absolute	222.63 ± 14.33*	221.82 ± 14.33	211.36 ± 14.33	220.86 ± 14.33	204.03 ± 14.33	186.69 ± 14.33
Relative	7.92 ± 0.44	7.83 ± 0.44	7.52 ± 0.44	7.88 ± 0.44	7.42 ± 0.44	7.05 ± 0.44
L. Testis						
Absolute	117.70 ± 2.64	114.47 ± 2.64	112.58 ± 2.64	114.59 ± 2.64	116.02 ± 2.64	113.37 ± 2.64
Relative	4.18 ± 0.09*	.06 ± 0.09	4.04 ± 0.09	4.11 ± 0.09	4.26 ± 0.09	4.30 ± 0.09
R. Testis						
Absolute	117.59 ± 2.62	115.51 ± 2.62	113.82 ± 2.62	119.32 ± 2.62	118.16 ± 2.62	114.59 ± 2.62
Relative	4.17 ± 0.09*	4.09 ± 0.09	4.08 ± 0.09	4.28 ± 0.09	4.34 ± 0.09	4.35 ± 0.09
Thymus						
Absolute	27.12 ± 1.69	24.33 ± 1.78[c]	24.31 ± 1.69	23.18 ± 1.69	22.72 ± 1.69	23.99 ± 1.69
Relative	0.96 ± 0.05	0.88 ± 0.06[c]	0.86 ± 0.05	0.83 ± 0.05	0.83 ± 0.05	0.91 ± 0.05
L. Epididymis						
Absolute	49.72 ± 2.17	49.73 ± 2.17	44.95 ± 2.17	47.80 ± 2.17	47.48 ± 2.17	44.54 ± 2.17
Relative	1.77 ± 0.08	1.77 ± 0.08	1.61 ± 0.08	1.71 ± 0.08	1.75 ± 0.08	1.69 ± 0.08
R. Epididymis						
Absolute	46.45 ± 1.58	43.80 ± 1.58	43.06 ± 1.58	45.73 ± 1.58	44.77 ± 1.58	43.48 ± 1.58
Relative	1.65 ± 0.06	1.55 ± 0.06	1.54 ± 0.06	1.64 ± 0.06	1.65 ± 0.06	1.65 ± 0.06
Female						
Necropsy Body Weight	21.9 ± 0.3	22.7 ± 0.4	21.3 ± 0.7[b]	21.6 ± 0.6	22.0 ± 0.4	22.2 ± 0.4
Heart						
Absolute	159.34 ± 7.37	147.80 ± 7.37	140.12 ± 7.77[c]	141.35 ± 7.37	149.74 ± 7.37	149.77 ± 7.37
Relative	7.24 ± 0.29	6.55 ± 0.29	6.55 ± 0.30[c]	6.53 ± 0.29	6.83 ± 0.29	6.75 ± 0.29
R. Kidney						
Absolute	160.85 ± 3.93	174.87 ± 3.93	166.21 ± 4.14[c]	158.31 ± 3.93	169.67 ± 3.93	171.28 ± 3.93
Relative	7.34 ± 0.13	7.74 ± 0.13	7.81 ± 0.14[c]	7.33 ± 0.13	7.73 ± 0.13	7.72 ± 0.13
Liver						

Absolute	826.43 ± 33.07***	864.17 ± 33.07	809.47 ± 34.86[c]	852.32 ± 33.07	851.06 ± 33.07	1,000.28 ± 33.07**
Relative	37.71 ± 0.92***	38.07 ± 0.92	37.71 ± 0.97[c]	39.46 ± 0.92	38.69 ± 0.92	44.99 ± 0.92***
Lung						
Absolute	199.10 ± 13.85	203.02 ± 13.85	179.13 ± 14.60[c]	211.78 ± 13.85	180.84 ± 13.85	178.86 ± 13.85
Relative	9.05 ± 0.51	8.92 ± 0.51	8.36 ± 0.54[c]	9.71 ± 0.51	8.21 ± 0.51	8.03 ± 0.51
Thymus						
Absolute	24.89 ± 2.26	27.79 ± 2.26	26.31 ± 2.38[c]	27.97 ± 2.26	23.39 ± 2.26	29.86 ± 2.26
Relative	1.13 ± 0.09	1.22 ± 0.09	1.21 ± 0.09[c]	1.27 ± 0.09	1.06 ± 0.09	1.34 ± 0.09

[a]Body weights, which are given in grams, were obtained just prior to euthanasia after an overnight fast and were generally lower than the animal removal weights for week 13 of the study reported in Appendix D which were obtained prior to the fast.
[b]Organ weights (absolute weights) are given in milligrams; organ-weight-to-body-weight ratios (relative weights) are given as mg organ weight/g body weight; necropsy body weights are given in grams. Values given as mean ± standard error. Asterisks denote significant dose trend (control column) or significant pairwise comparison to control group (Dunnett's test, other columns): $p \leq 0.05$ (*); $p \leq 0.01$ (**); $p \leq 0.001$ (***).
[c]n = 9.

Appendix F. Feed Consumption, Target Dose, and Water Consumption

Tables

Table F-1. Feed Consumption of Male Rats in the Three-month Feed Study of (+)-Usnic Acid

Week[a]	0 ppm			30 ppm			60 ppm			120 ppm			360 ppm			720 ppm		
	N[b]	Mean ± SE[c]	P Value[d]	N	Mean ± SE	P Value	N	Mean ± SE	P Value	N	Mean ± SE	P Value	N	Mean ± SE	P Value	N	Mean ± SE	P Value
1	10	21.2 ± 0.8	0.737	10	20.6 ± 0.8	0.934	10	19.0 ± 0.6	0.096	10	20.9 ± 0.6	0.997	10	19.9 ± 0.7	0.521	10	20.9 ± 0.7	0.997
2	10	21.6 ± 0.5	0.027	10	21.1 ± 0.8	0.992	10	19.6 ± 0.8	0.246	10	21.3 ± 1.0	0.998	10	21.4 ± 0.6	1.000	10	23.0 ± 1.0	0.616
3	10	21.5 ± 0.9	0.298	10	21.1 ± 0.4	0.999	10	20.4 ± 1.0	0.820	10	21.8 ± 0.9	0.998	10	22.1 ± 0.9	0.966	10	21.9 ± 0.6	0.994
4	10	22.4 ± 1.0	0.334	10	21.4 ± 1.1	0.871	10	20.2 ± 0.6	0.199	10	21.4 ± 0.6	0.861	10	20.7 ± 0.7	0.448	10	22.6 ± 0.8	1.000
5	10	23.4 ± 0.7	0.265	10	23.7 ± 0.9	0.997	10	22.8 ± 0.8	0.977	10	21.6 ± 0.6	0.323	10	23.2 ± 0.9	1.000	10	24.1 ± 0.6	0.944
6	10	22.8 ± 1.1	0.250	10	22.9 ± 0.6	1.000	10	21.2 ± 0.7	0.433	10	21.9 ± 0.9	0.867	10	22.5 ± 0.7	0.999	10	23.3 ± 0.6	0.986
7	10	23.8 ± 1.0	0.647	10	22.5 ± 1.1	0.766	10	22.5 ± 0.6	0.800	10	23.2 ± 0.8	0.992	10	23.3 ± 1.0	0.995	10	23.5 ± 1.0	1.000
8	10	23.8 ± 0.9	0.749	10	23.3 ± 1.3	0.996	10	21.4 ± 0.9	0.265	10	22.7 ± 0.8	0.875	10	23.4 ± 1.3	0.999	10	23.2 ± 0.7	0.987
9	10	22.9 ± 1.1	0.603	10	22.7 ± 0.9	1.000	10	23.2 ± 0.9	1.000	10	22.1 ± 1.2	0.985	10	24.5 ± 1.1	0.723	10	23.0 ± 1.2	1.000
10	10	22.3 ± 0.9	0.572	10	22.8 ± 0.7	0.993	10	20.6 ± 0.9	0.538	10	23.9 ± 1.3	0.604	10	22.8 ± 0.8	0.996	10	22.8 ± 0.7	0.996
11	10	22.0 ± 1.1	0.040	10	23.2 ± 0.7	0.810	10	21.1 ± 0.9	0.910	10	22.1 ± 1.1	1.000	10	22.8 ± 0.5	0.970	10	24.2 ± 0.7	0.277
12	10	23.1 ± 1.7	0.564	10	24.1 ± 1.0	0.985	10	23.0 ± 1.8	1.000	10	24.0 ± 1.2	0.993	10	23.4 ± 1.2	1.000	10	24.6 ± 1.5	0.932
13	10	24.2 ± 1.1	0.001	10	22.7 ± 0.9	0.846	10	23.4 ± 0.8	0.985	10	23.7 ± 1.2	0.999	10	25.6 ± 1.5	0.864	10	27.7 ± 1.4	0.128
Mean for Weeks																		
1–13		22.7 ± 0.3	0.001		22.5 ± 0.3	0.977		21.4 ± 0.3	0.011		22.3 ± 0.3	0.856		22.7 ± 0.3	1.000		23.5 ± 0.3	0.237

[a]Feed changed weekly and measured by cage.
[b]N = number of cages.
[c]Mean ± SE (g per day) = estimated least squares mean and standard error.
[d]p values in the 0 ppm column are the p values for the trend test; p values in the dosed columns are Dunnett's adjusted p values for pairwise comparisons of the dosed groups to the 0 ppm group.

Table F-2. Feed Consumption of Female Rats in the Three-month Feed Study of (+)-Usnic Acid

Week[a]	0 ppm			30 ppm			60 ppm			120 ppm			360 ppm			720 ppm		
	N[b]	Mean ± SE[c]	P Value[d]	N	Mean ± SE	P Value	N	Mean ± SE	P Value	N	Mean ± SE	P Value	N	Mean ± SE	P Value	N	Mean ± SE	P Value
1	10	16.6 ± 1.5	0.101	10	15.7 ± 0.7	0.971	10	15.0 ± 0.7	0.752	10	17.3 ± 1.5	0.988	10	14.8 ± 0.7	0.637	10	14.3 ± 0.7	0.402
2	10	15.4 ± 0.8	0.977	10	18.2 ± 1.0	0.050	10	15.6 ± 0.7	1.000	10	16.3 ± 0.9	0.905	10	15.4 ± 0.6	1.000	10	16.7 ± 0.3	0.684
3	10	17.5 ± 0.8	0.327	10	18.2 ± 0.6	0.971	10	18.7 ± 1.0	0.773	10	16.6 ± 1.1	0.935	10	17.6 ± 1.2	1.000	10	16.8 ± 0.5	0.981
4	10	18.5 ± 1.3	0.945	10	18.6 ± 0.9	1.000	10	17.9 ± 0.6	0.980	10	17.9 ± 0.7	0.981	10	18.2 ± 0.9	0.999	10	18.4 ± 0.6	1.000
5	10	17.6 ± 1.0	0.710	10	18.2 ± 0.9	0.987	10	17.1 ± 0.6	0.991	10	18.9 ± 1.1	0.759	10	19.0 ± 0.7	0.717	10	17.9 ± 0.9	0.999
6	10	18.1 ± 0.8	0.284	10	19.1 ± 1.1	0.887	10	19.5 ± 0.9	0.666	10	17.7 ± 1.0	0.996	10	17.8 ± 0.8	1.000	10	17.7 ± 0.7	0.999
7	10	19.8 ± 0.8	0.649	10	17.9 ± 1.1	0.567	10	18.7 ± 1.1	0.912	10	18.6 ± 0.7	0.903	10	19.2 ± 1.1	0.996	10	19.3 ± 1.3	0.997
8	10	17.8 ± 1.1	0.633	10	17.2 ± 0.8	0.992	10	18.2 ± 0.9	0.997	10	16.4 ± 0.9	0.754	10	18.4 ± 0.8	0.986	10	17.8 ± 0.7	1.000
9	10	17.6 ± 0.8	0.683	10	19.0 ± 1.3	0.782	10	18.0 ± 0.9	0.999	10	18.2 ± 0.9	0.992	10	19.4 ± 1.0	0.563	10	18.3 ± 0.9	0.983
10	10	17.1 ± 0.4	0.766	10	18.1 ± 0.6	0.880	10	17.5 ± 0.8	0.997	10	20.0 ± 1.5	0.087	10	16.9 ± 1.0	1.000	10	18.5 ± 0.7	0.683
11	10	20.4 ± 1.3	0.935	10	17.9 ± 0.9	0.231	10	16.4 ± 1.0	0.012	10	17.2 ± 0.9	0.064	10	17.9 ± 0.8	0.225	10	18.3 ± 0.8	0.362
12	10	16.9 ± 1.0	0.057	10	19.1 ± 1.0	0.481	10	16.2 ± 0.7	0.989	10	19.4 ± 1.3	0.372	10	18.7 ± 1.2	0.696	10	20.1 ± 1.2	0.163
13	10	18.7 ± 1.2	0.695	10	20.5 ± 1.1	0.623	10	18.0 ± 0.7	0.990	10	18.7 ± 1.1	1.000	10	19.2 ± 1.2	0.998	10	19.5 ± 1.2	0.974
Mean for Weeks																		
1–13		17.8 ± 0.3	0.821		18.3 ± 0.3	0.738		17.4 ± 0.3	0.810		17.9 ± 0.3	1.000		17.9 ± 0.3	1.000		18.0 ± 0.3	0.998

[a]Feed changed weekly and measured by cage.
[b]N = number of cages.
[c]Mean ± SE (g per day) = estimated least squares mean and standard error.
[d]p values in the 0 ppm column are the p values for the trend test; p values in the dosed columns are Dunnett's adjusted p values for pairwise comparisons of the dosed groups to the 0 ppm group.

