NATIONAL TOXICOLOGY PROGRAM
Toxicity Report Series
Number 62

NTP Technical Report on the Toxicity Studies of

Wy-14,643

(CAS No. 50892-23-4)

Administered in Feed to Sprague-Dawley Rats, B6C3F$_1$ Mice, and Syrian Hamsters

October, 2007

National Institutes of Health
Public Health Service
U.S. Department of Health and Human Services

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SUMMARY

Background

Wyeth-14,643 is a chemical that was developed by the pharmaceutical industry to lower serum cholesterol. It is not used in clinical applications. We studied the effects of Wyeth-14,643 on rats, mice, and hamsters because it was known that this chemical promotes the production of peroxisomes, organelles that contain a variety of enzymes involved in metabolism of lipids and cholesterol.

Methods

We gave groups of male rats, mice, and hamsters Wyeth-14,643 mixed in their food for three months. In each species, groups of 25 animals received either 5, 10, 50, 100, or 500 parts per million (ppm) of Wyeth-14,643 in feed. Other groups receiving undosed feed served as the control groups. Tissues from 35 sites were examined for each animal and measures of sperm motility were performed.

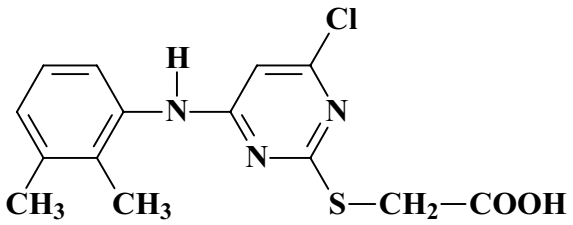
Results

All the animals survived until the end of the studies. All of the animal groups exposed to the chemical, except the groups receiving 5 ppm and the mice receiving 10 ppm, had lower body weights than their control groups although the feed consumption was generally similar in the various groups of each species. However, the liver weights of rats, mice, and hamsters fed Wyeth-14,643 were generally greater than those of the controls and liver foci were observed in three 100 ppm mice and one 500 ppm mouse. In all groups of animals exposed to Wyeth-14,643, there were significant increases in cytoplasmic alteration of the liver. In the examination of sperm motility, the weights of the cauda epididymis were decreased in all three species of rodents receiving 500 ppm, and in hamsters the testis weights and spermatid counts were decreased in all dosed groups.

Conclusions

Exposure to Wyeth-14,643 caused several changes in the livers of male rats, mice, and hamsters, including increased liver weights, increases in cytoplasmic alteration of the liver, and some liver foci. Wyeth-14,643 also had effects on the testes of exposed male rodents, decreasing the spermatid counts and the weights of the cauda epididymis.

ABSTRACT



WY-14,643

CAS No. 50892-23-4

Chemical Formula: $C_{14}H_{14}ClN_3O_2S$ Molecular Weight: 323.79

Synonym: [4-Chloro-6-(2,3-xylidino)-2-pyrimidinylthio]acetic acid

Wy-14,643 was selected for inclusion in a series of studies on peroxisome proliferators because it is known to produce considerable peroxisome proliferation and hepatocarcinogenicity in rats. Male Sprague-Dawley rats were exposed to Wy-14,643 (greater than 98% pure) in feed for up to 3 months; male B6C3F$_1$ mice and male Syrian hamsters were exposed to Wy-14,643 in feed for 2 weeks or up to 3 months. Animals were evaluated for clinical pathology, plasma concentrations of Wy-14,643, reproductive system effects, cell proliferation and peroxisomal enzyme analyses, and histopathology. Single and multiple-dose toxicokinetic studies of Wy-14,643 were conducted in additional groups of male Sprague-Dawley and Wistar Furth rats, B6C3F$_1$ mice, and Syrian hamsters. Genetic toxicology studies were conducted *in vivo* in Tg.AC mouse peripheral blood erythrocytes.

In the 2-week studies, groups of five mice were fed diets containing 0, 10, 50, 100, 500, or 1,000 ppm Wy-14,643 (equivalent to average daily doses of approximately 2 to 184 mg Wy-14,643/kg body weight). Groups of five hamsters were fed diets containing 0, 10, 100, 500, 1,000, or 5,000 ppm Wy-14,643 (equivalent to average daily doses of approximately 1 to 550 mg/kg). All animals survived to the end of the studies. The mean body weight gain of 500 ppm mice was significantly less than that of the controls; hamsters exposed to 100 ppm or greater lost weight during the study. Feed consumption by 500 ppm mice was greater than that by the controls. Liver weights of all exposed groups of mice and hamsters were generally significantly increased.

In the 2-week studies, an increase in peroxisomal enzyme activity occurred in 10 ppm mice; increases in peroxisomal β-oxidation, carnitine acetyltransferase, catalase, and acyl CoA oxidase occurred in all exposed mice compared to controls. Significantly increased BrdU-labeled hepatocyte percentages occurred in 100 and 1,000 ppm mice and 500 and 5,000 ppm hamsters; peroxisomal β-oxidation of lipids was increased in all exposed groups of mice and hamsters.

Gross lesions in the 2-week studies included liver foci in one 500 ppm mouse and one 1,000 ppm hamster and enlarged livers in one hamster in each of the 100 and 500 ppm groups and two 5,000 ppm hamsters. All 500 and 1,000 ppm mice had hepatocyte hypertrophy of the liver, and 1,000 ppm mice also had widespread individual cell necrosis. Minimal to mild multifocal vacuolation of the liver occurred in hamsters exposed to 500 ppm or greater.

In the 3-month core studies, groups of 10 male rats, mice, or hamsters were fed diets containing 0, 5, 10, 50, 100, or 500 ppm Wy-14,643 (equivalent to average daily doses of approximately 0.3 to 34 mg/kg for rats, 0.9 to 135 mg/kg for mice, and 0.4 to 42 mg/kg for hamsters). Groups of 15 male rats, mice, or hamsters designated for special studies received the same concentrations of Wy-14,643 for up to 13 weeks. Groups of six male rats, 36 male mice, or 12 male hamsters designated for plasma concentration studies were fed diets containing 50, 100, or 500 ppm Wy-14,643 for up to 9 weeks.

All core study animals survived to the end of the studies. Mean body weights were significantly decreased in all exposed groups except the 5 ppm groups and 10 ppm mice; hamsters in the 100 and 500 ppm groups lost weight during the study. Feed consumption by exposed rats and mice was generally similar to that by the controls; during week 14, hamsters exposed to 50 ppm or greater consumed slightly less feed than did the controls. The only clinical finding of toxicity was thinness of two 50 ppm and five 500 ppm hamsters. At all time points, the liver weights of exposed groups of core and special study rats, mice, and hamsters were generally significantly greater than those of the controls. Testis weights were significantly decreased in 500 ppm hamsters on day 34, in hamsters exposed to 5 ppm or greater at week 13 (special study), and in 100 and 500 ppm core study hamsters at the end of the study.

In the sperm motility evaluation, the cauda epididymis weight of 500 ppm rats, epididymis weights of 100 and 500 ppm rats and mice, and the testis weight of 500 ppm mice were significantly less than those of the controls. For hamsters, cauda epididymis, epididymis, and testis weights; spermatid heads per testis; and spermatid counts were significantly decreased in all exposed groups evaluated for sperm motility. Epididymal spermatozoal motility and concentration in the 100 and 500 ppm groups and spermatid heads per gram testis in the 500 ppm group were also significantly decreased. Serum concentrations of estradiol were significantly decreased in all exposed groups of hamsters, and concentrations of testosterone and luteinizing hormone were decreased in groups exposed to 50 ppm or greater.

At necropsy in the 3-month studies, liver foci were observed in three special study mice, including one 100 ppm mouse and one 500 ppm mouse on day 34 and one 100 ppm mouse at week 13. Liver discoloration and small testes were noted in 500 ppm hamsters on day 34, and hamsters exposed to 50 ppm or greater had enlarged livers and/or small testes at week 13 (special study) and at 3 months (core study). The incidences of cytoplasmic alteration in the liver were significantly increased in all exposed core groups of rats, mice, and hamsters; the severity of this lesion increased with increasing exposure concentration. The incidences of mitotic alteration of the liver in mice exposed to 50 ppm or greater and of liver pigmentation and oval cell hyperplasia in 500 ppm mice were significantly increased. Minimal regeneration of the corticomedullary junction of the renal tubule occurred in all exposed groups of rats. Significantly increased incidences of atrophy of the prostate gland, seminal vesicle, and testis occurred in 100 and 500 ppm hamsters. Degenerative myopathy of skeletal muscle was observed in the lumbar area and thigh of rats, mice, and hamsters and the lower leg of mice, primarily at 500 ppm.

Following single-dose gavage exposure to Wy-14,643, plasma concentrations were generally higher in mice than in rats, which in turn were higher than those in hamsters. This pattern of plasma concentrations was usually attributed to high bioavailability in mice, medium bioavailability in rats, and low bioavailabilty in hamsters following an oral exposure to Wy-14,643.

No increase in the frequency of micronucleated normochromatic erythrocytes was observed in the peripheral blood of male or female Tg.AC mice exposed to Wy-14,643 in feed or via dermal application for 6 months.

INTRODUCTION

CARCINOGENICITY

The term "peroxisome proliferator" denotes a drug or xenobiotic that induces proliferation of peroxisomes (microbodies), which are single-membrane cytoplasmic organelles with a finely granular matrix and are ubiquitous structures in plant and animal cells. These organelles function in intermediate metabolic pathways for the peroxisomal β-oxidation of fatty acids during the regulation of lipid homeostasis, and they contain hydrogen peroxide-generating oxidases and catalase that degrades hydrogen peroxide. The oxidases include α-hydroxy acid oxidase, D-amino acid oxidase, urate oxidase, acyl CoA oxidase, and the enzymes responsible for the peroxisomal β-oxidation of long chain fatty acids. Peroxisomes should not be confused with lysosomes which contain proteolytic enzymes and other acid hydrolases.

A wide variety of chemicals inducing peroxisome-associated enzymes have been shown to produce the sequelae of events in rodents associated with peroxisome proliferation. This condition includes enlarged livers associated with an increased number and size of hepatic peroxisomes and induction of peroxisomal and microsomal fatty acid-oxidizing enzymes including acyl CoA oxidase, carnitine acetyltransferase, and cytochrome P450 4A isozyme (Warren *et al.*, 1982; Reddy and Lalwani, 1983; Cerutti, 1985; Lake, 1995). Because peroxisomes contain several hydrogen peroxide-generating enzyme systems, it has been hypothesized that chronic exposure to peroxisome proliferators produces oxidative stress that results in the hepatocarcinogenicity observed in rodents chronically exposed to most peroxisome proliferators (Reddy *et al.*, 1980; Ashby *et al.*, 1994).

Various fibrate hypolipidemic drugs, herbicides, phthalate ester plasticizers, and endogenous long chain fatty acids cause peroxisome proliferation in rodents (Kawashima *et al.*, 1983; Reddy and Lalwani, 1983). In addition, the experimental cholesterol-lowering drug, Wy-14,643 (pirinixic acid), is a prototype chemical used to induce peroxisome proliferation, and exposure of male F344 rats to 0.1% Wy-14,643 in feed for 60 weeks resulted in a 100% incidence of hepatocellular carcinoma (Cayama *et al.*, 1978; Reddy *et al.*, 1979; Lalwani *et al.*, 1981; Rao *et al.*, 1984). Exposure of male CS mice to Wy-14,643 in feed at a concentration of 0.05% for 8.5 months or 1.1% for 6 months also produced a 100% incidence of hepatocellular carcinoma (Reddy *et al.*, 1979). Mechanistic studies conducted with similar exposure concentrations revealed a better correlation of hepatocarcinogenicity with cell proliferation than peroxisome proliferation (Marsman *et al.*, 1988). Other studies have indicated that Wy-14,643, gemfibrozil, and di(2-ethylhexyl)phthalate produce hepatocarcinogenicity in rats and mice (Fitzgerald *et al.*, 1981;

NTP, 1982; Cattley *et al.*, 1991). Hepatocellular carcinomas have occurred in rats administered 0.5% clofibrate in feed (Reddy and Qureshi, 1979; Svoboda and Azarnoff, 1979). Gemfibrozil (Fitzgerald *et al.*, 1981) and di(2-ethylhexyl)phthalate (Butterworth *et al.*, 1984) are nonmutagenic. Consequently, although biochemical and physiologic effects associated with hepatic peroxisome proliferation have been implicated in the etiology of liver toxicity and carcinogenicity and long-term exposure to certain peroxisome proliferators in sensitive species of rodents is associated with the development of hepatocellular carcinoma, the mechanism of peroxisome proliferator-induced tumorigenesis and the nature of its species-selectivity are not understood (Chen *et al.*, 1994; Roberts, 1999; Lake *et al.*, 2000). Since peroxisome proliferators and their metabolites are not directly mutagenic and neither bind to nor directly damage DNA, they are thought to cause cancer by nongenotoxic mechanisms. Exposure to peroxisome proliferators has been been associated with an increase in cell proliferation (Amacher *et al.*, 1997; Lake *et al.*, 2000), the modulation of growth regulatory genes and cell differentiation (Bronfman *et al.*, 1998; Vanden Heuvel *et al.*, 1998), a dysregulation of apoptosis (Chinetti *et al.*, 1998; Christensen *et al.*, 1998), and an increase in hepatic oxidative stress (Takagi *et al.*, 1990; Klaunig *et al.*, 1995; Sai-Kato *et al.*, 1995; Yeldandi *et al.*, 2000). These cellular changes, acting either alone or in combination, may account for the carcinogenic activity of peroxisome proliferators.

As indicated, oxidative stress is one mechanism through which peroxisome proliferators may cause hepatotoxicity. Peroxisome proliferator-induced increases in hepatic fatty acid oxidation have the potential to generate high levels of intracellular oxidants, and an additional oxidative burden is generated by a peroxisome proliferator-related activation of Kupffer's cells (Rose *et al.*, 1999). Changes in the intracellular redox state can modulate global cellular processes such as proliferation and apoptosis by multiple mechanisms (Sanchez *et al.*, 1996; Arrigo, 1999; Evans *et al.*, 2000). Oxidative damage to cellular macromolecules, particularly oxidant-mediated modifications of nuclear and mitochondrial DNA, are a second potential cause of peroxisome proliferator-associated liver pathology (Qu *et al.*, 1999). Adduct formation within nuclear DNA can influence gene expression through genetic (Burcham, 1998) and epigenetic (Rakitsky *et al.*, 2000) mechanisms, while lesions in mitochondrial DNA could be a source of mitochondrial dysfunction or dysgenesis (Shadel and Clayton, 1997). In addition, overproduction and leakage of hydrogen peroxide into the nucleus could result in genetic lesions such as sister chromatid exchanges and chromosomal aberrations, which might lead to the initiation of carcinogenicity (Cerutti, 1985).

Treatment of rodents with peroxisome proliferators causes large increases in the activity of the hydrogen peroxide-producing peroxisomal β-oxidation enzymes while causing only minimal increases in the activity of peroxisomal catalase and decreased activity of glutathione peroxidase (Lazarow, 1981; Furukawa *et al.*, 1983; Badr, 1992; Thottassery *et al.*, 1992). Consequently, it was hypothesized that an imbalance between hydrogen peroxide production and its degradation could lead to an increase in hydrogen peroxide-mediated oxidative damage that

eventually causes carcinogenesis in the liver of treated animals (Reddy *et al.*, 1980; Reddy, 1990). Alternately, several investigators suggested that hepatocarcinogenesis due to peroxisome proliferators may result from the promotion of spontaneously initiated cells and implicated DNA replication as a crucial factor in the carcinogenic activity of these compounds (Marsman *et al.*, 1988; Cattley *et al.*, 1991; Eacho *et al.*, 1991).

The oxidative stress theory of peroxisome proliferator-associated carcinogenesis, though attractive, has not been uniformly supported by studies that have quantitated tissue levels of oxidized macromolecules following peroxisome proliferator exposure (Sausen *et al.*, 1995; Huber *et al.*, 1997). Three problems with this experimental approach have been noted: first, the level of peroxisome proliferator-induced oxidative damage is small and difficult to differentiate from the background level of oxidation, some of which could occur during sample acquisition and preparation *ex vivo* (Otteneder and Lutz, 1999); second, the presence of increased levels of oxidized macromolecules implies, but does not necessarily prove, that biologically or pathologically significant oxidative stress was present in cells and tissues; and third, the significance of a given level of macromolecular damage is difficult to evaluate because the threshold at which oxidative damage triggers the cellular changes that cause neoplasia is unknown.

The cellular effects of the peroxisome proliferators are mediated primarily by their interactions with the peroxisome proliferator-activated receptor alpha (PPARα), a member of the PPAR subfamily of nuclear receptors (Gonzalez *et al.*, 1998). PPAR receptors modulate the transcription of multiple genes (pleiotropic) through direct interactions with peroxisome proliferator receptor elements in the regulatory regions of target genes. Additional mechanisms of transcriptional control, however, are also involved in peroxisome proliferator-related changes in gene expression (Kren *et al.*, 1996; Motojima, 1997). There are striking differences among mammals and between different species of rodents in the carcinogenicity of peroxisome proliferators. The molecular basis of these species differences is hypothesized to be a combination of quantitative differences in the hepatic expression of PPARα and qualitative differences in the pattern or functionality of the downstream events that are regulated by the receptor (Holden and Tugwood, 1999). A study of hepatic protein expression in tumor-sensitive mice and tumor-resistant Syrian hamsters exposed to Wy-14,643 for 14 days in feed showed significant quantitative changes in 49 liver proteins in mice and 35 in hamsters (Giometti *et al.*, 1998). Minimal overlap of the affected proteins in the sensitive and resistant species supports the qualitative difference hypothesis as a mechanism for species differences in tumor sensitivity and raises the prospect that the identification of differentially expressed specific hepatic protein constituents will reveal a biochemical basis for a tumor-sensitive phenotype.

Peroxisome proliferators with a great potential for human exposure include the hypolipidemic drugs such as clofibrate and gemfibrozil, plasticizers such as dibutyl phthalate and di(2-ethylhexyl)phthalate, solvents such as trichloroethylene, and chlorophenoxyacetic acid herbicides such as 2,4-dichlorophenoxyacetic acid. The molecular

mechanism by which hypolipidemic fibrates and antidiabetic thiazolidinediones exert their therapeutic effect in humans is similar to the way peroxisome proliferators exert their toxicity in rodents, namely by activation of the PPAR family of receptors. In response to exposure to a peroxisome proliferator, the mRNA and protein levels of numerous enzymes are increased in rodents, including the enzymes in the peroxisome *per se*, but also microsomal cytochrome CYP4A. The primary organs involved in this pleiotropic response are the liver, kidney, and heart. A receptor responsible for activating these diverse effects was identified as PPAR and was demonstrated to belong to the nuclear receptor superfamily that includes the estrogen, progesterone, and retinoic acid receptors. Members of the PPAR family of receptors include PPARα, PPARβ, and PPARγ, which have different tissue distributions, abundances, and functions in lipid metabolism during different stages of development. PPARγ mRNA has been detected in greatest amounts in human adipose, heart, placenta, lung, and kidney, but has also been identified in the human prostate, testis, and ovary (Lambe and Tugwood, 1996).

PPARα mediates gene activation through binding to a DNA response element (PPRE, a DR-1 response element) upstream from all genes that are known to respond to peroxisome proliferators. These include genes in the peroxisome mentioned above as well as cytochromes CYP4A and fatty acid binding protein. The other members of the PPAR subfamily (PPARβ and γ) bind to and activate similar PPREs but in different tissues. The PPAR-ligand complex binds to the PPRE upstream from the lipoprotein lipase (LPL) and apolipoprotein (apo) A-I and A-II genes in humans, whereas it binds upstream and activates different genes in rodents, namely those genes responsible for the peroxisome proliferation response. The increased LPL and apolipoprotein A-I and apolipoprotein A-II induction increase plasma high density lipoprotein (HDL) concentration and increase triglyceride mobilization. In rats, PPARα activation decreases apolipoprotein A-I and apolipoprotein A-II gene expression and lowers plasma HDL (Hennuyer *et al*., 1999). In humans, HDL cholesterol is elevated after fibrate treatment due to increased lipolysis of triglyceride-rich lipoproteins and redistribution of lipid components to HDL.

Although the PPRE is almost identical in rodents (TGACCTTTGTCCT) and humans (AGGTCAGCTGTCA), the location of the PPRE in the genome is different across species, resulting in vastly different genes being expressed following activation of the PPAR family (Roberts, 1999; Woodyatt *et al.*, 1999).

The human receptor appears to be activated by certain fatty acids and eicosanoids and thiazolidinedione antidiabetic drugs, although it appears to be only weakly activated by classical peroxisome proliferators such as Wy-14,643, nafenopin, and clofibric acid (Lambe and Tugwood, 1996). Endogenous ligands for PPARs include most straight-chain fatty acids, substituted fatty acids, the acyl CoA esters of fatty acids, and arachidonic acid-derived prostaglandins and eicosanoids (Schoonjans *et al.*, 1996).

As in rodents, fibrate drugs used in humans for the treatment of hyperlipidemia are thought to activate PPARα in the liver (Vu-Dac *et al.*, 1995; Auwerx *et al.*, 1996; Staels *et al.*, 1997). However, activation of PPARα in humans does not result in peroxisome proliferation but in increased apolipoprotein A-II and LPL transcription and reduced apolipoprotein C-III transcription, the mechanisms whereby these drugs lower serum triglycerides (Vu-Dac *et al.*, 1995; Auwerx *et al.*, 1996; Staels *et al.*, 1997) and induce fatty acid transport protein and acyl CoA synthetase (Martin *et al.*, 1997). Apolipoprotein C-III is a major component of very low density lipoproteins and inhibits LPL and clearance of lipoproteins by the liver.

The thiazolidinedione antidiabetic agents activate human PPARγ in adipose tissue where LPL expression is also increased (Auwerx *et al.*, 1996). LPL is transcriptionally activated and results in increased lipolytic activity and decreased serum triglycerides in humans without the increase in peroxisome activity due to the location of the PPRE upstream of the LPL gene (Auwerx *et al.*, 1996).

It is clear that humans possess a functional PPAR family of receptors. It is also clear that they regulate different genes than does the receptor family in rodents, and that the human PPAR receptor is activated by xenobiotic drugs and chemicals. In two recent reviews of the medical significance of PPARs, it was reported that since activation of PPARs does not induce peroxisomes in humans, the term peroxisome proliferator *per se* in a medical context is inappropriate (Roberts, 1999; Vamecq and Latruffe, 1999). The role of PPARα in the pathogenesis of disease in rodents and humans has been recently reviewed (Rusyn *et al*., 2006).

GENETIC TOXICITY

Little information is available on the genetic toxicity of Wy-14,643; therefore, a broad assessment of its mutagenic potential cannot be made. The compound does appear to be clastogenic in mammalian cell systems, however. Lefevre *et al.* (1994) demonstrated a dose-related increase in chromosomal aberrations in cultured Chinese hamster ovary cells treated with 518 to 907 µg/mL Wy-14,643, with and without S9 metabolic activation enzymes. The authors stated that clear evidence of cytotoxicity, manifested by a marked growth reduction in the treated cell cultures, was observed at the doses that produced positive responses. Tsutsui *et al.* (1993) reported induction of chromosomal aberrations in Syrian hamster embryo cells treated with 1 to 30 µM Wy-14,643 in the presence of S9. A third report of cytogenetic effects of Wy-14,643 presented evidence of induction of chromosomal aberrations, sister chromatid exchanges, and micronuclei in primary hepatocytes of rats or humans treated *in vitro* (Hwang *et al.*, 1993). However, independent evaluation of the data in this report is complicated by a number of factors, including high control rates for the endpoints evaluated, unusual protocol features, and the manner of data presentation.

NIEHS Extramural Mechanistic Studies

A small RO3 grant Request for Application (RFA-ES-98-003) was issued by the NIEHS to encourage investigator-initiated research that would utilize tissues from NTP contract studies of peroxisome proliferators. These extramural studies would complement the NTP studies by providing additional mechanistic information on the agents tested in order to improve the risk assessment process and thereby better protect the public health at little additional cost. These grants were a joint effort by the NTP and the Division of Extramural Research and Training designed to improve the collaboration between government and nongovernment scientists in assessing the toxicity of environmental agents. The purpose of this RFA was to utilize frozen and fixed tissues from the NTP toxicity studies of four peroxisome proliferators in three species in mechanistic investigations of peroxisome proliferator-induced toxicity. A detailed study design was provided on the NIEHS web site and included the species, exposure concentrations, endpoints measured, and tissues available for investigator-initiated studies. Investigators were requested to submit hypothesis-driven, mechanistically-based proposals to study biochemical and molecular endpoints that they believed would be related to or would predict liver cancer resulting from exposure to peroxisome proliferators. They were also instructed to use tissues from responsive (rodent) and less responsive (hamster) species and to justify tissue selection.

Applications accepted for funding included peroxisome proliferator-induced growth regulation; peroxisome proliferator-induced transcription factors; peroxisome proliferator-induced oxidative stress; mechanism of cell proliferation induced by peroxisome proliferators; effects of peroxisome proliferators on DNA methylation; and sequelae of Wy-14,643-induced oxidative stress. Abstracts of selected investigations funded by this Initiative are presented in Appendix K.

Study Rationale

The present studies were undertaken to further evaluate the oxidative stress theory of peroxisome proliferator-induced carcinogenesis and to identify hepatic proteins that are differentially expressed in sensitive and resistant rodent species. The NTP chose to evaluate oxidative stress by measuring the hepatic expression of proteins that are involved in the cellular response to oxidative damage. In addition to testing Wy-14,643, other peroxisome proliferators were also tested, including the therapeutically used hypolipidemic drug gemfibrozil, the phthalate plasticizer dibutyl phthalate, and the herbicide 2,4-dichlorophenoxyacetic acid.

Wy-14,643 was selected for inclusion in this series of studies on peroxisome proliferators because it is known to produce considerable peroxisome proliferation and hepatocarcinogenicity in rats. Rats and mice are commonly used in peroxisome proliferation studies. In addition to the liver, the testis is a common target organ in rodents exposed

to peroxisome proliferators. Several peroxisome proliferators have been shown to induce testicular Leydig cell tumors in rat strains other than the F344. Due to a high frequency of early onset, spontaneous testicular atrophy and/or Leydig cell tumors in F344 rats, induction of these lesions is difficult to detect; therefore, the Sprague-Dawley strain was selected for these studies. Based on the greater sensitivity of male animals to toxic effects and the large number of animals needed to conduct the series of peroxisome proliferation studies, these studies were performed using male rodents only. Hamsters were included in these studies because this species, like humans, is believed to be relatively resistant to peroxisome proliferation. For example, following lifelong exposure of Syrian hamsters to the peroxisome proliferator di(2-ethylhexyl)phthalate by inhalation and intraperitoneal injection, there were no significant increases in tumor incidences (Schmezer *et al*., 1988). In addition, these authors compared the ability of di(2-ethylhexyl)phthalate to produce DNA single-strand breaks in rat and hamster hepatocytes *in vitro*. Whereas di(2-ethylhexyl)phthalate produced toxicity and DNA single-strand breaks in rat hepatocytes at 5 µmole per tube, no toxicity or single-strand breaks were observed in hamster hepatocytes at up to 25 µmole per tube. A second report demonstrated that Sprague-Dawley rats, but not Syrian hamsters, fed diets containing the peroxisome proliferators nafenopin or Wy-14,643 for up to 60 weeks exhibited sustained hepatic cell proliferation and liver nodules and tumors (Lake *et al.*, 1993). These data indicated that hamsters are less sensitive than standard rodent models to the cell replication and enzyme induction of peroxisome proliferators, and it was thought that data from hamsters would thus aid in the understanding of the mechanism of toxicity by this class of compounds.

For the studies reported in this Toxicity Study Report, male Sprague-Dawley rats, B6C3F$_1$ mice, and Syrian hamsters were exposed to Wy-14,643 in feed for 2 weeks or up to 3 months. In addition to toxicity, cell proliferation, and hepatic peroxisomal enzyme studies, plasma Wy-14,643 concentration and toxicokinetic studies were performed. Genetic toxicology studies were conducted *in vivo* in peripheral blood erythrocytes from Tg.AC mice exposed to Wy-14,643 in feed or by dermal application for 6 months.

MATERIALS AND METHODS

PROCUREMENT AND CHARACTERIZATION OF WY-14,643

Wy-14,643 was obtained from Chemsyn Science Laboratories (Lenexa, KS) in two lots (91-314-72-07 and 91-314-100-33A), which were used throughout the 2-week and 3-month studies. Lots 91-314-72-07 and 91-314-100-33A were combined by the study laboratory, Battelle Columbus Laboratories (Columbus, OH), and assigned a new lot number (C041194). Identity, purity, and stability analyses were conducted by the analytical chemistry laboratory, Radian Corporation (Austin, TX), and the study laboratory (Appendix I). Reports on analyses performed in support of the Wy-14,643 studies are on file at the National Institute of Environmental Health Sciences.

The chemical, a white powder, was identified as Wy-14,643 by the analytical chemistry laboratory using infrared and proton nuclear magnetic resonance spectroscopy (lot 91-314-72-07) and by the study laboratory using infrared spectroscopy (lot 91-314-100-33A). The purity of lot 91-314-72-07 was determined by the analytical chemistry laboratory using high-performance liquid chromatography (HPLC), which indicated a major peak and no impurities. The overall purity of lot 91-314-72-07 was determined to be greater than 99%. For lot 91-314-100-33A, the manufacturer indicated a purity of 98% or greater using thermal analysis and HPLC. The study laboratory confirmed the purity of lot C041194 using HPLC, which indicated a major peak and two impurities with areas greater than 0.1% relative to the major peak area; smaller impurity peaks were also observed. The overall purity of lot C041194 was determined to be 98% or greater.

The manufacturer recommended storage under an inert atmosphere at 5° C, protected from light. The bulk chemical was stored at room temperature, protected from light, in amber glass bottles with Teflon®-lined caps. Stability was monitored during the studies with HPLC. No degradation of the bulk chemical was detected.

PREPARATION AND ANALYSIS OF DOSE FORMULATIONS

The dose formulations were prepared once (2-week studies) or approximately every 4 weeks (3-month studies) by mixing Wy-14,643 with feed (Table I2). Formulations were stored in plastic buckets at approximately 5° C, protected from light, for up to 21 days.

Homogeneity and stability studies of 10, 50, and 500 ppm dose formulations and stability studies of a 5 ppm dose formulation were performed by the analytical chemistry laboratory using HPLC. Homogeneity studies of 10 and

10,000 ppm dose formulations for the 2-week studies and the 5 and 500 ppm dose formulations for the 3-month studies were performed by the study laboratory with HPLC. Homogeneity was confirmed, and stability of dose formulations stored in glass vials with Teflon®-lined caps was confirmed for at least 23 days at –20° C and for 35 days at 4° ± 2° C or room temperature; dose formulations stored open to air and light were stable for 7 days.

Periodic analyses of the dose formulations were conducted by the study laboratory using HPLC. For the 2-week studies, the dose formulations were analyzed once; all dose formulations for mice and hamsters were within 10% of the target concentrations (Table I3). Animal room samples of these dose formulations were also analyzed; all animal room samples for mice and three of five for hamsters were within 10% of the target concentrations. For the 3-month studies, the dose formulations were analyzed at the beginning, midpoint, and end of the studies; animal room samples of these dose formulations were also analyzed (Table I4). Of the dose formulations analyzed, 19 of 20 were within 10% of the target concentrations; the single dose formulation that was outside the 10% criterion was considered suitable for use in the studies. All animal room samples were within 10% of the target concentrations.

2-Week Studies

Male B6C3F$_1$ mice were obtained from Taconic Farms, Inc. (Germantown, NY). Male Syrian hamsters were obtained from Frederick Cancer Research and Development Center (Frederick, MD). On receipt, the mice were 5 weeks old and the hamsters were 7 weeks old. Animals were quarantined for 13 days and were 7 (mice) or 9 (hamsters) weeks old on the first day of the studies. Before the studies began, five mice and five hamsters were randomly selected for parasite evaluation and gross observation for evidence of disease.

Groups of five male mice and male hamsters were fed diets containing 0, 10, 50 (mice), 100, 500, 1,000, or 5,000 (hamsters) ppm Wy-14,643 for 15 days. Feed and water were available *ad libitum*. Mice and hamsters were housed individually. Clinical findings were recorded twice per day for mice and hamsters. The animals were weighed initially, on day 8, and at the end of the studies. Feed consumption was recorded. Details of the study design and animal maintenance are summarized in Table 1.

On study day 10, mice in the 0, 10, 100, and 1,000 ppm groups and hamsters in the 0, 10, 500, and 5,000 ppm groups were implanted subcutaneously with osmotic minipumps (Model 2001, Alza Corp., Palo Alto, CA) prefilled with a 30 mg/mL solution of 5-bromo-2′-deoxyuridine (BrdU; Sigma Chemical Company, St. Louis, MO) in 0.01 N sodium hydroxide. The pumps were incubated in phosphate-buffered saline at 37° C for at least 4 hours and then implanted in animals anesthetized with 2% isoflurane between 1300 and 1600; the exact time of implantation in each animal was recorded. At necropsy, after 5 days (116 ± 3 hours) of BrdU exposure, the livers were evaluated for incorporation of BrdU.

