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

NTP Technical Report on the Hepatotoxicity Studies of the Liver Carcinogen

Methapyrilene Hydrochloride (CAS No. 135-23-9) Administered in Feed to Male F344/N Rats

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FOREWORD

The National Toxicology Program (NTP) is made up of four charter agencies of the U.S. Department of Health and Human Services (DHHS): the National Cancer Institute (NCI), National Institutes of Health; the National Institute of Environmental Health Sciences (NIEHS), National Institutes of Health; the National Center for Toxicological Research (NCTR), Food and Drug Administration; and the National Institute for Occupational Safety and Health (NIOSH), Centers for Disease Control and Prevention. In July 1981, the Carcinogenesis Bioassay Testing Program, NCI, was transferred to the NIEHS. The NTP coordinates the relevant programs, staff, and resources from these Public Health Service agencies relating to basic and applied research and to biological assay development and validation.

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The studies described in this Toxicity Study Report were performed under the direction of the NIEHS and were conducted in compliance with NTP laboratory health and safety requirements and must meet or exceed all applicable federal, state, and local health and safety regulations. Animal care and use were in accordance with the Public Health Service Policy on Humane Care and Use of Animals.

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Administered in Feed
to Male F344/N Rats

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July 2000

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U.S. Department of Health and Human Services
Public Health Service
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PEER REVIEW

The draft report on the toxicity studies of methapyrilene hydrochloride was evaluated by the reviewers listed below. These reviewers serve as independent scientists, not as representatives of any institution, company, or governmental agency. In this capacity, reviewers determine if the design and conditions of these NTP studies are appropriate and ensure that the Toxicity Study Report presents the experimental results and conclusions fully and clearly.

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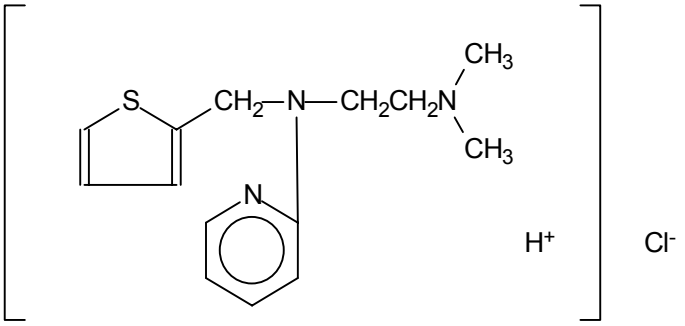
ABSTRACT

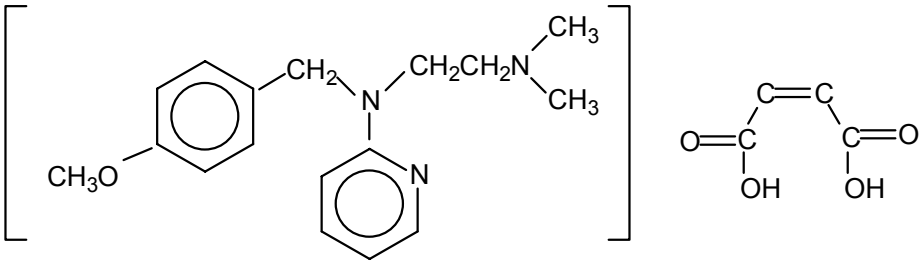


METHAPYRILENE HYDROCHLORIDE

CAS No. 135-23-9

Chemical Formula: $C_{14}H_{19}N_3S{\cdot}HCl$ Molecular Weight: 297.85

Synonyms: *N,N*-Dimethyl-*N′*-2-pyridinyl-*N′*-(2-thienylmethyl)-1,2-ethanediamine; 2-[(2-dimethyl-aminoethyl)-2-thenylamino]pyridine



PYRILAMINE MALEATE

CAS No. 59-33-6

Chemical Formula: $C_{17}H_{23}N_3O{\cdot}C_4H_4O_4$ Molecular Weight: 387.43

Synonyms: Paramal; paraminyl maleate; pymafed; pyra maleate; pyranilamine maleate; pyraninyl; renstamin; thylogen maleate
Trade Names: Anthisan; Dorantamin; Enrumay; Histan; Histatex; Histosol; Paraminyl Maleate; Stamine