Table F-3. Feed Consumption of Male Mice in the Three-month Feed Study of (+)-Usnic Acid

Week[a]	0 ppm			15 ppm			30 ppm			60 ppm			180 ppm			360 ppm		
	N[b]	Mean ± SE[c]	P Value[d]	N	Mean ± SE	P Value	N	Mean ± SE	P Value	N	Mean ± SE	P Value	N	Mean ± SE	P Value	N	Mean ± SE	P Value
1	10	6.6 ± 0.6	0.292	10	7.7 ± 1.0	0.723	10	8.4 ± 1.1	0.340	10	6.1 ± 0.3	0.992	10	6.2 ± 0.6	0.998	10	6.5 ± 0.7	1.000
2	10	8.3 ± 0.8	0.493	10	6.5 ± 0.7	0.417	10	8.4 ± 0.8	1.000	10	6.2 ± 0.6	0.264	10	7.1 ± 0.8	0.761	10	8.3 ± 1.0	1.000
3	10	6.5 ± 0.5	0.234	10	7.4 ± 0.7	0.764	10	7.3 ± 0.6	0.863	10	7.3 ± 0.7	0.806	10	6.7 ± 0.6	1.000	10	6.3 ± 0.4	0.999
4	10	6.6 ± 0.7	0.065	10	8.3 ± 1.3	0.573	10	9.9 ± 1.2	0.054	10	6.5 ± 0.8	1.000	10	6.9 ± 0.8	1.000	10	5.9 ± 0.6	0.989
5	10	7.4 ± 0.6	0.956	10	7.3 ± 0.7	1.000	10	6.3 ± 0.8	0.835	10	6.3 ± 0.7	0.831	10	7.0 ± 1.1	0.996	10	6.9 ± 1.1	0.990
6	10	7.0 ± 0.7	0.027	10	7.8 ± 0.9	0.888	10	8.2 ± 0.7	0.701	10	8.8 ± 1.0	0.310	10	6.7 ± 0.4	0.997	10	6.1 ± 0.3	0.843
7	10	6.6 ± 0.5	0.628	10	8.9 ± 1.0	0.170	10	7.0 ± 0.6	0.995	10	7.4 ± 0.8	0.938	10	7.3 ± 0.7	0.958	10	7.0 ± 1.0	0.995
8	10	8.0 ± 0.7	0.901	10	7.7 ± 1.2	0.999	10	6.5 ± 0.5	0.543	10	7.0 ± 0.8	0.852	10	7.0 ± 0.9	0.885	10	7.4 ± 0.5	0.977
9	10	7.6 ± 0.5	0.599	10	7.2 ± 1.0	0.997	10	7.1 ± 0.8	0.992	10	8.0 ± 1.0	0.998	10	6.5 ± 0.8	0.809	10	7.2 ± 0.6	0.995
10	10	7.2 ± 0.8	0.231	10	7.1 ± 1.0	1.000	10	6.7 ± 1.0	0.994	10	7.2 ± 0.8	1.000	10	6.8 ± 1.0	0.997	10	8.5 ± 1.0	0.785
11	10	7.3 ± 0.8	0.899	10	6.9 ± 0.5	0.998	10	8.0 ± 0.9	0.982	10	7.4 ± 1.4	1.000	10	6.9 ± 0.8	0.998	10	7.7 ± 1.2	0.999
12	10	6.4 ± 0.6	0.040	10	6.5 ± 0.3	1.000	10	6.3 ± 0.5	1.000	10	7.4 ± 0.9	0.784	10	7.1 ± 0.9	0.932	10	8.3 ± 1.0	0.242
13	10	8.2 ± 1.1	0.807	10	7.3 ± 0.7	0.946	10	7.8 ± 0.9	0.998	10	8.2 ± 1.1	1.000	10	8.7 ± 1.0	0.997	10	7.3 ± 0.9	0.948
Mean for Weeks																		
1–13		7.2 ± 0.2	0.376		7.4 ± 0.2	0.935		7.5 ± 0.2	0.806		7.2 ± 0.2	1.000		7.0 ± 0.2	0.951		7.2 ± 0.2	1.000

[a]Feed changed weekly and measured by cage.
[b]N = number of cages.
[c]Mean ± SE (g per day) = estimated least squares mean and standard error.
[d]p values in the 0 ppm column are the p values for the trend test; p values in the dosed columns are Dunnett's adjusted p values for pairwise comparisons of the dosed groups to the 0 ppm group.

Table F-4. Feed Consumption of Female Mice in the Three-month Feed Study of (+)-Usnic Acid

Week[a]	0 ppm			15 ppm			30 ppm			60 ppm			180 ppm			360 ppm		
	N[b]	Mean ± SE[c]	P Value[d]	N	Mean ± SE	P Value	N	Mean ± SE	P Value	N	Mean ± SE	P Value	N	Mean ± SE	P Value	N	Mean ± SE	P Value
1	10	6.3 ± 0.7	0.292	10	7.5 ± 0.7	0.571	10	6.8 ± 0.6	0.986	10	7.9 ± 0.8	0.316	10	5.9 ± 0.7	0.989	10	6.5 ± 0.3	1.000
2	10	6.1 ± 0.4	0.425	10	6.5 ± 0.5	0.988	10	8.0 ± 0.9	0.098	10	7.2 ± 0.3	0.592	10	7.2 ± 0.7	0.630	10	7.4 ± 0.5	0.456
3	10	7.5 ± 0.9	0.932	10	7.7 ± 0.7	1.000	10	7.3 ± 0.4	1.000	10	7.9 ± 0.9	0.998	10	7.2 ± 0.5	0.999	10	7.8 ± 1.0	1.000
4	10	7.2 ± 0.6	0.258	10	6.8 ± 0.7	0.994	9	6.9 ± 0.7	0.997	10	6.5 ± 0.6	0.920	10	7.8 ± 1.0	0.973	10	7.7 ± 0.8	0.991
5	10	7.4 ± 1.1	0.399	10	8.1 ± 0.9	0.967	9	7.0 ± 0.8	0.998	10	7.4 ± 0.7	1.000	10	6.3 ± 0.4	0.823	10	7.0 ± 1.0	0.997
6	10	7.5 ± 0.9	0.144	10	8.8 ± 0.9	0.802	9	6.4 ± 1.0	0.869	10	9.0 ± 1.4	0.699	10	5.9 ± 0.6	0.636	10	6.7 ± 0.9	0.951
7	10	8.8 ± 0.7	0.080	10	10.6 ± 1.1	0.455	9	6.6 ± 0.7	0.259	10	7.7 ± 0.9	0.827	10	6.7 ± 0.9	0.274	10	7.3 ± 0.9	0.605
8	10	8.3 ± 1.0	0.418	10	9.4 ± 1.8	0.903	9	7.9 ± 0.7	1.000	10	7.3 ± 0.8	0.945	10	7.2 ± 0.7	0.922	10	7.6 ± 1.0	0.993
9	10	6.4 ± 0.8	0.533	10	8.1 ± 0.8	0.390	9	7.7 ± 0.9	0.680	10	7.2 ± 0.7	0.932	10	7.3 ± 0.7	0.899	10	7.9 ± 0.9	0.502
10	10	6.4 ± 0.5	0.055	10	7.9 ± 1.0	0.572	9	7.7 ± 1.2	0.668	10	5.8 ± 0.5	0.991	10	7.9 ± 0.8	0.529	10	8.7 ± 0.8	0.153
11	10	7.9 ± 1.3	0.853	10	6.9 ± 1.0	0.919	9	7.0 ± 0.9	0.962	10	6.8 ± 0.5	0.887	10	6.6 ± 0.7	0.840	10	7.6 ± 1.3	1.000
12	10	7.8 ± 0.6	0.180	10	7.3 ± 0.6	0.986	9	6.6 ± 0.5	0.699	10	8.0 ± 0.9	0.999	10	7.8 ± 0.6	1.000	10	8.5 ± 1.0	0.941
13	10	7.9 ± 1.0	0.233	10	7.9 ± 0.6	1.000	9	7.4 ± 0.7	0.995	10	8.2 ± 0.6	0.999	10	8.8 ± 1.3	0.938	10	8.9 ± 1.0	0.902
Mean for Weeks																		
1–13		7.4 ± 0.2	0.883		8.0 ± 0.2	0.249		7.2 ± 0.2	0.983		7.5 ± 0.2	0.998		7.1 ± 0.2	0.936		7.7 ± 0.2	0.837

[a]Feed changed weekly and measured by cage.
[b]N = number of cages.
[c]Mean ± SE (g per day) = estimated least squares mean and standard error.
[d]p values in the 0 ppm column are the p values for the trend test; p values in the dosed columns are Dunnett's adjusted p values for pairwise comparisons of the dosed groups to the 0 ppm group.

Table F-5. Target and Observed Doses in Rats in the Three-month Feed Study of (+)-Usnic Acid

Feed Concentration (ppm)[a,b]	Target Dose (mg/kg/day)[c]	Observed Dose (mg/kg/day)[d]	
		Females	Males
30	2.5	2.91 ± 0.07	2.26 ± 0.08
60	5	5.51 ± 0.14	4.45 ± 0.13
120	10	11.64 ± 0.28	9.09 ± 0.31
360	30	35.64 ± 0.56	27.78 ± 0.80
720	60	76.98 ± 0.96	59.93 ± 1.80

[a]Feed concentrations are denoted by their (+)-usnic acid content as ppm added to feed.
[b]Doses were selected based on data obtained from 14-day feed studies (Appendix J) and historical data for the animal colonies.
[c]Target dose estimate was calculated from historical body weight and feed consumption data for the animal colonies.
[d]Observed values calculated from the observed weekly mean feed consumption and observed weekly mean body weights for surviving rats in each dosed group. Observed feed consumption values do not correct for spillage. Data presented as mean ± standard error for the 13 weekly values.

Table F-6. Target and Observed Doses in Mice in the Three-month Feed Study of (+)-Usnic Acid

Feed Concentration (ppm)[a,b]	Target Dose (mg/kg/day)[c]	Observed Dose (mg/kg/day)[d]	
		Females	Males
15	2.5	5.38 ± 0.20	4.04 ± 0.15
30	5	9.84 ± 0.28	8.25 ± 0.46
60	10	20.92 ± 0.80	15.58 ± 0.36
180	30	58.04 ± 1.59	46.82 ± 0.96
360	60	124.37 ± 2.04	98.88 ± 3.19

[a]Feed concentrations are denoted by their (+)-usnic acid content as ppm added to feed.
[b]Doses were selected based on data obtained from 14-day feed studies (Appendix J) and historical data for the animal colonies.
[c]Target dose estimate was calculated from historical body weight and feed consumption data for the animal colonies.
[d]Observed values calculated from the observed weekly mean feed consumption and observed weekly mean body weights for surviving mice in each dose group. Observed feed consumption values do not correct for spillage. Data presented as mean and standard error for the 13 weekly values.

Table F-7. Water Consumption of Male Rats in the Three-month Feed Study of (+)-Usnic Acid

Week[a]	0 ppm			30 ppm			60 ppm			120 ppm			360 ppm			720 ppm		
	N[b]	Mean ± SE[c]	P Value[d]	N	Mean ± SE	P Value	N	Mean ± SE	P Value	N	Mean ± SE	P Value	N	Mean ± SE	P Value	N	Mean ± SE	P Value
1	10	24.1 ± 0.7	0.332	10	23.5 ± 1.3	0.994	10	22.8 ± 1.0	0.870	10	23.3 ± 0.9	0.970	10	24.6 ± 1.1	0.998	10	24.5 ± 1.4	0.999
2	10	23.4 ± 1.1	0.016	10	23.8 ± 0.8	0.995	10	21.1 ± 0.7	0.146	10	22.2 ± 0.7	0.710	10	23.7 ± 0.4	0.999	10	24.8 ± 0.8	0.530
3	10	22.7 ± 1.0	0.000	10	22.4 ± 1.0	1.000	10	22.8 ± 0.9	1.000	10	22.6 ± 0.7	1.000	10	23.7 ± 0.7	0.861	10	26.8 ± 1.1	0.005
4	10	23.7 ± 0.8	0.000	10	23.3 ± 1.1	0.999	10	23.1 ± 2.3	0.997	10	22.2 ± 0.5	0.840	10	23.6 ± 0.9	1.000	10	32.2 ± 0.9	0.000
5	10	22.2 ± 0.8	0.000	10	21.6 ± 0.6	0.957	10	21.2 ± 0.8	0.722	10	21.3 ± 0.4	0.815	10	22.3 ± 0.3	1.000	10	27.9 ± 0.8	0.000
6	10	22.6 ± 0.5	0.000	10	21.4 ± 0.8	0.616	10	20.8 ± 0.4	0.247	10	21.9 ± 1.0	0.916	10	22.5 ± 0.6	1.000	10	27.2 ± 0.7	0.000
7	10	23.7 ± 1.0	0.000	10	21.4 ± 0.7	0.154	10	21.0 ± 0.6	0.063	10	22.2 ± 1.0	0.545	10	22.9 ± 0.6	0.930	10	25.8 ± 0.7	0.257
8	10	22.6 ± 0.5	0.000	10	22.5 ± 0.7	1.000	10	20.9 ± 0.6	0.276	10	23.2 ± 1.1	0.969	10	22.5 ± 0.5	1.000	10	25.7 ± 0.6	0.006
9	10	22.5 ± 1.0	0.000	10	21.4 ± 0.6	0.801	10	21.2 ± 0.5	0.650	10	23.2 ± 1.1	0.952	10	23.7 ± 0.5	0.696	10	25.2 ± 0.8	0.055
10	10	23.8 ± 1.1	0.000	10	22.2 ± 0.9	0.748	10	21.4 ± 1.9	0.360	10	21.2 ± 0.5	0.313	10	24.3 ± 0.8	0.998	10	26.6 ± 0.8	0.226
11	10	23.5 ± 1.5	0.000	10	22.4 ± 0.9	0.881	10	19.2 ± 0.6	0.005	10	21.7 ± 0.6	0.485	10	24.9 ± 0.9	0.736	10	27.7 ± 0.7	0.005
12	10	21.9 ± 1.1	0.000	10	22.1 ± 1.0	1.000	10	19.3 ± 1.0	0.181	10	21.1 ± 0.5	0.973	10	26.2 ± 1.1	0.004	10	25.9 ± 0.8	0.010
13	10	21.3 ± 0.8	0.000	10	20.7 ± 1.2	0.990	10	20.1 ± 0.9	0.876	10	21.0 ± 0.8	1.000	10	25.4 ± 1.0	0.013	10	25.5 ± 1.2	0.009
Mean for Weeks																		
1–13		22.9 ± 0.3	0.000		22.2 ± 0.3	0.407		21.1 ± 0.3	0.001		22.1 ± 0.3	0.256		23.9 ± 0.3	0.161		26.6 ± 0.3	0.000

[a]Water changed weekly and measured by cage.
[b]N = number of cages.
[c]Mean ± SE (g per day) = estimated least squares mean and standard error.
[d]p values in the 0 ppm column are the p values for the trend test; p values in the dosed columns are Dunnett's adjusted p values for pairwise comparisons of the dosed groups to the 0 ppm group.

Table F-8. Water Consumption of Female Rats in the Three-month Feed Study of (+)-Usnic Acid

Week[a]	0 ppm			30 ppm			60 ppm			120 ppm			360 ppm			720 ppm		
	N[b]	Mean ± SE[c]	P Value[d]	N	Mean ± SE	P Value	N	Mean ± SE	P Value	N	Mean ± SE	P Value	N	Mean ± SE	P Value	N	Mean ± SE	P Value
1	10	19.3 ± 1.0	0.614	10	18.9 ± 0.7	0.999	10	21.1 ± 1.0	0.502	10	20.3 ± 0.8	0.919	10	20.5 ± 1.1	0.822	10	19.0 ± 0.8	0.999
2	10	20.0 ± 0.5	0.211	10	19.8 ± 0.6	1.000	10	18.9 ± 0.7	0.767	10	19.9 ± 1.3	1.000	10	19.7 ± 0.7	0.999	10	20.9 ± 0.9	0.909
3	10	21.0 ± 0.6	0.347	10	20.8 ± 1.1	1.000	10	19.9 ± 0.4	0.795	10	19.9 ± 1.1	0.788	10	19.8 ± 0.6	0.745	10	19.8 ± 0.6	0.729
4	10	19.6 ± 0.6	0.008	10	20.4 ± 1.1	0.926	10	19.2 ± 0.8	0.991	10	19.4 ± 0.6	1.000	10	20.7 ± 0.6	0.808	10	22.0 ± 0.7	0.128
5	10	18.6 ± 0.6	0.000	10	19.6 ± 1.2	0.788	10	18.3 ± 0.6	0.999	10	19.0 ± 0.7	0.991	10	20.0 ± 0.6	0.550	10	24.6 ± 0.6	0.000
6	10	20.1 ± 1.3	0.001	10	19.5 ± 1.2	0.980	10	18.3 ± 0.5	0.412	10	17.8 ± 0.3	0.179	10	19.7 ± 0.4	0.999	10	22.2 ± 0.6	0.218
7	10	19.3 ± 1.1	0.101	10	18.3 ± 0.9	0.931	10	19.7 ± 1.8	1.000	10	18.9 ± 0.7	0.999	10	20.5 ± 0.6	0.892	10	20.8 ± 0.8	0.798
8	10	18.8 ± 1.1	0.000	10	18.2 ± 0.7	0.975	10	18.5 ± 0.7	0.998	10	17.7 ± 0.8	0.750	10	19.5 ± 0.4	0.943	10	21.8 ± 0.6	0.019
9	10	18.7 ± 0.7	0.000	10	19.4 ± 0.9	0.982	10	19.3 ± 1.1	0.994	10	19.7 ± 0.9	0.947	10	22.3 ± 1.1	0.058	10	25.0 ± 1.4	0.000
10	10	19.5 ± 0.7	0.000	10	20.4 ± 1.0	0.951	10	19.1 ± 1.2	0.999	10	18.7 ± 0.8	0.969	10	21.7 ± 0.9	0.346	10	24.6 ± 1.0	0.001
11	10	23.0 ± 2.2	0.000	10	19.3 ± 0.8	0.104	10	19.1 ± 1.1	0.073	10	19.7 ± 0.7	0.181	10	24.3 ± 0.8	0.920	10	25.9 ± 0.9	0.301
12	10	19.5 ± 0.9	0.000	10	20.0 ± 1.2	0.995	10	18.1 ± 0.8	0.751	10	20.1 ± 1.0	0.991	10	22.9 ± 0.7	0.043	10	23.7 ± 0.8	0.006
13	10	19.0 ± 1.3	0.004	10	20.6 ± 1.7	0.804	10	17.6 ± 0.7	0.907	10	19.3 ± 0.9	1.000	10	21.1 ± 1.1	0.635	10	23.0 ± 1.3	0.081
Mean for Weeks																		
1–13		19.7 ± 0.3	0.000		19.6 ± 0.3	1.000		19.0 ± 0.3	0.311		19.3 ± 0.3	0.718		21.0 ± 0.3	0.020		22.6 ± 0.3	0.000

Note: Water consumption was not analyzed for mice because large amounts of spillage occurred when cage lids were lifted, resulting in inaccurate measurements.
[a]Water changed weekly and measured by cage.
[b]N = number of cages.
[c]Mean ± SE (g per day) = estimated least squares mean and standard error.
[d]p values in the 0 ppm column are the p values for the trend test; p values in the dosed columns are Dunnett's adjusted p values for pairwise comparisons of the dosed groups to the 0 ppm group.