Necropsies were performed on all mice and hamsters. The right kidney, liver, and right testis were weighed. Histopathologic examinations of gross lesions and selected organs were performed on all mice in the 0 and 1,000 ppm groups and hamsters in the 0 and 5,000 ppm groups. Table 1 lists the organs examined.

During necropsy, a sample of three liver lobes from each animal implanted with an osmotic minipump was collected and reserved for peroxisome proliferation analyses, and another 250-mg sample was frozen and shipped to Argonne National Laboratory (Argonne, IL) for protein gel electrophoresis analyses (Giometti *et al.*, 1998). The remaining portion of the liver was fixed in 10% neutral buffered formalin for 48 hours. The formalin-fixed liver samples, as well as a transverse section of duodenum included as an internal control, were embedded in paraffin; tissues that could not be embedded after 48 hours of fixation were transferred to 70% ethanol. Two serial sections of each tissue were made; one slide was used for histopathologic examinations, and the second slide was stained with anti-BrdU antibody. Cell proliferation (labeled hepatocytes as a percentage of total hepatocytes) was measured by examining 2,000 hepatocyte nuclei (BrdU-labeled and unlabeled) from liver lobes of animals implanted with osmotic minipumps. Interlobe labeling variation was assessed by counting 2,000 hepatocyte nuclei from the left lobe of all implanted mice and hamsters in each exposure group, and the right median and right anterior lobes in the 0, 1,000 (mice), and 5,000 (hamsters) ppm groups. In addition, the right median and right anterior lobes were counted from a single hamster in each of the 10 and 500 ppm groups. Approximately 1-g (hamster) or 0.5-g (mouse) portions of the liver samples reserved for peroxisome proliferation analyses, as well as left lobe liver samples collected from mice in the 50 and 500 ppm groups and hamsters in the 100 and 1,000 ppm groups, were placed in 50 mM tris hydrochloride buffer (pH 8.0) and homogenized with a Teflon® pestle. Each homogenate was divided into at least 10 aliquots, frozen in dry ice ethanol, and stored for at least a day at approximately –70° C before analysis.

Peroxisome proliferation was determined in duplicate tissue extractions by measuring peroxisomal β-oxidation, catalase activity, and nonspecific carnitine acetyltransferase activity. For each assay, a liver sample from a rat exposed to 500 ppm Wy-14,643 for 1 week was included as a positive control. Peroxisomal oxidation was estimated by two methods: direct measurement of acyl coenzyme A oxidase activity (Small *et al.*, 1985) and measurement of the peroxisomal β-oxidation spiral (Lazarow, 1981). Acyl coenzyme A oxidase activity was measured by reacting liver homogenates with 0.05 mM dichlorofluorescein diacetate, 40 mM aminotriazole, 0.1 mg/mL horseradish peroxidase, 11 mM potassium phosphate buffer (pH 7.4), 0.02% Triton X-100, and 0.03 mM palmitoyl coenzyme A. The reaction was monitored spectrophotometrically at 490 nm for 5 minutes at 30° C using an ELISA reader. For measurement of the peroxisomal β-oxidation spiral, the liver homogenates were reacted with 50 mM tris hydrochloride (pH 8.0), 0.2 mM nicotinic acid adenine dinucleotide, 1 mM dithiothreitol, 0.75 µg/mL bovine serum albumin, 0.01 mM flavine adenine dinucleotide, 0.1 mM coenzyme A, 0.1 mg/mL Triton X-100, 1 mM potassium cyanide, and 0.01 mM palmitoyl coenzyme A. The reaction was monitored spectrophotometrically at 340 nm for 5 minutes at 30° C using an ELISA reader.

Nonspecific carnitine acetyltransferase activity was estimated by the method of Gray *et al.* (1982a,b). Liver homogenates were reacted with 0.25 mM acetyl coenzyme A, 0.156 mM 5,5′-dithiobis-(2-nitrobenzoic acid), 1.25 mM EDTA, and 50 mM tris hydrochloride (pH 8.0); the reaction was started with the addition of 3.125 mM DL-carnitine and was monitored with an ELISA reader at 405 nm for 20 minutes at 37° C. Peroxisomal catalase activity was estimated by a method derived from those of Van Lente and Pepoy (1990) and Yasmineh *et al.* (1992). Liver homogenates were diluted with a buffer of 50 mM potassium phosphate (pH 7.0) and 0.15 M ethanol and reacted with a mixture of 250 mM potassium phosphate, 1 M glucose, 6.85 M ethanol, 5.7 mM nicotinic acid adenine dinucleotide phosphate, yeast aldehyde dehydrogenase (30 units/mL), and deionized water. The reaction was monitored spectrophotometrically at 340 nm for 10 minutes at 30° C using an ELISA reader programmed to shake the microtiter plate before readings were conducted at 10-second intervals.

Protein concentrations were measured using the bicinchoninic method with bovine serum albumin as the standard (Smith *et al.*, 1985); commercially available reagents were used. Samples were incubated at 37° C for 30 minutes; the absorbance at 570 nm was then measured with a microtiter plate reader.

3-Month Studies

Male Sprague-Dawley rats were obtained from Harlan Sprague-Dawley, Inc. (Indianapolis, IN). Male B6C3F$_1$ mice were obtained from Taconic Farms, Inc. Male Syrian hamsters were obtained from Frederick Cancer Research and Development Center. On receipt, the rats, mice, and hamsters were approximately 4 to 6 weeks old. Animals were quarantined for 12 to 17 days and were approximately 7 to 9 weeks old on the first day of the studies. Before the studies began, five rats, mice, and hamsters were randomly selected for parasite evaluation and gross observation for evidence of disease. Blood was collected from five rats, mice, and hamsters 4 weeks after the studies began and at the end of the studies. The sera were analyzed for antibody titers to rodent viruses (Boorman *et al.*, 1986; Rao *et al.*, 1989a,b).

For the core studies, groups of 10 male rats, mice, and hamsters were fed diets containing 0, 5, 10, 50, 100, or 500 ppm Wy-14,643 for 14 weeks. Groups of 15 male rats, mice, and hamsters designated for special studies received the same concentrations of Wy-14,643 for up to 13 weeks. Groups of 6 male rats, 36 male mice, and 12 male hamsters designated for plasma concentration determinations were fed diets containing 50, 100, or 500 ppm Wy-14,643 for up to 9 weeks. Feed and water were available *ad libitum*. Core and special study rats were housed five per cage; plasma concentration study rats were housed three per cage. Mice and hamsters were housed individually. The animals were weighed initially, on day 8, and at the end of the studies. Clinical findings were recorded weekly for core and special study animals and before blood collections for plasma concentration study

animals. Feed consumption by core and plasma concentration study animals was recorded weekly. Details of the study design and animal maintenance are provided in Table 1.

On study days 1, 29, and 85, five special study rats, mice, and hamsters per group were implanted with osmotic minipumps as described for the 2-week studies. After 5 days (116 ± 3 hours) of BrdU exposure, the animals were evaluated for tissue incorporation of BrdU. A sample of the left liver lobe was collected and reserved for peroxisome proliferation analyses, and another sample (250 mg) was frozen until protein gel electrophoresis analyses could be performed (Giometti *et al.*, 1998). Approximately half of the left lobe was fixed in 10% neutral buffered formalin for 48 hours; the remaining tissue was frozen in liquid nitrogen. Slides were prepared and analyzed for cell proliferation as described for the 2-week studies. Approximately 1-g (rat and hamster) or 0.5-g (mouse) portions of the liver samples reserved for peroxisome proliferation analyses were prepared and analyzed as described for the 2-week studies. Portions of the liver homogenates of rats and mice evaluated during weeks 1 and 13 were analyzed for cyclin-dependent kinase activity and proliferating cell nuclear antigen concentration (week 13 only) using an indirect ELISA assay (Paracelsian, Inc., Ithaca, NY). Liver homogenates were thawed and centrifuged; the supernatant fractions were diluted to 1:100 with the buffer provided by the ELISA assay manufacturer and were then used to coat quadruplicate wells of a microtiter plate. Approximately 0.20 to 0.35 mg protein/mL was added to the wells. Each sample was analyzed in duplicate.

Over 24-hour collection periods during weeks 3, 5, and 9, blood was collected from one plasma concentration study rat, mouse, or hamster per group per time point at 0600, 0800, 1000, 1200, 1400, 1600, 1800, 2000, 2200, 2400, 0200, and 0400. Each rat was bled at two time points (12 hours apart) during each collection period. Each mouse was bled once during only one collection period. Each hamster was bled at one time point during each collection period. Animals were anesthetized with a carbon dioxide:oxygen mixture before blood was collected from the retroorbital sinus (rats and hamsters) or by cardiac puncture (mice). Blood was collected in tubes containing sodium heparin as an anticoagulant. The plasma was separated by centrifugation and was stored at approximately –20° C until analysis by CEDRA Corporation (Austin, TX) for Wy-14,643 concentration. The samples were analyzed within 24 days of collection with HPLC (Model 501; Waters-Millipore, Milford, MA) with ultraviolet detection at 254 nm, a ZORBAX® CN column (150 mm × 4.6 mm; Rockland Technologies, Inc., Newport, DE), and a mobile phase of 300 mL acetonitrile in 700 mL water, with 1.36 g potassium biphosphate added. The pH was adjusted to 3.0 with phosphoric acid, and the flow rate was 1.7 mL/minute.

Clinical pathology studies were performed on up to five special study or up to 10 core study rats, mice, and hamsters per group. Blood for clinical chemistry and reproductive hormone evaluations was collected from special study animals on day 34 and from core study animals at the end of the studies. The animals were anesthetized with a mixture of carbon dioxide and oxygen, and blood was withdrawn by cardiac puncture and placed in collection tubes

devoid of anticoagulant. The samples were allowed to clot and were then centrifuged; the serum was removed and stored at –70° C until analysis. Clinical chemistry variables were measured. Reproductive hormones were analyzed by AniLytics (Gaithersburg, MD). The clinical pathology parameters that were evaluated are listed in Table 1. Reagents were obtained from the equipment manufacturers.

At the end of the 3-month studies, samples were collected for sperm motility evaluations on core study rats, mice, and hamsters exposed to 0, 50, 100, or 500 ppm. The parameters that were evaluated are listed in Table 1. Animals were evaluated for sperm count and motility. The left testis and epididymis were isolated and weighed. The tail of the epididymis (cauda epididymis) was then removed from the epididymal body (corpus epididymis) and weighed. Test yolk (rats) or modified Tyrode's buffer (mice) was applied to slides and a small incision was made at the distal border of the cauda epididymis. The sperm effluxing from the incision were dispersed in the buffer on the slides, and the numbers of motile and nonmotile spermatozoa were counted for five fields per slide by two observers. Following completion of sperm motility estimates, each left cauda epididymis was placed in buffered saline solution. Caudae were finely minced, and the tissue was incubated in the saline solution and then heat fixed at 65° C. Sperm density was then determined microscopically with the aid of a hemacytometer. To quantify spermatogenesis, the testicular spermatid head count was determined by removing the tunica albuginea and homogenizing the left testis in phosphate-buffered saline containing 10% dimethyl sulfoxide. Homogenization-resistant spermatid nuclei were counted with a hemacytometer.

Necropsies were performed on all core and special study animals. The right kidney of core study animals and the liver and right testis of all animals were weighed. Tissues for microscopic examination were fixed and preserved in 10% neutral buffered formalin, processed and trimmed, embedded in paraffin, sectioned, and stained with hematoxylin and eosin. Complete histopathologic examinations were performed on all core study animals in the 0 and 500 ppm groups, and selected tissues were examined in the remaining groups. Table 1 lists the tissues and organs routinely examined. In addition, examinations of the muscles of the heart (0 and 500 ppm animals), lumbar area and thigh (all animals), lower leg (mice), and tongue (0 and 500 ppm animals, five per group) were conducted.

Upon completion of the laboratory pathologist's histopathologic evaluation, the slides, paraffin blocks, and residual wet tissues were sent to the NTP archives for inventory, slide/block match, and wet tissue audit. The slides, individual animal data records, and pathology tables were sent to an independent pathology laboratory where quality assessment was performed. Results were reviewed and evaluated by the NTP Pathology Working Group (PWG); the final diagnoses represent a consensus of contractor pathologists and the PWG. Details of these review procedures have been described by Maronpot and Boorman (1982) and Boorman *et al.* (1985).

TABLE 1
Experimental Design and Materials and Methods in the Feed Studies of Wy-14,643

2-Week Studies	3-Month Studies
Study Laboratory	
Battelle Columbus Laboratories (Columbus, OH)	Battelle Columbus Laboratories (Columbus, OH)
Strain and Species	
$B6C3F_1$ mice Syrian hamsters	Sprague-Dawley rats $B6C3F_1$ mice Syrian hamsters
Animal Source	
Mice: Taconic Farms, Inc. (Germantown, NY) Hamsters: Frederick Cancer Research and Development Center (Frederick, MD)	Rats: Harlan Sprague-Dawley, Inc. (Indianapolis, IN) Mice: Taconic Farms, Inc. (Germantown, NY) Hamsters: Frederick Cancer Research and Development Center (Frederick, MD)
Time Held Before Studies	
13 days	Rats: 12 (plasma concentration), 13 (special), or 14 (core) days Mice: 14 (plasma concentration), 15 (special), or 16 (core) days Hamsters: 16 (special) or 17 (plasma concentration, core) days
Average Age When Studies Began	
Mice: 7 weeks Hamsters: 9 weeks	Rats: 7 weeks Mice: 7 weeks Hamsters: 8-9 weeks
Date of First Exposure	
Mice: May 18, 1994 Hamsters: May 17, 1994	Rats: November 29 (plasma concentration) or 30 (special) or December 1 (core), 1994 Mice: December 6 (plasma concentration), 7 (special), or 8 (core), 1994 Hamsters: December 14 (special) or 15 (core, plasma concentration), 1994
Duration of Exposure	
15 days	9 (plasma concentration), 13 (special), or 14 (core) weeks
Date of Last Exposure	
Mice: June 1, 1994 Hamsters: May 31, 1994	Rats: January 25 (plasma concentration), February 27 (special), or March 2 (core), 1995 Mice: January 31 and February 1 (plasma concentration) or March 6 (special) or 9 (core), 1995 Hamsters: February 10 (plasma concentration) or 13 (special) or March 16 (core), 1995
Necropsy Dates	
Mice: June 1, 1994 Hamsters: May 31, 1994	Rats: December 5, 1994, or January 2 or February 27, 1995 (special); March 2, 1995 (core) Mice: December 12, 1994, or January 9 or March 6, 1995 (special); March 9, 1995 (core) Hamsters: December 19, 1994, or January 16 or March 13, 1995 (special); March 16, 1995 (core)
Average Age at Necropsy	
Mice: 9 weeks Hamsters: 11 weeks	Rats: 8, 12, or 20 weeks (special); 20 weeks (core) Mice: 7, 11, or 19 weeks (special); 20 weeks (core) Hamsters: 8-9, 12-13, or 20-21 weeks (special); 21-22 weeks (core)

TABLE 1
Experimental Design and Materials and Methods in the Feed Studies of Wy-14,643

2-Week Studies	3-Month Studies
Size of Study Groups	
5 males	Rats: 10 (core), 15 (special), or 6 (plasma concentration) males Mice: 10 (core), 15 (special), or 36 (plasma concentration) males Hamsters: 10 (core), 15 (special), or 12 (plasma concentration) males
Method of Distribution	
Animals were distributed randomly into groups of approximately equal initial mean body weights.	Same as 2-week studies
Animals per Cage	
1	Rats: 3 (plasma concentration) or 5 (core, special) Mice: 1 Hamsters: 1
Method of Animal Identification	
Mice: tail tattoo Hamsters: ear tag	Rats: tail tattoo Mice: tail tattoo and ear tag Hamsters: ear tag
Diet	
NIH-07 open formula meal diet (Zeigler Brothers, Inc., Gardners, PA), available *ad libitum*	NTP-2000 open formula meal diet (Zeigler Brothers, Inc., Gardners, PA), available *ad libitum*
Water	
Tap water (Columbus municipal supply) via automatic watering system (Edstrom Industries, Waterford, WI), available *ad libitum*	Same as 2-week studies
Cages	
Polycarbonate (Lab Products, Inc., Maywood, NJ), changed once per week	Polycarbonate (Lab Products, Inc., Maywood, NJ), changed twice per week (rats) or weekly (mice and hamsters)
Bedding	
Sani-Chips® hardwood chips (P.J. Murphy Forest Products Corp., Montville, NJ), changed at least once per week	Same as 2-week studies, except changed twice per week for rats
Cage Filters	
DuPont 2024 spun-bonded polyester (Snow Filtration Co., Cincinnati, OH), changed once	Same as 2-week studies, but changed every 2 weeks
Racks	
Stainless steel (Lab Products, Inc., Maywood, NJ), changed and rotated every 2 weeks	Stainless steel (Lab Products Inc., Seaford, DE), changed and rotated every 2 weeks
Animal Room Environment	
Temperature: 72° ± 3° F Relative humidity: 50% ± 15% Room fluorescent light: 12 hours/day Room air changes: at least 10/hour	Temperature: 72° ± 3° F Relative humidity: 50% ± 15% Room fluorescent light: 12 hours/day Room air changes: at least 10/hour
Exposure Concentrations	
Mice: 0, 10, 50, 100, 500, or 1,000 ppm in feed Hamsters: 0, 10, 100, 500, 1,000, or 5,000 ppm in feed	Core and special studies: 0, 5, 10, 50, 100, or 500 ppm in feed Plasma concentration study: 50, 100, or 500 ppm in feed

TABLE 1
Experimental Design and Materials and Methods in the Feed Studies of Wy-14,643

2-Week Studies	3-Month Studies
Type and Frequency of Observation	
Animals were observed and clinical findings were recorded twice daily. Animals were weighed initially, on day 8, and at the end of the studies. Feed consumption was recorded weekly.	Animals were observed twice daily and were weighed initially, weekly, and at the end of the studies. Clinical findings were recorded weekly for core and special study animals and before blood collections for plasma concentration study animals. Feed consumption was recorded weekly for core and plasma concentration study animals.
Method of Sacrifice	
Anesthetization with a carbon dioxide:oxygen mixture followed by exsanguination by cardiac puncture.	Anesthetization with a carbon dioxide:oxygen mixture followed by exsanguination by cardiac puncture
Necropsy	
Necropsies were performed on all animals. The following organs were weighed: right kidney, liver, and right testis.	Necropsies were performed on all animals in the core and special studies. The following organs were weighed: right kidney (core studies only), liver, and right testis.
Clinical Pathology	
None	Blood for clinical pathology analyses was collected by cardiac puncture from rats, mice, and hamsters anesthetized with a carbon dioxide:oxygen mixture. Animals in the special study groups were evaluated on day 34. Core study animals were evaluated at the end of the studies. ***Clinical chemistry:*** cholesterol and triglycerides (rats, mice, and hamsters); alanine aminotransferase, alkaline phosphatase, sorbitol dehydrogenase, and bile acids (rats and hamsters) ***Reproductive hormones:*** estradiol, testosterone, follicle-stimulating hormone, and luteinizing hormone.
Histopathology	
Histopathologic evaluations were performed on all mice in the 0 and 1,000 ppm groups and hamsters in the 0 and 5,000 ppm groups. In addition to gross lesions and tissue masses, the following tissues were examined: kidney, liver, pancreas, and testis. The liver was examined in the lower exposure groups.	Complete histopathologic evaluations were performed on core study rats, mice, and hamsters in the 0 and 500 ppm groups. In addition to gross lesions and tissue masses, the following tissues were examined: adrenal gland, bone and marrow, brain, esophagus, gallbladder (hamsters and mice), heart, large intestine (cecum, colon, rectum), small intestine (duodenum, jejunum, ileum), kidney, liver (left, right median, anterior right lobes), lung, lymph nodes (mandibular and mesenteric), mammary gland, nose, pancreas, parathyroid gland, pituitary gland, preputial gland, prostate gland, salivary gland, spleen, stomach (forestomach and glandular stomach), testis (with epididymis and seminal vesicle), thymus, thyroid gland, trachea, and urinary bladder. The liver, pancreas, and kidney (rats, mice, and hamsters); the preputial gland (rats, mice, and hamsters); the testis (mice and hamsters); and the prostate gland and seminal vesicle (hamsters) were examined in the remaining dosed groups. In addition, the heart (0 and 500 ppm animals), lumbar area and thigh (all animals), lower leg (mice), and tongue (0 and 500 ppm animals, five per group) were examined.
Sperm Motility Evaluations	
None	At the end of the studies, sperm samples were collected from core study rats, mice, and hamsters in the 0, 50, 100, and 500 ppm groups for sperm motility evaluations. The following parameters were evaluated: spermatid heads per testis and per gram testis, spermatid counts, and epididymal spermatozoal motility and concentration. The left cauda, left epididymis, and left testis were weighed.

TABLE 1
Experimental Design and Materials and Methods in the Feed Studies of Wy-14,643

2-Week Studies	3-Month Studies
Cell and Peroxisome Proliferation Analyses	
Osmotic minipumps containing 30 mg/mL 5-bromo-2′-deoxyuridine (BrdU) in 0.01 N sodium hydroxide were implanted in mice in the 0, 10, 100, and 1,000 ppm groups and hamsters in the 0, 10, 500, and 5,000 ppm groups on day 10. After 5 days, cell proliferation in one to three lobes of the liver was determined by measuring the incorporation of BrdU. Two thousand hepatocyte nuclei were counted in each lobe examined. Peroxisome proliferation in these animals and in 50 and 500 ppm mice and 100 and 1,000 ppm hamsters was determined by measuring the acyl coenzyme A oxidase activity, β oxidation spiral, nonspecific carnitine acetyltransferase activity, and peroxisomal catalase activity.	Osmotic minipumps containing 30 mg/mL BrdU in 0.01 N sodium hydroxide were implanted in five special study rats, mice, and hamsters per group on days 1, 29, and 85. After 5 days, cell proliferation in the liver was determined by measuring the incorporation of BrdU. Two thousand hepatocyte nuclei from the left liver lobe were examined for each animal. Peroxisome proliferation in these animals was determined by measuring the acyl coenzyme A oxidase activity, β oxidation spiral, nonspecific carnitine acetyltransferase activity, and peroxisomal catalase activity.
Liver Protein Analyses	
The livers of all mice and hamsters were evaluated by Argonne National Laboratory (Argonne, IL) using protein gel electrophoresis (Giometti *et al.*, 1998) and by the study laboratory using the bicinchoninic method (Smith *et al.*, 1985).	Liver samples from special study rats, mice, and hamsters were evaluated as described for the 2-week studies.
Cell Cycle Biomarker Analyses	
None	The livers of five special study rats and mice per group were analyzed for cyclin-dependent kinase activity during weeks 1 and 13 and for proliferating cell nuclear antigen concentration during week 13.
Determinations of Wy-14,643 in Plasma	
None	Blood was collected from the retroorbital sinus (rats and hamsters) or by cardiac puncture (mice) during weeks 3, 5, and 9. Blood was collected from one rat, mouse, or hamster per group per time point at 0600, 0800, 1000, 1200, 1400, 1600, 1800, 2000, 2200, 2400, 0200, and 0400. Each rat was bled at two time points (12 hours apart) during each collection period. Each mouse was bled once during only one collection period. Each hamster was bled at one time point during each collection period. All samples were analyzed for plasma concentrations of Wy-14,643.

STATISTICAL METHODS

Calculation and Analysis of Lesion Incidences

The incidences of nonneoplastic lesions are presented in Appendix A as the numbers of animals bearing such lesions at a specific anatomic site and the numbers of animals with that site examined microscopically. The Fisher exact test (Gart *et al.*, 1979), a procedure based on the overall proportion of affected animals, was used to determine significance.

Analysis of Continuous Variables

Two approaches were employed to assess the significance of pairwise comparisons between exposed and control groups in the analysis of continuous variables. Organ and body weight data, which historically have approximately

normal distributions, were analyzed with the parametric multiple comparison procedures of Dunnett (1955) and Williams (1971, 1972). Clinical chemistry, reproductive hormone, spermatid, epididymal spermatozoal, cell proliferation, and peroxisomal enzyme data, which have typically skewed distributions, were analyzed using the nonparametric multiple comparison methods of Shirley (1977) and Dunn (1964). Jonckheere's test (Jonckheere, 1954) was used to assess the significance of the dose-related trends and to determine whether a trend-sensitive test (Williams' or Shirley's test) was more appropriate for pairwise comparisons than a test that does not assume a monotonic dose-related trend (Dunnett's or Dunn's test). Prior to statistical analysis, extreme values identified by the outlier test of Dixon and Massey (1951) were examined by NTP personnel, and implausible values were eliminated from the analysis. Average severity values were analyzed for significance with the Mann-Whitney U test (Hollander and Wolfe, 1973). The percentages of BrdU-labeled hepatocytes in the median and right anterior liver lobes of male mice were analyzed with the Wilcoxon rank sum test (Conover, 1971).

Quality Assurance Methods

The 3-month studies were conducted in compliance with Food and Drug Administration Good Laboratory Practice Regulations (21 CFR, Part 58). The Quality Assurance Unit of Battelle Columbus Laboratories performed audits and inspections of protocols, procedures, data, and reports throughout the course of the studies.

Genetic Toxicology

Mouse Peripheral Blood Micronucleus Test Protocol

A detailed discussion of this assay is presented by MacGregor *et al.* (1990). Peripheral blood samples were obtained from male and female Tg.AC mice after 6 months of exposure to Wy-14,643 in feed (0, 10, 50, or 100 ppm) or by dermal (0, 2, 10, or 20 ppm) application. Smears were immediately prepared and fixed in absolute methanol. The methanol-fixed slides were sent to ILS, Inc. (Research Triangle Park, NC), where they were stained with acridine orange and coded. Slides were scanned to determine the frequency of micronuclei in 1,000 normochromatic erythrocytes (NCEs), and 1,000 cells were scored for the polychromatic erythrocytes (PCE) to NCE ratio in each of up to 14 animals per exposure group.

The results were tabulated as the mean of the pooled results from all animals within a treatment group plus or minus the standard error of the mean. The frequency of micronucleated cells among NCEs was analyzed by a statistical software package that tested for increasing trend over dose groups with a one-tailed Cochran-Armitage trend test, followed by pairwise comparisons between each dose group and the control group (ILS, 1990). In the presence of excess binomial variation, as detected by a binomial dispersion test, the binomial variance of the Cochran-Armitage test was adjusted upward in proportion to the excess variation. In the micronucleus test, an individual trial is

considered positive if the trend test P value is less than or equal to 0.025 or if the P value for any single exposed group is less than or equal to 0.025 divided by the number of exposed groups. A final call of positive for micronucleus induction is preferably based on reproducibly positive trials (as noted above). Results of the 6-month studies were accepted without repeat tests, because additional test data could not be obtained. Ultimately, the final call is determined by the scientific staff after considering the results of statistical analyses, the reproducibility of any effects observed, and the magnitudes of those effects.

Evaluation Protocol

These are the basic guidelines for arriving at an overall assay result for assays performed by the National Toxicology Program. Statistical as well as biological factors are considered. For an individual assay, the statistical procedures for data analysis have been described in the preceding protocol. There have been instances, however, in which multiple aliquots of a chemical were tested in the same assay, and different results were obtained among aliquots and/or among laboratories. Results from more than one aliquot or from more than one laboratory are not simply combined into an overall result. Rather, all the data are critically evaluated, particularly with regard to pertinent protocol variations, in determining the weight of evidence for an overall conclusion of chemical activity in an assay. In addition to multiple aliquots, the *in vitro* assays have another variable that must be considered in arriving at an overall test result. *In vitro* assays are conducted with and without exogenous metabolic activation. Results obtained in the absence of activation are not combined with results obtained in the presence of activation; each testing condition is evaluated separately. The results presented in the Abstract of this Toxicity Study Report represent a scientific judgement of the overall evidence for activity of the chemical in an assay.

RESULTS

RATS

3-MONTH STUDY

All core study rats survived to the end of the study (Table 2). Final mean body weights and body weight gains decreased with increasing exposure concentration, and the decreases were significant for all exposed groups except the 5 ppm group (Table 2 and Figure 1). Feed consumption by exposed animals was similar to that by the controls (Table 2). Exposure concentrations of 5, 10, 50, 100, and 500 ppm Wy-14,643 resulted in average daily doses of approximately 0.3, 0.6, 3, 7, and 34 mg Wy-14,643/kg body weight. There were no clinical findings of toxicity or exposure-related gross lesions noted at necropsy in core or special study rats.

TABLE 2
Survival, Body Weights, and Feed Consumption of Male Rats in the 3-Month Feed Study of Wy-14,643

Concentration (ppm)	Survival[a]	Mean Body Weight[b] (g)			Final Weight Relative to Controls (%)	Feed Consumption[c]	
		Initial	Final	Change		Week 1	Week 14
0	10/10	217 ± 3	423 ± 8	206 ± 7		20.2	21.4
5	10/10	216 ± 2	418 ± 7	202 ± 6	99	20.7	20.9
10	10/10	216 ± 3	394 ± 9**	178 ± 9**	93	20.7	20.7
50	10/10	213 ± 2	371 ± 7**	157 ± 6**	88	19.0	20.9
100	10/10	220 ± 2	352 ± 5**	132 ± 5**	83	18.7	21.1
500	10/10	218 ± 3	305 ± 7**	87 ± 6**	72	18.0	20.6

** Significantly different ($P \leq 0.01$) from the control group by Williams' test
[a] Number of animals surviving at 3 months/number initially in group
[b] Weights and weight changes are given as mean ± standard error.
[c] Feed consumption is expressed as grams per animal per day.

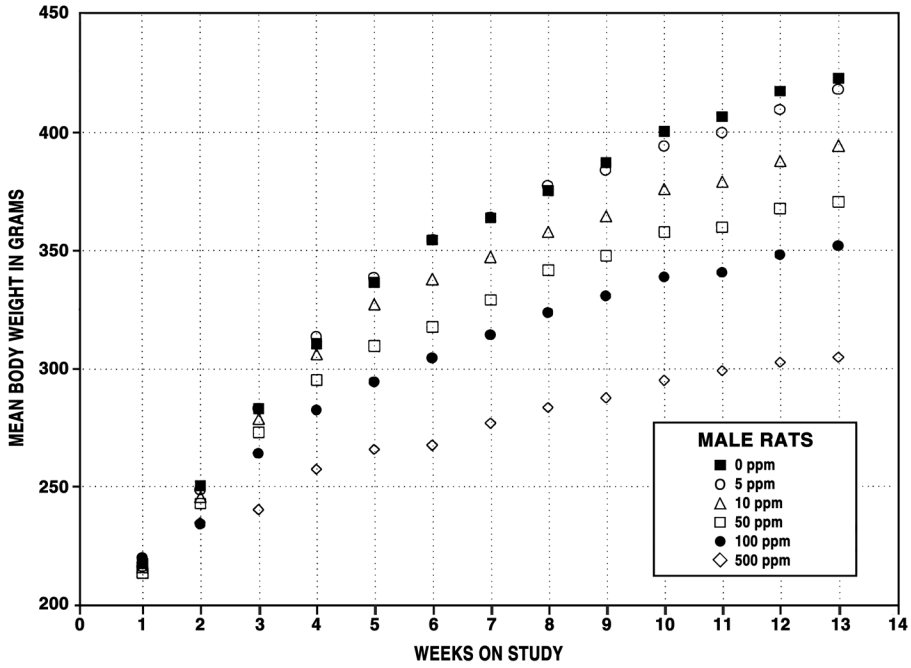


FIGURE 1
Body Weights of Male Rats Exposed to Wy-14,643 in Feed for 3 Months

The clinical chemistry data for male rats are listed in Tables 3 and B1. At day 34, there were treatment-related increases in serum alanine aminotransferase, sorbitol dehydrogenase, and alkaline phosphatase activities and bile salt concentrations. These changes occurred in multiple groups, affecting the 50, 100, and 500 ppm groups consistently. For markers of hepatocellular injury, the increase in alanine aminotransferase and sorbitol dehydrogenase activities ranged between 1.3- to 2.7-fold. For markers of cholestasis, the increase in alkaline phosphatase activity and bile salt concentration ranged from 1.3- to 3.3-fold. By week 14, increases in alanine aminotransferase and sorbitol dehydrogenase activities persisted in the 50, 100, and 500 ppm groups, but the increase ameliorated slightly to range from 1.1- to 1.4-fold. Increases in alkaline phosphatase activity and bile salt concentration also persisted, affecting all exposed groups with an increase from 1.3- to 4.1-fold. No changes occurred in the cholesterol, triglyceride, or reproductive hormone concentrations at either time point.