Appendix G. Reproductive Toxicology Studies

Tables

Table G-1. Summary of Reproductive Tissue Evaluations for Male Rats in the Three-month Feed Study of (+)-Usnic Acid

	0 ppm[a,b]	120 ppm	360 ppm	720 ppm
Weights (g)[c]				
Necropsy body weight	345.5 ± 5.27	338.7 ± 4.00	336.2 ± 5.73	320.3 ± 3.45**
L. cauda epididymis	0.222 ± 0.0062	0.206 ± 0.0080	0.225 ± 0.0062	0.241 ± 0.0157
L. epididymis	0.477 ± 0.0104	0.461 ± 0.0112	0.492 ± 0.0105	0.515 ± 0.0162
L. testis	1.574 ± 0.0164	1.578 ± 0.0173	1.603 ± 0.0268	1.652 ± 0.0152**
Spermatid Measurements[d]				
Spermatid heads (10^6/g testis)	134.24 ± 6.557	149.97 ± 9.901	148.08 ± 11.715	133.19 ± 14.652
Spermatid heads (10^6/testis)	211.29 ± 10.499	236.24 ± 15.363	238.03 ± 20.099	219.19 ± 23.884
Epididymal Spermatozoal Measurements[d]				
% Sperm motility	87.1 ± 1.24	86.7 ± 1.15	87.2 ± 0.81	85.5 ± 1.22
Sperm (10^6/g cauda epididymis)	839.8 ± 15.79	854.5 ± 15.46	833.4 ± 14.81	783.0 ± 42.92
Sperm (10^6/cauda epididymis)	186.1 ± 5.93	176.4 ± 7.94	187.1 ± 5.58	183.7 ± 5.00

[a]n = 10 for each group.
[b]Data are presented as mean ± standard error.
[c]Each dose is compared to the control with the William's test when a trend is present, $p \leq 0.01$ from Jonckheere's trend test, otherwise Dunnett's test is applied (** = $p \leq 0.01$).
[d]Each dose is compared to the control with Shirley's test when a trend is present, $p \leq 0.01$ from Jonckheere's trend test, otherwise Dunn's test is applied.

Table G-2. Estrous Cycle Characterization for Female Rats in the Three-month Feed Study of (+)-Usnic Acid

	0 ppm[a,b]	120 ppm	360 ppm	720 ppm
Necropsy Body Weight (g)[c]	198.2 ± 4.52	195.8 ± 3.95	184.8 ± 3.22	168.6 ± 2.24
Proportion of Regular Cycling Females[d]	10/10	10/10	10/10	7/10
Estrous Cycle Length (days)[e]	5.0 ± 0.00	5.0 ± 0.00	5.0 ± 0.00	5.2 ± 0.15*
Estrous Stages (% of cycle)[f]				
Diestrus	57.5	59.4	56.3	69.4**
Proestrus	19.4	20.6	20.6	13.1
Estrus	19.4	20.0	21.3	13.8
Metestrus	1.9	0.0	0.6	0.0
Uncertain diagnosis	1.9	0.0	1.3	3.8

[a]n = 10 for each group.
[b]Necropsy body weights and estrous cycle length data are presented as mean ± standard error.
[c]Statistically evaluated using the William's and Dunnett's tests
[d]Number of females with a regular cycle/number of females cycling.
[e]Statistically evaluated using the Shirley's and Dunn's tests (* $p \leq 0.05$).
[f]By multivariate analysis of variance, dosed females do not differ significantly from the vehicle control females in the relative length of time spent in the estrous stages. Tests for equality of transition probability matrices among all groups and between the vehicle control group and each dosed group indicated a significantly extended diestrus in the 720 ppm group relative to the control group ** $p \leq 0.01$). No other significant differences in transition probabilities among the groups were observed.

Table G-3. Summary of Reproductive Tissue Evaluations for Male Mice in the Three-month Feed Study of (+)-Usnic Acid

	0 ppm[a,b]	60 ppm	180 ppm	360 ppm
Weights (g)[c]				
Necropsy body weight	28.3 ± 0.62	28.0 ± 0.68	27.2 ± 0.65	26.4 ± 0.40
L. cauda epididymis	0.020 ± 0.0009	0.018 ± 0.0009	0.017 ± 0.0009	0.017 ± 0.0010
L. epididymis	0.050 ± 0.0015	0.048 ± 0.0021	0.047 ± 0.0017	0.045 ± 0.0013
L. testis	0.118 ± 0.0024	0.115 ± 0.0027	0.116 ± 0.0030	0.113 ± 0.0022
Spermatid Measurements[d]				
Spermatid heads (10^6/g testis)	222.01 ± 28.547	263.46 ± 15.699	222.59 ± 19.137	222.38 ± 14.054
Spermatid heads (10^6/testis)	26.51 ± 3.677	30.22 ± 2.008	25.62 ± 2.123	25.17 ± 1.587
Epididymal Spermatozoal Measurements[d]				
% Sperm motility	85.3 ± 1.15	84.4 ± 0.78	86.5 ± 1.11	84.6 ± 1.42
Sperm (10^6/g cauda epididymis)	856.9 ± 14.86	810.6 ± 30.55	840.3 ± 22.16	818.5 ± 13.17
Sperm (10^6/cauda epididymis)	17.2 ± 0.98	14.8 ± 1.06	14.5 ± 0.96	14.1 ± 0.87

[a]n = 10 for each group.
[b]Data are presented as mean ± standard error.
[c]Each dose is compared to the control with the Williams test when a trend is present, $p \leq 0.01$ from Jonckheere's trend test, otherwise Dunnett's test is applied.
[d]Each dose is compared to the control with the Shirley test when a trend is present, $p \leq 0.01$ from Jonckheere's trend test, otherwise Dunn's test is applied.

Table G-4. Estrous Cycle Characterization for Female Mice in the Three-month Feed Study of (+)-Usnic Acid

	0 ppm[a,b]	60 ppm	180 ppm	360 ppm
Necropsy Body Weight (g)[c]	21.9 ± 0.31	21.6 ± 0.59	22.0 ± 0.39	22.2 ± 0.45
Proportion of Regular Cycling Females[d]	10/10	10/10	10/10	10/10
Estrous Cycle Length (days)[e]	4.7 ± 0.60	4.3 ± 0.26	5.7 ± 0.94	4.4 ± 0.30
Estrous Stages (% of cycle)[f]				
Diestrus	48.8	53.1	49.4	46.9
Proestrus	3.8	3.1	5.6	2.5
Estrus	31.3	31.9	26.3	40.0
Metestrus	11.3	9.4	11.9	8.1
Uncertain diagnosis	5.0	2.5	6.9	2.5

[a]n = 10 for each group.
[b]Necropsy body weights and estrous cycle length data are presented as mean ± standard error.
[c]Statistically evaluated using the William's and Dunnett's tests.
[d]Number of females with a regular cycle/number of females cycling.
[e]Statistically evaluated using Shirley's and Dunn's tests.
[f]By multivariate analysis of variance, dosed females do not differ significantly from the vehicle control females in the relative length of time spent in the estrous stages. Tests for equality of transition probability matrices were conducted among all groups and between the vehicle control group and each dosed group. No significant differences in transition probabilities among the groups were observed.

Appendix H. Chemical Characteristics and Dose Formulation Studies

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H.1. Procurement and Characterization of Usnic Acid

(+)-Usnic acid [(*d*)-usnic acid, 2,6-diacetyl-7,9-dihydroxy-8,9b(*R*)-dimethyldibenzofuran-1,3(2*H*,9*bH*)-dione] was obtained from Sigma-Aldrich Co. (Milwaukee, WI) in one lot (02503HD). Identity, purity, and stability analyses were conducted by the study laboratory. Reports on the analyses performed in support of the study of usnic acid are on file at the National Center for Toxicological Research (NCTR).

The chemical, a bright-yellow powder, was identified as (+)-usnic acid by the study laboratory using ^{1}H and ^{13}C nuclear magnetic resonance (NMR), high-performance liquid chromatography (HPLC), gas chromatography, and HPLC-photodiode array (PDA) with electrospray ionization mass spectrometry (HPLC-/+ESI-MS, GC/EI-MS, and HPLC-PDA-\+ESI-MS) and MS/MS. All spectra were consistent with the structure of usnic acid and the GC/MS results showed one component which matched the NIST 2005 library for (+)-usnic acid (Figure H-1, Figure H-2, and Figure H-3).

The purity of lot 02503HD was determined by the study laboratory using HPLC-PDA (Waters, Milford, MA), with a mobile phase of 73% acetonitrile:27% water, 0.05% formic acid at a flow rate of 1.1 mL/minute through a Phenomenex Prodigy ODS-3 (250 mm × 4.6 mm, 5 µm, 100 Å pore size) C18 HPLC column for 30 minutes. Consistent with the extracted wavelengths from 200 to 600 nm, a Max-plot chromatogram was compared to the solvent blank and the estimated purity was determined to be 99.7%. Purity was verified using ^{1}H and ^{13}C NMR, which indicated a 2.6% impurity on a molar basis. The overall purity of lot 02503HD was determined to be approximately 98%.

(+)-Usnic acid was stable at normal temperature and pressures.

H.2. Preparation and Analyses of Dose Formulations

The dose formulations were prepared approximately every 6–8 weeks by hand blending a premix and blending with additional feed in a Patterson-Kelly twin-shell blender (Table H-1). Dose formulations were stored in stainless-steel feed cans at 2°C–8°C for up to 55 days.

Homogeneity and stability studies were performed on the 15 ppm dose formulations by the study laboratory using the HPLC-PDA method described above. Homogeneity and stability were confirmed for 14 days at room temperature and up to 17 weeks at 2°C–8°C.

Analyses of the dose formulations were conducted using the HPLC-PDA method described above. All dose formulations were analyzed (Table H-2). All dose formulations were within 10% of the target concentration.

Table H-1. Preparation and Storage of Dose Formulations in the Three-month Feed Studies of (+)-Usnic Acid

Preparation

A premix of usnic acid and feed was ground by hand with a mortar and pestle, then combined with the remaining feed and blended in a Patterson-Kelly twin-shell blender with the intensifier bar on for 30 minutes. Two batches of the 15 ppm dose formulation and three batches each of the 30, 60, 120, 180, 360, and 720 ppm dose formulations were prepared. The dose formulations were prepared approximately every 6–8 weeks. Cage feeders were changed weekly.

Chemical Lot Number

02503HD

Storage Conditions

Stored in stainless-steel feed cans at 2°C–8°C

Study Laboratory

National Center for Toxicological Research (Jefferson, AR)

Table H-2. Results of Analyses of Dose Formulations Administered to Rats and Mice in the Three-month Feed Studies of (+)-Usnic Acid

Date Prepared	Date Analyzed	Target Concentration (ppm)	Determined Concentration[a] (ppm)	Difference from Target (%)
Rats and Mice				
July 29, 2008	July 29, 2008	15[b]	14.3 ± 1.0	–4.4
		30	28.6 ± 0.6	–4.8
		60	55.1 ± 0.3	–8.1
		120	111 ± 0	–7.7
		180	168 ± 1	–6.6
		360	344 ± 1	–4.6
		720	688 ± 1	–4.5
September 9, 2008	September 9, 2008	30	27.3 ± 0.5	–9.1
		60	56.0 ± 0.6	–6.7
		120	118 ± 0	–1.5
		360	339 ± 8	–5.8
		720	674 ± 9	–6.4
September 30, 2008	September 30, 2008	15[c]	15.0 ± 0.5	–0.3
		180	181 ± 2	1
October 9, 2008	October 9, 2008	30	30.4 ± 1.1	1
		60	57.4 ± 0.7	–4.3
		120	115 ± 1	–3.9
		360	357 ± 6	–0.9
		720	699 ± 38	–2.9

[a]Results of three analyses (mean ± standard deviation). The limit of quantitation was estimated to be approximately 0.4 mg/kg diet.
[b]n = 9.
[c]n = 8.

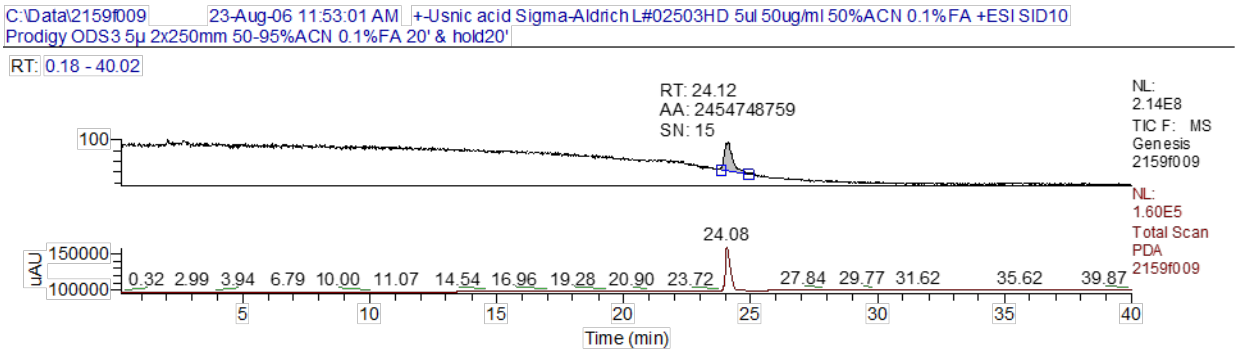

Figure H-1. Chromatogram of (+)-Usnic Acid

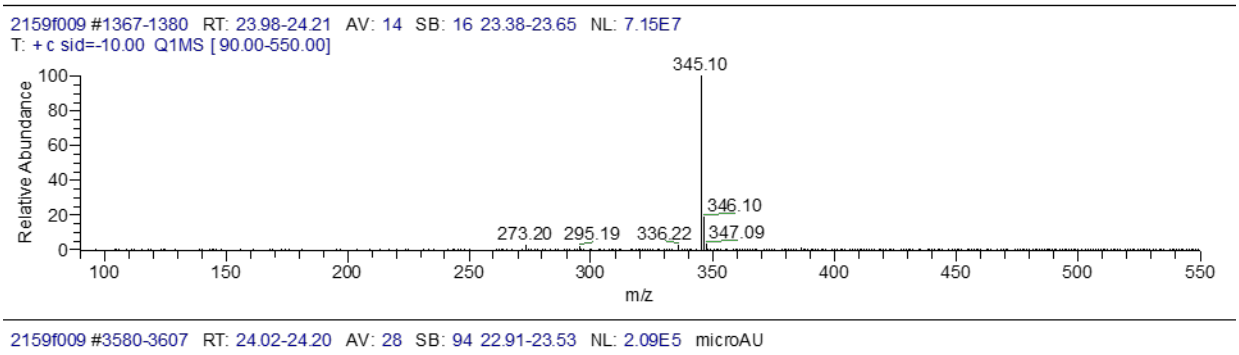

Figure H-2. Mass Spectrum of (+)-Usnic Acid

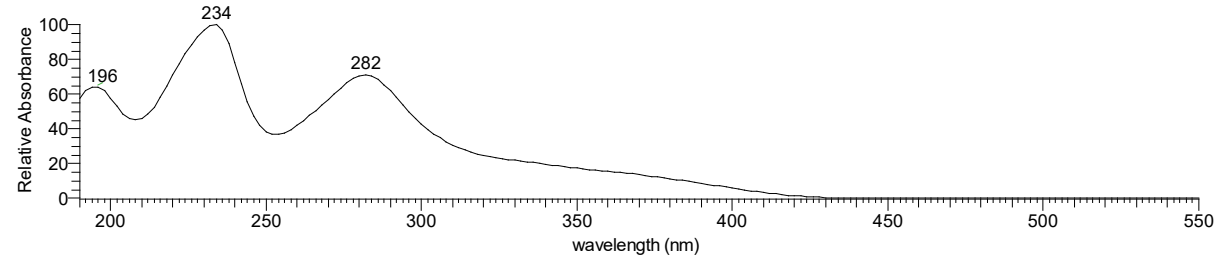

Figure H-3. Ultraviolet Spectrum of (+)-Usnic Acid

Appendix I. Ingredients, Nutrient Composition, and Contaminant Levels in NIH-41 Rodent Diet

Tables

Table I-1. Ingredients of NIH-41 Irradiated Diet

Ingredients	Percentage by Weight
Ground Whole Hard Wheat	34.9
Ground #2 Yellow Corn	21.0
Ground Whole oats	10.0
Wheat Middlings	10.0
Fish Meal (60% Protein)	9.0
Soy Oil	2.0
Soybean Meal (47.5% Protein)	5.0
Alfalfa Meal (17% Protein)	2.0
Corn Gluten Meal (60% Protein)	2.0
Dicalcium Phosphate	1.5
Brewers Dried Yeast	1.0
Premixes	0.5
Ground Limestone	0.5
Salt	0.5

Table I-2. Vitamins and Minerals in NIH-41 Irradiated Diet

	Amount	Source
Vitamins		
A	14,500,000 IU	Vitamin A palmitate or acetate
D_3	4,6000,000 IU	D-activated animal sterol
K	2.8 g	Menadione activity
dl-Alpha-tocopheryl Acetate	20,000 IU	
Choline	560 g	Choline chloride
Folic Acid	2.2 g	
Niacin	30.0 g	
d-Pantothenic Acid	18.0 g	d-Calcium pantothenate
Riboflavin Supplement	6.6 g	
Thiamin	10 g	Thiamin mononitrate
B_{12}	58.2 mg	
Pyridoxine	1.7 g	Pyridoxine hydrochloride
Biotin	113.5 mg	d-Biotin
Minerals		
Cobalt	400 mg	Cobalt carbonate
Copper	4 g	Copper sulfate
Iron	60 g	Iron sulfate
Magnesium	400 g	Magnesium oxide
Manganese	100 g	Manganese oxide
Zinc	10 g	Zinc oxide
Iodine	1,500 mg	Calcium iodate

Table I-3. Results of Analyses for Nutrients and Contaminants in NIH-41 Irradiated Diet[a]

Diet Sample SCR #	1456100001	1456100006	Average
Diet Lot #	042908M	072908M	
Nutrients			
Protein (% by wt.)	17.7	17.3	17.5
Total Fat (% by wt.)	7.90	9.45	8.68
Vitamin A, ppm	3.33	3.48	3.41
Vitamin B_1, ppm	27.6	16.1	21.9
Vitamin E, ppm	28.4	37.0	32.7
Contaminants			
Acrylamide, ppm	<LOQ	<LOQ	<LOQ
Aflatoxin-G_1, ppb	<MDL[c]	<MDL	<MDL

Aflatoxin-B_1, ppb	<MDL	<MDL	<MDL
Aflatoxin-B_2, ppb	<MDL	<MDL	<MDL
Aflatoxin-G_2, ppb	<MDL	<MDL	<MDL
Total Fumonisins, ppb	166	120	143
Volatiles (% by wt.)	8.35	9.45	8.68
Pb, ppm	0.28	0	0.14
Se, ppm	0.38	0.40	0.39
As, ppm	0.20	0.19	0.20
Cd, ppm	0	0.12	0.06

LOQ = limit of quantification (20 ppm); MDL = method detection limit (0.1 ppb for aflatoxins).
[a]Analyzed in lots that were used for the study.

Appendix J. Acute Toxicity

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J.1. Background

Acute toxicity (range-finding) studies, consisting of 2-week feed studies, were conducted as part of a National Center for Toxicological Research (NCTR) experimental study to investigate the acute toxicity of both (+)-usnic acid and *Usnea* lichens. This summary report focuses on the acute toxicity of (+)-usnic acid, and the effects of (+)-usnic acid on hepatic ATP concentration, which is a sensitive biomarker for mitochondrial uncoupling activity.

J.2. Experimental Methods

Animals: F344/N Nctr rats and B6C3F1/Nctr mice were provided by the NCTR breeding colonies and allocated to the experiment at 3 weeks of age. The experimental loading was staggered so that the animals were loaded in three replicates of either one or two per dose group at weekly intervals. Loading of mice preceded loading of rats. The animals were loaded on the Multigeneration Support System (MGSS) and assigned to dose groups at 6 weeks of age. The NCTR biometry staff provided a rack configuration and exposure randomization documents to control bias. Dosing, via feed, commenced at 8 weeks of age and proceeded for 14 days. The animal numbers in each dose group are listed in Table J-1 and Table J-2 for rats and mice, respectively.

The studies were run consecutively with staggered loading between December 5, 2006, and January 2, 2007, for the B6C3F1/Nctr mice and January 2, 2007, and January 30, 2007, for the F344/N Nctr rats. The animals were weighed weekly prior to dosing, and then twice weekly (i.e., every 3 or 4 days) during the dosing period so that any dose-related changes in body weight could be closely monitored. Dosed feed was allocated weekly in weighed amounts, weighed twice weekly, and the feed remaining measured so that daily feed consumption could be monitored. Morbid animals were immediately removed from the study and euthanatized.

The animals were sacrificed by decapitation and trunk blood was collected. Tissues were examined for gross abnormalities and observed lesions were processed for histopathological evaluation. These examinations were conducted under the supervision of the study pathologist. Gross examination data were recorded. The liver, kidneys, heart, and lungs from all animals were weighed wet as soon as possible after dissection.

All protocol-specified tissues were examined grossly, removed, and preserved in 10% neutral buffered formalin except eyes and testes, which were preserved in modified Davidson's fixative. The protocol-required tissues including all gross lesions were trimmed, processed, and embedded in Formula R®, sectioned at approximately 5 µm, and stained with hematoxylin and eosin. Tissues were examined microscopically and, when applicable, nonneoplastic lesions were graded for severity as 1 (minimal), 2 (mild), 3 (moderate), or 4 (marked).

Test article: (+)-Usnic acid (lot # 02503HD) was blended into powdered NIH-41 autoclaved rodent diet to achieve the required (+)-usnic acid concentration.

J.3. Results

Body weight: Exposure of male and female F344/N Nctr rats to (+)-usnic acid at doses of 1,250 and 2,500 ppm decreased body weight in both sexes (Figure J-1). These decreases were apparent

within 3 days of initiating dosing. The lower doses of (+)-usnic acid did not significantly reduce body weight or body weight gain. In B6C3F1/Nctr mice (Figure J-2), exposure to 1,200 ppm (+)-usnic acid resulted in large decreases in body weight in both males and females. There were smaller decreases in body weight in both male and female mice in the 600 ppm dosed group during the first week of exposure.

Survival: Exposure to 2,500 ppm (+)-usnic acid in feed was highly toxic for both male and female F344/N Nctr rats so that all 10 animals had either died or been removed as moribund by day 9 of exposure (Figure J-3). Two female rats and one male rat in the 1,250 ppm dosed group were removed by day 10 and day 11, respectively, due to morbidity and an additional morbid female was removed on day 14. All other animals on the study survived until terminal evaluation. B6C3F1/Nctr mice appeared to be more resilient to the effects of (+)-usnic acid than rats. All mice except three in the 1,200 ppm dosed group survived until terminal evaluation. In the 1,200 ppm dosed group, which corresponded to the same target dose of 200 mg/kg/day as the rat 2,500 ppm dose, two females and one male were removed due to morbidity by days 4, 8, and 6, respectively.

Histopathological effects of 2-week exposure to (+)-usnic acid: The histopathological effects of 2-week exposure of F344/N Nctr rats and B6C3F1/Nctr mice to (+)-usnic acid are listed in Table J-3 and Table J-4, respectively. Histopathological lesions were observed in both male and female F344/N Nctr rats exposed to either 1,250 or 2,500 ppm (+)-usnic acid in feed, but not at lower doses. Histopathological lesions were observed in both male and female B6C3F1/Nctr mice exposed to 1,200 ppm (+)-usnic acid in feed and in males exposed to 600 ppm, but not at lower doses. Thymic atrophy and gonadal lesions characteristic of extreme stress were observed in both rats and mice in the high dosed groups.

Hepatocellular alteration in this 2-week range-finding study included a variety of changes associated with hepatocellular toxicity. In both species, the affected animals displayed one or more of the following changes: cell swelling as well as cell contraction, cytoplasmic vacuolization or clearing, clumping (increased densities) of organelles, and in many animals, an increased cytoplasmic eosinophilia. Nuclear chromatin clumping with early karyorrhexis was occasionally observed; less frequently noted were single necrotic cells characterized by their dark appearance and being dislodged from their normal position. These changes represent patterns of cell degeneration with differences depending on the dose of toxin and the state of metabolism in the cell at the time of injury. The lesions described are part of a cascade of factors leading to irreversible degeneration and eventually necrosis.

Atrophy characterized by a decrease in the organ size was noted involving the thymus, seminal vesicles, and uterus (mice only) and was probably associated with decreased caloric intake (feed avoidance) and stress-associated metabolic changes. All other lesions were considered spontaneous background changes.

J.4. Adenosine 5’-Triphosphate Concentrations in Liver

Usnic acid is a known mitochondrial uncoupler and has been reported to decrease adenosine 5’-triphosphate (ATP) levels in cultured hepatocytes. As part of the 2-week range-finding study, ATP concentrations were evaluated in liver samples from both rats and mice exposed to (+)-usnic acid for 14 days.[89]

J.4.1. Methods

Hepatic ATP concentrations were determined using ATP Bioluminescent Assay kits (Sigma-Aldrich, St. Louis, MO, #FL-AA) on a Veritas 9100 Microplate Luminometer (Turner BioSystems, Sunnydale, CA). Liver extract (5%) was prepared in 2.5% trichloroacetic acid (TCA) and neutralized with 0.1 M Tris-Acetate buffer (pH 7.75) before using in a microtiter plate for ATP estimation. The luminescence data were converted to µmoles of ATP from standard solutions run with each assay plate. SAS (version 9.2, TS level 1M0) was used to produce means, standard error values and significant differences between dose groups via a Dunnett test evaluation and a linear trend test run under the SAS General Linear Models program.

J.4.2. Results

As shown in Table J-5, ATP concentrations in livers from both male and female F344/N Nctr rats were decreased by 2-week exposure to (+)-usnic acid. The decreases were greatest in the high dosed groups, which included moribund animals. In males, hepatic ATP content was significantly reduced in the 120, 360, 1,250, and 2,500 ppm dosed groups, but statistically significant decreases were not observed in the females despite the mean ATP content of the 1,250 dosed group being <50% of that of the control group. As shown in Table J-6, ATP concentrations in livers from both male and female B6C3F1/Nctr mice were decreased by 2-week exposure to (+)-usnic acid. The decreases were greatest in the high dosed groups, which included moribund animals. Statistically significant decreases were observed in the male 180, 600, and 1,200 ppm dosed groups and in the female 600 and 1,200 ppm dosed groups.

J.5. Serum and Hepatic Parameters Suggesting Increased Protein and Fat Catabolism in F344/N Nctr Rats Exposed to High Concentrations of (+)-Usnic Acid

As part of these 2-week range-finding toxicity studies, serum triglyceride and cholesterol concentrations were evaluated in trunk blood samples from both male and female F344/N Nctr rats that were sacrificed following 14 days of exposure or were removed from the study due to morbidity. In addition, serum leptin concentrations were evaluated in trunk blood from female F344/N Nctr rats that were sacrificed for the toxicokinetic study (measured by RIA) (Appendix K).

Hepatic tyrosine aminotransferase activity was assayed in samples of hepatic cytosol (100,000 g supernatant) prepared from livers of the male rats that either survived to their scheduled sacrifice or were removed early due to morbidity.

Exposure to high doses of (+)-usnic acid caused a significant decrease in serum leptin concentrations in the 360 and 1,250 ppm females (Figure J-5) and a significant increase in hepatic tyrosine aminotransferase activity in the 1,250 and 2,500 ppm males (Figure J-6). These changes are indicative of increased fat and protein catabolism, respectively. Serum triglyceride concentrations were also reduced in F344/N Nctr rats that were exposed to high concentrations of (+)-usnic acid in feed for 2 weeks (Table J-7). Serum triglyceride concentrations were significantly decreased in the 2,500 ppm males and in the 1,200 and 2,500 ppm females. Serum

cholesterol was significantly decreased in female but not male rats exposed to (+)-usnic acid at 2,500 ppm (Table J-7).

Table J-1. Two-week Range-finding Study for (+)-Usnic Acid in Rats

Dose[a]	Target Dose[b]	Males[c]	Females[c]
None	0	5	5
60	5	5	5
120	10	5	5
360	30	5	5
1,250	100	5	5
2,500	200	5	5
Totals		30	30

[a]Doses of usnic acid are given in ppm added to feed. The animals received dosed feed for 14 days prior to sacrifice.
[b]Approximate target dose in mg/kg/day calculated from NCTR historical mean body weight and feed consumption data.
[c]Number of animals used.

Table J-2. Two-week Range-finding Study for (+)-Usnic Acid in Mice

Dose[a]	Target Dose[b]	Males[c]	Females[c]
None	0	5	5
30	5	5	5
60	10	5	5
180	30	5	5
600	100	5	5
1,200	200	5	5
Totals		30	30

[a]Doses of usnic acid are given in ppm added to feed. The animals received dosed feed for 14 days prior to sacrifice.
[b]Approximate target dose in mg/kg/day calculated from NCTR historical mean body weight and feed consumption data.
[c]Number of animals used.