Plasma concentrations of Wy-14,643 in rats increased with increasing exposure concentration (Table C1). While volume constraints did not allow for measurement of Wy-14,643 in 3-month animals, there did not appear to be significant time-dependent differences in Wy-14,643 between samples tested at 3, 5, and 9 weeks at any exposure concentration (Table C1).

TABLE 3
Selected Clinical Chemistry Data in Male Rats in the 3-Month Feed Study of Wy-14,643[a]

	0 ppm	5 ppm	10 ppm	50 ppm	100 ppm	500 ppm
n						
Day 34	5	5	5	5	5	5
Week 14	10	10	10	10	10	10
Alanine aminotransferase (IU/L)						
Day 34	70 ± 7	113 ± 38	141 ± 49	94 ± 2*	110 ± 20*	146 ± 43**
Week 14	80 ± 3	84 ± 2	85 ± 4	98 ± 7*	89 ± 6	111 ± 6**
Alkaline phosphatase (IU/L)						
Day 34	615 ± 10	776 ± 53*	916 ± 53**	1,355 ± 124**	1,381 ± 26**	1,747 ± 150**
Week 14	495 ± 20	663 ± 31**	796 ± 37**	1,302 ± 38**	1,359 ± 85**	1,703 ± 136**
Sorbitol dehydrogenase (IU/L)						
Day 34	20 ± 1	25 ± 8	50 ± 26	33 ± 3*	31 ± 3*	53 ± 17*
Week 14	22 ± 1	21 ± 1	24 ± 2	30 ± 2**	24 ± 1*	25 ± 1*
Bile salts (μmol/L)						
Day 34	56.4 ± 5.8	53.8 ± 10.7	91.8 ± 17.7	83.8 ± 10.6	153.0 ± 20.6**	183.4 ± 21.0**
Week 14	35.2 ± 4.3	64.7 ± 8.1**	59.0 ± 5.0**	139.5 ± 15.2**	144.5 ± 25.2**	120.1 ± 17.3**

* Significantly different ($P \leq 0.05$) from the control group by Dunn's or Shirley's test
** Significantly different ($P \leq 0.01$) from the control group by Shirley's test
[a] Mean ± standard error. Statistical tests were performed on unrounded data.

At all time points, the absolute and relative liver weights of all exposed groups of core and special study rats were generally significantly greater than those of the controls (Tables D1 and D2). Histologically, hepatocyte cytoplasmic alteration was present in all dosed groups, and the severity of this lesion increased with increasing exposure concentrations (Tables 4 and A1). Affected hepatocytes were enlarged with abundant granular eosinophilic cytoplasm and indistinct sinusoids. Normal cytoplasmic vacuolation due to glycogen accumulation within hepatocytes was absent except in controls. The extent and severity of cytoplasmic alteration correlated well with biochemical assays for peroxisomal enzyme activity and the increase in liver weight. Peroxisomal β-oxidation of lipids generally increased with increasing exposure concentration, and peroxisomal β-oxidation on day 34 and at week 13 was higher than that on day 6 for all exposed groups (Table G1).

In agreement with the BrdU-labeling of hepatocytes, increased numbers of mitotic figures were present in livers of rats at 50 ppm and above (Tables 4 and A1). A few 500 mg/kg rat livers had increased apoptotic cells.

BrdU labeling of hepatocytes was increased in all exposed groups of rats by day 6 but was increased only in 50 ppm or greater rats on day 34 and at week 13 (Table F1). Later in the study, the magnitude of the labeling response was reduced; the labeling of hepatocytes in 5 ppm rats on day 6 was twofold higher than in 500 ppm rats on day 34 and threefold higher than in 500 ppm rats at week 13. These data were confirmed by similar exposure- and

TABLE 4
Incidences of Selected Nonneoplastic Lesions of Male Rats in the 3-Month Feed Study of Wy-14,643

	0 ppm	5 ppm	10 ppm	50 ppm	100 ppm	500 ppm
Liver[a]	10	10	10	10	10	10
Cytoplasmic Alteration[b]	0	9** (1.0)[c]	10** (2.0)	10** (2.8)	10** (2.9)	10** (3.0)
Mitotic Alteration	0	0	0	2 (2.5)	4* (2.0)	7** (2.1)
Kidney	10	9	10	9	10	10
Renal Tubule, Regeneration	0	1 (1.0)	4* (1.0)	4* (1.0)	9** (1.0)	5* (1.0)
Skeletal Muscle, Lumbar Area	10	10	10	10	10	10
Myopathy, Degenerative	0	0	0	0	1 (1.0)	9** (1.6)
Skeletal Muscle, Thigh	10	10	10	10	10	10
Myopathy, Degenerative	0	0	0	0	9** (1.6)	10** (2.6)

* Significantly different ($P \leq 0.05$) from the control group by the Fisher exact test
** $P \leq 0.01$
[a] Number of animals with tissue examined microscopically
[b] Number of animals with lesion
[c] Average severity grade of lesions in affected animals: 1=minimal, 2=mild, 3=moderate, 4=marked

time-dependent increases in cyclin-dependent kinase and proliferating cell nuclear antigen, two other markers in cells undergoing cell proliferation.

At 3 months, increased relative kidney weights occurred in all exposed core study groups (Table D2). Renal tubule regeneration of minimal severity at the cortico-medullary junction occurred in all exposed groups with significantly increased incidences in rats exposed to 10 ppm or greater (Tables 4 and A1).

Significant increases of degenerative myopathy occurred in the skeletal muscle of the lumbar area in 500 ppm rats and the thigh in 100 and 500 ppm rats, and the severities were increased at 500 ppm (Table 4).

At 34 days, decreased absolute testis weight occurred in the 50 to 500 ppm special study groups; at 3 months, increased relative testis weight occurred in the 50 to 500 ppm core and special study groups (Tables D1 and D2). The cauda epididymis weight of the 500 ppm group and epididymis weights of the 100 and 500 ppm groups were significantly less than those of the controls (Table E1). There were no significant differences in spermatid or epididymal spermatozoal measurements between exposed and control rats. Changes in reproductive hormones did not appear to be exposure-related (Table B1).

MICE

2-WEEK STUDY

All mice survived to the end of the study (Table 5). The mean body weight gain of 500 ppm mice was significantly less than that of the controls (Table 5). During the second week of the study, feed consumption by 500 ppm mice was greater than that by the controls; no feed consumption could be determined for the 1,000 ppm group due to feed scattering (Table 5). Exposure concentrations of 10, 50, 100, 500, and 1,000 ppm resulted in average daily doses of approximately 2, 9, 16, 105, and 184 mg/kg. There were no clinical findings of toxicity.

The liver weights of all exposed groups of mice were generally significantly increased (Table D3).

Wy-14,643 produced a dose-related increase in peroxisomal enzyme activity in mice, starting at the 10 ppm exposure level (Table G2). Peroxisomal β-oxidation, carnitine acetyltransferase, catalase, and acyl CoA oxidase were increased in all exposed groups compared to controls. The percentages of BrdU-labeled hepatocytes were increased in 100 and 1,000 ppm mice (Table F2).

Hepatocyte hypertrophy was observed microscopically in the liver of mice exposed to 50 ppm or greater, and the incidences were significantly increased in the 500 and 1,000 ppm groups (0/5, 0/5, 1/5, 2/5, 5/5, 5/5). Four of five mice in the 1,000 ppm group also had widespread individual cell necrosis.

TABLE 5
Survival, Body Weights, and Feed Consumption of Male Mice in the 2-Week Feed Study of Wy-14,643

Concentration (ppm)	Survival[a]	Mean Body Weight[b] (g)			Final Weight Relative to Controls (%)	Feed Consumption[c]	
		Initial	Final	Change		Day 8	Day 15
0	5/5	22.8 ± 0.4	26.9 ± 0.5	4.1 ± 0.4		4.4	4.8
10	5/5	23.0 ± 0.2	26.1 ± 0.9	3.1 ± 0.7	97	4.0	5.5
50	5/5	22.9 ± 0.2	26.1 ± 0.3	3.2 ± 0.2	97	4.4	5.1
100	5/5	22.7 ± 0.4	27.4 ± 0.3	4.6 ± 0.3	102	4.0	4.8
500	5/5	22.8 ± 0.3	24.0 ± 1.5	1.2 ± 1.2*	89	4.2	6.0
1,000	5/5	22.6 ± 0.3	25.3 ± 0.2	2.7 ± 0.2	94	4.4	—[d]

* Significantly different (P≤0.05) from the control group by Dunnett's test
[a] Number of animals surviving at 2 weeks/number initially in group
[b] Weights and weight changes are given as mean ± standard error.
[c] Feed consumption is expressed as grams per animal per day.
[d] No data were available due to feed scattering.

3-Month Study

All core study mice survived to the end of the study (Table 6). Final mean body weights and body weight gains generally decreased with increasing exposure concentration, and the decreases were significant for mice exposed to 50 ppm or greater (Table 6 and Figure 2). Feed consumption by exposed and control mice was similar; no feed consumption could be determined for the 500 ppm group during week 14 due to feed scattering (Table 6). Exposure concentrations of 5, 10, 50, 100, and 500 ppm resulted in average daily doses of approximately 0.9, 2, 12, 22, and 135 mg/kg. There were no clinical findings of toxicity.

Plasma concentrations of Wy-14,643 in mice increased with increasing exposure concentration (Table C2). While volume constraints did not allow for measurement of Wy-14,643 in 3-month animals, there did not appear to be significant time-dependent differences in Wy-14,643 between samples tested at 3, 5, and 9 weeks at any exposure concentration (Table C2).

Table 6
Survival, Body Weights, and Feed Consumption of Male Mice in the 3-Month Feed Study of Wy-14,643

Concentration (ppm)	Survival[a]	Mean Body Weight[b] (g)			Final Weight Relative to Controls (%)	Feed Consumption[c]	
		Initial	Final	Change		Week 2	Week 14
0	10/10	22.3 ± 0.3	33.3 ± 0.7	11.0 ± 0.8		4.9	4.5
5	10/10	22.2 ± 0.2	32.0 ± 0.5	9.8 ± 0.5	96	4.3	4.6
10	10/10	22.0 ± 0.2	32.2 ± 0.5	10.1 ± 0.5	96	5.7	5.0
50	10/10	21.9 ± 0.3	30.7 ± 0.7**	8.8 ± 0.5**	92	5.2	4.7
100	10/10	21.6 ± 0.3	30.1 ± 0.3**	8.5 ± 0.2**	90	5.3	5.3
500	10/10	22.4 ± 0.3	24.6 ± 0.3**	2.2 ± 0.4**	74	4.6	—[d]

** Significantly different (P≤0.01) from the control group by Williams' test
[a] Number of animals surviving at 3 months/number initially in group
[b] Weights and weight changes are given as mean ± standard error.
[c] Feed consumption is expressed as grams per animal per day.
[d] No data were available due to feed scattering.

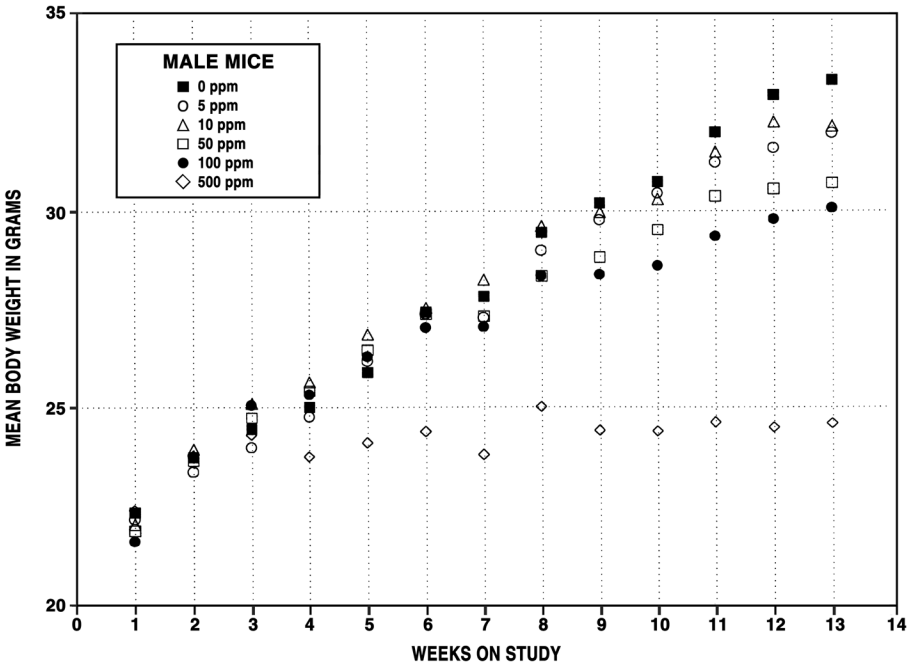


FIGURE 2
Body Weights of Male Mice Exposed to Wy-14,643 in Feed for 3 Months

The clinical chemistry data for male mice are listed in Tables 7 and B2. On day 34, a slight (1.2-fold) increase in cholesterol concentration occurred in 500 ppm animals. Conversely, triglyceride concentrations were decreased in all exposed groups; the decrease ranged from 30% to 55%. By week 14, the slight increase in cholesterol concentration persisted and occurred in essentially all exposed groups with increases from 1.05- to 1.35-fold. The decreases in triglyceride concentrations also persisted, affecting all exposed groups with decreases from 46% to 65%. At week 14, there was an increase in estradiol concentration (~1.3-fold) and a decrease in follicle-stimulating hormone concentration (~65%) in 500 ppm male mice.

At all time points, the liver weights of all exposed groups of core and special study mice were generally significantly greater than those of the controls (Tables D4 and D5). Cytoplasmic alteration characterized by hypertrophied hepatocytes with eosinophilic granular cytoplasm was present in all treated mice with an exposure concentration-related increase in severity (Tables 8 and A2). Exposure concentration-related increases in frequency and severity of mitoses were present in the liver of mice exposed to 50 ppm or greater. Mild to marked numbers of apoptosis were present at 100 and 500 ppm with an exposure concentration-related increase in severity.

TABLE 7
Selected Clinical Chemistry and Reproductive Hormone Data in Male Mice in the 3-Month Feed Study of Wy-14,643[a]

	0 ppm	5 ppm	10 ppm	50 ppm	100 ppm	500 ppm
Clinical Chemistry						
n						
Day 34	5	5	5	4	5	5
Week 14	10	10	10	10	10	10
Cholesterol (mg/dL)						
Day 34	158 ± 14	188 ± 8	182 ± 6	182 ± 5	177 ± 3	192 ± 4*
Week 14	173 ± 6	228 ± 5**	233 ± 6**	206 ± 11	183 ± 4	211 ± 5*
Triglycerides (mg/dL)						
Day 34	165 ± 14	102 ± 10*	108 ± 12*	86 ± 6**	74 ± 9**	114 ± 20**
Week 14	177 ± 12	96 ± 7**	81 ± 5**	76 ± 8**	73 ± 7**	63 ± 3**
Reproductive Hormone Analyses						
n						
Day 34	4	5	5	4	5	4
Week 14	10	9	10	10	10	10
Estradiol (pg/mL)						
Day 34	50.8 ± 6.3	61.8 ± 3.5	65.1 ± 1.6	60.9 ± 0.6	65.2 ± 2.7	67.5 ± 8.4
Week 14	59.1 ± 5.5[b]	68.3 ± 0.9	69.8 ± 1.0	62.7 ± 2.2	60.2 ± 5.0[b]	77.6 ± 2.1**
Follicle-stimulating hormone (ng/mL)						
Day 34	22.9 ± 0.7[c]	20.8 ± 4.7[c]	27.0 ± 1.8	23.9 ± 1.1[d]	18.8 ± 0.7[c]	—[e]
Week 14	26.8 ± 2.1[f]	23.6 ± 1.7	24.5 ± 0.9[g]	27.0 ± 1.8[b]	30.7 ± 7.6[g]	9.3 ± 0.8**[g]

* Significantly different ($P \leq 0.05$) from the control group by Dunn's or Shirley's test
** $P \leq 0.01$
[a] Mean ± standard error. Statistical tests were performed on unrounded data.
[b] n=9
[c] n=3
[d] n=2
[e] Not measured at this exposure concentration
[f] n=7
[g] n=8

Minimal hepatic pigmentation consistent with cholestasis and oval cell proliferation was present in all mice exposed to 500 ppm and a few 100 ppm mice (Tables 8 and A2). Cytoplasmic lipid vacuolization was present in all exposed mice and was not observed in any control mice, and the severity increased with increasing exposure concentration. There was a clear increase in peroxisomal enzyme activity in all exposed groups. Histomorphological changes are consistent with prolonged peroxisome proliferation similar to chronic studies of other peroxisome proliferators and concomitant hepatic toxicity (cytoplasmic vacuolation and oval cell hyperplasia) with a proliferative response.

TABLE 8
Incidences of Selected Nonneoplastic Lesions in Male Mice in the 3-Month Feed Study of Wy-14,643

	0 ppm	5 ppm	10 ppm	50 ppm	100 ppm	500 ppm
Liver[a]	10	10	10	10	10	10
Cytoplasmic Alteration[b]	0	10** (1.0)[c]	10** (1.2)	10** (2.3)	10** (2.6)	10** (3.2)
Mitotic Alteration	0	0	0	4* (1.0)	7** (1.4)	10** (3.0)
Apoptosis Alteration	0	0	0	0	6** (1.8)	10** (3.6)
Pigmentation	0	0	0	0	3 (1.0)	10** (1.0)
Oval Cell, Hyperplasia	0	0	0	0	0	10** (1.0)
Skeletal Muscle, Lumbar Area	10	10	10	10	10	10
Myopathy, Degenerative	0	0	0	0	0	10** (1.0)
Skeletal Muscle, Thigh	9	8	9	8	8	9
Myopathy, Degenerative	0	0	0	1 (1.0)	0	9** (1.7)
Skeletal Muscle, Lower Leg	9	8	9	8	8	9
Myopathy, Degenerative	0	0	0	2 (1.0)	3 (1.0)	9** (1.2)

* Significantly different ($P \leq 0.05$) from the control group by the Fisher exact test
** $P \leq 0.01$
[a] Number of animals with tissue examined microscopically
[b] Number of animals with lesion
[c] Average severity grade of lesions in affected animals: 1=minimal, 2=mild, 3=moderate, 4=marked

All exposed groups had an increase in BrdU-labeled hepatocytes as well as increased levels of cyclin-dependent kinase and proliferating cell nuclear antigen, indicative of a proliferative response (Table F3). The percentages of BrdU-labeled hepatocytes were increased in mice exposed to concentrations as low as 10 ppm at 6 and 34 days and at 50 ppm or greater at 13 weeks. These data were confirmed by similar exposure- and time-dependent increases in cyclin-dependent kinase and proliferating cell nuclear antigen. As in rats, peroxisomal β-oxidation of lipids increased with increasing exposure concentration; in 100 and 500 ppm mice, peroxisomal β-oxidation on day 34 and at the end of the study was greater than on day 6 (Table G3).

In skeletal muscle, significantly increased incidences of minimal to mild degenerative myopathy occurred in 500 ppm mice in the lumbar area, thigh, and lower leg (Table 8).

The testis weight of the 500 ppm group and epididymis weights of the 100 and 500 ppm groups were significantly less than those of the controls (Table E2). There were no significant differences in spermatid or epididymal spermatozoal measurements between exposed and control mice. Few alterations in reproductive hormone levels were observed (Table B2).

Other organ weight differences between exposed and control rats reflected body weight differences.

HAMSTERS

2-WEEK STUDY

All hamsters survived to the end of the study (Table 9). The final mean body weights and body weight gains of hamsters exposed to 100 ppm or greater were significantly less than those of the controls; these groups lost weight during the study (Table 9). Feed consumption by exposed and control hamsters was generally similar; high feed consumption values for some groups were attributed to feed scattering (Table 9). No feed consumption could be determined for the 1,000 ppm group on day 8 due to feed scattering. Exposure concentrations of 10, 100, 500, 1,000, and 5,000 ppm resulted in average daily doses of approximately 1, 8, 55, 80, and 550 mg/kg. There were no clinical findings of toxicity. The liver weights of all exposed groups of hamsters were significantly increased (Table D6). The percentages of BrdU-labeled hepatocytes were significantly increased in 500 and 5,000 ppm hamsters (Table F4). Peroxisomal β-oxidation of lipids was increased in all exposed groups (Table G4).

At necropsy, one hamster in each of the 100 and 500 ppm groups and two 5,000 ppm hamsters had enlarged livers; multiple liver foci were observed in one 1,000 ppm hamster. Minimal to mild multifocal vacuolation was observed microscopically in the liver of four 500 ppm hamsters, three 1,000 ppm hamsters, and three 5,000 ppm hamsters. Hepatocyte hypertrophy was observed in one hamster in each of the 500 and 5,000 ppm groups.

TABLE 9
Survival, Body Weights, and Feed Consumption of Male Hamsters in the 2-Week Feed Study of Wy-14,643

Concentration (ppm)	Survival[a]	Mean Body Weight[b] (g)			Final Weight Relative to Controls (%)	Feed Consumption[c]	
		Initial	Final	Change		Day 8	Day 15
0	5/5	106 ± 3	115 ± 4	9 ± 1		9.6	9.8
10	5/5	109 ± 2	115 ± 3	6 ± 2	100	7.7	8.9
100	5/5	107 ± 2	95 ± 3**	–12 ± 2**	83	10.4	6.5
500	5/5	107 ± 1	99 ± 3**	–8 ± 1**	86	8.9	11.3
1,000	5/5	106 ± 2	87 ± 3**	–19 ± 3**	76	—[d]	7.0
5,000	5/5	107 ± 2	94 ± 3**	–13 ± 2**	82	12.2	8.7

** Significantly different (P≤0.01) from the control group by Williams' test
[a] Number of animals surviving at 2 weeks/number initially in group
[b] Weights and weight changes are given as mean ± standard error.
[c] Feed consumption is expressed as grams per animal per day.
[d] No data were available due to feed scattering.

3-Month Study

All core study hamsters survived to the end of the study (Table 10). Final mean body weights and body weight gains decreased with increasing exposure concentration, and the decreases were significant in groups exposed to 10 ppm or greater (Table 10 and Figure 3). Hamsters in the 100 and 500 ppm groups lost weight during the study. During week 14, hamsters exposed to 50 ppm or greater consumed slightly less feed than did the controls (Table 10). Exposure concentrations of 5, 10, 50, 100, and 500 ppm resulted in average daily doses of approximately 0.4, 0.7, 4, 9, and 42 mg/kg. The only clinical finding of toxicity was thinness of two 50 ppm and five 500 ppm hamsters.

Table 10
Survival, Body Weights, and Feed Consumption of Male Hamsters in the 3-Month Feed Study of Wy-14,643

Concentration (ppm)	Survival[a]	Mean Body Weight[b] (g)			Final Weight Relative to Controls (%)	Feed Consumption[c]	
		Initial	Final	Change		Week 2	Week 14
0	10/10	88 ± 2	119 ± 4	30 ± 3		8.0	8.2
5	10/10	90 ± 2	118 ± 3	28 ± 3	99	7.9	7.2
10	10/10	88 ± 2	104 ± 3**	16 ± 1**	88	6.6	7.6
50	10/10	88 ± 3	88 ± 4**	0 ± 3**	74	7.8	6.3
100	10/10	89 ± 2	87 ± 3**	–2 ± 3**	74	8.6	6.2
500	10/10	89 ± 2	77 ± 3**	–12 ± 3**	65	9.6	5.2

** Significantly different (P≤0.01) from the control group by Williams' test
[a] Number of animals surviving at 3 months/number initially in group
[b] Weights and weight changes are given as mean ± standard error.
[c] Feed consumption is expressed as grams per animal per day.

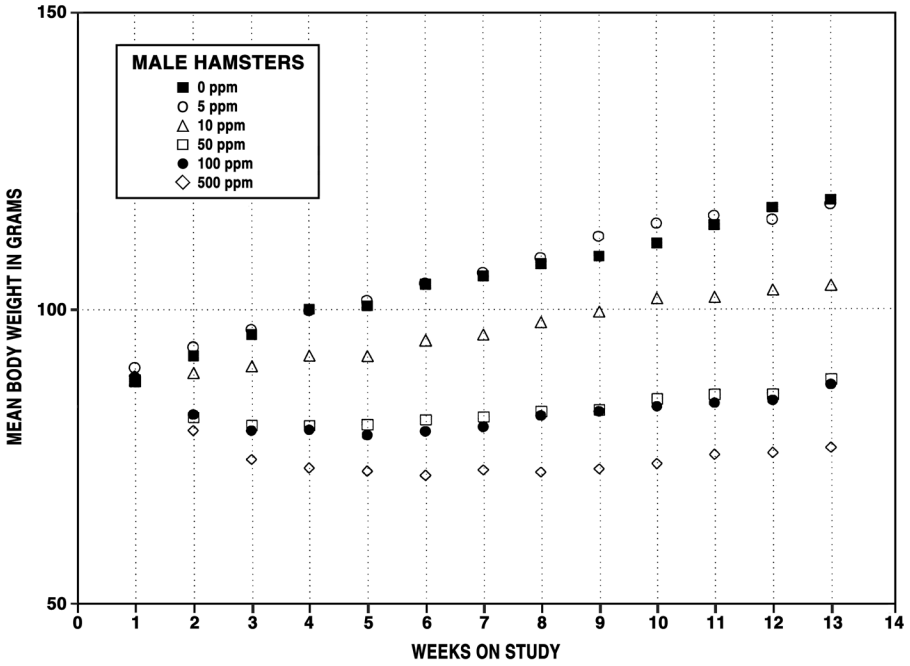


FIGURE 3
Body Weights of Male Hamsters Exposed to Wy-14,643 in Feed for 3 Months

The clinical chemistry data for male hamsters are listed in Tables 11 and B3. At day 34, a treatment-related decrease in cholesterol concentration occurred in the 10, 50, 100, and 500 ppm groups (15% to 45%). Triglyceride concentrations were also decreased in the 50, 100, and 500 ppm groups with decreases from 50% to 65%. At this time point, there were decreases in estradiol, testosterone, and luteinizing hormone concentrations in the 50, 100, and 500 ppm groups. For estradiol concentration, the decreases were mild at approximately 20%. For testosterone and luteinizing hormone concentrations, the decreases were marked at 97% to 100%.

At week 14, the decrease in cholesterol concentration persisted and occurred in all exposed groups with decreases from 35% to 56%. The decreases in triglyceride concentrations also persisted, affecting all exposed groups with decreases from 25% to 70%. At this time point, there were also increases in the markers of hepatocellular injury in the 50, 100, and 500 ppm groups; increases in serum alanine aminotransferase and sorbitol dehydrogenase activities and bile salt concentrations ranged from 1.3- to 2.5-fold. The decreases in reproductive hormone concentrations that occurred at day 34 were also present at week 14. For estradiol concentration, the decreases occurred in all exposed groups but remained mild, ranging from 12% to 30%. Similar to day 34, the decreases for testosterone and luteinizing hormone concentrations occurred in the 50, 100, and 500 ppm groups and were moderate to marked; testosterone was decreased 79% to 93% and luteinizing hormone was decreased 24% to 66%.

TABLE 11
Selected Clinical Chemistry and Reproductive Hormone Data in Male Hamsters in the 3-Month Feed Study of Wy-14,643[a]

	0 ppm	5 ppm	10 ppm	50 ppm	100 ppm	500 ppm
n						
Day 34	5	5	5	5	5	5
Week 14	10	10	10	10	10	10
Clinical Chemistry						
Cholesterol (mg/dL)						
Day 34	140 ± 19	124 ± 4	118 ± 5**	87 ± 3**	77 ± 3**	78 ± 4**
Week 14	157 ± 4	104 ± 4**	101 ± 8**[b]	76 ± 4**	69 ± 4**	90 ± 5**[b]
Triglycerides (mg/dL)						
Day 34	233 ± 38	199 ± 33	185 ± 6	113 ± 16**	87 ± 12**	82 ± 10**
Week 14	207 ± 9	145 ± 8**	157 ± 32**[b]	87 ± 8**	65 ± 5**	63 ± 5**[b]
Alanine aminotransferase (IU/L)						
Day 34	70 ± 11[c]	53 ± 5	61 ± 11	75 ± 8	78 ± 7	103 ± 15
Week 14	69 ± 4	61 ± 7	65 ± 8	96 ± 6*	104 ± 13*	162 ± 30**
Sorbitol dehydrogenase (IU/L)						
Day 34	54 ± 9	51 ± 4	56 ± 11	58 ± 6	48 ± 10	67 ± 13
Week 14	57 ± 4	55 ± 8	50 ± 3	75 ± 5*	81 ± 17	142 ± 40**
Bile salts (µmol/L)						
Day 34	17.8 ± 8.6	10.2 ± 2.5	16.0 ± 5.8	12.0 ± 1.4	18.4 ± 8.0	14.8 ± 2.4
Week 14	10.0 ± 1.1	12.4 ± 1.3	10.2 ± 1.4	15.2 ± 1.6*	16.7 ± 2.9*	22.7 ± 2.6**
Reproductive Hormone Analyses						
Estradiol (pg/mL)						
Day 34	54.1 ± 3.2	53.4 ± 2.3	48.4 ± 3.5	44.7 ± .98*	44.9 ± 3.2*	43.2 ± 3.0*
Week 14	53.3 ± 1.6	42.2 ± 1.5**	46.9 ± 4.5**	40.6 ± 1.7**	37.6 ± 1.8**	41.9 ± 1.4**[b]
Testosterone (ng/mL)						
Day 34	0.7 ± 0.2	0.8 ± 0.5	0.7 ± 0.3	0.0 ± 0.0**	0.0 ± 0.0**	0.0 ± 0.0**
Week 14	1.4 ± 0.3	1.9 ± 0.6	1.1 ± 0.4	0.3 ± 0.2*	0.3 ± 0.2**	0.1 ± 0.1**
Luteinizing hormone (ng/mL)						
Day 34	1.03 ± 0.41	1.20 ± 0.70	0.33 ± 0.14	0.03 ± 0.03**	0.00 ± 0.00**	0.02 ± 0.01**
Week 14	2.14 ± 0.42	3.62 ± 0.83[d]	2.31 ± 0.47	1.03 ± 0.20*	0.73 ± 0.08**	1.63 ± 0.43*

* Significantly different (P≤0.05) from the control group by Dunn's or Shirley's test
** Significantly different (P≤0.01) from the control group by Shirley's test
[a] Mean ± standard error. Statistical tests were performed on unrounded data.
[b] n=10
[c] n=4
[d] n=8

Plasma concentrations of Wy-14,643 in hamsters increased with increasing exposure concentration (Table C3). While volume constraints did not allow for measurement of Wy-14,643 in 3-month animals, there did not appear to be significant time-dependent differences in Wy-14,643 between samples tested at 3, 5, and 9 weeks at any exposure concentration (Table C3).

At all time points, the liver weights of all exposed groups of core and special study hamsters were significantly greater than those of the controls (Tables D7 and D8). On gross examination at necropsy, liver discoloration was observed in 500 ppm hamsters; microscopically, pigmentation was observed only in two control and one 5 ppm hamsters.

Percentages of BrdU-labeled hepatocytes were significantly increased in all exposed groups on day 6 and in 50 ppm or greater groups on day 34 and at the end of the study (Table F5). Similar to rats but not to mice, the magnitude of the increased hepatocyte proliferation in hamsters decreased over time, and the magnitude in 500 ppm hamsters at 13 weeks was approximately 50% of that on day 6. As in rats and mice, peroxisomal β-oxidation of lipids increased with increasing exposure concentration; peroxisomal β-oxidation also generally increased with time (Table G5).

Panlobular cytoplasmic alteration consisting of enlarged hepatocytes with eosinophilic granular cytoplasm was observed in all exposed hamsters and had an exposure concentration-dependent increase in severity ranging from mild in the 5 ppm group to marked in the 500 ppm group (Tables 12 and A3). This change is consistent with the observed increase in peroxisomal enzyme activity. Despite the increased BrdU labeling of hepatocytes, there was no apparent hepatocellular hyperplasia. Increased numbers of mitotic figures and apoptosis were minimal and rarely recognized. Mild cytoplasmic lipid vacuolization was present in the 50 ppm or greater groups, and the incidence was significantly increased at 100 and 500 ppm.

Significantly increased incidences of minimal degenerative myopathy occurred in skeletal muscle of the lumbar area and thigh in 100 and 500 ppm hamsters (Table 12).

Testis weights were significantly decreased in 500 ppm special study hamsters at day 34 (Table D7). At 3 months, testis weights were significantly decreased in special study hamsters exposed to 50 ppm or greater and in core study hamsters exposed to 100 or 500 ppm (Tables D7 and D8). Cauda epididymis, epididymis, and testis weights; spermatid heads per testis; and spermatid counts were significantly decreased in all exposed groups evaluated for sperm motility (Table E3). Epididymal spermatozoal motility and concentration in the 100 and 500 ppm groups and spermatid heads per gram testis in the 500 ppm group were also significantly decreased. Spermatid and epididymal spermatozoal measurements of 50 and 100 ppm hamsters were similar to those of the controls (Table E4). Cauda epididymis and epididymis weights of the 50 and 100 ppm groups and the testis weight of the 50 ppm group were again less than those of the controls. At the end of the study, serum estradiol concentrations were significantly less in all exposed groups of hamsters than in the controls (Table B3). In addition, testosterone and luteinizing hormone concentrations in groups exposed to 50 ppm or greater were significantly less than those in the controls.