Table J-3. Incidence (%) of Nonneoplastic Lesions in Rats in the Two-week Study of (+)-Usnic Acid[a]

	0 ppm	60 ppm	120 ppm	360 ppm	1,250 ppm	2,500 ppm
Male						
Liver, Cellular Alteration	0	0	0	0	100	100
Thymus Atrophy	0	0	0	0	0	40
Seminal Vesicle Atrophy	0	0	20	0	100	80
Female						
Liver, Cellular Alteration	0	0	0	0	100	80
Thymus Atrophy	0	0	0	0	0	20

[a]Incidence (%) based on animals per group.

Table J-4. Incidence (%) of Nonneoplastic Lesions in Mice in the Two-week Study of (+)-Usnic Acid[a]

	0 ppm	30 ppm	60 ppm	180 ppm	600 ppm	1,200 ppm
Male						
Liver, Cellular Alteration	0	0	0	0	80	80
Thymus Atrophy	0	0	0	0	20	80
Seminal Vesicle Atrophy	0	0	0	0	20	80
Female						
Liver, Cellular Alteration	0	0	0	0	0	60
Thymus Atrophy	0	0	0	0	0	20
Uterine Atrophy	0	0	0	0	0	40

[a]Incidence (%) based on animals per group.

Table J-5. Hepatic Adenosine 5'-Triphosphate Concentrations in Rats Exposed to (+)-Usnic Acid for Two Weeks[a]

	0 ppm	60 ppm (5)[b]	120 ppm (10)	360 ppm (30)	1,250 ppm (100)	2,500 ppm (200)
Male						
Observed Dose[c]	0	5.0	9.3	27.5	85.4	239
ATP (µmol/g)	1.46 ± 0.03 (4)[d]	1.28 ± 0.25 (4)	0.85 ± 0.07 (3)	0.56 ± 0.06 (5)	0.80 ± 0.24 (3)	0.40 ± 0.19 (2)
	p = 0.003[e]	p = 0.486	p = 0.028	p ≤ 0.001	p = 0.018	p = 0.002
Female						
Observed Dose	0	5.6	10.5	30.6	93.1	182
ATP (µmol/g)	1.09 ± 0.13 (3)	1.02 ± 0.31 (5)	0.81 ± 0.14 (3)	0.60 ± 0.14 (5)	0.50 ± 0.06 (4)	0.26 (1)
	p = 0.062	p = 0.688	p = 0.436	p = 0.157	p = 0.110	

ATP = adenosine 5'-triphosphate.
[a]Livers from terminal sacrifice and moribund animals were evaluated. Livers from dead animals were not evaluated. Values are expressed as mean ± standard error with sample number in parentheses.
[b]Target dose in mg/kg/day.
[c]Actual dose calculated from observed body weight and feed consumption data.
[d]Number of samples examined shown in parentheses. Liver samples from some of the rats were not available for assay due to problems with freezer storage.
[e]Significance given with the control group is the dose trend; that given with other dose groups is the difference from the control group on a one-tailed Dunnett test.

Table J-6. Hepatic Adenosine 5'-Triphosphate Concentrations in Mice Exposed to (+)-Usnic Acid for Two Weeks[a]

	0 ppm	30 ppm (5)[b]	60 ppm (10)	180 ppm (30)	600 ppm (100)	1,200 ppm (200)
Male						
Observed Dose[c]	0	8.4	15.4	44.1	145	336
ATP (µmol/g)	1.64 ± 0.21 (4)[d]	1.75 ± 0.19 (5)	1.32 ± 0.16 (5)	0.85 ± 0.22 (5)	0.96 ± 0.17 (5)	0.71 ± 0.16 (5)
	p ≤ 0.001[e]	p = 0.910	p = 0.332	p = 0.016	p = 0.039	p = 0.005
Female						
Observed Dose	0	9.4	19.6	50.1	195	326
ATP (µmol/g)	1.21 ± 0.17 (5)	0.86 ± 0.13 (5)	1.05 ± 0.08 (5)	0.79 ± 0.18 (5)	0.64 ± 0.16 (5)	0.64 ± 0.11 (4)
	p = 0.020	p = 0.167	p = 0.519	p = 0.091	p = 0.021	p = 0.027

ATP = adenosine 5'-triphosphate.
[a]Livers from terminal sacrifice and moribund animals were evaluated. Livers from dead animals were not evaluated. Values are expressed as mean ± standard error with sample number in parentheses.
[b]Target dose in mg/kg/day.
[c]Actual dose calculated from observed body weight and feed consumption data.
[d]Number of samples examined shown in parentheses.
[e]Significance given with the control group is the dose trend; that given with other dose groups is the difference from the control group on a one-tailed Dunnett test.

Table J-7. Serum Triglyceride and Cholesterol Concentrations in Rats Exposed to (+)-Usnic Acid for Two Weeks[a]

	0 ppm	60 ppm (5)	120 ppm (10)	360 ppm (30)	1,200 ppm (100)	2,500 ppm (200)
Male						
Triglyceride[b]	96.2 ± 9.8 (5)	126 ± 23 (5)	95.2 ± 12.2 (5)	126 ± 23 (5)	51.2 ± 7.1 (5)	41.0 ± 2.7 (3)
	p = 0.0015[c]	p = 0.99	p = 0.83	p = 0.99	p = 0.102	p = 0.081
Cholesterol[d]	81.8 ± 5.9 (5)	85.2 ± 5.5 (5)	82.8 ± 3.4 (5)	114 ± 10 (5)	102 ± 6.7 (5)	86.3 ± 1.2 (3)
	p = 0.78	p = 0.93	p = 0.87	p = 1.0	p = 0.99	p = 0.94
Female						
Triglyceride	81.4 ± 11.1 (5)	71.2 ± 8.3 (5)	66.2 ± 4.4 (5)	64.0 ± 4.9 (5)	54.0 ± 6.9 (5)	34.3 ± 3.4 (3)
	p = 0.0006	p = 0.43	p = 0.23	p = 0.17	p = 0.027	p = 0.001
Cholesterol	106 ± 5.7 (5)	102 ± 1.9 (5)	115 ± 5.9 (5)	123 ± 3.7 (5)	90.4 ± 2.4 (5)	68.3 ± 8.6 (3)
	p ≤ 0.0001	p = 0.66	p = 1.0	p = 1.0	p = 0.051	p = 0.0001

[a]Values are expressed as mean ± standard error with sample number in parentheses.
[b]Activity given as units/L.
[c]p values listed under the control group values denote trend test significance, and those beneath the dosed group values denote significance of Dunnett test pairwise comparisons between the feed controls and that dosed group. Two-tailed Dunnett tests were used.
[d]Concentrations given as mg/dL.

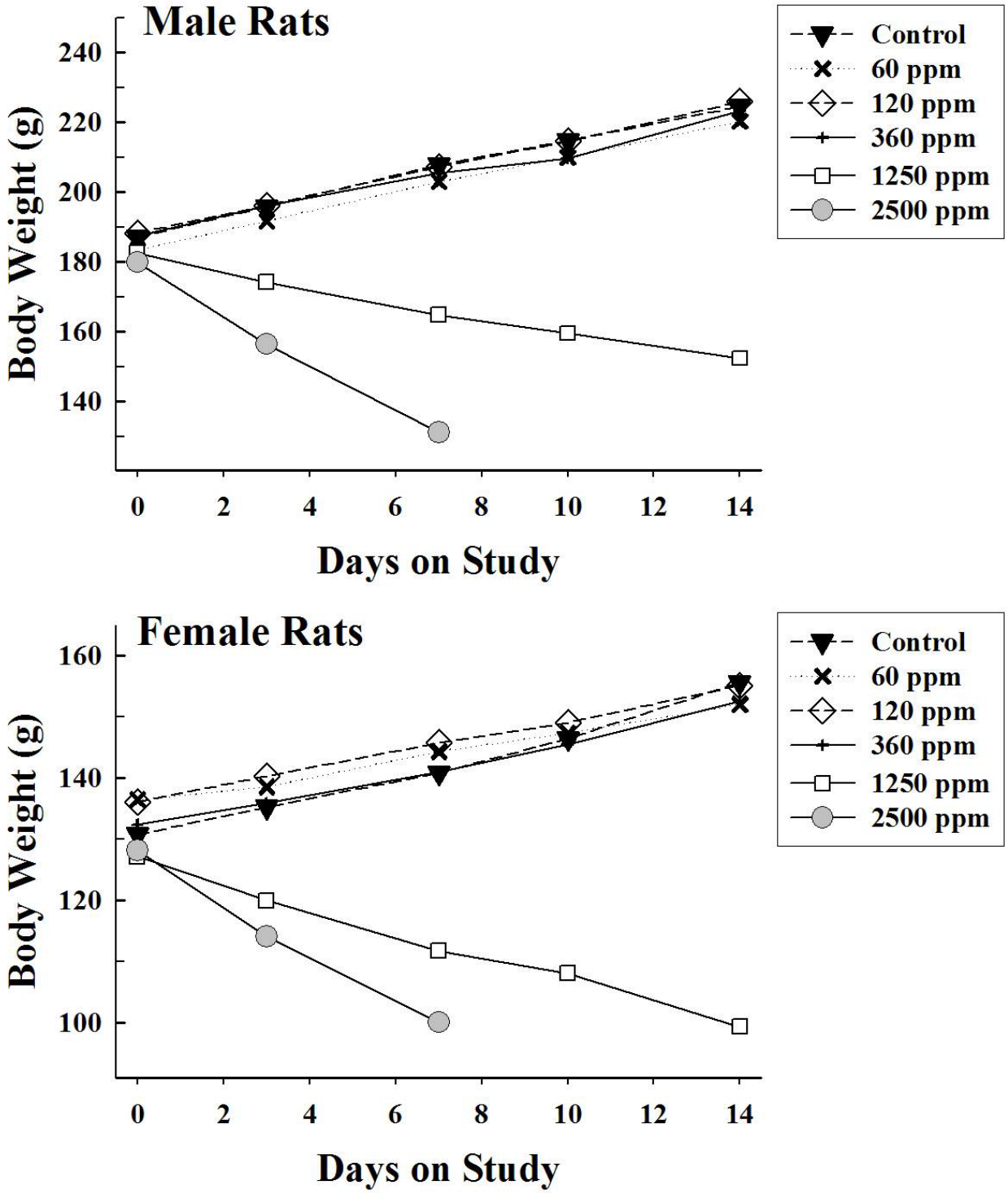

Figure J-1. Effect of Two-week Exposure to (+)-Usnic Acid in Feed on Mean Body Weight in Rats

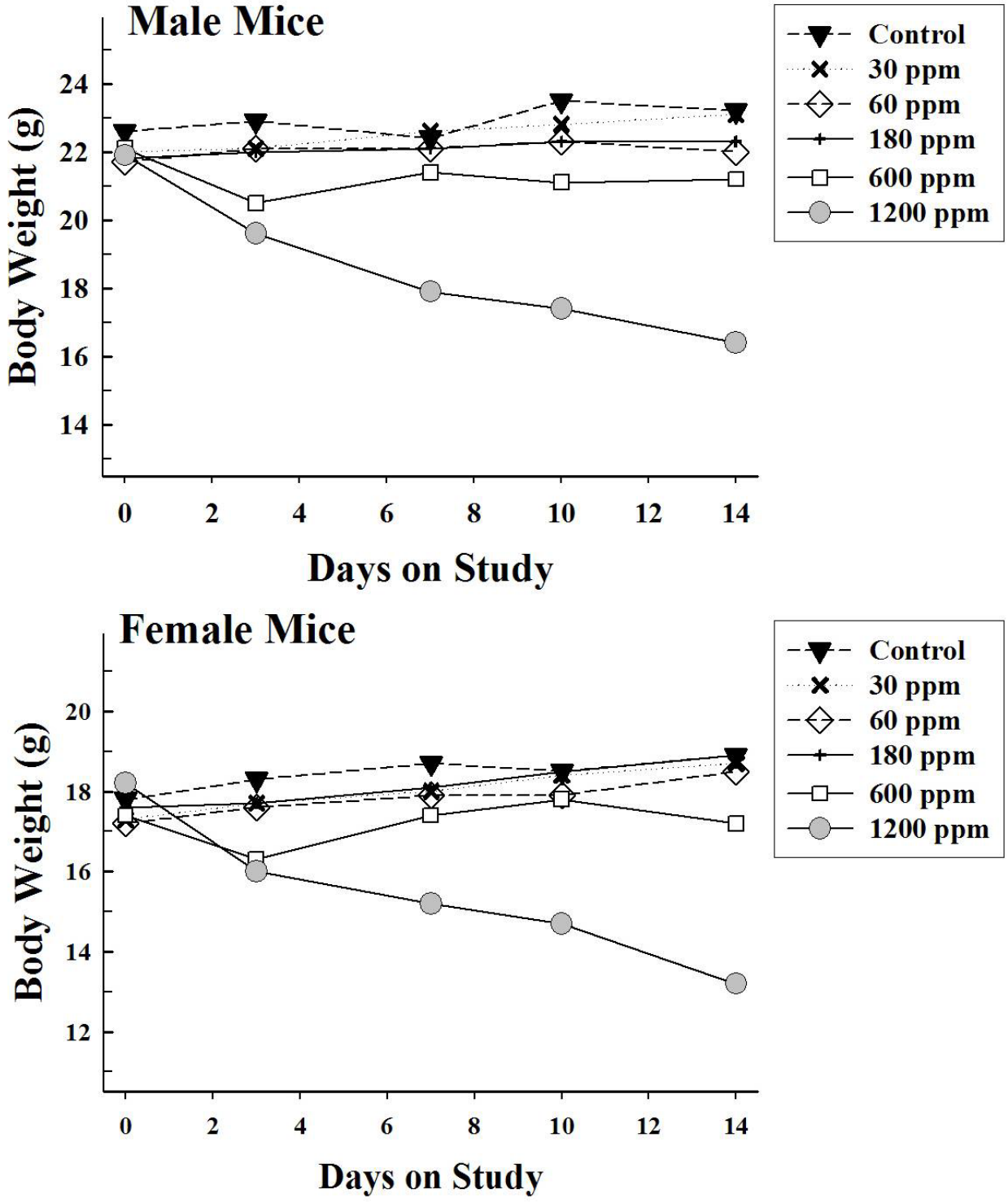

Figure J-2. Effect of Two-week Exposure to (+)-Usnic Acid in Feed on Mean Body Weight in Mice

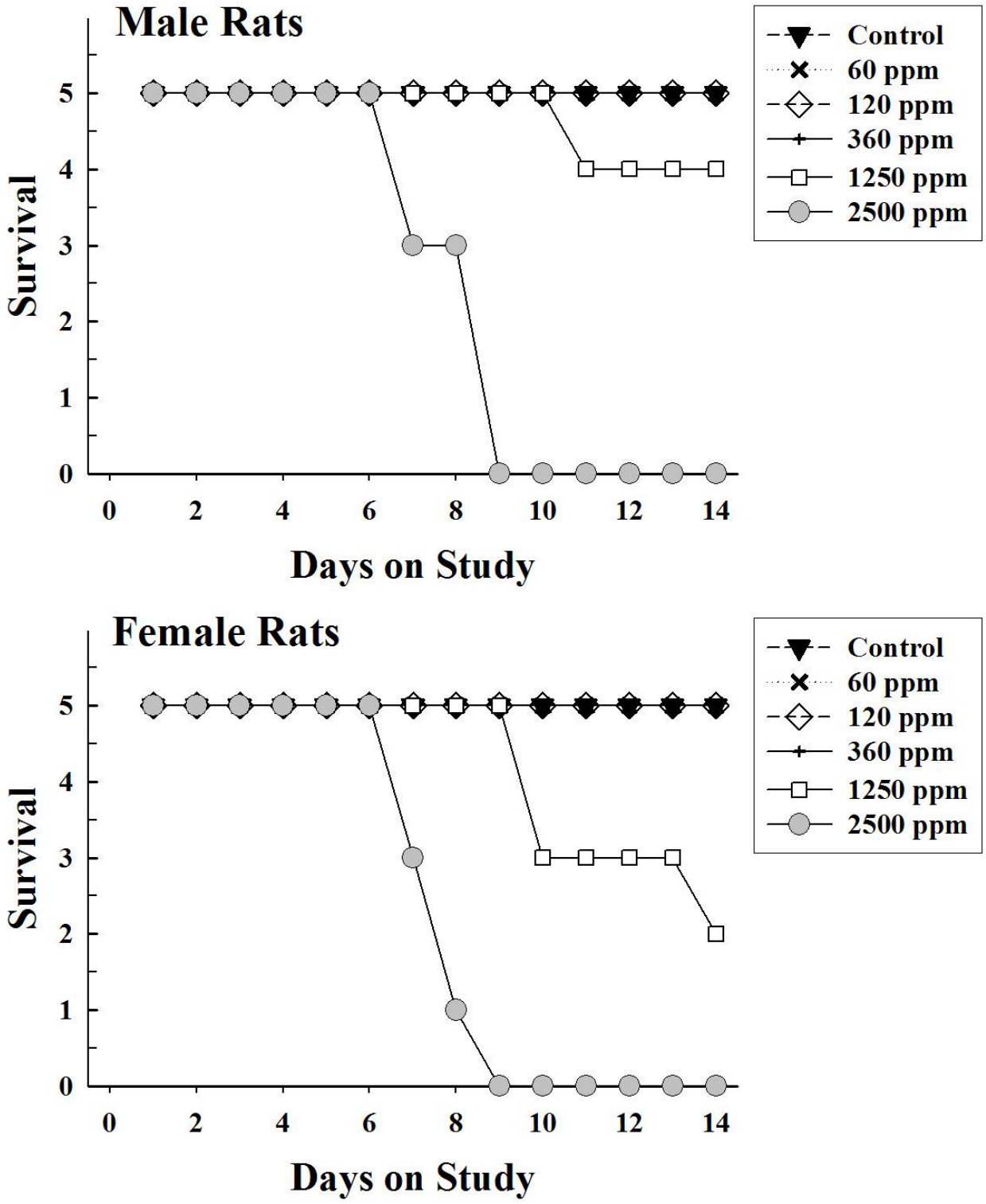

Figure J-3. Survival of Rats Exposed to (+)-Usnic Acid in Feed for Two Weeks

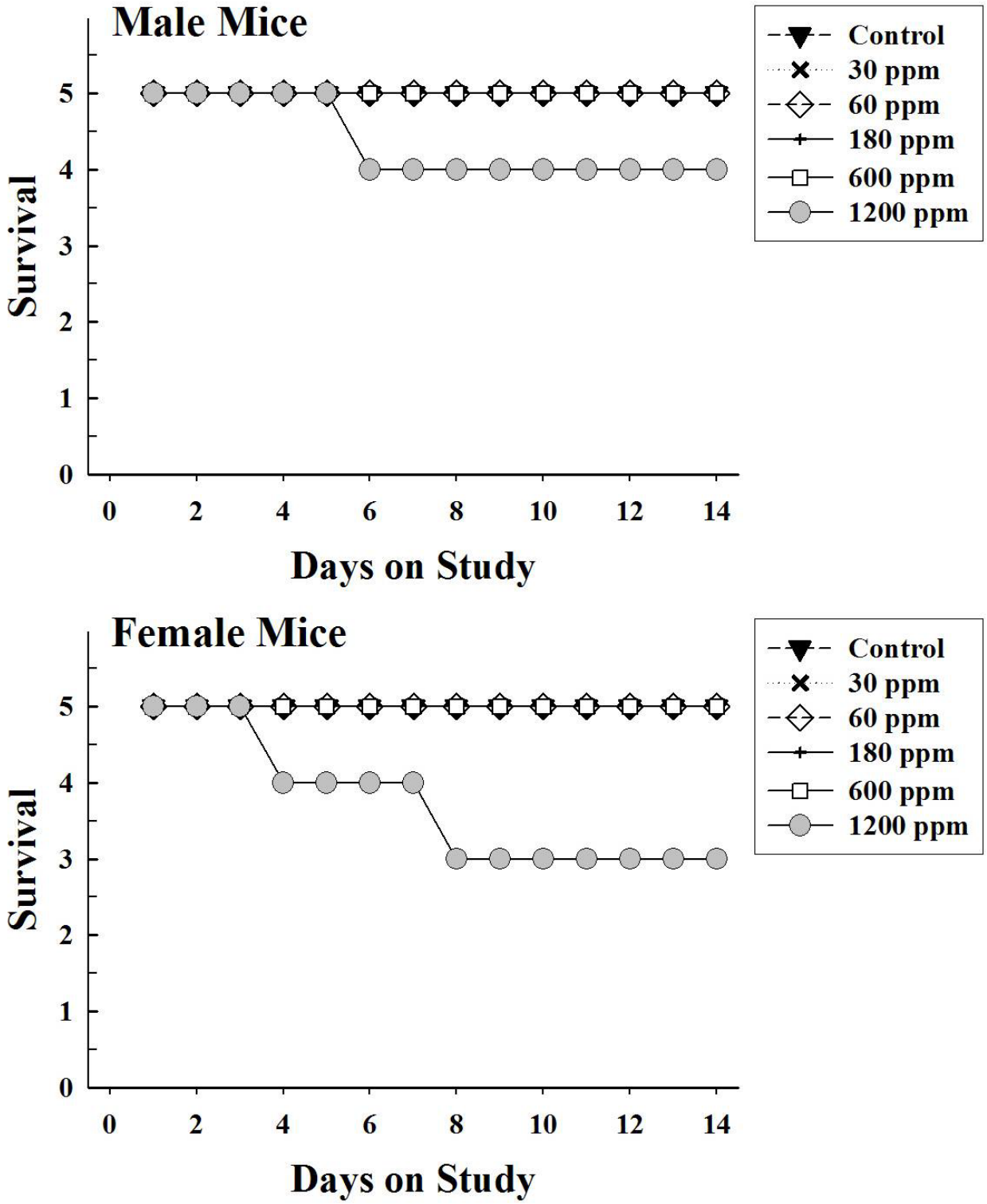

Figure J-4. Survival of Mice Exposed to (+)-Usnic Acid in Feed for Two Weeks

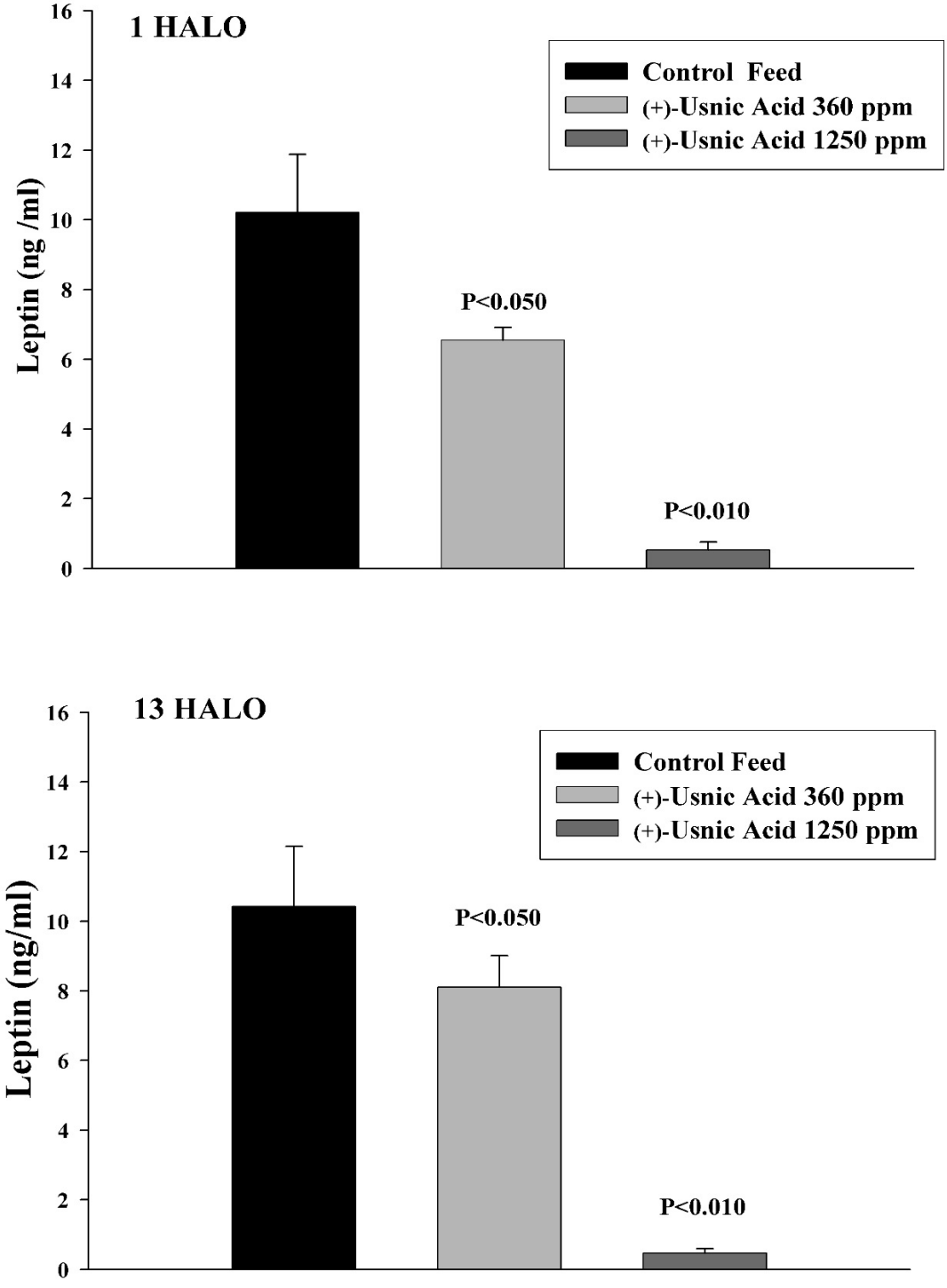

Figure J-5. Serum Leptin Concentrations in Female Rats Exposed to (+)-Usnic Acid for Two Weeks

HALO denotes hours after lights on.

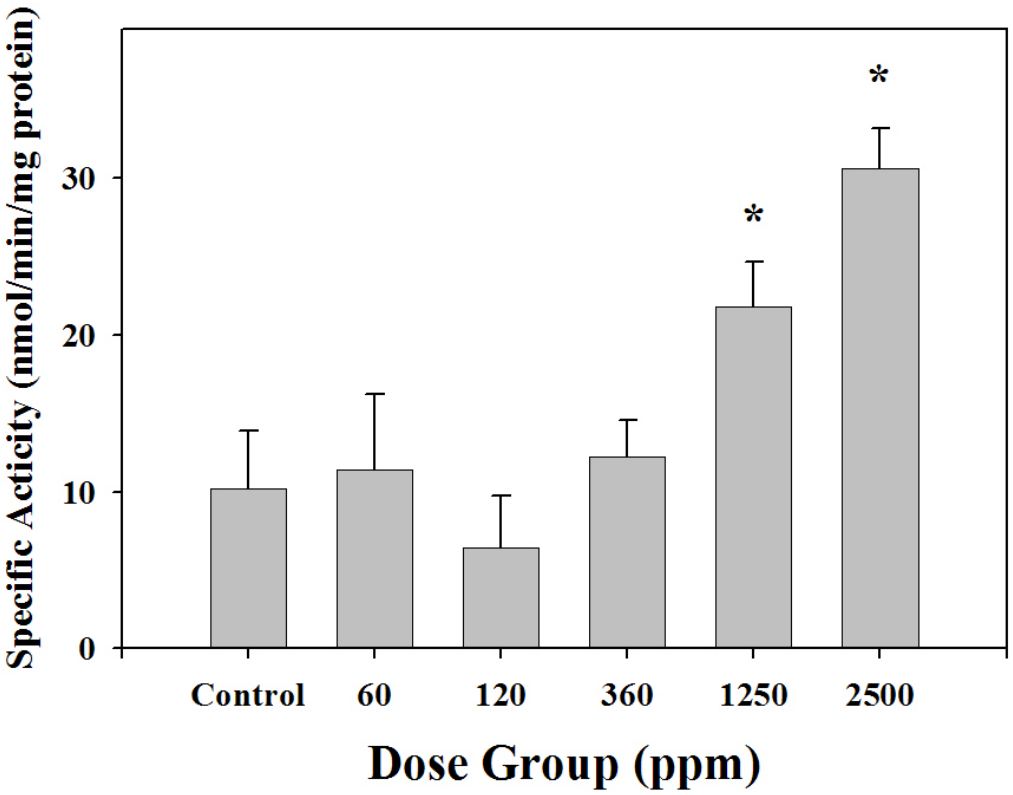

Figure J-6. Hepatic Tyrosine Aminotransferase in Cytosol from Male Rats Exposed to (+)-Usnic Acid for Two Weeks

Asterisks denote significant pairwise comparison to control group (Dunnett test); statistically significant at $p \leq 0.05$ (*).

Appendix K. Toxicokinetic Studies

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Tables

Figures

K.1. Background

Toxicokinetic studies were performed to complement the 2-week range-finding feed studies and were primarily designed to establish the steady-state concentrations of usnic acid in the liver of F344/N Nctr rats and B6C3F1/Nctr mice following a 2-week exposure to either (+)-usnic acid or *Usnea* lichens in feed. The data were required to compare the in vitro and in vivo hepatotoxicity of usnic acid. The studies therefore utilized individual animals for each time point rather than taking serial blood samples so that liver and other tissues could be collected. This method also had the advantage that the animal's feeding behavior was not disrupted as would have occurred if serial blood samples had been collected.

K.2. Materials and Methods

Eight-week-old F344/N Nctr rats and B6C3F1/Nctr mice were fed either (+)-usnic acid or *Usnea* lichens in feed, as was used for the 2-week range-finding studies (Appendix J of this report and Appendix J of NTP TOX 105[110]). Dose groups and sacrifice time points for the rats and mice are listed in Table K-1 and Table K-2, respectively. A major objective of this study was to provide data on hepatic concentrations of (+/-)-usnic acid following exposure in feed throughout the daily feeding cycle, which required animals to be euthanized for each time point. Because of the large number of animals required and the lack of significant sex differences in observed effects on body weight and survival (Appendix J), only one sex from each species was evaluated (i.e., female rats and male mice). Feed (powdered NIH-41) and water were provided ad libitum. The animals were housed one per cage and kept on a 12-hour light and dark cycle, but each experimental group was divided between different animal rooms on light cycles that were 11 hours out of phase so that the required circadian sacrifices could be conducted within normal work hours. The animals were serially sacrificed by decapitation on the 13th day of dosing, at 4-hour intervals starting at 1 hour after lights off (HALO). Liver and serum were collected from the sacrificed animals and stored at −80°C until analysis.