TABLE 12
Incidences of Selected Nonneoplastic Lesions in Male Hamsters in the 3-Month Feed Study of Wy-14,643

	0 ppm	5 ppm	10 ppm	50 ppm	100 ppm	500 ppm
Liver[a]	10	10	10	10	10	10
Cytoplasmic Alteration[b]	0	10** (2.0)[c]	10** (2.3)	10** (3.4)	10** (3.4)	10** (3.5)
Cytoplasmic Vacuolization	0	0	0	1 (2.0)	5* (1.8)	4* (1.8)
Skeletal Muscle, Lumbar Area	10	10	10	10	10	10
Myopathy, Degenerative	0	0	0	0	9** (1.0)	9** (1.0)
Skeletal Muscle, Thigh	10	10	10	10	10	10
Myopathy, Degenerative	0	0	0	0	8** (1.0)	9** (1.0)
Prostate Gland	10	10	10	10	10	9
Atrophy	1 (2.0)	1 (2.0)	0	3 (2.0)	10** (2.9)	9** (3.4)
Seminal Vesicle	10	10	10	10	10	10
Atrophy	1 (2.0)	1 (2.0)	0	3 (2.0)	10** (2.8)	10** (2.8)
Testes	10	10	10	10	10	10
Atrophy	2 (2.5)	2 (2.5)	1 (1.0)	4 (2.5)	7** (2.9)	10** (3.0)

* Significantly different ($P \leq 0.05$) from the control group by the Fisher exact test
** $P \leq 0.01$
[a] Number of animals with tissue examined microscopically
[b] Number of animals with lesion
[c] Average severity grade of lesions in affected animals: 1=minimal, 2=mild, 3=moderate, 4=marked

Consistent with sperm and hormonal measurements, there was atrophy of the testes and accessory sex glands (Tables 12, A1, and B3), probably secondary to the significant exposure concentration-related decrease in body weight gain in hamsters. The documented pancreatitis, renal mineralization, and nephropathy are not considered treatment related (Table A3).

TOXICOKINETIC STUDIES

Toxicokinetics and estimates of the internal dose and linear dose range of Wy-14,643 administered as a single intravenous or gavage dose or *ad libitum* for 9 or 10 days in feed to male Wistar Furth rats, $B6C3F_1$ mice, and Syrian hamsters were conducted (Appendix J). Observed plasma concentrations of Wy-14,643 from the feed studies were compared with simulated concentrations using mathematical models based on the results of single-dose intravenous and gavage studies.

In multiple exposure feed studies, the exposure concentrations were 50 and 500 ppm (rats and mice) and 100 and 1,000 ppm (hamsters). In these studies, the average daily dose of Wy-14,643 was proportional to the concentration

of Wy-14,643 in feed, indicating that palatability of the formulated diet was not a problem. The highest observed plasma concentrations of Wy-14,643 (C_{max}) following 9 or 10 days of feeding at the low exposure concentrations were 0.15 to 0.73 µg/mL, 0.088 to 1.3 µg/mL, and not detected to 0.45 µg/mL for rats, mice, and hamsters, respectively; at the high exposure concentrations, they were 2.4 to 9.0 µg/mL, 1.6 to 8.4 µg/mL, and 0.53 to 4.0 µg/mL for rats, mice, and hamsters, respectively.

Single-dose intravenous and oral gavage studies were performed in Sprague-Dawley rats, B6C3F$_1$ mice, and Syrian hamsters. Following a single gavage dose of 2, 2, or 3 mg/kg in rats, mice, and hamsters, bioavailability (F) was 0.34, 0.98, and 0.24; C_{max} was 1.42, 6.94, and 2.88 µg/mL plasma; and clearance (Cl_{app}) was 8.48, 1.51, and 15.8 mL/minute per kg for rats, mice, and hamsters, respectively. Following a single gavage dose of 5, 8, or 10 mg/kg, F was 0.53, 0.44, and 0.16; C_{max} was 5.13, 20.5, and 6.04 µg/mL plasma; and Cl_{app} was 5.48, 3.37, and 23.2 mL/minute per kg for rats, mice, and hamsters, respectively. Based on examination of areas under the concentration versus time curves (AUC), exposure to Wy-14,643 appeared to be dose proportional at doses of 1 to 5 mg/kg in rats and 1 to 10 mg/kg in hamsters. However, dose proportionality was not observed in mice over a dose range of 2 to 8 mg/kg.

Comparing the plasma concentrations following a single oral gavage dose to plasma concentrations achieved after repeated administration in the diet, all dietary exposures would be anticipated to lie in the linear range. Simulations of plasma concentrations after repeated dietary exposure were made using mathematical models comprising the compartmental models of the single-dose toxicokinetic data, feed consumption patterns and quantities, and the method of superposition. Using a one-compartment model derived from the intravenous and low dose oral gavage data, and a two-compartment model derived from the high dose oral gavage toxicokinetic data in the Sprague-Dawley rat, simulations under predicted plasma concentrations observed at both exposure concentrations in the Wistar Furth rat. Using oral gavage toxicokinetic data from the B6C3F$_1$ mouse and a two-compartment model derived from either the intravenous and low oral gavage data or high intravenous and high oral gavage data, simulations under predicted the plasma concentrations observed at both exposure concentrations. For the rat and mouse simulations, the general shape of the concentration-time profiles were similar for the predicted and observed data.

GENETIC TOXICOLOGY

No increase in the frequency of micronucleated normochromatic erythrocytes was observed in the peripheral blood of male or female Tg.AC mice exposed to Wy-14,643 in feed (Table H1) or via dermal application (Table H2) for 6 months. In neither study was a significant alteration in the percent polychromatic erythrocytes induced by chemical treatment, indicating no toxic effects in the bone marrow of treated animals.

DISCUSSION

All rats survived to the end of the 3-month study. However, significant exposure-related decreases in body weight gains occurred in rats exposed to 10 ppm or greater; the decreases were not unexpected and were similar to other investigators' findings (Marsman *et al.*, 1988). The decreases in body weight gains in the current 3-month study were likely due to the increase in peroxisomal β-oxidation of lipids that resulted from the effects of peroxisome proliferation. BrdU labeling of hepatocytes was increased in all exposed groups of rats by day 6 but was increased only in 50 ppm or greater rats on day 34 and at week 13. Later in the study, however, the magnitude of the labeling response was reduced; the labeling of hepatocytes in 5 ppm rats on day 6 was twofold higher than in 500 ppm rats on day 34 and threefold higher than in 500 ppm rats at week 13. These data were confirmed by similar exposure- and time-dependent increases in cyclin-dependent kinase and proliferating cell nuclear antigen, two other markers in cells undergoing cell proliferation. Few peroxisome proliferators produce a sustained proliferative response, with maximum response in 1 to 2 weeks then returning to basal levels in 1 to 2 months. Wy-14,643 is unusual in that it is able to produce a sustained higher level of hepatocyte proliferation (Marsman *et al.*, 1988).

The incidences of cytoplasmic alteration of the liver were significantly increased in all exposed groups of rats, and the severity of this lesion increased in an exposure-dependent fashion. The incidences of regeneration of the corticomedullary junction of the renal tubule were significantly increased in rats exposed to 10 ppm or greater. The cauda epididymis weight of the 500 ppm group and the epididymis weights of the 100 and 500 ppm groups were less than those of the controls, and this may have been due to peroxisomal proliferation in the epididymis. Increased hepatic catabolism of steroid hormones did not seem to be a factor because there were few changes in serum reproductive hormones. The incidences of degenerative myopathy of skeletal muscle were significantly increased in the lumbar area of 500 ppm rats and the thigh of 100 and 500 ppm rats.

All mice survived to the end of the 2-week and 3-month studies. The mean body weight gain of 500 ppm mice was significantly less than that of the controls in the 2-week study. Final mean body weights and body weight gains of 50 ppm or greater mice were significantly decreased in the 3-month study, probably due to increased lipid catabolism secondary to peroxisome proliferation. No clinical signs of toxicity were noted in either study. Hepatocyte hypertrophy in 50 ppm or greater mice and necrosis in 1,000 ppm mice occurred at 2 weeks and were possibly related to hypertrophy and alterations in nutrient and oxygen flow across the sinusoid spaces. The percentages of BrdU-labeled hepatocytes were increased in 100 and 1,000 ppm mice at 2 weeks and in mice exposed to

concentrations as low as 10 ppm at 6 and 34 days and at 50 ppm or greater at 3 months. These data were confirmed by similar exposure- and time-dependent increases in cyclin-dependent kinase and proliferating cell nuclear antigen, two markers in cells undergoing cell proliferation (Holmes *et al*., 2002). Interestingly, the magnitude of the proliferation response in mice was sustained from day 6 through 3 months, unlike that in rats, which returned to basal levels of proliferation much more rapidly. This indicates that mice should be more sensitive to the hepatocarcinogenic action of Wy-14,643 than rats; bioassays in mice have not confirmed this, although hepatic foci have occurred in a few mice but not rats after exposure. Hepatic foci are thought to be precursors of neoplastic transformation in the liver (Pitot *et al.*, 1991).

Similar to rats, the testis weight of 500 ppm mice and epididymis weights of 100 and 500 ppm mice were significantly less than those of the controls in the 3-month study. Few alterations in serum reproductive hormones were observed. Some of the 100 and 500 ppm special study mice exhibited liver foci. All exposed core study mice had cytoplasmic alteration of the liver, and the severity increased with increasing exposure concentration. Mitotic alteration of the liver occurred in mice exposed to 50 ppm or greater, and pigmentation and oval cell hyperplasia occurred in 500 ppm mice. These lesions, characteristic of exposure to hepatocarcinogens and to the higher exposure concentrations used in this study, suggest that mice would be expected to develop liver neoplasms if chronically exposed to Wy-14,643. The incidences of degenerative myopathy of skeletal muscle were significantly increased in the lumbar area, the thigh, and the lower leg of 500 ppm mice.

All hamsters survived to the end of the 2-week and 3-month studies. Reduced weight gain occurred in hamsters exposed to 100 ppm or greater by the end of the 2-week study and in hamsters exposed to 10 ppm or greater by the end of the 3-month study, again probably due to increased lipid catabolism secondary to peroxisome proliferation. Minimal clinical toxicity occurred in either study. Multifocal vacuolization and/or hypertrophy of the liver occurred in some hamsters exposed to 500 ppm or greater in the 2-week study. In the 3-month study, exposed hamsters had cytoplasmic alteration of the liver, and the severity of this lesion increased with increasing exposure concentration. The percentages of BrdU-labeled hepatocytes were significantly increased in 500 and 5,000 ppm hamsters in the 2-week study; in the 3-month study, they were significantly increased in all exposed groups of hamsters on day 6 and in 50 ppm or greater hamsters on day 34 and at week 13. Similar to rats but not to mice, the magnitude of the increased hepatocyte proliferation in hamsters decreased somewhat over time, and the magnitude in 500 ppm hamsters at 13 weeks was approximately 50% that on day 6. This suggests that the response to chronic exposure to Wy-14,643 by hamsters would be similar to that by rats, but not by mice.

Cauda epididymis, epididymis, and testis weights; spermatid heads per testis; spermatid counts; and sperm motility were generally decreased in the 3-month study. Prostate gland, seminal vesicle, and testicular atrophy occurred in

100 and 500 ppm hamsters. Several reproductive hormone concentrations (estradiol, testosterone, and luteinizing hormone) were decreased. These decreases may have been due, in part, to increased hepatic catabolism of reproductive hormones that are due to the pleotropic induction of cytochrome P450 isozymes that catabolize steroid hormones. The incidences of degenerative myopathy of skeletal muscle were significantly increased in the lumbar area and in the thigh of 100 and 500 ppm hamsters.

The current studies confirm and extend the responsiveness of rats and mice to the induction of peroxisomes and hepatocyte proliferation after dietary exposure to Wy-14,643 observed by other investigators. The magnitude and duration of these responses in mice were new and interesting and suggest that mice may be as or more responsive than rats to developing hepatic neoplasia following chronic dietary exposure to Wy-14,643. It is tempting to postulate that the reason rats and hamsters are similar is because they have similar bioavailability, 0.34 for rats and 0.24 for hamsters, and the reason for the sustained response in mice was due to a much higher bioavailability (0.98); such speculation would overinterpret the data. Most studies of Wy-14,643 have been conducted in rats, not mice, so there is a paucity of data for valid comparisons. Therefore, until a proper bioassay is conducted in mice, the above will remain a postulation.

The hamsters' response to peroxisome and hepatocyte proliferation was unexpected. The hamster was selected as a model species because it was considered to be unresponsive or less responsive to the effects of peroxisome proliferators. Thus, hamsters would then provide an animal model to study the differences in molecular and cellular biology between responsive species, specifically rats and mice, and an unresponsive species, hamsters. Hamsters were considered to be unresponsive to peroxisome proliferators since Syrian hamsters showed no increase in tumor incidence after a lifetime exposure to the peroxisome proliferator, di(2-ethylhexyl)phthalate (DEHP), by inhalation or intraperitoneal injection (Schmezer *et al.*, 1988). These authors also compared the ability of DEHP to produce DNA single-strand breaks in rat and hamster hepatocytes *in vitro*. Whereas DEHP produced toxicity and DNA single-strand breaks in rat hepatocytes at 5 µmole/tube, no toxicity or single-strand breaks occurred in hamster hepatocytes up to 25 µmole/tube. A second report demonstrated that Syrian hamsters fed diets containing the peroxisome proliferators nafenopin or Wy-14,643 for up to 60 weeks did not sustain hepatic cell proliferation or induction of liver nodules or tumors, whereas Sprague-Dawley rats fed a similar diet for 60 weeks did exhibit sustained hepatic cell proliferation and liver nodules and tumors (Lake *et al.*, 1993). These data indicated that hamsters were less sensitive to the standard responses of cell replication and enzyme induction that peroxisome proliferators, as a class, produce in rodent models. However, data in the current studies were collected at earlier time points and demonstrated that hamsters exposed to Wy-14,643 show peroxisome and hepatocyte proliferation responses similar to those in rats and mice. Whether Wy-14,643 can cause increases in the incidences of hepatic neoplasms in hamsters similar to that in rats is open to question. The Lake *et al.* (1993) study may not have used an

adequate exposure duration to conclude that hamsters are not sensitive to the carcinogenicity of Wy-14,643 over longer periods, nor is it established that the hamster is an adequate model for carcinogenesis testing *per se*.

For the rats and hamsters (liver biomarkers were not evaluated in the mice due to serum availability), the increases in serum alanine aminotransferase and sorbitol dehydrogenase activity would suggest a treatment-related hepatocellular effect. Increases in serum activities of alanine aminotransferase and sorbitol dehydrogenase are used as markers of hepatocellular necrosis or increased cell membrane permeability (Clampitt and Hart, 1978; Boyd, 1983; Hoffmann *et al*., 1989). And while no evidence of hepatocellular necrosis was observed microscopically in these studies, there was microscopic evidence of a treatment-related hepatocellular cytoplasmic alteration (in rats, mice, and hamsters), and this finding may, in part, help explain the increase observed for the aforementioned biomarkers, in rats and hamsters, related to an increase in hepatocyte membrane permeability. It has been demonstrated that increases in liver alanine aminotransferase (ALT) can occur as a result of enzyme induction and that increases in liver ALT could be reflected in increased serum activity (Hoffmann *et al.*, 1999). Interestingly, rats administered 100 mg Wy-14,643/kg per day by gavage for 4 consecutive days did not demonstrate any increases in serum alanine aminotransferase or sorbitol dehydrogenase activities (O'Brien *et al.*, 2002). The authors also demonstrated that intra-hepatic alanine aminotransferase activity was not affected by Wy-14,643 treatment. Since the treatment regimen was very short, morphological and/or biochemical hepatocellular effects may not have had time to develop, but regardless of the lack of morphological changes, there was no evidence of enzyme induction related to compound administration. Additionally, it has been said that hepatic injury appears to be the only source of increased serum sorbitol dehydrogenase activity (Kramer, 1989). Further, in a liver protein profiling mouse study (Chu *et al.*, 2004), male C57BL/6J mice demonstrated a 50% decrease in intra-hepatic sorbitol dehydrogenase protein after 2 weeks of Wy-14,643 exposure in the diet. Thus, it appears the increases in the activities of serum alanine aminotransferase and sorbitol dehydrogenase were related to increased hepatocellular membrane permeability and corresponds to the hepatic effects observed microscopically. While the same doses were affected in the rat and hamster studies, the rats developed these enzyme changes sooner than the hamsters and this species difference may be related to the observation that rats are more sensitive to certain effects of peroxisome proliferators than hamsters (Styles *et al.*, 1988; James and Roberts, 1996; O'Brien *et al*., 2001).

For the rats and hamsters, dose-related increases in bile salt concentrations, a marker of cholestasis, occurred. Additionally, serum alkaline phosphatase activity (another biomarker of cholestasis) increased in the rat study but not the hamsters. While evaluation of bile salt concentration is often used as a marker of cholestasis, serum concentrations can be affected by mechanisms other than cholestasis. For example, altered enterohepatic circulation, impaired hepatic function, and non-cholestatic liver injury can elevate circulating bile acid concentrations (Hofmann, 1988; Bai *et al.*, 1992). In contrast, serum alkaline phosphatase activity, another marker of cholestasis, increases

minimally in response to hepatocellular damage (Hoffmann *et al.*, 1989; Bai *et al.*, 1992). Further, rats administered 100 mg Wy-14,643/kg per day by gavage for 4 days demonstrated increases (~2.3-fold) in alkaline phosphatase activity without evidence of morphological liver changes (O'Brien *et al.*, 2002). In that study, the authors also demonstrated that treatment of rats for five consecutive days with 50 mg Wy-14,643/kg per day resulted in a threefold increase in intra-hepatic alkaline phosphatase activity and, thus, concluded the increases in serum activity were related to induction of the enzyme by the treatment. Interestingly, Wy-14,643 appears to suppress bile acid synthesis and alters the quantitative and qualitative output of bile acids and cholesterol in the bile (Hunt *et al*., 2000; Post *et al*., 2001). Therefore, the increase in serum bile salt concentration, occurring in both species, would be consistent with a hepatic effect and may correspond to the effects observed for the alanine aminotransferase, sorbitol dehydrogenase, and microscopically. Conversely, the increases in alkaline phosphatase activity observed in the rat study would be consistent with enzyme induction and suggests that the hamsters were either not susceptible or not as sensitive to the development of this alteration.

Peroxisome proliferators are a diverse group of chemicals that induce hypolipidemia and a variety of liver effects (e.g., alterations in bile acid synthesis, induction of enzymes of fatty acid metabolism, liver carcinogenesis). There is variation in response that depends on the compound tested and the species evaluated, and for this series of studies, there was a clear difference between the species regarding a lipid (triglycerides and cholesterol) lowering effect of Wy-14,643. At the same exposure concentrations, hamsters demonstrated decreases in serum cholesterol and triglyceride concentrations, mice demonstrated decreases in triglyceride but increases in cholesterol, and rats demonstrated no effect. The mechanism(s) for these observed variations in response is unknown. But it has been reported that for peroxisome proliferation, rats and mice are considered sensitive species, hamsters are weakly responsive, and dogs, guinea pigs and non-human primates are nonresponsive. Additionally, there is some variation in the response within species. In SV/129 mice, Wy-14,643 administered in the feed did not alter plasma cholesterol concentration (Knight *et al*., 2003). In another study, Akiyama *et al*. (2001) demonstrated decreases in triglyceride, but not cholesterol, concentrations in SV/129 and C57BL/6N mice administered Wy-14,643 in the diet. In a rat study, male F344 rats of various ages (4, 10, 50, or 100 weeks of age) were administered 250 mg Wy-14643/kg by gavage and then sampled 48 hours later (Youssef *et al*., 2003). Rats at 4 weeks of age demonstrated no changes in serum triglyceride or cholesterol concentrations. Animals at 10, 50, or 100 weeks of age, however, demonstrated changes for these serum variables related to treatment. Ten-week old rats demonstrated decreases in both serum triglyceride and cholesterol; at 50 weeks of age, only triglycerides demonstrated decreases; at 100 weeks of age, serum cholesterol concentration was increased but there was no change in the serum triglycerides. Since the rats in the present study were approximately 13 or 22 weeks of age at the sampling times, some decrease or change in these serum lipid markers might have been expected. However, this study utilized Sprague-Dawley rats, thus, strain differences may account for the lack of decreases in the serum triglyceride and cholesterol concentrations.

For this series of studies, there was a clear difference between the species regarding a reproductive hormone profile (testosterone, estradiol, follicle-stimulating hormone, and luteinizing hormone) related to Wy-14,643 administration in male animals. At the same exposure concentrations and treatment regimen, hamsters demonstrated decreases in serum testosterone, estradiol, and luteinizing hormone concentrations, mice demonstrated decreases in follicle-stimulating hormone but increases in estradiol, and rats demonstrated no effect. The mechanism for these differences was unknown. Testosterone production is primarily influenced by the effects of the pituitary gonadotropin luteinizing hormone on Leydig's cells (Reimers, 1999). It has been observed that Wy-14,643 treatment of rat granulosa cells in cell culture resulted in decreased estradiol production and aromatase mRNA levels (Lovekamp and Davis, 2001). It would appear that the decrease in estradiol production is related to a reduction in aromatase, the rate-limiting enzyme that converts testosterone to estradiol. Aromatase is under the stimulatory influence of androgens and follicle-stimulating hormone. In part, this may help explain the effects noted *in vivo*, and it might be expected that estradiol concentrations would decrease; this did occur for hamsters, but the opposite occurred in mice, and there was no effect in rats. In hamsters, if Wy-14,643 induced a decrease in luteinizing hormone, this might have lowered testosterone concentrations, and lower testosterone and suppressed aromatase may have resulted in lower estradiol concentrations. This does not explain the alterations, or lack thereof, in mice and rats.

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APPENDIX A
SUMMARY OF NONNEOPLASTIC LESIONS IN RATS, MICE, AND HAMSTERS

TABLE A1 Summary of the Incidence of Nonneoplastic Lesions in Male Rats in the 3-Month Feed Study of Wy-14,643 . . . . . A-2
TABLE A2 Summary of the Incidence of Nonneoplastic Lesions in Male Mice in the 3-Month Feed Study of Wy-14,643 . . . . . A-4
TABLE A3 Summary of the Incidence of Nonneoplastic Lesions in Male Hamsters in the 3-Month Feed Study of Wy-14,643 . . . . . A-6

Table A1
Summary of the Incidence of Nonneoplastic Lesions in Male Rats in the 3-Month Feed Study of Wy-14,643[a]

	0 ppm		5 ppm		10 ppm		50 ppm		100 ppm		500 ppm	
Disposition Summary												
Animals initially in study	10		10		10		10		10		10	
Survivors												
Terminal sacrifice	10		10		10		10		10		10	
Animals examined microscopically	10		10		10		10		10		10	
Alimentary System												
Liver	(10)		(10)		(10)		(10)		(10)		(10)	
Cytoplasmic alteration			9	(90%)	10	(100%)	10	(100%)	10	(100%)	10	(100%)
Infiltration cellular, mononuclear cell	6	(60%)	8	(80%)	6	(60%)	5	(50%)	6	(60%)	6	(60%)
Necrosis, focal					1	(10%)	1	(10%)				
Bile duct, hyperplasia			1	(10%)								
Hepatocyte, mitosis							2	(20%)	4	(40%)	7	(70%)
Pancreas	(10)		(10)		(10)		(10)		(9)		(10)	
Inflammation, chronic	1	(10%)			1	(10%)						
Stomach, forestomach	(10)										(10)	
Inflammation, acute, focal											1	(10%)
Cardiovascular System												
Heart	(10)										(10)	
Cardiomyopathy	5	(50%)									2	(20%)
Endocrine System												
Parathyroid gland	(10)										(4)	
Syncytial alteration											1	(25%)
Thyroid gland	(10)										(10)	
Cyst	1	(10%)										
General Body System												
None												
Genital System												
Preputial gland	(8)		(10)		(8)		(8)		(7)		(7)	
Inflammation, chronic	8	(100%)	10	(100%)	8	(100%)	8	(100%)	7	(100%)	6	(86%)
Prostate	(10)								(1)		(10)	
Inflammation, chronic	1	(10%)							1	(100%)		
Hematopoietic System												
None												
Integumentary System												
None												

TABLE A1
Summary of the Incidence of Nonneoplastic Lesions in Male Rats in the 3-Month Feed Study of Wy-14,643

	0 ppm		5 ppm		10 ppm		50 ppm		100 ppm		500 ppm	
Musculoskeletal System												
None												
Nervous System												
None												
Respiratory System												
Lung	(10)										(10)	
Hemorrhage	2	(20%)									2	(20%)
Alveolus, infiltration cellular, histiocyte	4	(40%)									3	(30%)
Interstitium, inflammation											1	(10%)
Interstitium, inflammation, chronic											1	(10%)
Nose	(10)										(10)	
Inflammation, chronic, focal	1	(10%)										
Special Senses System												
None												
Urinary System												
Kidney	(10)		(9)		(10)		(9)		(10)		(10)	
Nephropathy	8	(80%)	9	(100%)	8	(80%)	8	(89%)	7	(70%)	8	(80%)
Corticomedullary junction, renal tubule, regeneration			1	(11%)	4	(40%)	4	(44%)	9	(90%)	5	(50%)
Urinary bladder	(10)										(10)	
Infiltration cellular	1	(10%)										

[a] Number of animals examined microscopically at the site and the number of animals with lesion

Table A2
Summary of the Incidence of Nonneoplastic Lesions in Male Mice in the 3-Month Feed Study of Wy-14,643[a]

	0 ppm		5 ppm		10 ppm		50 ppm		100 ppm		500 ppm	
Disposition Summary												
Animals initially in study	10		10		10		10		10		10	
Survivors												
Terminal sacrifice	10		10		10		10		10		10	
Animals examined microscopically	10		10		10		10		10		10	
Alimentary System												
Liver	(10)		(10)		(10)		(10)		(10)		(10)	
Clear cell focus	1	(10%)										
Cytoplasmic alteration			10	(100%)	10	(100%)	10	(100%)	10	(100%)	10	(100%)
Infiltration cellular, mononuclear cell	3	(30%)	1	(10%)	1	(10%)	2	(20%)			1	(10%)
Infiltration cellular, mixed cell	5	(50%)	2	(20%)	6	(60%)	3	(30%)	7	(70%)		
Necrosis, focal					1	(10%)	1	(10%)	2	(20%)		
Pigmentation									3	(30%)	10	(100%)
Vacuolization cytoplasmic									2	(20%)	3	(30%)
Hepatocyte, apoptosis									6	(60%)	10	(100%)
Hepatocyte, mitosis							4	(40%)	7	(70%)	10	(100%)
Oval cell, hyperplasia											10	(100%)
Pancreas	(10)		(5)		(10)		(10)		(10)		(10)	
Inflammation, chronic							1	(10%)	1	(10%)	3	(30%)
Stomach, glandular	(10)										(10)	
Inflammation, chronic active	2	(20%)										
Cardiovascular System												
None												
Endocrine System												
None												
General Body System												
None												
Genital System												
Preputial gland	(10)		(4)		(8)		(10)		(10)		(10)	
Inflammation, chronic			3	(75%)	1	(13%)	4	(40%)	2	(20%)		
Testes	(10)		(5)		(10)		(10)		(10)		(10)	
Atrophy									1	(10%)		
Hematopoietic System												
None												
Integumentary System												
None												
Musculoskeletal System												
None												

TABLE A2
Summary of the Incidence of Nonneoplastic Lesions in Male Mice in the 3-Month Feed Study of Wy-14,643

	0 ppm		5 ppm		10 ppm		50 ppm		100 ppm		500 ppm	
Nervous System												
None												
Respiratory System												
Lung	(10)										(10)	
Congestion											1	(10%)
Hemorrhage											5	(50%)
Special Senses System												
None												
Urinary System												
Kidney	(10)		(5)		(10)		(10)		(10)		(10)	
Nephropathy			3	(60%)	4	(40%)	4	(40%)	3	(30%)		
Urinary bladder	(10)										(10)	
Infiltration cellular	3	(30%)									1	(10%)
Inflammation, chronic active	1	(10%)									1	(10%)

[a] Number of animals examined microscopically at the site and the number of animals with lesion

Table A3
Summary of the Incidence of Nonneoplastic Lesions in Male Hamsters in the 3-Month Feed Study of Wy-14,643[a]

	0 ppm		5 ppm		10 ppm		50 ppm		100 ppm		500 ppm	
Disposition Summary												
Animals initially in study	10		10		10		10		10		10	
Survivors												
Terminal sacrifice	10		10		10		10		10		10	
Animals examined microscopically	10		10		10		10		10		10	
Alimentary System												
Gallbladder	(10)										(10)	
Infiltration cellular, mixed cell	8	(80%)									7	(70%)
Liver	(10)		(10)		(10)		(10)		(10)		(10)	
Cytoplasmic alteration			10	(100%)	10	(100%)	10	(100%)	10	(100%)	10	(100%)
Infiltration cellular, mixed cell	10	(100%)	10	(100%)	10	(100%)	10	(100%)	9	(90%)	10	(100%)
Pigmentation	2	(20%)	1	(10%)								
Vacuolization cytoplasmic							2	(20%)	5	(50%)	4	(40%)
Pancreas	(10)		(10)		(10)		(9)		(10)		(10)	
Atrophy	1	(10%)									1	(10%)
Inflammation, chronic	7	(70%)	7	(70%)	9	(90%)	9	(100%)	7	(70%)	8	(80%)
Cardiovascular System												
None												
Endocrine System												
None												
General Body System												
None												
Genital System												
Epididymis	(10)		(2)								(10)	
Degeneration			2	(100%)								
Prostate	(10)		(10)		(10)		(10)		(10)		(9)	
Atrophy	1	(10%)	1	(10%)			3	(30%)	10	(100%)	9	(100%)
Inflammation, chronic			1	(10%)								
Seminal vesicle	(10)		(10)		(10)		(10)		(10)		(10)	
Atrophy	1	(10%)	1	(10%)			3	(30%)	10	(100%)	10	(100%)
Testes	(10)		(10)		(10)		(10)		(10)		(10)	
Atrophy	2	(20%)	2	(20%)	1	(10%)	4	(40%)	7	(70%)	10	(100%)
Hematopoietic System												
None												
Integumentary System												
None												
Musculoskeletal System												
None												

TABLE A3
Summary of the Incidence of Nonneoplastic Lesions in Male Hamsters in the 3-Month Feed Study of Wy-14,643

	0 ppm		5 ppm		10 ppm		50 ppm		100 ppm		500 ppm	
Nervous System												
None												
Respiratory System												
Lung	(10)										(10)	
Hemorrhage	2	(20%)									1	(10%)
Special Senses System												
None												
Urinary System												
Kidney	(10)		(10)		(9)		(10)		(10)		(10)	
Mineralization	1	(10%)	2	(20%)	3	(33%)	2	(20%)			1	(10%)
Nephropathy	5	(50%)	3	(30%)	6	(67%)	2	(20%)	3	(30%)	1	(10%)
Urinary bladder	(10)										(10)	
Infiltration cellular	1	(10%)										
Mineralization	1	(10%)										

[a] Number of animals examined microscopically at the site and the number of animals with lesion

APPENDIX B
CLINICAL PATHOLOGY RESULTS

TABLE B1 Clinical Chemistry and Reproductive Hormone Data for Male Rats in the 3-Month Feed Study of Wy-14,643 . . . . . B-2
TABLE B2 Clinical Chemistry and Reproductive Hormone Data for Male Mice in the 3-Month Feed Study of Wy-14,643 . . . . . B-3
TABLE B3 Clinical Chemistry and Reproductive Hormone Data for Male Hamsters in the 3-Month Feed Study of Wy-14,643 . . . . . B-4

TABLE B1
Clinical Chemistry and Reproductive Hormone Data for Male Rats in the 3-Month Feed Study of Wy-14,643[a]

	0 ppm	5 ppm	10 ppm	50 ppm	100 ppm	500 ppm
n						
Day 34	5	5	5	5	5	5
Week 14	10	10	10	10	10	10
Clinical Chemistry						
Cholesterol (mg/dL)						
Day 34	119 ± 7	114 ± 9	113 ± 2	109 ± 4	108 ± 6	112 ± 6
Week 14	125 ± 5	125 ± 5	131 ± 3	123 ± 9	134 ± 7	119 ± 7
Triglycerides (mg/dL)						
Day 34	92 ± 10	101 ± 9	125 ± 12	118 ± 6	111 ± 6	132 ± 11
Week 14	122 ± 8	123 ± 12	125 ± 10	158 ± 10*	144 ± 8	127 ± 11
Alanine aminotransferase (IU/L)						
Day 34	70 ± 7	113 ± 38	141 ± 49	94 ± 2*	110 ± 20*	146 ± 43**
Week 14	80 ± 3	84 ± 2	85 ± 4	98 ± 7*	89 ± 6	111 ± 6**
Alkaline phosphatase (IU/L)						
Day 34	615 ± 10	776 ± 53*	916 ± 53**	1,355 ± 124**	1,381 ± 26**	1,747 ± 150**
Week 14	495 ± 20	663 ± 31**	796 ± 37**	1,302 ± 38**	1,359 ± 85**	1,703 ± 136**
Sorbitol dehydrogenase (IU/L)						
Day 34	20 ± 1	25 ± 8	50 ± 26	33 ± 3*	31 ± 3*	53 ± 17*
Week 14	22 ± 1	21 ± 1	24 ± 2	30 ± 2**	24 ± 1*	25 ± 1*
Bile salts (µmol/L)						
Day 34	56.4 ± 5.8	53.8 ± 10.7	91.8 ± 17.7	83.8 ± 10.6	153.0 ± 20.6**	183.4 ± 21.0**
Week 14	35.2 ± 4.3	64.7 ± 8.1**	59.0 ± 5.0**	139.5 ± 15.2**	144.5 ± 25.2**	120.1 ± 17.3**
Reproductive Hormone Analyses						
Estradiol (pg/mL)						
Day 34	100.8 ± 3.2	113.5 ± 4.3	111.9 ± 2.6	108.3 ± 7.1	88.1 ± 3.3	93.4 ± 5.7
Week 14	102.2 ± 1.7	114.7 ± 2.3	113.6 ± 1.5	104.1 ± 3.0	101.3 ± 2.6	95.9 ± 2.9
Testosterone (ng/mL)						
Day 34	3.0 ± 0.5	2.3 ± 1.0	1.5 ± 0.3	1.6 ± 1.1	1.7 ± 0.5	0.9 ± 0.4
Week 14	0.8 ± 0.2	1.0 ± 0.2	1.3 ± 0.2	1.0 ± 0.3	1.4 ± 0.4	1.8 ± 0.3
Follicle-stimulating hormone (ng/mL)						
Day 34	7.42 ± 0.41	9.61 ± 0.94	7.84 ± 0.37	8.93 ± 0.70	10.14 ± 0.58*	9.56 ± 0.27
Week 14	8.59 ± 0.35	9.85 ± 0.66	9.53 ± 0.39	7.47 ± 0.24	7.11 ± 0.52	9.82 ± 0.65
Luteinizing hormone (ng/mL)						
Day 34	0.28 ± 0.05	0.67 ± 0.20	0.63 ± 0.03	0.66 ± 0.08	1.03 ± 0.40*	0.52 ± 0.17
Week 14	0.43 ± 0.05	0.46 ± 0.03	0.51 ± 0.09	0.57 ± 0.09	0.31 ± 0.11	0.70 ± 0.14

* Significantly different ($P \leq 0.05$) from the control group by Dunn's or Shirley's test
** Significantly different ($P \leq 0.01$) from the control group by Shirley's test
[a] Mean ± standard error. Statistical tests were performed on unrounded data.

TABLE B2
Clinical Chemistry and Reproductive Hormone Data for Male Mice in the 3-Month Feed Study of Wy-14,643[a]

	0 ppm	5 ppm	10 ppm	50 ppm	100 ppm	500 ppm
Clinical Chemistry						
n						
Day 34	5	5	5	4	5	5
Week 14	10	10	10	10	10	10
Cholesterol (mg/dL)						
Day 34	158 ± 14	188 ± 8	182 ± 6	182 ± 5	177 ± 3	192 ± 4*
Week 14	173 ± 6	228 ± 5**	233 ± 6**	206 ± 11	183 ± 4	211 ± 5*
Triglycerides (mg/dL)						
Day 34	165 ± 14	102 ± 10*	108 ± 12*	86 ± 6**	74 ± 9**	114 ± 20**
Week 14	177 ± 12	96 ± 7**	81 ± 5**	76 ± 8**	73 ± 7**	63 ± 3**
Reproductive Hormone Analyses						
n						
Day 34	4	5	5	4	5	4
Week 14	10	9	10	10	10	10
Estradiol (pg/mL)						
Day 34	50.8 ± 6.3	61.8 ± 3.5	65.1 ± 1.6	60.9 ± 0.6	65.2 ± 2.7	67.5 ± 8.4
Week 14	59.1 ± 5.5[b]	68.3 ± 0.9	69.8 ± 1.0	62.7 ± 2.2	60.2 ± 5.0[b]	77.6 ± 2.1**
Testosterone (ng/mL)						
Day 34	0.32 ± 0.10	0.20 ± 0.05	0.32 ± 0.03	0.21 ± 0.03	0.40 ± 0.09	0.67 ± 0.18[c]
Week 14	11.70 ± 3.58	7.94 ± 3.96	8.56 ± 3.30	2.40 ± 1.23	4.80 ± 2.23	3.97 ± 1.78
Follicle-stimulating hormone (ng/mL)						
Day 34	22.9 ± 0.7[c]	20.8 ± 4.7[c]	27.0 ± 1.8	23.9 ± 1.1[d]	18.8 ± 0.7[c]	—[e]
Week 14	26.8 ± 2.1[f]	23.6 ± 1.7	24.5 ± 0.9[g]	27.0 ± 1.8[b]	30.7 ± 7.6[g]	9.3 ± 0.8**[g]
Luteinizing hormone (ng/mL)						
Day 34	0.22[h]	—	0.84 ± 0.24[d]	0.65 ± 0.14[d]	0.29 ± 0.15[d]	—
Week 14	0.14 ± 0.07[i]	0.30 ± 0.08	0.29 ± 0.14[i]	0.41 ± 0.14[i]	0.24 ± 0.10[j]	0.21 ± 0.02[j]

* Significantly different (P≤0.05) from the control group by Dunn's or Shirley's test
** P≤0.01
[a] Mean ± standard error. Statistical tests were performed on unrounded data.
[b] n=9
[c] n=3
[d] n=2
[e] Not measured at this exposure concentration
[f] n=7
[g] n=8
[h] n=1; no standard error calculated
[i] n=5
[j] n=6

TABLE B3
Clinical Chemistry and Reproductive Hormone Data for Male Hamsters in the 3-Month Feed Study of Wy-14,643[a]

	0 ppm	5 ppm	10 ppm	50 ppm	100 ppm	500 ppm
n						
Day 34	5	5	5	5	5	5
Week 14	10	10	9	10	10	9
Clinical Chemistry						
Cholesterol (mg/dL)						
Day 34	140 ± 19	124 ± 4	118 ± 5**	87 ± 3**	77 ± 3**	78 ± 4**
Week 14	157 ± 4	104 ± 4**	101 ± 8**[b]	76 ± 4**	69 ± 4**	90 ± 5**[b]
Triglycerides (mg/dL)						
Day 34	233 ± 38	199 ± 33	185 ± 6	113 ± 16**	87 ± 12**	82 ± 10**
Week 14	207 ± 9	145 ± 8**	157 ± 32**[b]	87 ± 8**	65 ± 5**	63 ± 5**[b]
Alanine aminotransferase (IU/L)						
Day 34	70 ± 11[c]	53 ± 5	61 ± 11	75 ± 8	78 ± 7	103 ± 15
Week 14	69 ± 4	61 ± 7	65 ± 8	96 ± 6*	104 ± 13*	162 ± 30**
Alkaline phosphatase (IU/L)						
Day 34	255 ± 13	216 ± 13	196 ± 9*	217 ± 9	241 ± 12	296 ± 14
Week 14	204 ± 9	159 ± 6	133 ± 12	208 ± 11	209 ± 7	223 ± 8
Sorbitol dehydrogenase (IU/L)						
Day 34	54 ± 9	51 ± 4	56 ± 11	58 ± 6	48 ± 10	67 ± 13
Week 14	57 ± 4	55 ± 8	50 ± 3	75 ± 5*	81 ± 17	142 ± 40**
Bile salts (µmol/L)						
Day 34	17.8 ± 8.6	10.2 ± 2.5	16.0 ± 5.8	12.0 ± 1.4	18.4 ± 8.0	14.8 ± 2.4
Week 14	10.0 ± 1.1	12.4 ± 1.3	10.2 ± 1.4	15.2 ± 1.6*	16.7 ± 2.9*	22.7 ± 2.6**
Reproductive Hormone Analyses						
Estradiol (pg/mL)						
Day 34	54.1 ± 3.2	53.4 ± 2.3	48.4 ± 3.5	44.7 ± .98*	44.9 ± 3.2*	43.2 ± 3.0*
Week 14	53.3 ± 1.6	42.2 ± 1.5**	46.9 ± 4.5**	40.6 ± 1.7**	37.6 ± 1.8**	41.9 ± 1.4**[b]
Testosterone (ng/mL)						
Day 34	0.7 ± 0.2	0.8 ± 0.5	0.7 ± 0.3	0.0 ± 0.0**	0.0 ± 0.0**	0.0 ± 0.0**
Week 14	1.4 ± 0.3	1.9 ± 0.6	1.1 ± 0.4	0.3 ± 0.2*	0.3 ± 0.2**	0.1 ± 0.1**
Follicle-stimulating hormone (ng/mL)						
Day 34	1.65 ± 0.37	2.39 ± 0.66	1.31 ± 0.36	0.87 ± 0.33	1.54 ± 0.40	1.99 ± 0.77
Week 14	10.27 ± 0.48	10.34 ± 0.70[d]	8.6 ± 0.45	8.44 ± 0.81	9.78 ± 0.89	9.98 ± 0.65
Luteinizing hormone (ng/mL)						
Day 34	1.03 ± 0.41	1.20 ± 0.70	0.33 ± 0.14	0.03 ± 0.03**	0.00 ± 0.00**	0.02 ± 0.01**
Week 14	2.14 ± 0.42	3.62 ± 0.83[e]	2.31 ± 0.47	1.03 ± 0.20*	0.73 ± 0.08**	1.63 ± 0.43*

* Significantly different (P≤0.05) from the control group by Dunn's or Shirley's test
** Significantly different (P≤0.01) from the control group by Shirley's test
[a] Mean ± standard error. Statistical tests were performed on unrounded data.
[b] n=10
[c] n=4
[d] n=9
[e] n=8

APPENDIX C
DETERMINATIONS OF WY-14,643 IN PLASMA

TABLE C1 Plasma Concentrations of Wy-14,643 in Male Rats after 2, 4, or 8 Weeks of Exposure to Wy-14,643 in Feed C-2
TABLE C2 Plasma Concentrations of Wy-14,643 in Male Mice after 2, 4, or 8 Weeks of Exposure to Wy-14,643 in Feed C-3
TABLE C3 Plasma Concentrations of Wy-14,643 in Male Hamsters after 2, 4, or 8 Weeks of Exposure to Wy-14,643 in Feed C-4

TABLE C1
Plasma Concentrations of Wy-14,643 in Male Rats after 2, 4, or 8 Weeks of Exposure to Wy-14,643 in Feed[a]

Time of Collection	50 ppm	100 ppm	500 ppm
Week 3			
0600	1.24	2.58	13.1
0800	0.634	1.75	8.98
1000	0.513	2.26	7.84
1200	0.788	0.984	7.91
1400	0.102	0.741	2.88
1600	0.481	0.597	5.10
1800	0.275	1.15	3.89
2000	0.534	1.48	5.87
2200	0.682	1.32	5.51
2400	0.817	1.47	10.9
0200	1.39	1.69	11.5
0400	1.02	1.38	6.94
Week 5			
0600	0.822	2.20	10.6
0800	0.792	1.04	6.19
1000	0.813	2.09	11.0
1200	0.589	1.34	7.85
1400	0.645	1.30	5.45
1600	0.562	1.15	3.84
1800	0.0928[b]	1.03	0.951
2000	0.670	1.99	5.04
2200	0.761	1.18	7.07
2400	0.607	1.42	9.96
0200	0.809	1.29	10.1
0400	0.750	1.42	7.75
Week 9			
0600	0.860	1.78	9.62
0800	0.647	1.18	8.14
1000	0.610	1.11	8.33
1200	0.585	0.684	2.95
1400	0.817	0.888	2.12
1600	0.564	1.14	2.64
1800	0.429	0.922	5.89
2000	0.522	1.76	6.60
2200	0.865	1.35	9.86
2400	0.838	1.36	48.2[c]
0200	0.652	1.62	8.11
0400	0.655	1.16	4.33

[a] Data are given in µg/mL for individual animals.
[b] Estimated concentration. The measured value was less than the experimental limit of quantitation (0.100 µg/mL) but greater than the limit of detection (0.0133 µg/mL).
[c] Anomalous data

TABLE C2
Plasma Concentrations of Wy-14,643 in Male Mice after 2, 4, or 8 Weeks of Exposure to Wy-14,643 in Feed[a]

Time of Collection	50 ppm	100 ppm	500 ppm
Week 3			
0600	0.484	0.614	2.59
0800	0.383	0.637	2.88
1000	—[b]	0.167[c,d]	3.28
1200	—	0.831	1.52
1400	0.236[c,d]	0.111[c,d]	5.13
1600	0.0546[c,d]	0.531	0.416
1800	0.228	2.07	4.75
2000	0.484	1.86	6.38
2200	0.704	1.19	8.67
2400	0.486	1.22	5.22
0200	0.572	0.756	8.98
0400	—	1.79	4.05
Week 5			
0600	0.426	0.723	5.66
0800	0.382	0.728	4.06
1000	0.109[c,d]	—	2.01
1200	0.0266[c]	0.219	1.31
1400	0.123	—	0.0307[c]
1600	—	0.0477[c]	3.10
1800	0.319	0.338	3.70
2000	0.546	0.584	9.64
2200	0.760	0.996	3.12
2400	0.427	1.49	6.70
0200	0.224	1.07	2.03
0400	0.286	1.45	5.05
Week 9			
0600	0.281	0.550	2.23
0800	0.110	1.11	0.493
1000	0.0550[c]	0.232	2.92
1200	0.0346[c,d]	—	1.44
1400	0.0452[c]	—	2.51
1600	0.0486[c]	0.451	2.83
1800	0.131	0.552	2.33
2000	0.440	0.945	5.62
2200	0.692	0.876	6.35
2400	0.483	1.61	6.30
0200	0.734	1.30	2.91
0400	0.178	0.839	4.10

[a] Data are given in µg/mL for individual animals.
[b] Less than the limit of detection (0.0133 µg/mL)
[c] Estimated concentration. The measured value was less than the experimental limit of quantitation (0.100 µg/mL) but greater than the limit of detection.
[d] Normalized data, corrected for required dilution of insufficient (<0.2 mL) sample volume

TABLE C3
Plasma Concentrations of Wy-14,643 in Male Hamsters after 2, 4, or 8 Weeks of Exposure to Wy-14,643 in Feed[a]

Time of Collection	50 ppm	100 ppm	500 ppm
Week 3			
0600	0.187	0.463	1.87
0800	0.194	0.294	1.45
1000	0.0769[b]	0.269	1.24
1200	0.137	0.150	1.41
1400	0.0689[b]	0.220	1.17
1600	0.0892[b]	0.161	0.717
1800	0.0977[b]	0.0811[b]	0.920
2000	0.140	0.467	1.11
2200	0.0817[b]	0.285	2.28
2400	0.121	0.391	2.11
0200	0.145	0.281	1.70
0400	0.265	0.201	2.64
Week 5			
0600	0.215[b,c]	0.240	0.805
0800	0.126[b,c]	0.203	0.895
1000	0.101	0.202	0.991
1200	0.0832[b,c]	0.140[b,c]	1.09
1400	0.0815[b]	0.209	1.41
1600	0.130[b,c]	0.0718[b]	1.29
1800	0.0993[b]	0.177	0.215
2000	0.0768[b]	0.370	1.73
2200	0.0999[b]	0.354	1.43
2400	0.117	0.177	1.24
0200	0.138	0.232	1.26
0400	0.0913[b]	0.227	1.13
Week 9			
0600	0.237	0.518	2.72
0800	0.170	0.286	1.43
1000	0.192	0.372	1.47
1200	0.250	0.0691[b]	1.98
1400	2.38[d]	0.445	0.135[b,c]
1600	0.200	0.0981[b]	1.29
1800	0.134	0.190	1.64
2000	0.244	0.266	1.62
2200	0.504	0.544	1.51
2400	0.207	0.260	2.61
0200	0.309	0.209	5.33
0400	0.370	0.0967[b]	1.72

[a] Data are given in µg/mL for individual animals.
[b] Estimated concentration. The measured value was less than the experimental limit of quantitation (0.100 µg/mL) but greater than the limit of detection (0.0101 µg/mL).
[c] Normalized data, corrected for required dilution of insufficient (<0.2 mL) sample volume
[d] Anomalous data

APPENDIX D
ORGAN WEIGHTS
AND ORGAN-WEIGHT-TO-BODY-WEIGHT RATIOS

TABLE D1 Organ Weights and Organ-Weight-to-Body-Weight Ratios for Special Study Male Rats in the 3-Month Feed Study of Wy-14,643 . . . . . D-2
TABLE D2 Organ Weights and Organ-Weight-to-Body-Weight Ratios for Core Study Male Rats in the 3-Month Feed Study of Wy-14,643 . . . . . D-3
TABLE D3 Organ Weights and Organ-Weight-to-Body-Weight Ratios for Male Mice in the 2-Week Feed Study of Wy-14,643 . . . . . D-3
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TABLE D1
Organ Weights and Organ-Weight-to-Body-Weight Ratios for Special Study Male Rats in the 3-Month Feed Study of Wy-14,643[a]

	0 ppm	5 ppm	10 ppm	50 ppm	100 ppm	500 ppm
Day 6						
n	5	5	5	5	5	5
Necropsy body wt	239 ± 5	242 ± 3	236 ± 4	227 ± 4	233 ± 6	232 ± 3
Liver						
Absolute	9.876 ± 0.468	11.880 ± 0.334**	13.074 ± 0.264**	15.002 ± 0.437**	15.157 ± 0.598**	15.541 ± 0.294**
Relative	41.28 ± 1.30	49.09 ± 1.39**	55.39 ± 0.71**	65.95 ± 1.02**	65.01 ± 1.05**	67.04 ± 0.83**
R. Testis						
Absolute	1.509 ± 0.020	1.576 ± 0.024	1.592 ± 0.033	1.513 ± 0.041	1.609 ± 0.066	1.517 ± 0.016
Relative	6.33 ± 0.10	6.51 ± 0.14	6.74 ± 0.08	6.66 ± 0.21	6.92 ± 0.34	6.55 ± 0.08
Day 34						
n	5	5	5	5	5	5
Necropsy body wt	348 ± 5	346 ± 10	335 ± 6	318 ± 6**	303 ± 9**	286 ± 7**
Liver						
Absolute	12.406 ± 0.323	15.352 ± 0.382**	16.722 ± 0.578**	20.128 ± 0.258**	20.538 ± 0.798**	20.427 ± 0.822**
Relative	35.61 ± 0.55	44.36 ± 0.81**	49.88 ± 1.41**	63.45 ± 1.45**	67.68 ± 1.05**	71.29 ± 1.33**
R. Testis						
Absolute	1.884 ± 0.054	1.768 ± 0.044	1.809 ± 0.063	1.703 ± 0.040*	1.671 ± 0.015**	1.636 ± 0.048**
Relative	5.41 ± 0.14	5.13 ± 0.23	5.41 ± 0.23	5.37 ± 0.15	5.53 ± 0.14	5.72 ± 0.16
Month 3						
n	5	4	5	5	5	5
Necropsy body wt	427 ± 11	419 ± 9	422 ± 8	378 ± 10**	370 ± 11**	332 ± 18**
Liver						
Absolute	12.904 ± 0.772	15.644 ± 0.709	17.715 ± 0.592**	21.068 ± 1.040**	24.450 ± 1.031**	22.357 ± 1.882**
Relative	30.13 ± 1.01	37.32 ± 0.99**	41.98 ± 1.02**	55.66 ± 1.44**	65.99 ± 1.80**	67.06 ± 2.63**
R. Testis						
Absolute	1.870 ± 0.065	1.987 ± 0.100	1.913 ± 0.036	1.932 ± 0.049	1.885 ± 0.049	1.881 ± 0.074
Relative	4.38 ± 0.05	4.75 ± 0.25	4.54 ± 0.10	5.12 ± 0.07**	5.10 ± 0.10**	5.72 ± 0.34**

* Significantly different ($P \leq 0.05$) from the control group by Williams' test
** $P \leq 0.01$
[a] Organ weights (absolute weights) and body weights are given in grams; organ-weight-to-body-weight ratios (relative weights) are given as mg organ weight/g body weight (mean ± standard error).

TABLE D2
Organ Weights and Organ-Weight-to-Body-Weight Ratios for Core Study Male Rats in the 3-Month Feed Study of Wy-14,643[a]

	0 ppm	5 ppm	10 ppm	50 ppm	100 ppm	500 ppm
n	10	10	10	10	10	10
Necropsy body wt	432 ± 8	429 ± 7	404 ± 10*	377 ± 8**	359 ± 6**	313 ± 8**
R. Kidney						
Absolute	1.153 ± 0.033	1.237 ± 0.027	1.236 ± 0.029	1.218 ± 0.032	1.263 ± 0.030*	1.155 ± 0.024
Relative	2.67 ± 0.05	2.89 ± 0.06**	3.06 ± 0.04**	3.23 ± 0.05**	3.52 ± 0.05**	3.69 ± 0.07**
Liver						
Absolute	13.091 ± 0.341	15.413 ± 0.310**	16.427 ± 0.559**	20.386 ± 0.475**	21.994 ± 0.567**	21.079 ± 0.756**
Relative	30.31 ± 0.39	35.93 ± 0.40**	40.56 ± 0.50**	54.02 ± 0.70**	61.28 ± 0.88**	67.21 ± 1.29**
R. Testis						
Absolute	1.964 ± 0.045	1.925 ± 0.031	1.853 ± 0.039	1.960 ± 0.050	1.913 ± 0.037	1.875 ± 0.032
Relative	4.56 ± 0.09	4.49 ± 0.08	4.59 ± 0.07	5.20 ± 0.12**	5.34 ± 0.09**	6.01 ± 0.17**

* Significantly different (P≤0.05) from the control group by Williams' or Dunnett's test
** Significantly different (P≤0.01) from the control group by Williams' test
[a] Organ weights (absolute weights) and body weights are given in grams; organ-weight-to-body-weight ratios (relative weights) are given as mg organ weight/g body weight (mean ± standard error).

TABLE D3
Organ Weights and Organ-Weight-to-Body-Weight Ratios for Male Mice in the 2-Week Feed Study of Wy-14,643[a]

	0 ppm	10 ppm	50 ppm	100 ppm	500 ppm	1,000 ppm
n	5	5	5	5	5	5
Necropsy body wt	26.9 ± 0.5	26.1 ± 0.9	26.1 ± 0.3	27.4 ± 0.3	24.0 ± 1.5	25.3 ± 0.2
R. Kidney						
Absolute	0.265 ± 0.005	0.274 ± 0.008	0.268 ± 0.006	0.265 ± 0.003	0.258 ± 0.015	0.229 ± 0.002*
Relative	9.844 ± 0.104	10.528 ± 0.270	10.302 ± 0.232	9.678 ± 0.052	10.795 ± 0.303*	9.047 ± 0.142
Liver						
Absolute	1.518 ± 0.031	1.910 ± 0.058	2.489 ± 0.041**	2.723 ± 0.045**	3.068 ± 0.323**	3.781 ± 0.073**
Relative	56.542 ± 1.810	73.388 ± 1.543**	95.547 ± 1.878**	99.529 ± 1.377**	126.275 ± 7.358**	149.561 ± 2.389**
R. Testis						
Absolute	0.102 ± 0.001	0.097 ± 0.004	0.099 ± 0.003	0.102 ± 0.003	0.094 ± 0.004	0.099 ± 0.003
Relative	3.792 ± 0.113	3.767 ± 0.257	3.790 ± 0.089	3.737 ± 0.101	3.979 ± 0.319	3.934 ± 0.120

* Significantly different (P≤0.05) from the control group by Dunnett's test
** Significantly different (P≤0.01) from the control group by Williams' test
[a] Organ weights (absolute weights) and body weights are given in grams; organ-weight-to-body-weight ratios (relative weights) are given as mg organ weight/g body weight (mean ± standard error).

TABLE D4
Organ Weights and Organ-Weight-to-Body-Weight Ratios for Special Study Male Mice in the 3-Month Feed Study of Wy-14,643[a]

	0 ppm	5 ppm	10 ppm	50 ppm	100 ppm	500 ppm
Day 6						
n	5	5	5	5	5	5
Necropsy body wt	24.6 ± 0.3	25.0 ± 0.4	24.5 ± 0.5	25.4 ± 0.5	25.0 ± 0.3	24.4 ± 0.2
Liver						
Absolute	1.278 ± 0.039	1.612 ± 0.036**	1.685 ± 0.026**	2.070 ± 0.061**	2.220 ± 0.055**	2.286 ± 0.049**
Relative	51.93 ± 1.17	64.53 ± 0.64**	68.75 ± 0.61**	81.43 ± 0.89**	88.72 ± 1.68**	93.81 ± 1.71**
R. Testis						
Absolute	0.093 ± 0.002	0.088 ± 0.003	0.091 ± 0.006	0.087 ± 0.007	0.092 ± 0.002	0.101 ± 0.003
Relative	3.77 ± 0.08	3.53 ± 0.09	3.71 ± 0.20	3.42 ± 0.23	3.68 ± 0.05	4.13 ± 0.15
Day 34						
n	5	5	5	4	5	5
Necropsy body wt	26.1 ± 1.4	25.8 ± 0.6	28.0 ± 0.5	28.7 ± 0.6	27.9 ± 0.3	24.5 ± 1.2
Liver						
Absolute	1.200 ± 0.108	1.436 ± 0.071	1.808 ± 0.028**	2.393 ± 0.052**	2.645 ± 0.019**	3.158 ± 0.228**
Relative	45.66 ± 2.46	55.53 ± 1.67**	64.50 ± 0.47**	83.40 ± 0.58**	94.94 ± 0.66**	128.38 ± 3.21**
R. Testis						
Absolute	0.097 ± 0.004	0.101 ± 0.005	0.105 ± 0.005	0.102 ± 0.005	0.102 ± 0.002	0.087 ± 0.004
Relative	3.74 ± 0.16	3.92 ± 0.23	3.76 ± 0.16	3.55 ± 0.18	3.67 ± 0.08	3.60 ± 0.23
Month 3						
n	5	5	4	5	5	5
Necropsy body wt	30.9 ± 1.0	31.9 ± 0.9	30.3 ± 1.3	30.9 ± 0.3	31.8 ± 0.6	25.2 ± 0.6**
Liver						
Absolute	1.314 ± 0.048	1.682 ± 0.064**	1.650 ± 0.156*	2.490 ± 0.063**	3.154 ± 0.092**	3.289 ± 0.088**
Relative	42.51 ± 0.67	52.75 ± 0.84**	54.19 ± 3.50**	80.60 ± 1.44**	99.20 ± 1.60**	130.63 ± 2.19**
R. Testis						
Absolute	0.108 ± 0.007	0.112 ± 0.002	0.113 ± 0.003	0.109 ± 0.004	0.113 ± 0.002	0.096 ± 0.001
Relative	3.50 ± 0.19	3.51 ± 0.09	3.75 ± 0.20	3.54 ± 0.13	3.57 ± 0.09	3.83 ± 0.15

* Significantly different ($P \leq 0.05$) from the control group by Williams' test
** Significantly different ($P \leq 0.01$) from the control group by Williams' or Dunnett's test
[a] Organ weights (absolute weights) and body weights are given in grams; organ-weight-to-body-weight ratios (relative weights) are given as mg organ weight/g body weight (mean ± standard error).

TABLE D5
Organ Weights and Organ-Weight-to-Body-Weight Ratios for Core Study Male Mice in the 3-Month Feed Study of Wy-14,643[a]

	0 ppm	5 ppm	10 ppm	50 ppm	100 ppm	500 ppm
n	10	10	10	10	10	10
Necropsy body wt	33.6 ± 0.8	32.8 ± 0.4	32.5 ± 0.5	31.6 ± 0.9*	31.3 ± 0.3**	26.2 ± 0.3**
R. Kidney						
Absolute	0.275 ± 0.007	0.253 ± 0.003*	0.256 ± 0.003	0.263 ± 0.007	0.280 ± 0.005	0.228 ± 0.006**
Relative	8.18 ± 0.13	7.73 ± 0.08	7.89 ± 0.11	8.34 ± 0.23	8.93 ± 0.14**	8.70 ± 0.16**
Liver						
Absolute	1.406 ± 0.027	1.689 ± 0.029**	1.852 ± 0.031**	2.493 ± 0.081**	2.989 ± 0.047**	3.529 ± 0.073**
Relative	41.90 ± 0.75	51.58 ± 0.75**	56.92 ± 0.50**	78.86 ± 1.45**	95.57 ± 1.23**	134.48 ± 1.97**
R. Testis						
Absolute	0.125 ± 0.002	0.123 ± 0.002	0.120 ± 0.002	0.115 ± 0.004*	0.113 ± 0.004**	0.104 ± 0.003**
Relative	3.73 ± 0.10	3.74 ± 0.06	3.68 ± 0.07	3.65 ± 0.13	3.61 ± 0.14	3.94 ± 0.08

* Significantly different (P≤0.05) from the control group by Williams' or Dunnett's test
** P≤0.01
[a] Organ weights (absolute weights) and body weights are given in grams; organ-weight-to-body-weight ratios (relative weights) are given as mg organ weight/g body weight (mean ± standard error).

TABLE D6
Organ Weights and Organ-Weight-to-Body-Weight Ratios for Male Hamsters in the 2-Week Feed Study of Wy-14,643[a]

	0 ppm	10 ppm	100 ppm	500 ppm	1,000 ppm	5,000 ppm
n	5	4	5	5	5	5
Necropsy body wt	115 ± 4	115 ± 3	95 ± 3**	99 ± 3**	87 ± 3**	94 ± 3**
R. Kidney						
Absolute	0.503 ± 0.030	0.553 ± 0.014	0.518 ± 0.030	0.530 ± 0.005	0.504 ± 0.020	0.548 ± 0.022
Relative	4.366 ± 0.151	4.801 ± 0.159	5.465 ± 0.308**	5.363 ± 0.103**	5.771 ± 0.045**	5.804 ± 0.079**
Liver						
Absolute	6.162 ± 0.234	8.129 ± 0.290**[b]	8.488 ± 0.413**	10.599 ± 0.131**	9.170 ± 0.477**	10.285 ± 0.339**
Relative	53.646 ± 0.694	70.820 ± 1.977**[b]	89.297 ± 2.554**	107.179 ± 1.860**	104.796 ± 1.848**	109.129 ± 1.701**
R. Testis						
Absolute	1.766 ± 0.103	1.809 ± 0.070	1.554 ± 0.080	1.658 ± 0.085	1.435 ± 0.205	1.644 ± 0.114
Relative	15.389 ± 0.758	15.677 ± 0.512	16.419 ± 0.959	16.722 ± 0.694	16.188 ± 1.828	17.371 ± 0.761

** Significantly different (P≤0.01) from the control group by Williams' test
[a] Organ weights (absolute weights) and body weights are given in grams; organ-weight-to-body-weight ratios (relative weights) are given as mg organ weight/g body weight (mean ± standard error).
[b] n=5

TABLE D7
Organ Weights and Organ-Weight-to-Body-Weight Ratios for Special Study Male Hamsters in the 3-Month Feed Study of Wy-14,643[a]

	0 ppm	5 ppm	10 ppm	50 ppm	100 ppm	500 ppm
n	5	5	5	5	5	5
Day 6						
Necropsy body wt	87 ± 1	91 ± 4	91 ± 4	85 ± 3	86 ± 3	85 ± 3
Liver						
Absolute	4.243 ± 0.044	4.975 ± 0.158*	5.303 ± 0.209**	5.697 ± 0.301**	5.644 ± 0.254**	5.697 ± 0.189**
Relative	49.02 ± 0.75	54.52 ± 1.44*	58.52 ± 1.81**	66.88 ± 2.42**	65.34 ± 1.36**	67.62 ± 2.77**
R. Testis						
Absolute	1.336 ± 0.066	1.488 ± 0.051	1.410 ± 0.049	1.348 ± 0.064	1.445 ± 0.030	1.309 ± 0.110
Relative	15.46 ± 0.89	16.30 ± 0.36	15.56 ± 0.26	15.87 ± 0.69	16.80 ± 0.54	15.41 ± 0.95
Day 34						
Necropsy body wt	103 ± 3	100 ± 5	99 ± 3	85 ± 2**	79 ± 4**	75 ± 2**
Liver						
Absolute	4.259 ± 0.167	5.634 ± 0.364**	6.316 ± 0.114**	7.470 ± 0.292**	7.273 ± 0.568**	9.058 ± 0.345**
Relative	41.51 ± 1.62	56.53 ± 1.27**	63.69 ± 0.68**	87.33 ± 1.59**	92.02 ± 3.65**	121.29 ± 2.96**
R. Testis						
Absolute	1.659 ± 0.068	1.563 ± 0.111	1.701 ± 0.092	1.440 ± 0.071	1.227 ± 0.149*	0.708 ± 0.183**
Relative	16.16 ± 0.57	15.70 ± 0.76	17.10 ± 0.55	16.86 ± 0.77	15.40 ± 1.35	9.47 ± 2.40**
Month 3						
Necropsy body wt	123 ± 3	117 ± 2	104 ± 4**	90 ± 5**	91 ± 6**	80 ± 2**
Liver						
Absolute	4.599 ± 0.171	6.415 ± 0.217**	6.126 ± 0.334**	7.910 ± 0.448**	9.583 ± 0.651**	10.463 ± 0.318**
Relative	37.35 ± 0.97	54.83 ± 1.32**	58.88 ± 2.70**	87.76 ± 1.24**	105.02 ± 1.62**	130.17 ± 3.18**
R. Testis						
Absolute	1.684 ± 0.097	1.234 ± 0.200	1.015 ± 0.251*	0.442 ± 0.222**	0.432 ± 0.120**	0.226 ± 0.055**
Relative	13.64 ± 0.49	10.57 ± 1.76	10.00 ± 2.56	4.75 ± 2.30**	4.65 ± 1.15**	2.81 ± 0.66**

* Significantly different ($P \leq 0.05$) from the control group by Williams' test
** Significantly different ($P \leq 0.01$) from the control group by Williams' or Dunnett's test
[a] Organ weights (absolute weights) and body weights are given in grams; organ-weight-to-body-weight ratios (relative weights) are given as mg organ weight/g body weight (mean ± standard error).