Two methods were used to determine hepatic usnic acid concentrations. Method 1 was a macro method, which incorporated an enzyme hydrolysis stage to determine whether conjugated usnic acid was present in the tissue. It was used to analyze the rat liver samples and the initial analysis of mouse liver. Subsequently, Method 2, which incorporated an internal standard, required less tissue, and allowed higher throughput, was developed to complete the analysis of mouse liver and to evaluate both rat and mouse serum. Both methods gave similar values when individual liver samples were compared. Neither method resolved (−)-usnic acid from (+)-usnic acid, therefore, the detected usnic acid is referred to as (+/−)-usnic acid. However, when chiral column separation was used to resolve the usnic acid enantiomers in samples of the *Usnea* lichen preparations used in this study, the relative concentrations were 97.5% ± 0.2% (+)-usnic acid and 2.5% ± 0.2% (−)-usnic acid (Appendix H). Because inter-conversion of the enantiomers is not expected to occur in vivo, it was therefore assumed that the (+/−)-usnic acid present in tissue samples from animals exposed to (+)-usnic acid was essentially 100% (+)-usnic acid, and that present in tissue samples from animals exposed to *Usnea* lichens was essentially >97% (+)-usnic acid.

K.2.1. Method 1

Liver samples (0.5–1.0 g) were homogenized in sufficient homogenization buffer (0.2 M sodium phosphate dibasic [Sigma-Aldrich, trihydrate] adjusted to pH 4.6 with formic acid) to produce a 10% (w/v) homogenate, using an Ultra-Torrax homogenizer followed by ultra-sonication with a Vibra-Cell sonicator at 100 kJ (5–10 seconds). *Helix pomatia* β-glucuronidase (Sigma-Aldrich, H-5, 400 units/mg) was reconstituted with 0.02 M ammonium acetate buffer to make a stock enzyme solution containing 40,000 units/mL. Aliquots of β-glucuronidase solution were added to 1 mL aliquots of liver homogenate to produce final β-glucuronidase concentrations of 4,000 to 24,000 units/mL and were incubated in a water bath at 39°C for 20 hours. For nonhydrolyzed controls, equal volumes of acetate buffer were substituted for the β-glucuronidase solution. After incubation, the samples were extracted with 3 × 3 mL of ethyl acetate and the combined extracts evaporated under nitrogen at 40°C. The residue was reconstituted with 2 mL of acetonitrile: ethyl acetate (75:25) acidified with 0.6% formic acid. The residue solutions were filtered through 0.45 µm nylon syringe filters into amber HPLC vials.

Samples were analyzed using a Waters HPLC-PDA system, which included a Model 600E controller, 717plus autoinjector and a 996 photodiode array detector. Injections (35 µL) were passed through a 250 × 4.60 mm (4 µm particle) Phenomenex Prodigy 5 µm ODS-3 100 Å column maintained at 35°C. Mobile phase consisted of 73% acetonitrile in 0.05% formic acid in water. The flow rate was held at 1.1 mL/minute for 30 minutes and usnic acid peaks were detected at 232 nm. Recovery of (+)-usnic acid was 100% with or without enzyme from spiked control tissue.

K.2.2. Method 2

This method utilized dexamethasone as an internal standard and a Waters Acquity HPLC system. Weighed samples of frozen liver, weighing approximately 50 mg, were homogenized in 950 µL of homogenization buffer (0.2 M sodium phosphate brought to pH 4.6 with formic acid) using a Vibra-Cell sonicator at 100 kJ (5–10 seconds). Internal standard (30 pmol of dexamethasone-21-acetate (Sigma-Aldrich) in 30 µL of acetonitrile) was added to each sonicate, followed by a further 5-second sonication. Three 300 µL aliquots of the resulting sonicates were extracted three times with 1 mL of ethyl acetate and the pooled ethyl acetate extracts from each aliquot were evaporated to dryness under nitrogen at 40°C. The dried sample extracts were resuspended in 200 µL Mobile Phase A/B, 20/80 (see below) and filtered through 0.22 µm polyvinylidene difluoride (PVDF) filters (*Ultrafree* centrifugal filters, Millipore Inc., Billerica, MA). For each sample replicate, 40 µL of filtrate was mixed with 40 µL Mobile Phase A (see below) in an ultra-performance liquid chromatography (UPLC) sample vial and the resulting mixture analyzed.

The UPLC system consisted of an Acquity sample manager, a solvent manager and photodiode array modules (Waters Inc., Milford, MA) and utilized an Acquity BEH C18, 1.7 µm, 2.1 × 50 mm UPLC column in conjunction with a BEH C18, 1.7 µm, 2.1 × 5 mm Acquity Vanguard precolumn. Mobile Phase A was water/acetonitrile/acetic acid (94.5/5.0/0.5, v/v/v) and Mobile Phase B was acetonitrile/acetic acid (99.5/0.5, v/v) and the (+/−)-usnic acid and dexamethasone-21-acetate peaks were resolved with a binary linear gradient of 40% B to 100% B between 1 and 4 minutes of a 10-minute sample cycle time with a flow rate of 0.25 mL/min. The sample runs were returned to initial conditions at 7 minutes. The column was maintained at ambient temperature and sample injection volume was 5 µL. The peaks were monitored at

258 nm. Sample recovery was calculated using standards wherein 10 pmol of dexamethasone-21-acetate were added directly to the UPLC sample vial and (+)-usnic acid standard curves were constructed for each sample batch by adding known concentrations of (+)-usnic acid to homogenates of liver from untreated rats or mice to give a concentration range equivalent to 20–300 µM in liver.

K.2.3. Assay of (+/−)-Usnic Acid in Serum

For rat samples, 25 µL of thawed serum was mixed with 10 pmol of dexamethasone-12-acetate in 10 µL acetonitrile, 3.5 µL of 1 M sodium acetate (adjusted to pH 5.0 with acetic acid) and 500 µL of acetonitrile. The mixtures were sonicated for 5 minutes in a sonicator bath, and then centrifuged at 1,200 g for 5 minutes. Mouse serum samples were processed in the same way, except 12.5 µL of sample serum and 12.5 µL of commercial mouse serum were used due to the limited volumes of mouse sample serum that were available. After centrifugation, a 400 µL aliquot of each supernatant was evaporated to dryness in a centrifugal vacuum evaporator (Savant SpeedVac, Thermo Scientific). The dried sample extracts were resuspended in 100 µL Mobile Phase A/B, 20/80 (see Method 2) and filtered through 0.22 µm PVDF filters. For each sample replicate, 40 µL of filtrate was mixed with 40 µL Mobile Phase A (see Method 2) in a UPLC sample vial and the resulting mixture analyzed by the UPLC method that was used for liver samples (see Method 2). The (+)-usnic acid standard curves were constructed for each sample batch by adding known concentrations of (+)-usnic acid to serum obtained from untreated rats or mice (Innovative Research Inc., Novi, MI) to give a concentration range equivalent to 20–300 µM in sample serum.

K.3. Results

K.3.1. Rat Liver

Hepatic concentrations of (+/−)-usnic acid in female F344/N Nctr rats exposed to either 360 or 1,250 ppm (+)-usnic acid or ground *Usnea* lichens at a dose equivalent to 360 ppm (+/−)-usnic acid are shown in Figure K-1. For each exposure dose, (+/−)-usnic acid concentrations appeared to have reached a steady state and did not significantly vary with the circadian time point at which the animal was sacrificed. Increasing the (+)-usnic acid dose 3.5-fold from 360 to 1,250 ppm only increased hepatic concentrations of (+/−)-usnic acid from approximately 75–80 nmol/g wet weight (µM cellular concentration) to approximately 90–95 nmol/g wet weight (µM). Interestingly, hepatic concentrations of (+/−)-usnic acid in rats exposed to feed containing *Usnea* lichens at a concentration that provided 360 ppm of (+/−)-usnic acid exceeded that of both the 360 and 1,250 ppm (+)-usnic acid dosed groups. Hydrolysis of liver homogenates with β-glucuronidase did not increase hepatic (+/−)-usnic acid concentrations, which suggested that only negligible amounts of usnic acid were glucuronidated. The actual mean daily doses of pure (+)-usnic acid or (+/−)- usnic acid in *Usnea* lichens were calculated from observed feed consumption and body weight data and are compared with the target doses in Table K-3. Actual doses were slightly higher than target for both the rats and mice.

K.3.2. Rat Serum

Serum concentrations of (+/−)-usnic acid in female F344/N Nctr rats exposed to either 360 or 1,250 ppm (+)-usnic acid or ground *Usnea* lichens at a dose of 360 ppm (+/−)-usnic acid are

shown in Figure K-2. While mean serum (+/−)-usnic acid concentrations were similar to hepatic concentrations for the 360 ppm (+)-usnic acid and 360 ppm *Usnea* lichens dosed groups, serum concentrations were greater and more variable than hepatic concentrations for the 1,250 ppm dosed group and ranged between 170 and 240 μM at different time points.

K.3.3. Mouse Liver

Hepatic concentrations of (+/−)-usnic acid in male B6C3F1/Nctr mice exposed to either 180 or 600 ppm (+)-usnic acid or ground *Usnea* lichens at a dose equivalent to 180 ppm (+/−)-usnic acid are shown in Figure K-3. For each exposure dose, (+/−)-usnic acid concentrations appeared to have reached a steady state and did not significantly vary with the circadian time point at which the animal was sacrificed. In contrast to rats, the hepatic concentrations of (+/−)-usnic acid in both the 180 ppm (+)-usnic acid and the 180 ppm *Usnea* lichens dosed groups in mice were similar and ranged between 38 and 58 nmol/g wet weight (μM cellular concentration). The hepatic concentration of (+/−)-usnic acid in the 600 ppm (+)-usnic acid was greater and ranged between 85 and 115 nmol/g wet weight (μM). Hydrolysis of liver homogenates with β-glucuronidase did not increase hepatic (+/−)-usnic acid concentrations, which suggested that only negligible amounts of usnic acid were glucuronidated.

K.3.4. Mouse Serum

Serum concentrations of (+/−)-usnic acid in male B6C3F1/Nctr mice exposed to either 180 or 600 ppm (+)-usnic acid or ground *Usnea* lichens at a dose of 180 ppm (+/−)-usnic acid are shown in Figure K-4. Serum (+/−)-usnic acid concentrations were greater than hepatic concentrations for the 180 and 600 ppm (+)-usnic acid-exposed groups; and serum concentrations were greater than hepatic concentrations for the 180 ppm *Usnea* lichens exposed group, which ranged between 60 and 100 μM.

K.4. Discussion

The study utilized doses of 360 and 180 ppm (+)-usnic acid for F344/N Nctr rats and B6C3F1/Nctr mice respectively, which resulted in hepatic and serum (+/−)-usnic acid concentrations that appeared to have reached steady-state levels that ranged between 40 and 100 μM. These doses, which were designed to deliver 30 mg (+)-usnic acid per kg/day, did not produce hepatotoxicity in the 2-week exposure studies (Appendix J). The study also utilized doses of 1,250 and 600 ppm (+)-usnic acid for F344/N Nctr rats and B6C3F1/Nctr mice respectively, which resulted in hepatic and serum (+/−)-usnic acid concentrations that appeared to have reached steady-state levels that ranged between 85 and 115 μM in liver and 150 and 250 μM in serum. These doses, which were designed to deliver 100 mg (+)-usnic acid per kg/day, did produce hepatotoxicity in some cases in the 2-week exposure studies. Exposure of isolated rodent hepatocytes to (+)-usnic in vitro has been reported to result in adenosine 5'-triphosphate depletion and complete cytotoxicity after 24 hours at doses >2 μM.[25; 60; 89] Taken together, the observations suggest that (+)-usnic acid is much less toxic to hepatocytes in vivo than in vitro.

Exposure to (+)-usnic acid in ground *Usnea* lichens resulted in greater (+/−)-usnic acid concentrations in rat liver and serum and in mouse serum than from exposure to equivalent concentrations of pure (+)-usnic acid. This observation was particularly evident in rat liver wherein exposure to 360 ppm (+/−)-usnic acid as *Usnea* lichens resulted in greater

concentrations than did exposure to 1,250 ppm of pure (+)-usnic acid, which suggests that additional components in the ground lichens reduce the hepatic clearance of (+/−)-usnic acid. Feed consumption was similar across the three dosed groups.

Table K-1. Toxicokinetics Study of (+)-Usnic Acid in Rats

Group	Dose[a] (ppm in Feed)	Sample Time[b] (HALO)	Number of Rats[c] (Female)
1	360 (+)-usnic acid	1	4
2	360 (+)-usnic acid	5	4
3	360 (+)-usnic acid	9	4
4	360 (+)-usnic acid	13	4
5	360 (+)-usnic acid	17	4
6	360 (+)-usnic acid	21	4
7	360 *Usnea* lichens[d]	1	4
8	360 *Usnea* lichens	5	4
9	360 *Usnea* lichens	9	4
10	360 *Usnea* lichens	13	4
11	360 *Usnea* lichens	17	4
12	360 *Usnea* lichens	21	4
13	1,250 (+)-usnic acid	1	4
14	1,250 (+)-usnic acid	5	4
15	1,250 (+)-usnic acid	9	4
16	1,250 (+)-usnic acid	13	4
17	1,250 (+)-usnic acid	17	4
18	1,250 (+)-usnic acid	21	4
Totals			72

[a]The rats were dosed via the feed as with the 2-week studies. Doses were selected from the 2-week study data.
[b]The animals were sacrificed at 4-hour intervals starting 1 HALO (hours after lights on) on the 13th day of dosing.
[c]Only females were evaluated as significant sex differences were not observed in the 2-week study.
[d]*Usnea* lichens was added to feed to provide the listed concentration of (+/−)-usnic acid.

Table K-2. Toxicokinetics Study of (+)-Usnic Acid in Mice

Group	Dose[a] (ppm in Feed)	Sample Time[b] (HALO)	Number of Mice[c] (Male)
1	180 (+)-usnic acid	1	4
2	180 (+)-usnic acid	5	4
3	180 (+)-usnic acid	9	4
4	180 (+)-usnic acid	13	4
5	180 (+)-usnic acid	17	4
6	180 (+)-usnic acid	21	4
7	180 *Usnea* lichens[d]	1	4
8	180 *Usnea* lichens	5	4
9	180 *Usnea* lichens	9	4
10	180 *Usnea* lichens	13	4
11	180 *Usnea* lichens	17	4
12	180 *Usnea* lichens	21	4
13	600 (+)-usnic acid	1	4
14	600 (+)-usnic acid	5	4
15	600 (+)-usnic acid	9	4
16	600 (+)-usnic acid	13	4
17	600 (+)-usnic acid	17	4
18	600 (+)-usnic acid	21	4
Totals			72

[a]The mice were dosed via the feed as with the 2-week studies. Doses were selected from the 2-week study data.
[b]The animals were sacrificed at 4-hour intervals starting 1 HALO (hours after lights on) on the 13th day of dosing.
[c]Only males were evaluated because significant sex differences were not observed in the 2-week study.
[d]*Usnea* lichens was added to feed to provide the listed concentration of (+/−)-usnic acid.

Table K-3. Comparison of Observed Actual Doses to Target Doses for the Toxicokinetics Study of (+)-Usnic Acid

	Target Dose[a] (mg/kg/day)	Actual Dose Week 1[b] (mg/kg/day)	Actual Dose Week 2[b] (mg/kg/day)	Average for 14 Days (mg/kg/day)
Female Rats				
(+)-Usnic Acid 360 ppm	30	40.9	44.2	42.6
(+)-Usnic Acid 1,250 ppm	100	107.3	124.3	115.8
(+/−)-Usnic Acid 360 ppm[c]	30	39.0	43.1	41.1
Male Mice				
(+)-Usnic Acid 180 ppm	30	35.1	36.9	36.0
(+)-Usnic Acid 600 ppm	100	119.6	135.2	127.4
(+/−)-Usnic Acid 180 ppm[d]	30	36.6	36.8	36.7

[a]Calculated from historical body weight and feed consumption data.
[b]Calculated from observed body weight and feed consumption data.
[c]Given as *Usnea* lichens powder standardized to 360 ppm (+/−)-usnic acid.
[d]Given as *Usnea* lichens powder standardized to 180 ppm (+/−)-usnic acid.