TABLE D8
Organ Weights and Organ-Weight-to-Body-Weight Ratios for Core Study Male Hamsters in the 3-Month Feed Study of Wy-14,643[a]

	0 ppm	5 ppm	10 ppm	50 ppm	100 ppm	500 ppm
n	10	10	10	10	10	10
Necropsy body wt	121 ± 4	119 ± 3	106 ± 3**	90 ± 4**	89 ± 2**	78 ± 3**
R. Kidney						
Absolute	0.432 ± 0.013	0.464 ± 0.008	0.465 ± 0.016	0.446 ± 0.009	0.476 ± 0.011*	0.452 ± 0.010
Relative	3.59 ± 0.06	3.93 ± 0.11	4.38 ± 0.09**	5.04 ± 0.28**	5.33 ± 0.11**	5.82 ± 0.12**
Liver						
Absolute	4.608 ± 0.198	6.015 ± 0.201**	6.138 ± 0.217**	7.435 ± 0.355**	8.584 ± 0.309**	9.429 ± 0.396**
Relative	38.07 ± 0.74	50.51 ± 0.65**	57.69 ± 0.92**	82.37 ± 1.61**	96.01 ± 2.38**	120.51 ± 1.94**
R. Testis						
Absolute	1.526 ± 0.197	1.292 ± 0.139	1.373 ± 0.086	0.854 ± 0.164**	0.560 ± 0.150**	0.327 ± 0.037**
Relative	12.49 ± 1.52	11.06 ± 1.28	12.83 ± 0.59	9.14 ± 1.66	6.07 ± 1.50**	4.22 ± 0.49**

* Significantly different ($P \leq 0.05$) from the control group by Dunnett's test
** Significantly different ($P \leq 0.01$) from the control group by Williams' test
[a] Organ weights (absolute weights) and body weights are given in grams; organ-weight-to-body-weight ratios (relative weights) are given as mg organ weight/g body weight (mean ± standard error).

APPENDIX E
REPRODUCTIVE TISSUE EVALUATIONS

TABLE E1 Summary of Reproductive Tissue Evaluations for Male Rats in the 3-Month Feed Study of Wy-14,643 . . . . . E-2
TABLE E2 Summary of Reproductive Tissue Evaluations for Male Mice in the 3-Month Feed Study of Wy-14,643 . . . . . E-2
TABLE E3 Summary of Reproductive Tissue Evaluations for Male Hamsters in the 3-Month Feed Study of Wy-14,643 . . . . . E-3
TABLE E4 Summary of Reproductive Tissue Evaluations for Male Hamsters in the 3-Month Feed Study of Wy-14,643: Immature Animals with No Sperm Present E-4

TABLE E1
Summary of Reproductive Tissue Evaluations for Male Rats in the 3-Month Feed Study of Wy-14,643[a]

	0 ppm	50 ppm	100 ppm	500 ppm
n	10	10	10	10
Weights (g)				
Necropsy body wt	432 ± 8	377 ± 8**	359 ± 6**	313 ± 8**
L. Cauda epididymis	0.2180 ± 0.0085	0.2156 ± 0.0059	0.1963 ± 0.0094	0.1897 ± 0.0070*
L. Epididymis	0.6521 ± 0.0120	0.6166 ± 0.0144	0.5922 ± 0.0125**	0.5667 ± 0.0155**
L. Testis	1.9861 ± 0.0411	1.9506 ± 0.0522	1.8608 ± 0.0296	1.8947 ± 0.0369
Spermatid measurements				
Spermatid heads (10^7/g testis)	6.008 ± 0.225	5.946 ± 0.311	6.390 ± 0.291	5.739 ± 0.240
Spermatid heads (10^7/testis)	11.960 ± 0.559	11.485 ± 0.424	11.850 ± 0.484	10.865 ± 0.489
Spermatid count (mean/10^{-4} mL suspension)	59.800 ± 2.793	57.425 ± 2.121	59.250 ± 2.422	54.325 ± 2.445
Epididymal spermatozoal measurements				
Motility (%)	68.90 ± 0.82	66.60 ± 2.41	70.12 ± 0.49	67.55 ± 0.99
Concentration (10^6/g cauda epididymal tissue)	428 ± 28	441 ± 35	502 ± 25	466 ± 28

* Significantly different (P≤0.05) from the control group by Williams' test
** (P≤0.01)
[a] Data are presented as mean ± standard error. Differences from the control group are not significant by Dunnett's test (testis weight) or Dunn's test (spermatid and epididymal spermatozoal measurements).

TABLE E2
Summary of Reproductive Tissue Evaluations for Male Mice in the 3-Month Feed Study of Wy-14,643[a]

	0 ppm	50 ppm	100 ppm	500 ppm
n	10	10	10	10
Weights (g)				
Necropsy body wt	33.6 ± 0.8	31.6 ± 0.9*	31.3 ± 0.3*	26.2 ± 0.3**
L. Cauda epididymis	0.0128 ± 0.008	0.0131 ± 0.0007	0.0116 ± 0.0004	0.0114 ± 0.0006
L. Epididymis	0.0402 ± 0.0008	0.0396 ± 0.0013	0.0368 ± 0.0010*	0.0330 ± 0.0010**
L. Testis	0.1191 ± 0.0031	0.1098 ± 0.0032	0.1133 ± 0.0018	0.1002 ± 0.0023**
Spermatid measurements				
Spermatid heads (10^7/g testis)	14.592 ± 1.517	16.176 ± 0.998	15.315 ± 0.636	13.718 ± 1.600
Spermatid heads (10^7/testis)	1.724 ± 0.169	1.780 ± 0.121	1.732 ± 0.070	1.364 ± 0.150
Spermatid count (mean/10^{-4} mL suspension)	53.850 ± 5.277	55.625 ± 3.790	54.150 ± 2.168	42.600 ± 4.679
Epididymal spermatozoal measurements				
Motility (%)	71.12 ± 0.94	70.68 ± 0.76	71.75 ± 0.81	71.90 ± 0.94
Concentration (10^6/g cauda epididymal tissue)	544 ± 66	550 ± 72	653 ± 100	494 ± 73

* Significantly different (P≤0.05) from the control group by Williams' test
** (P≤0.01)
[a] Data are presented as mean ± standard error. Differences from the control group are not significant by Dunnett's test (cauda epididymal weight) or Dunn's test (spermatid and epididymal spermatozoal measurements).

TABLE E3
Summary of Reproductive Tissue Evaluations for Male Hamsters in the 3-Month Feed Study of Wy-14,643[a]

	0 ppm	50 ppm	100 ppm	500 ppm
n	10	10	10	10
Weights (g)				
Necropsy body wt	121 ± 4	90 ± 4**	89 ± 2**	78 ± 3**
L. Cauda epididymis	0.2177 ± 0.0359	0.0987 ± 0.0212**	0.0670 ± 0.0209**	0.0213 ± 0.0014**
L. Epididymis	0.5794 ± 0.0660	0.2884 ± 0.0552**	0.2097 ± 0.0566**	0.0828 ± 0.0060**
L. Testis	1.4831 ± 0.2011	0.8237 ± 0.1571**	0.6449 ± 0.1661**	0.3186 ± 0.0332**
Spermatid measurements				
Spermatid heads (10^7/g testis)	5.519 ± 0.977	4.618 ± 1.157	2.813 ± 1.222	0.519 ± 0.178**
Spermatid heads (10^7/testis)	9.705 ± 1.785	5.335 ± 1.589*	3.525 ± 1.666*	0.200 ± 0.084**
Spermatid count (mean/10^{-4} mL suspension)	48.525 ± 8.926	26.675 ± 7.947*	17.625 ± 8.332*	1.000 ± 0.422**
Epididymal spermatozoal measurements				
Motility (%)	64.79 ± 7.23	50.21 ± 10.98	27.76 ± 11.34**	0.00 ± 0.00**
Concentration (10^6/g cauda epididymal tissue)	796 ± 104	489 ± 126	279 ± 114**	0 ± 0**

* Significantly different ($P \leq 0.05$) from the control group by Shirley's test
** Significantly different ($P \leq 0.01$) from the control group by Williams' test (body and tissue weights) or by Shirley's test (spermatid and epididymal spermatozoal measurements)
[a] Data are presented as mean ± standard error.

TABLE E4
Summary of Reproductive Tissue Evaluations for Male Hamsters in the 3-Month Feed Study of Wy-14,643: Immature Animals with no Sperm Present

	0 ppm	50 ppm	100 ppm
n	9	7	4
Weights (g)			
Necropsy body wt	121 ± 4	95 ± 1**	94 ± 4**
L. Cauda epididymis	0.2402 ± 0.0313	0.1333 ± 0.0173*	0.1385 ± 0.0212*
L. Epididymis	0.6296 ± 0.0479	0.3843 ± 0.0378**	0.4007 ± 0.0599**
L. Testis	1.6276 ± 0.1564	1.1021 ± 0.0992*	1.2124 ± 0.1477
Spermatid measurements			
Spermatid heads (10^7/g testis)	6.132 ± 0.851	6.436 ± 1.006	6.823 ± 1.496
Spermatid heads (10^7/testis)	10.783 ± 1.591	7.593 ± 1.606	8.725 ± 2.409
Spermatid count (mean/10^{-4} mL suspension)	53.917 ± 7.954	37.964 ± 8.031	43.625 ± 12.044
Epididymal spermatozoal measurements			
Motility (%)	71.99 ± 0.78	71.73 ± 0.93	69.40 ± 1.17
Concentration (10^6/g cauda epididymal tissue)	884 ± 61	699 ± 98	697 ± 14

* Significantly different ($P \leq 0.05$) from the control group by Williams' test (cauda epididymal weight) or by Dunnett's test (testis weight)
** Significantly different ($P \leq 0.01$) from the control group by Williams' test
[a] Data are presented as mean ± standard error. Differences from the control group for spermatid and epididymal spermatozoal measurements are not significant by Dunn's test.

APPENDIX F
CELL PROLIFERATION INDICES

TABLE F1 Cell Proliferation Indices in Left Lobe Hepatocytes of Special Study Male Rats in the 3-Month Feed Study of Wy-14,643 . . . . . F-2
TABLE F2 Percentages of BrdU-Labeled Hepatocytes in Male Mice in the 2-Week Feed Study of Wy-14,643 . . . . . F-3
TABLE F3 Cell Proliferation Indices in Left Lobe Hepatocytes of Special Study Male Mice in the 3-Month Feed Study of Wy-14,643 . . . . . F-3
TABLE F4 Percentages of BrdU-Labeled Hepatocytes in Male Hamsters in the 2-Week Feed Study of Wy-14,643 . . . . . F-4
TABLE F5 Percentages of BrdU-Labeled Left Lobe Hepatocytes in Special Study Male Hamsters in the 3-Month Feed Study of Wy-14,643 . . . . . F-4

Table F1
Cell Proliferation Indices in Left Lobe Hepatocytes of Special Study Male Rats in the 3-Month Feed Study of Wy-14,643[a]

	0 ppm	5 ppm	10 ppm	50 ppm	100 ppm	500 ppm
n						
Day 6	5	5	5	5	5	5
Day 34	5	5	5	5	5	5
Week 13	5	4	5	5	5	5
BrdU-labeled hepatocytes (%)						
Day 6	3.9 ± 0.6	16.2 ± 1.7**	22.5 ± 1.0**	36.1 ± 2.1**	36.6 ± 1.9**	39.1 ± 2.2**
Day 34	1.1 ± 0.1	1.4 ± 0.2	1.4 ± 0.1	3.3 ± 0.2**	7.5 ± 0.4**	8.1 ± 1.0**
Week 13	0.9 ± 0.1	1.0 ± 0.1	0.9 ± 0.1	2.2 ± 0.1**	3.5 ± 0.2**	5.0 ± 0.9**
Cyclin-dependent kinase (fmol/mg liver)						
Day 6	22.5 ± 3.5	76.3 ± 3.4**	104.5 ± 3.8**	146.4 ± 6.9**	148.6 ± 4.8**	163.0 ± 4.1**
Week 13	25.7 ± 0.7	63.0 ± 4.0*	91.5 ± 5.4**	135.6 ± 11.8**	165.1 ± 6.0**	164.9 ± 16.5**
Proliferating cell nuclear antigen (fmol/mg liver)						
Week 13	2.8 ± 0.2	8.4 ± 0.3*	13.5 ± 0.8**	47.1 ± 5.0**	52.8 ± 3.9**	48.9 ± 3.2**

* Significantly different ($P \leq 0.05$) from the control group by Shirley's test
** $P \leq 0.01$
[a] Data are presented as mean ± standard error. BrdU=bromodeoxyuridine

TABLE F2
Percentages of BrdU-Labeled Hepatocytes in Male Mice in the 2-Week Feed Study of Wy-14,643[a]

	0 ppm	10 ppm	100 ppm	1,000 ppm
n	5	5	5	5
Left lobe	1.0 ± 0.1	1.5 ± 0.5	2.8 ± 0.5*	18.1 ± 1.8**
Right median lobe	1.1 ± 0.2	—[b]	—	17.9 ± 1.7**
Right anterior lobe	1.4 ± 0.2	—	—	15.2 ± 1.6**

* Significantly different (P≤0.05) from the control group by Shirley's test
** Significantly different (P≤0.01) from the control group by Shirley's test (left lobe) or the Wilcoxon rank sum test
[a] Data are presented as mean ± standard error. BrdU=bromodeoxyuridine
[b] Not measured at this exposure concentration

TABLE F3
Cell Proliferation Indices in Left Lobe Hepatocytes of Special Study Male Mice in the 3-Month Feed Study of Wy-14,643[a]

	0 ppm	5 ppm	10 ppm	50 ppm	100 ppm	500 ppm
n						
Day 6	5	5	5	5	5	5
Day 34	5	5	5	4	5	5
Week 13	5	5	4	5	5	5
BrdU-labeled hepatocytes (%)						
Day 6	0.9 ± 0.2	1.8 ± 0.4	3.3 ± 0.3**	7.7 ± 1.1**	15.7 ± 0.8**	18.5 ± 3.6**
Day 34	0.7 ± 0.2	1.5 ± 0.5	2.0 ± 0.4*	2.6 ± 0.2**	10.1 ± 1.0**	22.4 ± 1.2**
Week 13	0.8 ± 0.1	1.0 ± 0.2	1.1 ± 0.4	5.0 ± 1.3*	13.9 ± 5.7**	15.9 ± 2.2**
Cyclin-dependent kinase (fmol/mg liver)						
Day 6	40.6 ± 1.9	51.8 ± 1.6**	73.1 ± 3.7**	81.9 ± 2.6**	91.1 ± 2.9**	102.2 ± 4.5**
Week 13	26.1 ± 0.7	36.6 ± 1.3**	43.3 ± 3.8**	73.8 ± 3.9**	108.1 ± 3.6**	148.6 ± 5.8**
Proliferating cell nuclear antigen (fmol/mg liver)						
Week 13	1.8 ± 0.2	5.4 ± 0.9*	12.0 ± 2.8**	35.4 ± 1.4**	54.5 ± 2.4**	76.4 ± 4.3**

* Significantly different (P≤0.05) from the control group by Shirley's test
** P≤0.01
[a] Data are presented as mean ± standard error. BrdU=bromodeoxyuridine

TABLE F4
Percentages of BrdU-Labeled Hepatocytes in Male Hamsters in the 2-Week Feed Study of Wy-14,643[a]

	0 ppm	10 ppm	500 ppm	5,000 ppm
n	5	5	5	5
Left lobe	0.1 ± 0.1	0.3 ± 0.1	7.2 ± 1.5**	7.0 ± 1.8**
Right median lobe	0.1 ± 0.0	0.4[b]	6.2[b]	8.0 ± 2.1**
Right anterior lobe	0.0 ± 0.0	0.6[b]	8.1[b]	7.1 ± 1.4**

** Significantly different (P≤0.01) from the control group by Shirley's test
[a] Data are presented as mean ± standard error. BrdU=bromodeoxyuridine
[b] Only one sample was examined at this exposure concentration; no standard error was calculated.

TABLE F5
Percentages of BrdU-Labeled Left Lobe Hepatocytes in Special Study Male Hamsters in the 3-Month Feed Study of Wy-14,643[a]

	0 ppm	5 ppm	10 ppm	50 ppm	100 ppm	500 ppm
n	5	5	5	5	5	5
Day 6	1.3 ± 0.5	5.4 ± 2.3*	5.7 ± 1.4*	9.3 ± 2.8**	8.8 ± 1.6**	9.8 ± 2.1**
Day 34	1.1 ± 0.1	0.8 ± 0.1	1.1 ± 0.1	2.4 ± 0.3*	2.5 ± 0.5*	3.5 ± 0.5**
Week 13	1.7 ± 0.3	1.9 ± 0.4	2.5 ± 0.3	2.9 ± 0.3*	6.4 ± 1.5**	4.4 ± 0.6**

* Significantly different (P≤0.05) from the control group by Shirley's test
** P≤0.01
[a] Data are presented as mean ± standard error. BrdU=bromodeoxyuridine

APPENDIX G
PEROXISOMAL ENZYME ANALYSIS RESULTS

TABLE G1 Peroxisomal Enzyme Activities in Left Lobe Hepatocytes of Special Study Male Rats in the 3-Month Feed Study of Wy-14,643 . . . . . G-2
TABLE G2 Peroxisomal Enzyme Activities in Hepatocytes of Male Mice in the 2-Week Feed Study of Wy-14,643 . . . . . G-3
TABLE G3 Peroxisomal Enzyme Activities in Left Lobe Hepatocytes of Special Study Male Mice in the 3-Month Feed Study of Wy-14,643 . . . . . G-3
TABLE G4 Peroxisomal Enzyme Activities in Hepatocytes of Male Hamsters in the 2-Week Feed Study of Wy-14,643 . . . . . G-4
TABLE G5 Peroxisomal Enzyme Activities in Left Lobe Hepatocytes of Special Study Male Hamsters in the 3-Month Feed Study of Wy-14,643 . . . . . G-4

TABLE G1
Peroxisomal Enzyme Activities in Left Lobe Hepatocytes of Special Study Male Rats in the 3-Month Feed Study of Wy-14,643[a]

	0 ppm	5 ppm	10 ppm	50 ppm	100 ppm	500 ppm
n						
Day 6	10	10	10	10	10	10
Day 34	10	10	10	10	10	10
Week 13	10	8	10	10	10	10
Acyl CoA oxidase (nmol DCF/minute per mg)						
Day 6	1.5 ± 0.0	7.8 ± 0.4**	11.3 ± 0.4**	15.2 ± 1.0**	14.0 ± 1.1**	13.9 ± 0.9**
Day 34	1.6 ± 0.1[b]	7.6 ± 0.4**	10.5 ± 1.0**	15.8 ± 0.9**	16.3 ± 0.7**	17.1 ± 0.7**
Week 13	1.6 ± 0.0[b]	7.6 ± 0.4**	8.4 ± 0.2**	16.6 ± 0.5**	18.0 ± 1.2**[b]	17.1 ± 0.6**[b]
Peroxisomal β-oxidation (Lazarow method) (nmol NADH/minute per mg)						
Day 6	1.4 ± 0.1[c]	8.9 ± 0.4*[b]	13.2 ± 1.0**[d]	25.4 ± 1.5**	24.5 ± 1.6**	30.9 ± 0.9**
Day 34	1.8 ± 0.0[e]	17.1 ± 1.0**	23.6 ± 1.0**	43.8 ± 1.4**[b]	42.9 ± 2.4**	41.4 ± 1.7**
Week 13	1.7 ± 0.1	16.9 ± 1.0**	21.6 ± 0.8**	39.3 ± 0.7**	47.0 ± 1.7**	37.5 ± 2.2**
Carnitine acetyltransferase (nmol reduced CoA/minute per mg)						
Day 6	0.7 ± 0.1[b]	16.5 ± 0.6**	29.0 ± 1.1**	43.7 ± 1.6**	48.2 ± 2.5**	46.9 ± 2.5**
Day 34	0.6 ± 0.1	41.9 ± 2.2**	76.4 ± 4.2**[b]	109.9 ± 7.5**[d]	103.8 ± 6.0**[b]	105.9 ± 5.9**[b]
Week 13	0.9 ± 0.2	40.1 ± 1.8**	62.5 ± 5.2**	106.4 ± 7.1**[d]	102.5 ± 7.3**	112.4 ± 6.1**
Catalase (nmol NADPH/minute per mg)						
Day 6	211 ± 8	223 ± 6	255 ± 8**	303 ± 19**	295 ± 19**	310 ± 22**
Day 34	272 ± 7	257 ± 11	321 ± 6**	393 ± 10**	368 ± 8**	377 ± 13**
Week 13	256 ± 8	298 ± 8**	318 ± 7**	362 ± 7**	339 ± 22**	381 ± 9**

* Significantly different ($P \leq 0.05$) from the control group by Shirley's test
** $P \leq 0.01$
[a] Data are presented as mean ± standard error of duplicate tissue extractions of each liver sample. CoA= coenzyme A; DCF=dichlorofluorescein diacetate; NADH=nicotinic acid adenine dinucleotide; NADPH=nicotinic acid adenine dinucleotide phosphate
[b] n=9
[c] n=3
[d] n=8
[e] n=6

TABLE G2
Peroxisomal Enzyme Activities in Hepatocytes of Male Mice in the 2-Week Feed Study of Wy-14,643[a]

	0 ppm	10 ppm	50 ppm	100 ppm	500 ppm	1,000 ppm	Positive Control
n	30	30	10	30	10	30	23
Acyl CoA oxidase							
(nmol DCF/minute per mg)	1.3 ± 0.0[b]	14.2 ± 0.6**[c]	27.9 ± 2.0**	32.6 ± 1.3**[b]	32.5 ± 3.2**	40.8 ± 1.4**[b]	26.0 ± 0.7
Peroxisomal β-oxidation (Lazarow method)							
(nmol NADH/minute per mg)	1.2 ± 0.1[d]	17.1 ± 1.0**	30.1 ± 3.5**[e]	44.0 ± 1.6**[f]	56.6 ± 1.3**[g]	70.5 ± 2.5**[h]	36.0 ± 0.9
Carnitine acetyltransferase							
(nmol reduced CoA/minute per mg)	2.1 ± 0.1	19.8 ± 0.6**	37.8 ± 1.9**	52.4 ± 0.9**	55.8 ± 3.6**	75.2 ± 1.5**	73.8 ± 2.3
Catalase							
(nmol NADPH/minute per mg)	106 ± 7[i]	257 ± 5**[c]	345 ± 13**	361 ± 11**[c]	350 ± 23**	349 ± 7**[c]	431 ± 9[j]

** Significantly different (P≤0.01) from the untreated control group by Shirley's test
[a] Data are presented as mean ± standard error of duplicate tissue extractions of one or three liver lobes from each animal. Positive control rats received 500 ppm Wy-14,643. No pairwise comparisons were made between positive control rats and untreated control mice. CoA= coenzyme A; DCF=dichlorofluorescein diacetate; NADH=nicotinic acid adenine dinucleotide; NADPH=nicotinic acid adenine dinucleotide phosphate
[b] n=25 [c] n=28 [d] n=17 [e] n=7 [f] n=24 [g] n=9 [h] n=23 [i] n=22 [j] n=20

TABLE G3
Peroxisomal Enzyme Activities in Left Lobe Hepatocytes of Special Study Male Mice in the 3-Month Feed Study of Wy-14,643[a]

	0 ppm	5 ppm	10 ppm	50 ppm	100 ppm	500 ppm
n						
Day 6	8	10	10	10	10	10
Day 34	10	10	10	8	10	10
Week 13	10	10	8	10	10	10
Acyl CoA oxidase (nmol DCF/minute per mg)						
Day 6	1.1 ± 0.0	8.1 ± 0.3**	12.7 ± 0.4**	17.6 ± 1.3**[b]	15.0 ± 1.4**[c]	16.4 ± 1.9**[c]
Day 34	1.1 ± 0.1	7.7 ± 0.4**	9.8 ± 0.3**	16.7 ± 0.9**[d]	18.4 ± 0.9**[c]	21.7 ± 0.9**[d]
Week 13	1.6 ± 0.3	6.8 ± 0.3**	8.7 ± 1.4**	17.2 ± 0.6**[e]	19.8 ± 0.7**[d]	19.2 ± 0.9**[c]
Peroxisomal β-oxidation (Lazarow method) (nmol NADH/minute per mg)						
Day 6	1.3 ± 0.1	11.5 ± 0.5**	22.2 ± 0.5**	35.6 ± 0.9**	39.0 ± 1.3**	39.6 ± 0.6**
Day 34	1.7 ± 0.3[b]	10.3 ± 0.6**	20.4 ± 0.7**	39.3 ± 2.2**	47.4 ± 1.1**	65.3 ± 5.3**
Week 13	1.3 ± 0.2	11.7 ± 0.9**[e]	18.4 ± 0.7**[f]	39.2 ± 1.0**	45.0 ± 1.8**	59.8 ± 1.2**
Carnitine acetyltransferase (nmol reduced CoA/minute per mg)						
Day 6	1.5 ± 0.1[g]	11.5 ± 0.5**	21.2 ± 0.4**	34.7 ± 0.8**	47.9 ± 1.7**	47.5 ± 1.0**
Day 34	2.0 ± 0.1	12.4 ± 0.3**	20.4 ± 2.1**	55.5 ± 3.9**	54.4 ± 1.9**	59.8 ± 1.3**
Week 13	1.6 ± 0.1	12.0 ± 0.8**	17.9 ± 2.1**	45.6 ± 1.9**	58.8 ± 3.5**	61.9 ± 2.4**
Catalase (nmol NADPH/minute per mg)						
Day 6	97 ± 8	217 ± 9**	288 ± 12**	290 ± 7**	292 ± 8**	279 ± 13**
Day 34	85 ± 9[e]	185 ± 9**	256 ± 10**	358 ± 8**	334 ± 9**	276 ± 20**
Week 13	88 ± 10	187 ± 13**	198 ± 29**	297 ± 16**	313 ± 7**	282 ± 8**

** Significantly different (P≤0.01) from the control group by Shirley's test
[a] Data are presented as mean ± standard error of duplicate tissue extractions of each liver sample. CoA= coenzyme A; DCF=dichlorofluorescein diacetate; NADH=nicotinic acid adenine dinucleotide; NADPH=nicotinic acid adenine dinucleotide phosphate
[b] n=7 [c] n=8 [d] n=6 [e] n=9 [f] n=5 [g] n=10

TABLE G4
Peroxisomal Enzyme Activities in Hepatocytes of Male Hamsters in the 2-Week Feed Study of Wy-14,643[a]

	0 ppm	10 ppm	100 ppm	500 ppm	1,000 ppm	5,000 ppm	Positive Control
n	28	29	10	29	10	29	23
Acyl CoA oxidase							
(nmol DCF/minute per mg)	4.0 ± 0.2[b]	12.3 ± 0.7**	38.1 ± 3.8**	43.3 ± 2.0**[c]	38.3 ± 2.7**	43.7 ± 2.3**[c]	26.0 ± 0.7
Peroxisomal β-oxidation (Lazarow method)							
(nmol NADH/minute per mg)	3.4 ± 0.2[d]	11.5 ± 0.8**[e]	41.7 ± 2.9**[f]	47.4 ±1.2**[g]	53.7 ± 1.5**	50.5 ± 1.6**[h]	36.0 ± 0.9
Carnitine acetyltransferase							
(nmol reduced CoA/minute per mg)	7.5 ± 0.4[i]	44.8 ± 2.7**	104.7 ± 3.5**[j]	120.1 ± 6.9**[k]	146.3 ± 6.2**[l]	132.4 ± 4.2**[m]	73.8 ± 2.3
Catalase							
(nmol NADPH/minute per mg)	241 ± 7	222 ± 9[n]	306 ± 12**	267 ± 9	284 ± 24	241 ± 9	431 ± 9[h]

** Significantly different (P≤0.01) from the untreated control group by Dunn's or Shirley's test
[a] Data are presented as mean ± standard error of duplicate tissue extractions of one or three liver lobes from each animal. Positive control rats received 500 ppm Wy-14,643. No pairwise comparisons were made between positive control rats and untreated control mice. CoA= coenzyme A; DCF=dichlorofluorescein diacetate; NADH=nicotinic acid adenine dinucleotide; NADPH=nicotinic acid adenine dinucleotide phosphate
[b] n=26 [c] n=24 [d] n=19 [e] n=16 [f] n=7 [g] n=23 [h] n=20 [i] n=13 [j] n=14
[k] n=22 [l] n=9 [m] n=28 [n] n=30

TABLE G5
Peroxisomal Enzyme Activities in Left Lobe Hepatocytes of Special Study Male Hamsters in the 3-Month Feed Study of Wy-14,643[a]

	0 ppm	5 ppm	10 ppm	50 ppm	100 ppm	500 ppm
n	10	10	10	10	10	10
Acyl CoA oxidase (nmol DCF/minute per mg)						
Day 6	2.3 ± 0.1	5.7 ± 0.4**	8.2 ± 0.3**	13.5 ± 1.1**	13.7 ± 0.7**	16.2 ± 1.4**[b]
Day 34	2.8 ± 0.2	5.9 ± 0.4**	8.7 ± 0.5**	24.8 ± 2.3**[c]	28.6 ± 1.2**[c]	32.6 ± 1.7**[c]
Week 13	2.6 ± 0.1	6.5 ± 0.3**	9.3 ± 0.4**	26.3 ± 1.3**[c]	24.9 ± 2.2**[d]	26.9 ± 3.4**[d]
Peroxisomal β-oxidation (Lazarow method) (nmol NADH/minute per mg)						
Day 6	2.9 ± 0.2[e]	7.6 ± 0.8**[d]	10.4 ± 0.3**	18.0 ± 1.1**[d]	19.6 ± 0.6**[d]	23.4 ± 1.1**
Day 34	3.7 ± 0.3[f]	9.7 ± 0.5**[b]	16.1 ± 1.5**[d]	46.2 ± 2.1**	53.8 ± 2.6**	63.3 ± 2.4**
Week 13	3.2 ± 0.2	11.2 ± 0.4**	18.4 ± 1.9**	44.2 ± 0.7**	48.9 ± 2.0**	63.0 ± 2.9**
Carnitine acetyltransferase (nmol reduced CoA/minute per mg)						
Day 6	7.8 ± 0.2[b]	24.3 ± 1.0**	40.7 ± 2.5**	61.6 ± 3.3**[f]	66.9 ± 2.6**[b]	74.0 ± 2.8**[b]
Day 34	7.5 ± 0.3	52.7 ± 3.7**[d]	92.0 ± 3.0**[b]	152.6 ± 9.4**[d]	169.6 ± 10.2**[f]	194.8 ± 8.7**[g]
Week 13	6.5 ± 0.2[b]	61.1 ± 3.2**[f]	89.2 ± 4.4**	140.6 ± 7.2**	164.0 ± 6.8**[b]	155.0 ± 10.9**[f]
Catalase (nmol NADPH/minute per mg)						
Day 6	269 ± 11	257 ± 11	255 ± 7	266 ± 8	264 ± 7	330 ± 35
Day 34	284 ± 8	258 ± 5	270 ± 19	302 ± 6	304 ± 8	245 ± 6*
Week 13	302 ± 6	282 ± 3*	314 ± 10	302 ± 9	269 ± 4**	234 ± 10**[g]

* Significantly different (P≤0.05) from the control group by Dunn's or Shirley's test
** Significantly different (P≤0.01) from the control group by Shirley's test
[a] Data are presented as mean ± standard error of duplicate tissue extractions of each liver sample. CoA= coenzyme A; DCF=dichlorofluorescein diacetate; NADH=nicotinic acid adenine dinucleotide; NADPH=nicotinic acid adenine dinucleotide phosphate
[b] n=9 [c] n=5 [d] n=7 [e] n=11 [f] n=8 [g] n=6

APPENDIX H
GENETIC TOXICOLOGY

TABLE H1 Frequency of Micronuclei in Normochromatic Peripheral Blood Erythrocytes of Tg.AC Mice Following Administration of Wy-14,643 in Feed for 6 Months H-2
TABLE H2 Frequency of Micronuclei in Normochromatic Peripheral Blood Erythrocytes of Tg.AC Mice Following Dermal Application of Wy-14,643 for 6 Months H-3

TABLE H1
Frequency of Micronuclei in Normochromatic Peripheral Blood Erythrocytes of Tg.AC Mice Following Administration of Wy-14,643 in Feed for 6 Months[a]

Concentration (ppm)	Number of Mice with Erythrocytes Scored	Micronucleated NCEs/ 1,000 NCEs[b]	P Value[c]	% PCEs
Male				
0	11	2.18 ± 0.35		2.6
10	14	2.00 ± 0.30	0.6229	2.6
50	12	3.17 ± 0.49	0.0751	2.7
100	14	2.64 ± 0.44	0.2316	3.1
		P=0.111[d]		
Female				
0	11	2.18 ± 0.40		2.8
10	11	2.27 ± 0.24	0.4431	2.6
50	12	2.33 ± 0.41	0.4045	2.4
100	12	2.33 ± 0.43	0.4045	2.7
		P=0.412		

[a] Study was performed at ILS, Inc. The detailed protocol is presented by MacGregor *et al*. (1990). NCE=normochromatic erythrocyte; PCEs=polychromatic erythrocyte
[b] Mean ± standard error
[c] Pairwise comparison with the controls, significant at P≤0.008 (ILS, 1990)
[d] Significance of micronucleated NCEs/1,000 NCEs tested by the one-tailed trend test, significant at P≤0.025 (ILS, 1990)

TABLE H2
Frequency of Micronuclei in Normochromatic Peripheral Blood Erythrocytes of Tg.AC Mice Following Dermal Administration of Wy-14,643 for 6 Months[a]

Concentration (ppm)	Number of Mice with Erythrocytes Scored	Micronucleated NCEs/ 1,000 NCEs[b]	P Value[c]	% PCEs
Male				
0	10	3.20 ± 0.55		3.7
2	10	3.30 ± 0.45	0.4506	3.6
10	10	3.90 ± 0.38	0.2026	3.1
20	10	4.60 ± 0.62	0.0561	3.1
		P=0.034[d]		
Female				
0	10	2.00 ± 0.37		4.0
2	10	3.10 ± 0.50	0.0615	3.6
10	10	2.90 ± 0.55	0.0990	3.9
20	9	3.20 ± 0.44	0.0478	3.2
		P=0.124		

[a] Study was performed at ILS, Inc. The detailed protocol is presented by MacGregor *et al*. (1990). NCE=normochromatic erythrocyte, PCE=polychromatic erythrocyte
[b] Mean ± standard error
[c] Pairwise comparison with the controls, significant at P≤0.008 (ILS, 1990)
[d] Significance of micronucleated NCEs/1,000 NCEs tested by the one-tailed trend test, significant at P≤0.025 (ILS, 1990)

APPENDIX I
CHEMICAL CHARACTERIZATION AND DOSE FORMULATION STUDIES

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CHEMICAL CHARACTERIZATION AND DOSE FORMULATION STUDIES

Procurement and Characterization of Wy-14,643

Wy-14,643 was obtained from Chemsyn Science Laboratories (Lenexa, KS) in two lots (91-314-72-07 and 91-314-100-33A), which were used throughout the 2-week and 3-month studies. Lots 91-314-72-07 and 91-314-100-33A were combined by the study laboratory, Battelle Columbus Laboratories (Columbus, OH), and assigned a new lot number (C041194). Identity and purity analyses were conducted by the analytical chemistry laboratory, Radian Corporation (Austin, TX), and the study laboratory. Reports on analyses performed in support of the Wy-14,643 studies are on file at the National Institute of Environmental Health Sciences.