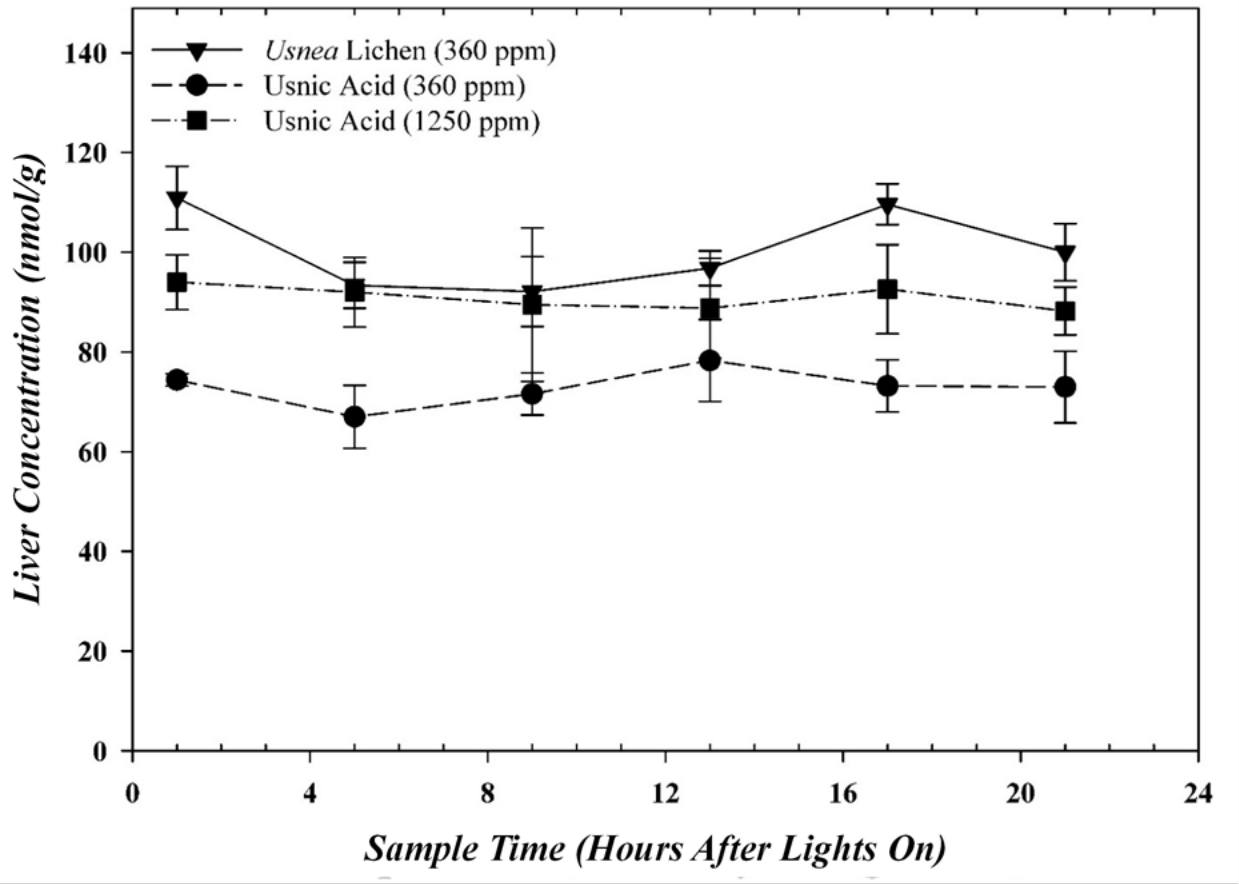

Figure K-1. Concentrations of (+/−)-Usnic Acid in Livers from Female Rats Exposed to Either (+)-Usnic Acid or Ground *Usnea* Lichens in Feed

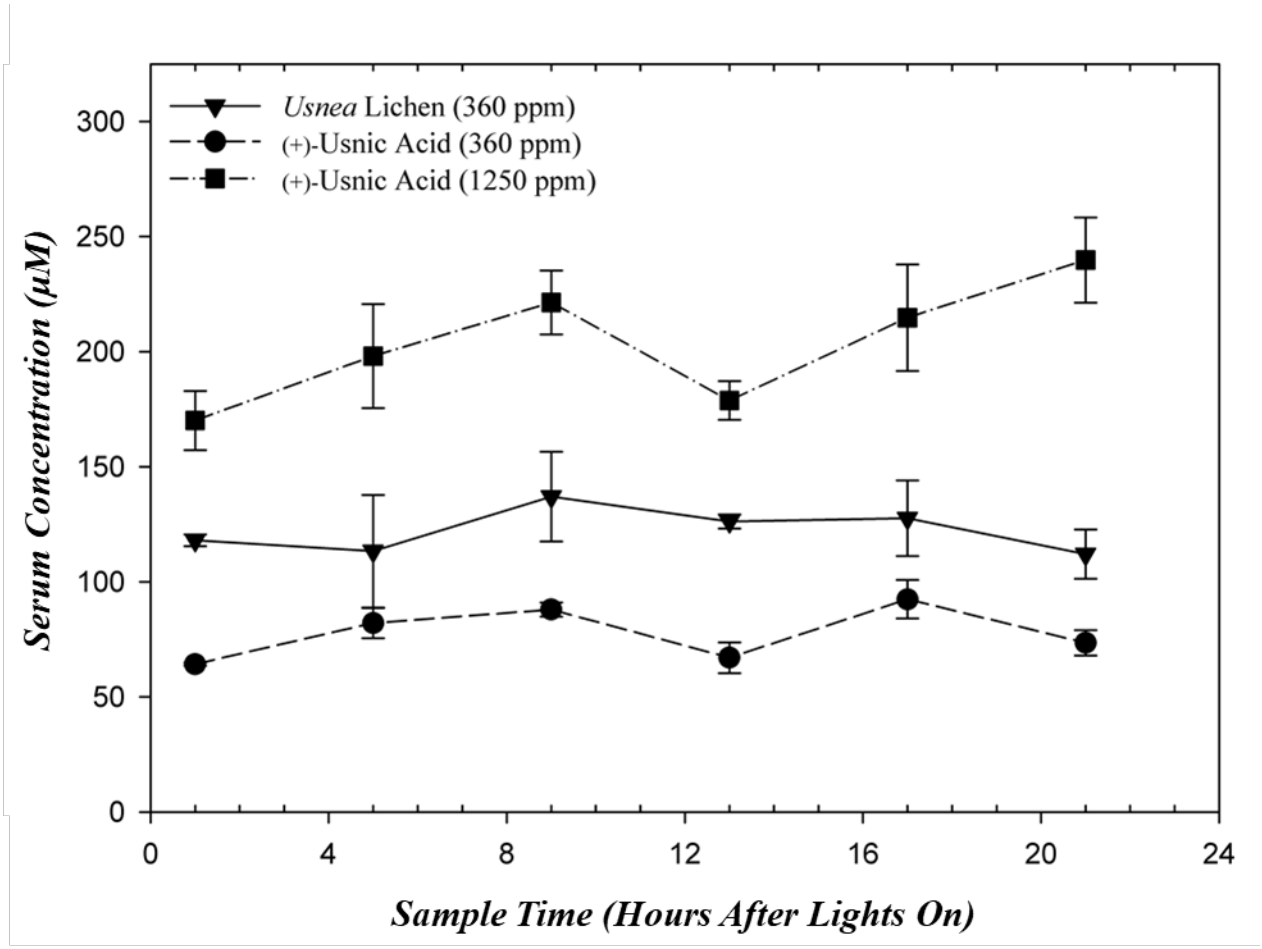

Figure K-2. Serum Concentrations of (+/−)-Usnic Acid in Female Rats Exposed to Either (+)-Usnic Acid or Ground *Usnea* Lichens in Feed

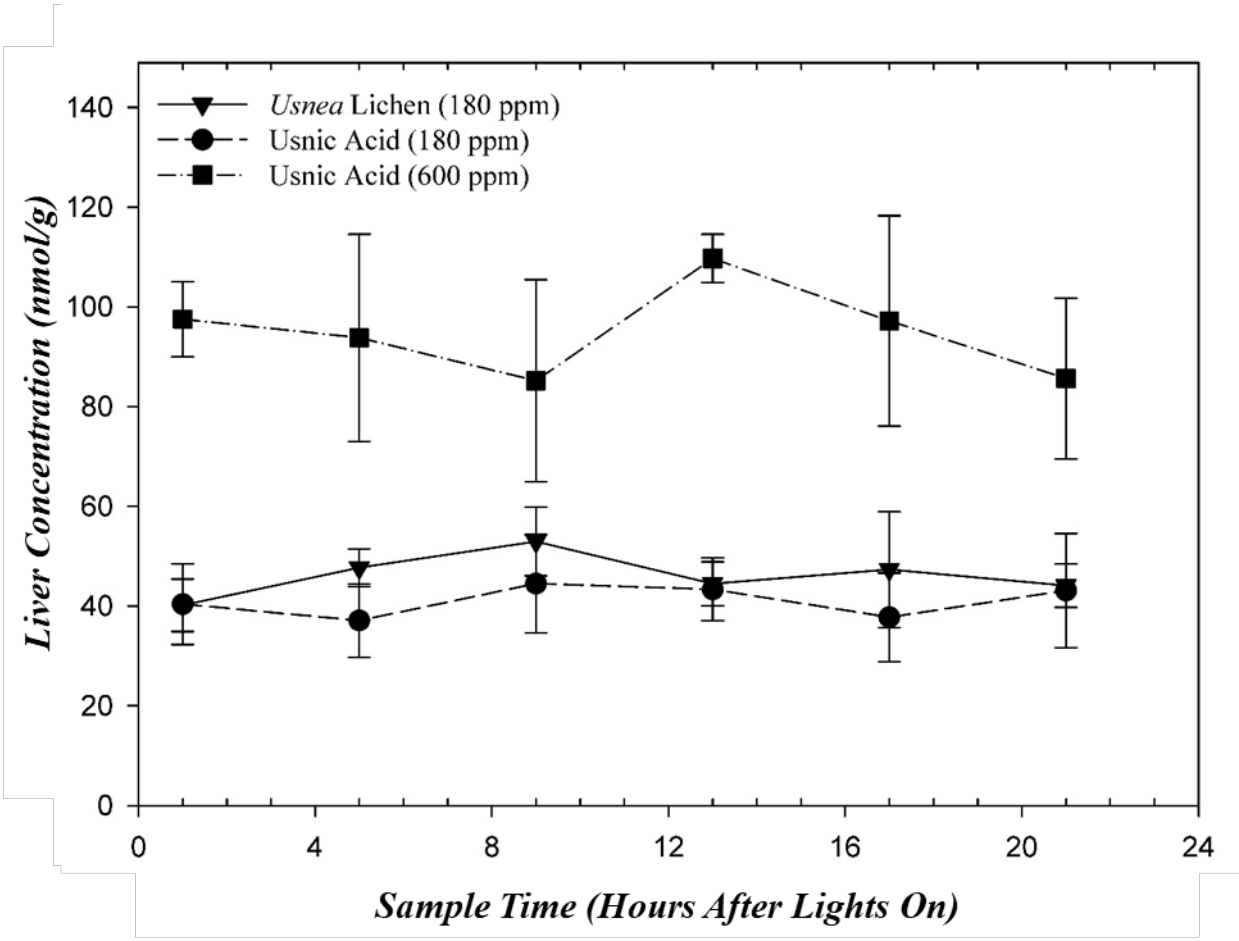

Figure K-3. Concentrations of (+/−)-Usnic Acid in Livers from Male Mice Exposed to Either (+)-Usnic Acid or Ground *Usnea* Lichens in Feed

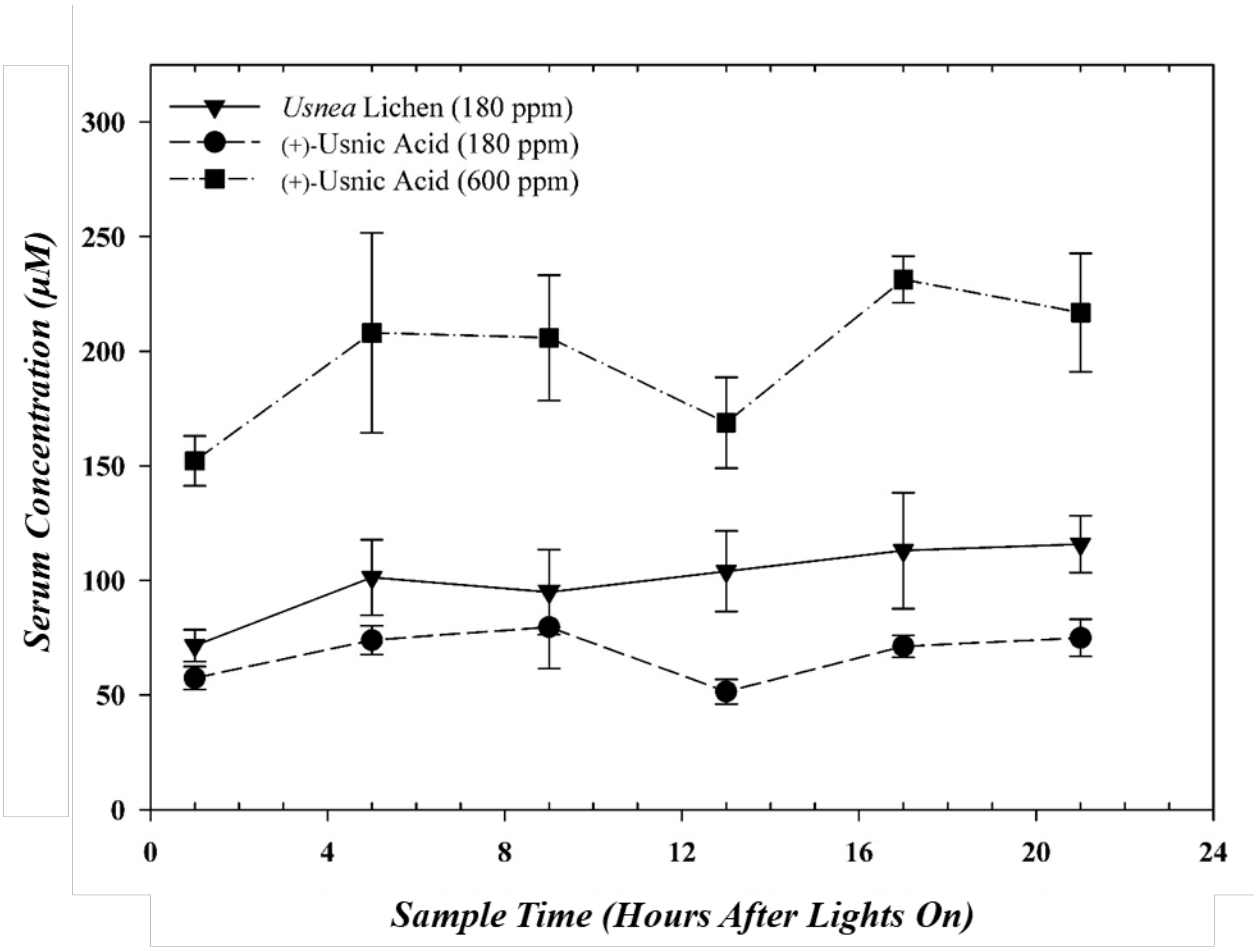

Figure K-4. Serum Concentrations of (+/−)-Usnic Acid in Male Mice Exposed to Either (+)-Usnic Acid or Ground *Usnea* Lichens in Feed

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