The chemical, a white powder, was identified as Wy-14,643 by the analytical chemistry laboratory using infrared and proton nuclear magnetic resonance spectroscopy (lot 91-314-72-07) and by the study laboratory using infrared spectroscopy (lot 91-314-100-33A). All spectra were consistent with the structure of Wy-14,643. The infrared and nuclear magnetic resonance spectra are presented in Figures I1 and I2.

The purity of lot 91-314-72-07 was determined by the analytical chemistry laboratory using high-performance liquid chromatography (HPLC) by system A (Table I1). HPLC indicated a major peak and no impurities. The overall purity of lot 91-314-72-07 was determined to be greater than 99%.

For lot 91-314-100-33A, the manufacturer indicated a purity of 98% or greater using thermal analysis and HPLC by system B. The study laboratory confirmed the purity of lot C041194 using HPLC by system C. HPLC indicated a major peak and two impurities with areas greater than 0.1% relative to the major peak area; smaller impurity peaks were also observed. The overall purity of lot C041194 was determined to be 98% or greater.

The manufacturer recommended storage under an inert atmosphere at 5° C, protected from light. The bulk chemical was stored at room temperature, protected from light, in amber glass bottles with Teflon®-lined caps. Periodic analyses of the bulk chemical were performed during the studies with HPLC by system C; no degradation was detected.

Preparation and Analysis of Dose Formulations

The dose formulations were prepared once (2-week studies) or approximately every 4 weeks (3-month studies) by mixing Wy-14,643 with feed (Table I2). A premix was prepared by hand; the premixes for the 10, 50, and 100 ppm formulations in the 2-week studies and all dose formulations in the 3-month studies were ground in a mill with a 1-mm screen. The premix was blended with additional feed in a Patterson-Kelly® twin-shell blender for 15 minutes using an intensifier bar for the first 5 minutes (2-week studies) or for the entire mixing period (3-month studies). Formulations were stored in plastic buckets at approximately 5° C, protected from light, for up to 21 days.

Homogeneity and stability studies of 10, 50, and 500 ppm dose formulations and stability studies of a 5 ppm dose formulation were performed by the analytical chemistry laboratory using HPLC by systems A and D. Homogeneity studies of 10 and 10,000 ppm dose formulations for the 2-week studies and the 5 and 500 ppm dose formulations for the 3-month studies were performed by the study laboratory with HPLC by system C. Homogeneity was confirmed, and stability of dose formulations stored in glass vials with Teflon®-lined caps was confirmed for at least 23 days at –20° C and for 35 days at 4° ± 2° C or at room temperature; dose formulations stored open to air and light were stable for 7 days.

Periodic analyses of the dose formulations were conducted by the study laboratory using HPLC by system C. For the 2-week studies, the dose formulations were analyzed once; all dose formulations for mice and hamsters were within 10% of the target concentrations (Table I3). Animal room samples of these dose formulations were also analyzed; all animal room samples for mice and three of five for hamsters were within 10% of the target concentrations. For the 3-month studies, the dose formulations were analyzed at the beginning, midpoint, and end of the studies; animal room samples of these dose formulations were also analyzed (Table I4). Of the dose formulations analyzed, 19 of 20 were within 10% of the target concentrations; the single dose formulation that was outside the 10% criterion was considered suitable for use in the studies. All animal room samples were within 10% of the target concentrations.

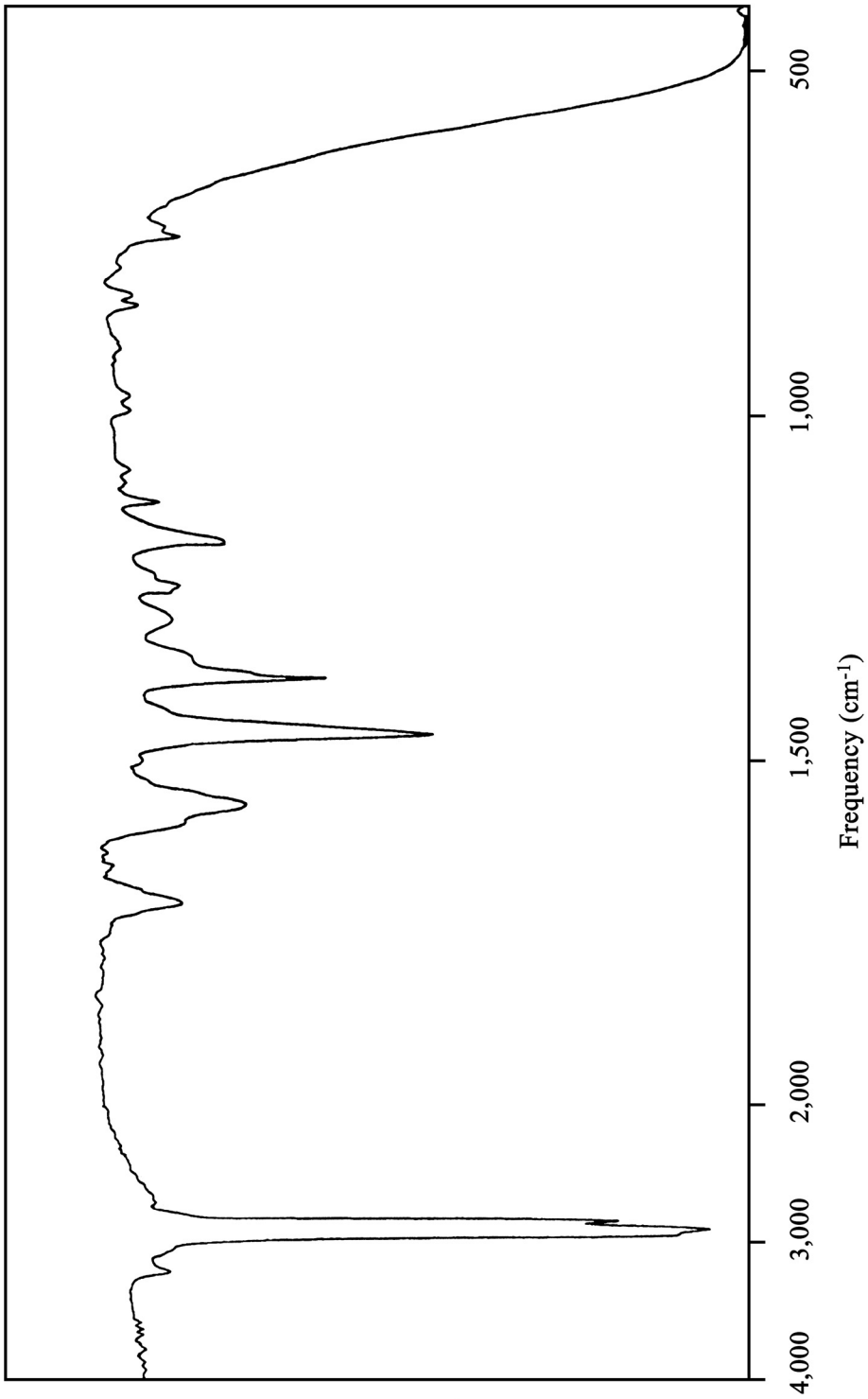

Figure I1
Infrared Absorption Spectrum of Wy-14,643

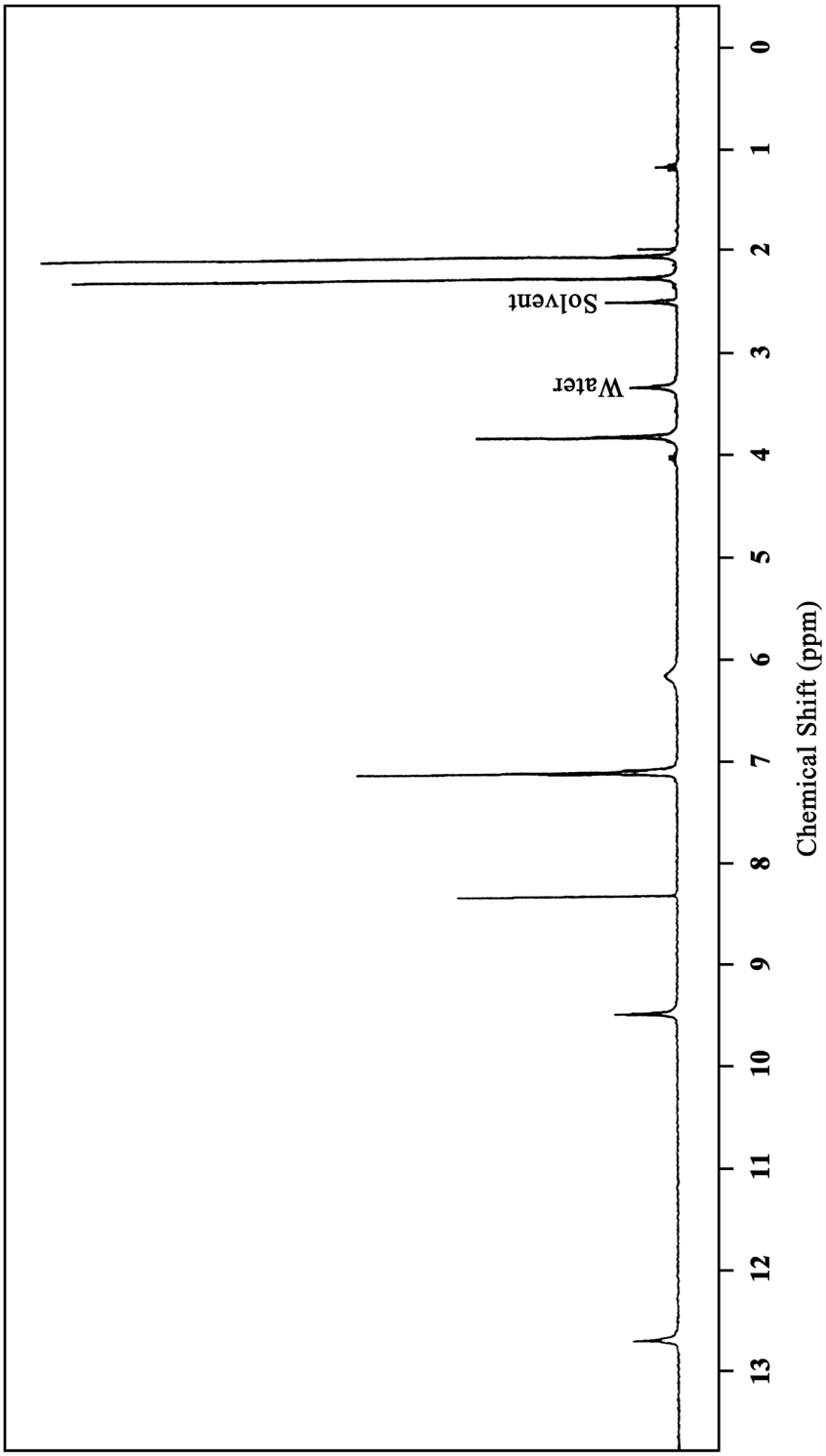

Figure I2
Proton Nuclear Magnetic Resonance Spectrum of Wy-14,643

Table I1
High-Performance Liquid Chromatography Systems Used in the Feed Studies of Wy-14,643

Detection System	Column	Solvent System
System A		
Ultraviolet (254 nm)	Partisil 5 ODS-3, 100 mm × 4.6 mm, 5-µm particle size (Whatman, Inc., Clifton, NJ)	A) Methanol and B) 0.0075 M heptane sulfonic acid buffer (60% A:40% B); flow rate 1.0 mL/minute
System B		
Ultraviolet (254 nm)	Phenomenex Partisil 5 ODS-3, 250 mm × 4.6 mm (Phenomenex, Torrance, CA)	A) Methanol and B) 0.0075 M heptane sulfonic acid buffer (pH 3.4) (60% A:40% B); flow rate 1.0 mL/minute
System C		
Ultraviolet (254 nm)	Metachem Inertsil ODS-2, 150 mm × 4.6 mm, 5-µm particle size (Metachem Technologies, Inc., Lake Forest, CA)	A) Methanol and B) 0.0075 M heptane sulfonic acid buffer (pH 3.4); 68% A:32% B, isocratic, or 85% A:15% B, isocratic; flow rate 1.0 mL/minute
System D		
Ultraviolet (254 nm)	Partisil ODS-3, 150 mm × 3.9 mm, 5-µm particle size (Whatman, Inc.)	A) Methanol and B) 0.0075 M heptane sulfonic acid buffer (pH 3.4); 40% A:60% B for 20 minutes, then 60% A:40% B; flow rate 1.5 mL/minute

[a] High-performance liquid chromatographs were manufactured by Hewlett-Packard (Palo Alto, CA) (system A), Varian, Inc. (Walnut Creek, CA) (systems B and D), and Spectra Physics (San Jose, CA) (system C).

TABLE I2
Preparation and Storage of Dose Formulations in the Feed Studies of Wy-14,643

2-Week Studies	3-Month Studies
Preparation	
A premix of feed and Wy-14,643 was prepared by hand; the premixes for the 10, 50, and 500 ppm dose formulations were then ground in a mill with a 1-mm screen (equivalent to an 18-mesh sieve). The premix was layered with the remaining feed in a twin-shell blender and blended with the intensifier bar on for the first 5 minutes and off for 10 minutes. Dose formulations were prepared once.	Same as for 2-week studies, except premixes for all dose formulations were ground, and intensifier bar was on for the entire mixing period. Dose formulations were prepared approximately every 4 weeks.
Chemical Lot Number	
C041194	C041194
Maximum Storage Time	
21 days	21 days
Storage Conditions	
Stored in plastic buckets, protected from light, at approximately 5° C	Same as 2-week studies
Study Laboratory	
Battelle Columbus Laboratories (Columbus, OH)	Battelle Columbus Laboratories (Columbus, OH)

TABLE I3
Results of Analyses of Dose Formulations Administered to Mice and Hamsters in the 2-Week Feed Studies of Wy-14,643

Date Prepared	Date Analyzed	Target Concentration (ppm)	Determined Concentration[a] (ppm)	Difference from Target (%)
Mice				
May 11, 1994	May 12-13, 1994	10	9.31	–7
		50	46.0	–8
		100	94.3	–6
		500	537	+7
		1,000	1,097	+10
	June 7-8, 1994[b]	10	9.55	–4
		50	47.1	–6
		100	103	+3
		500	541	+8
		1,000	1,078	+8
Hamsters				
May 11, 1994	May 12-13, 1994	10	9.31	–7
		100	94.3	–6
		500	548	+10
		1,000	1,097	+10
		5,000	5,190	+4
	June 7-8, 1994[b]	10	9.78	–2
		100	105	+5
		500	566	+13
		1,000	1,093	+9
		5,000	6,310	+26

[a] Results of duplicate analyses
[b] Animal room samples

TABLE I4
Results of Analyses of Dose Formulations Administered to Rats, Mice, and Hamsters in the 3-Month Feed Studies of Wy-14,643

Date Prepared	Date Analyzed	Target Concentration (ppm)	Determined Concentration[a] (ppm)	Difference from Target (%)
November 17, 1994	November 21-22, 1994	5	5.29	+6
		10	9.69	–3
		50	50.8	+2
		100	107	+7
		500	505	+1
	December 12-13, 1994[b]	5	4.84	–3
		10	9.93	–1
		50	48.7	–3
		100	105	+5
		500	546	+9
January 3, 1995	January 31-February 1, 1995[c]	5	4.63	–7
		10	9.68	–3
		50	48.8	–2
		100	90.7	–9
		500	486	–3
January 17, 1995	January 19-24, 1995	5	5.11	+2
		10	9.53	–5
		50	45.9	–8
		100	99.0	–1
		500	495	–1
February 28, 1995	March 1-2, 1995	5	4.26	–15
		10	9.59	–4
		50	51.9	+4
		100	98.5	–1
		500	504	+1
	May 18-22, 1995[d]	5	4.82	–4
		10	9.55	–4
		50	48.0	–4
		100	90.4	–10
		500	499	0

[a] Results of duplicate analyses
[b] Animal room samples for rats
[c] Dose formulations prepared on January 3, 1995, were used 1 day past their expiration date; samples from feed storage containers were collected on last day of use and analyzed to confirm that use past the expiration date had no impact on the studies.
[d] Animal room samples for hamsters

APPENDIX J
TOXICOKINETIC STUDIES

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TOXICOKINETIC STUDIES

INTRODUCTION

Toxicokinetic studies were performed in male Sprague-Dawley and Wistar Furth rats, B6C3F$_1$ mice, and Syrian hamsters to obtain estimates of basic kinetic parameters and to establish a dose range over which plasma kinetics are linear following a single intravenous or gavage dose of Wy-14,643 and to determine internal doses after repeated administration of Wy-14,643 in the diet. Prestart studies with a single intravenous injection or gavage dose were conducted to establish doses and blood collection time points for the subsequent definitive feed and single-dose intravenous injection and gavage toxicokinetic studies. The studies were performed by Research Triangle Institute (Research Triangle Park, NC).

MATERIALS AND METHODS

Wy-14,643 (lot 91-314-72-07) was obtained from Chemsyn Science Laboratories (Lenexa, KS). Analyses of the bulk chemical are described in Appendix I. Intravenous and gavage dose formulations were prepared by Radian Corporation (Austin, TX) by mixing Wy-14,643 with an emulphor solution (80:10:10 deionized water:ethanol:emulphor) or with 0.5% methylcellulose in deionized water. The mixtures were sonicated and stirred with a magnetic stir bar (except prestart emulphor mixtures), and the methylcellulose mixtures were homogenized. Dosed feed formulations were prepared by the toxicokinetic study laboratory by mixing Wy-14,643 with feed. A premix was prepared by hand and then blended with additional feed in a Patterson-Kelly® twin-shell blender for 15 minutes using an intensifier bar for the initial 5 minutes. All intravenous, gavage, and feed dose formulations were analyzed and found to be within 10% of the target concentrations.

For the definitive studies, male Sprague-Dawley rats were obtained from Harlan Sprague-Dawley, Inc. Male Wistar Furth rats, B6C3F$_1$ mice, and Syrian hamsters were obtained from Frederick Cancer Research and Development Center. Animals were quarantined for 1 week (rats and mice) or at least 5 weeks (hamsters) and were approximately 11 weeks old when the studies began. Animals received certified NIH-07 pelleted or ground feed (Zeigler Brothers, Inc., Gardners, PA) and deionized, filtered drinking water *ad libitum*. Animals were individually housed in polycarbonate cages (Lab Products, Inc., Rochelle Park, NJ) with Ab-Sorb-Dri® cage litter (Lab Products, Inc., Garfield, NJ). Animals were monitored for morbidity and mortality. Body weights and feed consumption were recorded daily. Body weights were used to calculate dosing volumes. Groups of eight Wistar Furth rats and mice were administered 50 or 500 ppm and groups of eight hamsters were administered 100 or 1,000 ppm Wy-14,643 in feed for 9 or 10 days. Groups of 13 Sprague-Dawley rats and mice, and 12 hamsters were administered a single intravenous injection of 2 mg/kg (rats and mice) or 3 mg/kg (hamsters) at a dosing volume of 2 mL/kg for rats and hamsters or 4 mL/kg for mice. Rats and mice were injected in the lateral tail vein, and hamsters were injected in the cephalic vein. Groups of 12 Sprague-Dawley rats and mice, and 10 (1 and 3 mg/kg dose group) or 13 (10 mg/kg dose group) hamsters were administered a single gavage dose of 1, 2, or 5 mg/kg (rats); 2, 4, or 8 mg/kg (mice); or 1, 3, or 10 mg/kg (hamsters) at a dosing volume of 5 mL/kg for rats and hamsters or 10 mL/kg for mice. Sprague-Dawley rats were anesthetized by exposure to a mixture of carbon dioxide and oxygen, and blood was collected from alternate retroorbital sinuses at time points greater than 2 hours apart; the rats were then killed with carbon dioxide anesthesia. Wistar Furth rats, mice, and hamsters were killed by exposure to carbon dioxide, and blood samples were collected by cardiac puncture. Blood was collected from one animal (feed studies) or from three animals (intravenous injection and gavage studies) per time point. For the feed studies, blood was collected from Wistar Furth rats in one or both exposed groups at 1200, 1500, 1700, 1800, 2000, 0000, 0200 (high dose only), 0400, 0500, 0600, and 0700; from mice at 1200, 1400, 1500 (high dose only), 1600, 1700, 1800, 2100 (high dose only), 0000 (high dose only), 0100, 0200, 0300 (low dose only), 0700, and 0900; and from hamsters at 1400, 1600, 1800, 2000, 2200, 0000, 0200, 0600, 0800, 1000, and 1200 (high dose only). Blood was collected from Sprague-Dawley rats 2.5 (intravenous injection only), 5, 15, 30, 60, 120, 180 (not at 5 mg/kg), 240, 300 (not at 5 mg/kg), 360, 420 (not at 5 mg/kg), 480, and 600 minutes after intravenous or gavage dosing, with blood collected at the additional time points of 900, 1,200, and 1,440 minutes after gavage

dosing for the 5 mg/kg group. For mice, blood was collected at 5, 15, 30, 60, 90, 120, 240, 360 (intravenous and 2 mg/kg gavage), 480, 600, 900 (intravenous and 2 mg/kg gavage), 1,080 (4 and 8 mg/kg oral gavage), 1,200 (2 mg/kg intravenous and 2 mg/kg gavage), 1,260 (4 mg/kg gavage), 1,440 (intravenous, 4, and 8 mg/kg gavage), and 1,800 (8 mg/kg gavage) minutes after intravenous injection or gavage dosing. For hamsters, blood was collected at 2.5, 5, 10, 20, 30, 60, 120, 150, 180, 240, 300, and 360 minutes after intravenous injection and 7.5, 10, 20, 40, 60, 90, 120, 180, 240, and 360 minutes following a gavage dose of 1 mg/kg or 5, 7.5, 15, 30, 60, 120, 180, 240, 360, and 480 minutes after a gavage dose of 3 and 10 mg/kg. Blood was also collected at 600, 900, and 1,200 minutes following a gavage dose of 10 mg/kg. Blood samples from rats (feed study), mice, and hamsters were collected into heparinized glass syringes; blood from toxicokinetic study rats was drawn into heparinized hematocrit tubes. All blood samples were transferred to silylated, heparinized glass test tubes and chilled in an ice bath prior to centrifugation for 10 minutes; the plasma was transferred to silylated amber glass vials with Teflon®-lined caps and frozen at –20° C until analyses were performed.

Plasma samples were analyzed at CEDRA Corporation, Inc. (Austin, TX). Plasma samples (0.2 mL) were spiked with indomethacin, an internal standard, and then combined with 100 µL of 0.20 M hydrochloric acid in saturated brine and 2 mL of 20% isopropanol in cyclohexane. Samples were vortexed, and 1.5 mL of the upper organic phase was transferred and evaporated. The residue was reconstituted in 150 µL methanol and analyzed with a Waters Model 501 high-performance liquid chromatograph (Waters-Millipore, Milford, MA) with ultraviolet detection at 254 nm and a Zorbax® CN column (150 mm × 4.6 mm; Rockland Technologies, Inc., Newport, DE). The mobile phase was 700 mL water:300 mL acetonitrile:0.01 M potassium phosphate, with the pH adjusted to 3.0 with phosphoric acid; the flow rate was 1.7 mL/minute. The limit of detection (LOD) for this method was found to be 0.0259 µg/mL plasma.

Nondetectable data were treated as missing. Means, standard deviations, and weights were calculated using EXCEL (Microsoft Corporation, Redmond, WA). When a mean for a given timepoint was less than the LOD or estimated limit of quantitation (ELOQ), it was not used for modeling. For the intravenous studies, C_{max} was calculated by back extrapolation of a linear regression of the initial timepoints on the plasma concentration-time curve to time zero using EXCEL.

Toxicokinetic data were analyzed with noncompartmental modeling techniques (PCNONLIN Software Models 200 and 201, Version 4.2, SCI Software, Lexington, KY). The estimated parameters included maximum observed plasma concentration (C_{max}); time at which C_{max} was observed (t_{max}); terminal elimination half-life ($t_{½β}$); area under the plasma concentration × time curve extrapolated to infinity (AUC_{∞}), until 24 hours ($AUC_{24\ hours}$), or until the last time point (AUC_{last}); clearance (Cl for intravenous dosing; $Cl_{apparent}$ for gavage dosing), calculated as Dose/AUC; area under the first moment of the plasma concentration × time curve (AUMC), calculated as AUC × time; mean residence time (MRT), calculated as AUMC/AUC; and bioavailability (F) of a gavage dose, calculated as $(Dose_{intravenous} \times AUC_{\infty,gavage})/(Dose_{gavage} \times AUC_{\infty,intravenous})$.

RESULTS

Pilot Comparative Studies

The plasma profiles for male Wistar Furth and Sprague-Dawley rats following a 2.14 mg/kg gavage dose are shown in Table J1 and Figure J1.

Definitive Studies

9- or 10-Day Multiple Exposure Feed Studies

Observed mean plasma concentration versus time data are shown in Table J2. Plasma concentrations of Wy-14,643 varied with the diurnal variation in feed consumption for rats and mice. Mean plasma concentrations (C_p) at the

low exposure concentration were 0.465 ± 0.206, 0.457 ± 0.373, and 0.212 ± 0.126 µg/mL for rats, mice, and hamsters, respectively.

Average daily doses of Wy-14,643 (mg/kg per day) were calculated based on daily body weights and feed consumption (Table J3). Average daily doses ranged from 3.17 ± 0.17 to 4.17 ± 0.40 mg/kg for low exposure concentration rats and 24.12 ± 1.94 to 33.40 ± 2.76 mg/kg for high exposure concentration rats. At both exposure concentrations in the rat study, the most feed consumption occurred on the first day of exposure and tended to decrease during the study. Average daily doses for low exposure concentration mice ranged from 6.72 ± 1.37 to 8.98 ± 0.87 mg/kg. For high exposure concentration mice, the average daily dose range was 47.32 ± 12.47 to 69.83 ± 6.80 mg/kg. At both exposure concentrations in the mouse study, the lowest average daily dose occurred on the first day of exposure and may have indicated a slight problem with palatability which corrected itself by day 3 of the study. Hamsters consumed an average daily dose of 5.00 ± 0.87 to 9.25 ± 6.36 mg/kg at the low exposure concentration and 35.71 ± 10.01 to 71.63 ± 40.81 mg/kg at the high exposure concentration. The least feed consumption occurred on day 6 followed by increases for the remainder of the study, although daily doses at the end of the study tended to be lower than those at the beginning of the study. Mean daily doses over the course of each study were 3.63 and 28.77 mg/kg per day for rats, 8.15 and 63.39 mg/kg for mice, and 6.11 and 51.30 mg/kg for hamsters at the low and high exposure concentrations, respectively.

Single Dose Toxicokinetic Studies

Mean plasma concentration versus time data for intravenous and gavage doses of Wy-14,643 in Sprague-Dawley rats are presented in Table J4. Wy-14,643 was rapidly absorbed and then eliminated from plasma by approximately 10 hours after dosing. In general, the elimination phase of each gavage dose paralleled that of the intravenous dose, although the gavage doses showed some variability with respect to plasma Wy-14,643 concentrations at the later time points. Elimination half-lives were 48.9 minutes for the intravenous dose and 155, 99.2, and 129 minutes for the low, mid, and high gavage doses, respectively (Table J5). There was a suggestion of a plateau in plasma concentrations between 60 and 360 minutes following gavage dosing that was not present in the intravenous time course which may have resulted in the longer elimination times for the gavage doses. Cl was 2.89 mL/minute per kg after an intravenous dose. AUC, F, and Cl_{app} values indicated that the kinetics of Wy-14,643 after gavage administration were dose proportional. With gavage administration, AUC increased with dose, while F and Cl_{app} remained relatively constant, although the Cl_{app} values (5.48 to 8.48 mL/minute per kg) were much higher than with the intravenous dose.

Mean plasma concentration versus time data for intravenous and oral gavage doses of Wy-14,643 in mice are presented in Table J6. It can be seen from the time course that, in general, Wy-14,643 was rapidly absorbed and then eliminated from plasma by approximately 10 hours after dosing. Elimination kinetics after the low intravenous and gavage doses were complex; after an initial decline to 6 hours, plasma concentrations increased again between 6 and 8 hours and then decreased to below the ELOQ by 11 hours. This pattern was absent from the higher gavage doses. At the higher doses, Wy-14,643 plasma concentrations dropped to the ELOQ by 8 hours and became flat at or below the ELOQ out to 20 hours. After an intravenous dose, Wy-14,643 disappeared from plasma with an elimination half-life of 243 minutes (Table J5). Elimination half-lives for the low, mid, and high gavage doses were 64.6, 61.6, and 67.0 minutes, respectively. Clearance was 1.48 and 1.51 mL/minute per kg after the intravenous and low gavage doses, respectively. Cl_{app} values at the two higher gavage doses were double that of the low dose with values of 3.30 and 3.37 mL/minute per kg, respectively. Post-gavage AUC values for the 4 and 8 mg/kg doses were proportional to each other but not to the 2 mg/kg dose. This trend was reflected in the bioavailability values, which were 0.98 for the 2 mg/kg gavage dose and 0.45 and 0.44 for the 4 and 8 mg/kg gavage doses, respectively.

Mean plasma concentration versus time data for intravenous and gavage doses of Wy-14,643 in hamsters are presented in Table J7. Wy-14,643 was rapidly absorbed and then eliminated from plasma by about 6 hours after an intravenous or gavage dose. In general, the elimination profiles for the gavage doses paralleled each other and

were similar to the intravenous time course. Wy-14,643 disappeared from plasma with an elimination half-life of 108 minutes after an intravenous dose (Table J5). Elimination half-lives were similar for all gavage doses but were approximately one half that of the intravenous dose. There was a suggestion of a small plateau in plasma concentrations of Wy-14,643 from 30 to 60 minutes after dosing that may have resulted in the different elimination half-lives for the intravenous and gavage doses. Cl after an intravenous dose was 3.72 mL/minute per kg. AUC, F, and Cl_{app} values indicated that the kinetics of Wy-14,643 after gavage administration were dose proportional. With gavage administration, AUC increased with dose, while F and Cl_{app} remained relatively constant, although the Cl_{app} values (15.8 to 23.2 mL/minute per kg) were much higher than with the intravenous dose.

DISCUSSION

Very few studies of Wy-14,643 toxicokinetics are available in the published literature. Fahl *et al.* (1983) demonstrated that metabolites of Wy-14,643, but not the parent compound, are present in the milk of rat dams within 2 hours of a bolus oral dose. The studies reported here provide information for comparing pharmacokinetic parameters in Sprague-Dawley and Wistar Furth rats, $B6C3F_1$ mice, and Syrian hamsters. We also evaluated the ability of kinetic parameters after an oral bolus dose to predict concentrations in plasma following chronic administration in feed.

Based on the ratios of AUC for intravenous injection and gavage dosing in the definitive single-dose toxicokinetic studies, bioavailability of Wy-14,643 was 34% for rats, 98% for mice, and 24% for hamsters at a dose of 2 (rats and mice) or 3 (hamsters) mg/kg. Maximum plasma concentrations at these doses were 1.42 µg/mL for rats, 6.94 µg/mL for mice, and 2.88 µg/mL for hamsters. A comparison of plasma concentrations after single gavage doses with concentrations following 9 or 10 days of exposure in feed indicates that the dietary doses for all three species in the 3-month feed studies would lie in the linear kinetic range.

Following oral gavage in the definitive studies, AUC values were dose-linear in both strains of rats and in hamsters at all doses and in mice at doses above 4 mg/kg. Elimination half-life ($t_{½β}$) was not significantly different at any dose in rats, mice, or hamsters, which suggests that the nonlinear behavior observed in mice may be related to nonlinear absorption in that species. Mice showed a similar pattern for clearance values, with the low intravenous and gavage doses yielding similar clearance values and gavage doses of 4 mg/kg and above having Cl_{app} about two times greater. Within each rat strain, Cl_{app} values following oral gavage were similar at all doses, which is consistent with the linear dose response suggested by the AUC values. Cl_{app} in Wistar Furth rats, however, was slightly lower than that in Sprague-Dawley rats. In Sprague-Dawley rats, Cl_{app} for gavage doses was two to three times greater than that of an intravenous dose, suggesting a significant first-pass effect; Wistar Furth rats did not show this difference between the routes of administration. Clearance values in hamsters following oral gavage of Wy-14,643 were similar at all doses, were three to ten times greater than in rats and mice, and were much higher than after intravenous dosing even at similar doses, which suggests a pronounced first-pass effect. Bioavailability of gavage doses of Wy-14,643 for each species reflected the trend in the AUC values. Bioavailability was similar at all doses in each strain of rats, however, it was higher for Wistar Furth rats than for Sprague-Dawley rats (0.70 versus 0.44). For mice, bioavailability was high at the lowest dose but dropped by 50% at the two higher doses, possibly reflecting reduced absorption at the higher doses. Hamsters had the lowest bioavailability of the three species, averaging 0.20 for all doses tested. The low bioavailability in hamsters may be related to the significant first-pass effect observed in that species. C_{max} values, normalized to dose, were relatively constant at all oral gavage doses tested in the three species, but they were highest in mice and lowest in hamsters. Enterohepatic recirculation was not evident in the concentration-time profiles for any dose in either rat strain or in the hamsters. In mice, however, an increase in plasma concentrations after 6 hours followed by a broad plateau between 8 and 12 hours may indicate that enterohepatic recirculation may be occurring with the low intravenous and gavage doses. However, the absence of the increase in plasma concentrations at late times following the other gavage doses in mice may argue against this conclusion.

Measurements of AUC and C_p for the 9- or 10-day multiple exposure feed studies indicated that the kinetics following feeding were approximately exposure concentration-linear for each species. Values for C_p were similar between rats and mice but were 50% lower in hamsters at both exposure concentrations, even though the average daily dosage for hamsters was almost double that of rats. Values for $AUC_{24\ hours}$ were also 50% lower in hamsters than those found in rats or mice. The differences in $AUC_{24\ hours}$ and C_p values could not be explained by differences in feed consumption or average daily dose between rats, mice, and hamsters but may be related to the high Cl_{app} observed in hamsters when compared to rats and mice following an oral dose.

REFERENCE

Fahl, W.E., Lalwani, N.D., Reddy, M.K., and Reddy, J.K. (1983). Induction of peroxisomal enzymes in livers of neonatal rats exposed to lactating mothers treated with hypolipidaemic drugs. Role of drug metabolite transfer in milk. *Biochem. J.* **210**, 875-883.

TABLE J1
Plasma Concentrations of Wy-14,643 in Male Wistar Furth and Sprague-Dawley Rats after a Single Intravenous Injection or Gavage Dose of Wy-14,643: Comparative Studies[a]

Time after Dosing (minutes)	Wistar Furth Rats			Sprague-Dawley Rats
	2.14 mg/kg Intravenous	2.14 mg/kg Gavage	21.4 mg/kg Gavage	2.14 mg/kg Gavage
5	23.8	0.321	3.26	0.100[b]
10		1.80	14.8	0.514
15	14.4			1.96
20		7.16	51.5	
30	5.20	7.08	74.9	1.35
40		4.63	44.0	
60	2.73	3.70	31.4	1.43
90		1.36	20.3	
120	1.12	1.54	12.4	1.65
180	0.334			
240		0.290	2.92	0.847
360	ND			0.762
480		0.112[b]	1.09	
600		ND	0.520	0.439
720	ND			
900				0.0904[b]
1,080	ND	ND	ND	0.0983[b]
1,440	ND	ND	ND	ND
1,800	ND			

[a] Plasma concentrations are presented as µg/mL. ND=not detectable; limit of detection estimated to be 0.0259 µg/mL
[b] Less than the experimental limit of quantitation (0.2 µg/mL) but greater than the limit of detection (0.0259 µg/mL)

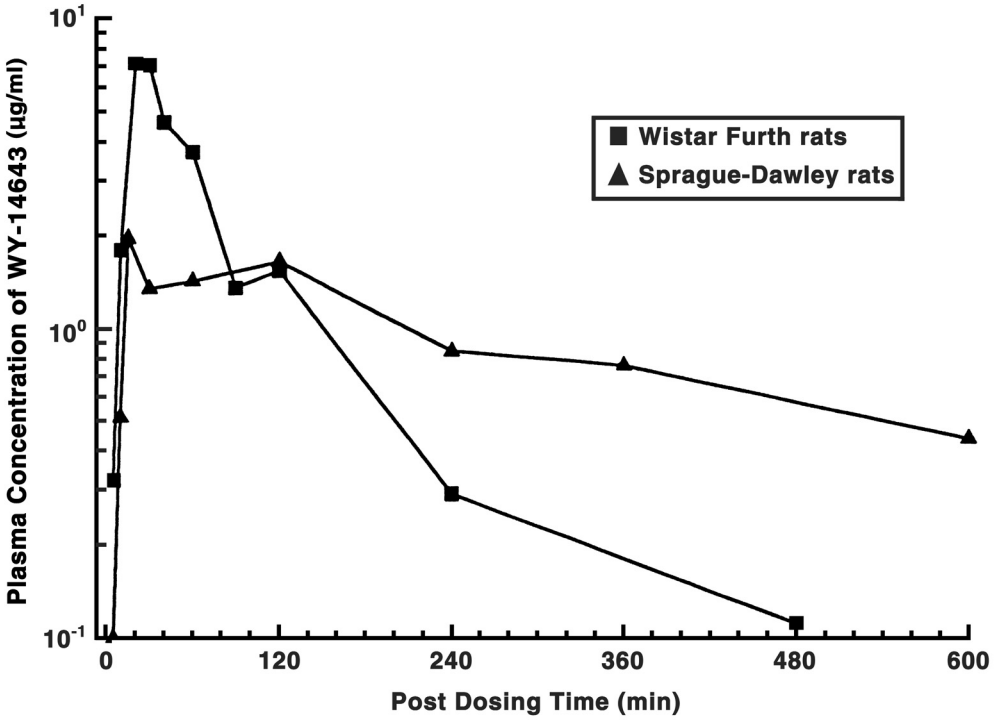

FIGURE J1
Plasma Concentrations of Wy-14,643 in Male Wistar Furth and Sprague-Dawley Rats after a Single Gavage Dose of 2.14 mg/kg Wy-14,643: Comparative Studies

TABLE J2
Plasma Concentrations of Wy-14,643 in Male Wistar Furth Rats, B6C3F$_1$ Mice, and Syrian Hamsters Following Exposure to Wy-14,643 in Feed for 9 or 10 Days[a]

Time of Collection	Wistar Furth Rats		B6C3F$_1$ Mice		Syrian Hamsters	
	50 ppm	500 ppm	50 ppm	500 ppm	100 ppm	1,000 ppm
1200	0.154	2.38	0.259	3.72		
1400			0.0879	4.02	0.111	1.61
1500	0.336	5.54		4.78		
1600			0.329	1.57	0.0714	1.69
1700	0.201	3.70	0.151	6.74		
1800	0.460	3.31	1.26		0.0950	0.882
2000	0.465	5.15			0.210	0.534
2100				4.76		
2200					0.151	1.40
0000	0.715	6.45		2.24	ND	3.60
0100			0.828	7.38		
0200		9.04	0.697	8.42	0.340	1.46
0300			0.474			
0400	0.356	7.05				
0500	0.557	5.55				
0600	0.671	6.10			0.191	3.83
0700	0.734	5.17	0.112	3.16		
0800					0.293	2.70
0900			0.368	5.71		
1000					0.447	4.04
1200						3.87

[a] Plasma concentrations are presented in µg/mL. ND=not detectable; limit of detection estimated to be 0.0579 µg/mL for hamsters

TABLE J3
Average Daily Doses of Wy-14,643 in Wistar Furth Rats, B6C3F$_1$ Mice, and Syrian Hamsters in the 9- or 10-Day Multiple Exposure Feed Studies of Wy-14,643[a]

Day of Study	Wistar Furth Rats		B6C3F$_1$ Mice		Syrian Hamsters	
	50 ppm[b]	500 ppm[c]	50 ppm[b]	500 ppm[c]	100 ppm[b]	1,000 ppm[c]
Days 1-2	4.17 ± 0.40	33.40 ± 2.76	6.72 ± 1.37	47.32 ± 12.47	9.25 ± 6.36	54.46 ± 28.84
Days 2-3	4.04 ± 0.24	32.74 ± 2.27	7.97 ± 1.46	66.49 ± 4.71	8.12 ± 8.30	53.60 ± 26.22
Days 3-4	4.01 ± 0.24	31.03 ± 2.46	7.86 ± 0.66	66.84 ± 4.03	5.78 ± 2.77	71.63 ± 40.81
Days 4-5	3.59 ± 0.32	28.04 ± 3.12	7.79 ± 1.66	53.79 ± 7.10	7.18 ± 4.25	59.36 ± 25.45
Days 5-6	3.70 ± 0.27	30.34 ± 2.39	8.94 ± 0.88	69.83 ± 6.80	5.41 ± 1.76	51.17 ± 18.83
Days 6-7	3.45 ± 0.31	27.19 ± 2.75	8.98 ± 0.87	67.56 ± 4.75	5.00 ± 0.87	35.71 ± 10.01
Days 7-8	3.46 ± 0.22	27.96 ± 2.95	8.47 ± 0.63	62.92 ± 8.79	5.61 ± 1.69	45.97 ± 24.13
Days 8-9	3.17 ± 0.17	24.12 ± 1.94	7.06 ± 1.68	56.33 ± 7.32	5.68 ± 1.39	41.66 ± 15.85
Days 9-end	2.06 ± 1.92	14.79 ± 7.57	3.90 ± 3.86	30.70 ± 22.21	2.50 ± 1.67	22.40 ± 19.75
Mean[d]	3.63	28.77	8.15	63.39	6.11	51.30

[a] Milligrams of Wy-14,643 consumed per kilogram body weight per day (mean ± standard deviation)
[b] n=10
[c] n=11
[d] Overall group mean dose excluding days 1-2 and 9-end

TABLE J4
Plasma Concentrations of Wy-14,643 in Male Sprague-Dawley Rats after a Single Intravenous Injection or Gavage Dose of Wy-14,643[a]

Time after Dosing (minutes)	2 mg/kg Intravenous	1 mg/kg Gavage	2 mg/kg Gavage	5 mg/kg Gavage
2.5	29.4 ± 1.1			
5	14.0 ± 10[b]	0.054 ± 0.019	0.359 ± 0.077	0.81 ± 0.45
15	11.9 ± 4.8	0.406 ± 0.070	0.914 ± 0.026	5.1 ± 4.7
30	5.09 ± 0.12	0.96 ± 0.41	1.42 ± 0.23	3.01 ± 0.95
60	2.41 ± 0.24	0.71 ± 0.15	0.81 ± 0.26	4.75 ± 0.68
120	0.74 ± 0.11	0.45 ± 0.23	0.68 ± 0.23	2.43 ± 0.88
180	0.311 ± 0.050	0.13 ± 0.07	0.59 ± 0.33	
240	0.24 ± 0.22	0.261 ± 0.066[c]	0.230 ± 0.061	1.07 ± 0.12
300	0.048 ± 0.013[c]	0.19 ± 0.14	0.45 ± 0.12	
360	0.0265[d]	0.133 ± 0.098[c]	0.162 ± 0.041	0.75 ± 0.25
420	ND	0.09 ± 0.02	0.102 ± 0.062[c]	
480	ND	0.110 ± 0.074	0.14 ± 0.15	0.28 ± 0.13
600	ND	0.0471[d]	0.0400[d]	0.087 ± 0.055
900				0.0619[d]
1,200				ND
1,440				0.129[d]

[a] Plasma concentrations are presented as mean ± standard deviation (µg/mL) for three animals per time point. ND=not detectable in any of the three samples; limit of detection estimated to be 0.0259 µg/mL
[b] n=2
[c] One of three samples was below the limit of detection.
[d] Two of three samples were below the limit of detection.

TABLE J5
Noncompartmental Analyses of Wy-14,643 Plasma Concentration-versus-Time Profiles for Male Sprague-Dawley and Wistar Furth Rats, B6C3F$_1$ Mice, and Syrian Hamsters after a Single Intravenous Injection, Gavage, or Feed Dose of Wy-14,643[a]

Route Dose	C_{max} (µg/mL)	t_{max} (min)[b]	$t_{½β}$ (min)	$AUC_∞$ (µg/mL×min)	Clearance (mL/min/kg)	F	$MRT_∞$ (min)
Sprague-Dawley Rats							
Intravenous injection (mg/kg)							
2	61.6	—[c]	48.9	693	2.89	—	38.9
Gavage (mg/kg)							
1	0.961	30	155	157	6.35	0.45	221[d]
2	1.42	30	99.2	236	8.48	0.34	185
5	5.13	15	129	912	5.48	0.53	246
Wistar Furth Rats							
Feed (ppm)							
50	0.734	7:00	NA	661	NA	NA	NA
500	9.04	2:00	NA	7,470	NA	NA	NA
B6C3F$_1$ Mice							
Intravenous injection (mg/kg)							
2	15.7	—	243	1,353[d]	1.48	—	242[d]
Gavage (mg/kg)							
2	6.94	30	64.6	1,325	1.51	0.98	415
4	14.5	15	61.6	1,211	3.30	0.45	83.9
8	20.5	15	67.0	2,376	3.37	0.44	100
Feed (ppm)							
50	1.26	18:00	NA	777	NA	NA	NA
500	8.42	2:00	NA	7,060	NA	NA	NA
Syrian Hamsters							
Intravenous injection (mg/kg)							
3	62.4	—	108	806	3.72	—	38.1[d]
Gavage (mg/kg)							
1	0.485	10	51.7	50[d]	20.1	0.19	97.1[d]
3	2.88	15	78.6	190	15.8	0.24	102[d]
10	6.04	15	51.1	431	23.2	0.16	100
Feed (ppm)							
100	0.447	10:00	NA	301	NA	NA	NA
1,000	4.04	10:00	NA	3,390	NA	NA	NA

[a] C_{max}=maximum mean plasma concentration; t_{max}=time of maximum mean plasma concentration; $t_{½β}$=terminal elimination half-life; AUC=area under the plasma concentration × time curve; F=bioavailability; MRT=mean residence time; NA=not available
[b] For feed studies, t_{max} is reported as 24-hour clock time.
[c] Not applicable
[d] Estimate$_{(0-T)}$/estimate$_∞$ is less than 0.90.

TABLE J6
Plasma Concentrations of Wy-14,643 in Male B6C3F$_1$ Mice after a Single Intravenous Injection or Gavage Dose of Wy-14,643[a]

Time after Dosing (minutes)	2 mg/kg Intravenous	2 mg/kg Gavage	4 mg/kg Gavage	8 mg/kg Gavage
5	15.2 ± 0.6	2.31 ± 0.28	8.05 ± 4.59	13.50 ± 5.12
15	14.2 ± 1.9	4.18 ± 0.46	14.5 ± 2.7	20.5 ± 4.4
30	8.08 ± 0.91	6.94 ± 0.98	12.6 ± 2.3	19.3 ± 5.4
60	4.72 ± 0.93	4.19 ± 0.78	4.80 ± 0.84	13.1 ± 1.7
90	3.17 ± 0.54	2.51 ± 0.44	5.27 ± 1.19	7.15 ± 0.24
120	2.61 ± 0.56	1.22 ± 0.44	3.15 ± 0.40	7.59 ± 0.85
240	0.278 ± 0.022	1.47 ± 1.02	0.747 ± 0.323	2.05 ± 0.80
360	0.26 ± 0.31	0.0955 ± 0.0150		
480	0.99 ± 0.60	0.325 ± 0.004[b]	0.0609 ± 0.0156	0.181 ± 0.067
600	0.55 ± 0.52	1.19 ± 0.61	0.0570[c]	0.053 ± 0.027[b]
900	0.305[c]	1.03 ± 0.08[b]		
1,080			0.0273[c]	0.088 ± 0.059[b]
1,200	0.86 ± 0.87[b]	0.0806[c]		
1,260			0.043 ± 0.014[b]	
1,440	0.734[c]		ND	ND
1,800				0.107[c]

[a] Plasma concentrations are presented as mean ± standard deviation (µg/mL) for three animals per time point. ND=not detectable in any of the three samples; limit of detection estimated to be 0.0259 µg/mL
[b] One of three samples was below the limit of detection.
[c] Two of three samples were below the limit of detection.

TABLE J7
Plasma Concentrations of Wy-14,643 in Male Syrian Hamsters after a Single Intravenous Injection or Gavage Dose of Wy-14,643[a]

Time after Dosing (minutes)	3 mg/kg Intravenous	1 mg/kg Gavage	3 mg/kg Gavage	10 mg/kg Gavage
2.5	46.6 ± 4.7			
5	32.9 ± 3.5		0.57 ± 0.29	2.41 ± 1.66
7.5		0.41 ± 0.22	0.95 ± 0.54	4.49 ± 3.58
10	18.4 ± 0.4	0.49 ± 0.23		
15			2.88 ± 0.87	6.04 ± 2.85
20	9.60 ± 0.46	0.429 ± 0.037		
30	4.99 ± 0.66		1.17 ± 0.61	3.14 ± 1.01
40		0.26 ± 0.10		
60	1.89 ± 0.29	0.213 ± 0.050	1.24 ± 0.13	2.24 ± 1.29
90		0.31 ± 0.12		
120	0.461 ± 0.085	0.193 ± 0.034	0.374 ± 0.020	0.716 ± 0.149
150	0.262 ± 0.056			
180	0.191 ± 0.030	0.092 ± 0.028	0.328 ± 0.082	1.14 ± 0.37
240	0.0883 ± 0.0010	ND	0.165 ± 0.036	0.298 ± 0.067
300	0.109 ± 0.077			
360	0.109[b]	ND	0.0653[b]	0.0893 ± 0.0211
480			ND	0.0823 ± 0.0348
600				ND
900				ND
1,200				ND

[a] Plasma concentrations are presented as mean ± standard deviation (µg/mL) for three animals per time point. ND=not detectable in any of the three samples; limit of detection estimated to be 0.0579 µg/mL

[b] Two of three samples were below the limit of detection.

APPENDIX K
NIEHS EXTRAMURAL MECHANISTIC STUDIES

OUTCOMES OF SELECTED STUDIES FUNDED BY RFA-ES-98-003 . . . . . K-2
REFERENCES K-5

NIEHS EXTRAMURAL MECHANISTIC STUDIES

OUTCOMES OF SELECTED STUDIES FUNDED BY RFA-ES-98-003

Study A: Effects of Peroxisome Proliferators on Glutathione and Glutathione-Related Enzymes in Rats and Hamsters (O'Brien *et al.*, 2001a)

Peroxisome proliferators cause hepatomegaly, peroxisome proliferation, and hepatocarcinogenesis in rats and mice. Conversely, hamsters are less responsive to these compounds. Peroxisome proliferators increase peroxisomal beta-oxidation and P450 4A subfamily activity, which has been hypothesized to result in oxidative stress. It was hypothesized that differential modulation of glutathione-related defenses could account for the resulting difference in species susceptibility following peroxisome proliferator administration. Accordingly, glutathione *S*-transferase, glutathione peroxidase, and glutathione reductase activities and total glutathione were measured in male Sprague-Dawley rats and Syrian hamsters administered two doses of three known peroxisome proliferators (dibutyl phthalate, gemfibrozil, and Wy-14,643) in feed for 6, 34, or 90 days. In rats, decreases in glutathione reductase, glutathione *S*-transferase, and selenium-dependent glutathione peroxidase were observed at various time points following treatment. In hamsters, higher basal levels of activities for glutathione reductase, glutathione *S*-transferase, and selenium-dependent glutathione peroxidase were observed when compared to rats. Hamsters also showed treatment-associated decreases in glutathione reductase and glutathione *S*-transferase activities. Interestingly, selenium-dependent glutathione peroxidase activity was increased in hamsters following treatment with Wy-14,643 and dibutyl phthalate. Treatment with Wy-14,643 for 90 days resulted in no change in glutathione peroxidase-1 mRNA in rats and increased glutathione peroxidase-1 mRNA in hamsters. This divergence in the hydrogen peroxide detoxification ability between rats and hamsters could be a contributing factor in the proposed oxidative stress mechanism of peroxisome proliferators observed in responsive and nonresponsive species. Although the basal activities of glutathione *S*-transferase and glutathione reductase were higher in the naive hamster, the response to peroxisome proliferators was similar to that of rats. Conversely, selenium-dependent glutathione peroxidase was increased and decreased in hamsters and rats, respectively, indicating a different capacity for controlling excess hydrogen peroxide in the cytosol and mitochondria between these species.

Study B: Differential Activation of Hepatic NF-kappaB in Rats and Hamsters by the Peroxisome Proliferators Wy-14,643, Gemfibrozil and Dibutyl Phthalate

(Tharappel *et al.*, 2001)

Nuclear factor-κB (NF-kappa B) is an oxidative stress-activated transcription factor involved in the regulation of cell proliferation and apoptosis. In a previous study, the authors found that the peroxisome proliferator ciprofibrate activates nuclear factor-κB in the liver of rats and mice. In the current study, the effects of three other peroxisome proliferators on nuclear factor-κB activation were studied in rats and Syrian hamsters using electrophoretic mobility shift assays with confirmation by supershift assays. The peroxisome proliferators WY-14,643, gemfibrozil, and dibutyl phthalate were administered in feed to animals for 6, 34, or 90 days. Wy-14,643 increased the DNA binding activity of nuclear factor-κB in rat livers more than gemfibrozil or dibutyl phthalate. No differences occurred in hepatic nuclear factor-κB levels in control or treated hamsters, demonstrating species-specific differences in hepatic nuclear factor-κB activation by peroxisome proliferators. These results indicate that species susceptible to the carcinogenicity of chronic exposure to peroxisome proliferators exhibit high levels of oxidative stress and the concomitant induction of nuclear factor-κB, whereas species that do not experience oxidant stress or induction of nuclear factor-κB are less susceptible.

Study C: Peroxisome Proliferators Do Not Activate the Transcription Factors AP-1, Early Growth Response-1, or Heat Shock Factors 1 and 2 in Rats or Hamsters

(O'Brien *et al.*, 2002)

Previous studies indicated that differential modulation of the oxidative stress-associated transcription factor nuclear factor-κB may contribute to the observed differences in species susceptibility when exposed to peroxisome proliferators. In this study, other oxidative stress-associated transcription factors (AP-1, early growth response gene 1, and heat shock factors 1 and 2) were studied to determine if these factors were regulated in the same way. Activation of these transcription factors was measured using gel mobility shift assays on hepatic nuclear extracts obtained from rats and Syrian hamsters administered the peroxisome proliferators dibutyl phthalate (5,000 or 16,000 ppm), Wy-14,643 (50 or 500 ppm) or gemfibrozil (1,000 or 16,000 ppm for rats; 6,000 or 24,000 ppm for hamsters) in feed for 6, 34 or 90 days. No consistent dose-responsive changes were observed in DNA binding activities of these transcription factors, which suggests that these factors are not involved in increased cell proliferation following exposure to peroxisome proliferators.

Study D: Effects of Peroxisome Proliferators on Antioxidant Enzymes and Antioxidant Vitamins in Rats and Hamsters (O'Brien *et al.*, 2001b)

Peroxisome proliferators cause hepatomegaly, peroxisome proliferation and hepatocarcinogenesis in rats and mice, whereas hamsters are less responsive to this class of chemicals. Peroxisome proliferators increase the activities of enzymes involved in peroxisomal beta-oxidation and omega-hydroxylation of fatty acids, which has been hypothesized to result in oxidative stress. The hypothesis tested in this study was that differential modulation of antioxidant enzymes and vitamins might account for differences in species susceptibility to peroxisome proliferators. Accordingly, the activities of DT-diaphorase and superoxide dismutase and the hepatic content of ascorbic acid and alpha-tocopherol were analyzed in male Sprague-Dawley rats and Syrian hamsters administered two doses of the peroxisome proliferators dibutyl phthalate, gemfibrozil, or Wy-14,643 in feed for 6, 34, or 90 days. In untreated animals, the activity of DT-diaphorase was much higher in hamsters than rats, but the activities of superoxide dismutase and content of ascorbic acid and alpha-tocopherol were similar between the species. In Wy-14,643-treated rats and hamsters, decreases in alpha-tocopherol content and superoxide dismutase activity were observed. DT-diaphorase activity was decreased in rats at all time points and doses; hamsters were sporadically affected. Dibutyl phthalate-treated rats and hamsters demonstrated increased superoxide dismutase activity at 6 days; in rats, however, superoxide dismutase activity decreased at 90 days and alpha-tocopherol content was decreased throughout. In gemfibrozil-treated rats and hamsters, alpha-tocopherol content decreased and DT-diaphorase activity increased. No consistent trend was observed in total ascorbic acid content for rats or hamsters after treatment with any of the peroxisome proliferators. These data suggest that both rats and hamsters are compromised in antioxidant capabilities following peroxisome proliferator treatment and additional hypotheses for species susceptibility should be considered.

Study E: Hepatic Expression of Polymerase beta, Ref-1, PCNA, and Bax in WY 14,643-exposed Rats and Hamsters (Holmes *et al.,* 2002)

Using immunoblotting, the hepatic levels of three protein markers of oxidative stress, polymerase beta, Ref-1, and proliferating cell nuclear antigen (PCNA), and of the pro-apoptotic protein, Bax, were measured in detergent-extracted whole liver homogenates obtained from Sprague-Dawley rats (rodents susceptible to peroxisome proliferator-induced liver tumors) and Syrian hamsters (rodents relatively resistant to peroxisome proliferator-induced liver tumors) after exposure to the peroxisome proliferator WY 14,643 (500 ppm) in feed for 6 or 34 days. In treated rats, there was a marked increase in the abundance of a 45-kDalton variant of polymerase beta immunoreactivity and significant increases in the expression of Ref-1 and PCNA. In contrast, treated hamsters expressed only trace levels of the polymerase beta variant and significant decreases in the expression of Ref-1 and PCNA. Long-term exposure yielded marked decreases in Bax expression in both rats and hamsters. Dose-response studies in rats showed significant increases in hepatic expression of polymerase beta and Ref-1 after

6 days of exposure to WY 14,643 at 5 and 50 ppm, respectively. Analysis of subcellular fractions of rat liver showed pathological increases in levels of polymerase beta, Ref-1, and PCNA, especially prominent in mitochondria-enriched particulate liver subfractions. These results indicate that WY 14,643 exposure is associated with an increase in oxidative stress to the liver and liver mitochondria are a major target of WY 14,643-associated liver damage. These data are consistent with the hypothesis that the chronic overexpression of mutagenic or oncogenic effectors like polymerase beta and Ref-1 in a setting of increased hepatocyte proliferation and decreased apoptosis may facilitate peroxisome proliferator-induced hepatocellular carcinoma in the rat.

Study F: Effect of Peroxisome Proliferators on the Methylation and Protein Level of the c-myc Protooncogene in B6C3F$_1$ Mice Liver (Ge *et al.,* 2002)

The peroxisome proliferators Wy-14,643, 2,4-dichlorophenoxyacetic acid, dibutyl phthalate, and gemfibrozil were evaluated for their ability to alter the methylation and expression of the c-*myc* protooncogene. Wy-14,643 (5 to 500 ppm), 2,4-dichlorophenoxyacetic acid (1,680 ppm), dibutyl phthalate (20,000 ppm), or gemfibrozil (8,000 ppm) were administered in feed to male B6C3F$_1$ mice for 6 days. All four peroxisome proliferators caused hypomethylation of the c-*myc* gene in the liver. Wy-14,643 appeared to be the most efficacious with a threshold between 10 and 50 ppm. The level of c-*myc* protein was increased by Wy-14,643 but not the other peroxisome proliferators. Female B6C3F$_1$ mice were administered 50 mg/kg Wy-14,643 by gavage 16 hours after receiving a partial (2/3) hepatectomy, and hypomethylation of the gene occurred 24 hours later. Hypomethylation did not occur in Wy-14,643-treated mice following a sham operation. These results support the hypothesis that peroxisome proliferators prevent methylation of hemimethylated sites formed by DNA replication.

Study G: Expression of Base Excision Repair Enzymes in Rat and Mouse Liver is Induced by Peroxisome Proliferators and is Dependent upon Carcinogenic Potency (Rusyn *et al.*, 2000)

Elevated and sustained cell replication, together with a decrease in apoptosis, is considered the main mechanism of hepatic tumor promotion due to peroxisome proliferators. In contrast, the role of oxidative stress and DNA damage in carcinogenesis is less well understood. In view of the possible induction of DNA damage by peroxisome proliferators, DNA repair mechanisms may be an important factor to consider in the mechanism of action of these compounds. The ability of peroxisome proliferators to induce expression of base excision repair enzymes was studied. The peroxisome proliferator, Wy-14,643 increased the expression of several base excision repair enzymes in a dose- and time-dependent manner. Importantly, the expression of enzymes that do not repair oxidative DNA damage was not changed. Moreover, less potent members of the peroxisome proliferator group had much weaker or no effects on the expression of DNA base excision repair enzymes when compared to Wy-14,643. Collectively, these data suggest that DNA base excision repair may be an important factor in peroxisome proliferator-induced carcinogenesis and that induction of DNA repair might provide further evidence supporting a role of oxidative DNA damage by peroxisome proliferators.

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