Methapyrilene hydrochloride is a histamine H_1-receptor antagonist that was an active ingredient in many over-the-counter cold and allergy medications. In the mid- to late 1970s, studies in rats suggested that methapyrilene hydrochloride was a hepatocarcinogen, and the drug was removed from these preparations. In most cases, methapyrilene hydrochloride was replaced by pyrilamine maleate, a structurally similar analogue. As part of

a program to investigate mechanisms of toxicity whereby structurally similar chemicals produce different toxicities, these chemicals were studied for induction of cell proliferation and protein alterations by two-dimensional gel electrophoresis in the liver of F344/N rats. A complete toxicologic evaluation was not needed for this research-oriented study. Rather, the goal of the present study was to provide retrospective data from subchronic toxicity studies with the known rat carcinogen methapyriline hydrochloride that could then be used to predict the potential carcinogenicity of unknown chemical agents and that could also be compared with similar data on the structural analogue pyrilamine maleate. Pyrilamine maleate differs from methapyrilene hydrochloride in the substitution of the thienyl ring with a paramethoxyphenyl ring. Pyrilamine maleate has been shown to produce an equivocal increase in the incidences of liver neoplasms in rats in 2-year feed studies, but only at 2,000 ppm, indicating that its potency, if any, to produce neoplasms is much less than that of methapyriline hydrochloride. The hepatocarcinogenic peroxisome proliferator Wy-14,643 was included in this study as a positive control that is known to induce cell proliferation, as well as protein alterations, in the liver.

In the 14-week study of methapyrilene hydrochloride, groups of 40 male F344/N rats were given 0, 50, 100, 250, or 1,000 ppm methapyrilene hydrochloride, 1,000 ppm pyrilamine maleate (negative control), or 50 ppm Wy-14,643 (positive control) in feed. Rats in all groups were administered bromodeoxyuridine (BrdU) by osmotic minipump for the assessment of hepatocyte proliferation. Ten rats from each group were evaluated on days 15, 29, and 43 and at 14 weeks. At these times, samples of liver tissue were analyzed for evidence of cell proliferation via BrdU labeling and proliferating cell nuclear antigen (PCNA) labeling.

There were no exposure-related deaths. Low mean body weights were generally observed in the 1,000 ppm methapyriline hydrochloride group and in the positive control group. Final mean body weights and mean body weight gains of rats exposed to 1,000 ppm methapyrilene hydrochloride were significantly less than those of the untreated control group at all time points. The final mean body weights of rats in the positive control group were significantly less than those of the untreated control group for rats evaluated on days 29 and 43 and at week 14; the mean body weight gains of rats in the positive control group were significantly less than those of the untreated control group on day 29 and at week 14.

Feed consumption by rats exposed to 1,000 ppm methapyrilene hydrochloride was significantly less than that by the untreated control group throughout the study. The predominant clinical observation related to methapyrilene hydrochloride exposure was thinness in rats exposed to 1,000 ppm; this finding was first observed on day 29.

On days 29 and 43 and at 14 weeks, the absolute liver weights of rats exposed to 1,000 ppm methapyrilene hydrochloride were significantly less than those of the untreated control group. At all time points, the relative liver

weights of rats exposed to 1,000 ppm methapyrilene hydrochloride and the absolute and relative liver weights of positive control rats were significantly greater than those of the untreated control group. No significant differences in liver weights were observed between the negative and untreated control groups at any time point.

Hepatic lesions were observed predominantly in the 250 and 1,000 ppm methapyrilene hydrochloride groups and in the positive control group. The incidences of bile duct hyperplasia, hepatocyte necrosis, hepatocyte mitosis, and hepatocyte hypertrophy in rats in the 1,000 ppm group were significantly greater than those in the untreated control group at all time points. The severities of hepatocyte hypertrophy and hepatocyte mitosis in 1,000 ppm rats were generally mild to moderate; the lesions occurring in 250 ppm animals were less severe. At each time point, the incidence of bile duct hyperplasia in 250 ppm rats was significantly greater than that in the untreated control group. The incidences of hepatocyte mitosis on days 15 and 29 and the incidences of hepatocyte necrosis on days 29 and 43 in rats in the 250 ppm group were significantly greater than those in the untreated control group. Incidences of pigmentation in the 250 and 1,000 ppm methapyrilene hydrochloride groups were significantly greater than those in the untreated control group on days 29 and 43 and at 14 weeks.

In the positive control group, the incidences of granulomatous inflammation were significantly greater than those in the untreated control group on days 15, 29, and 43. The incidences of hepatocyte hypertrophy and hepatocyte mitosis in the positive control group were significantly greater than those in the untreated control group on days 15, 29, and 43. The incidence of hepatocyte hypertrophy was also significantly increased in the positive control group at 14 weeks. The severity of hepatocyte hypertrophy in the 1,000 ppm methapyrilene hydrochloride group was generally greater than that in the positive control group at each time point.

In general, methapyriline hydrochloride produced a dramatic and sustained increase in hepatic cell proliferation over 14 weeks, whereas pyrilamine maleate at the same concentration produced few if any effects. Wy-14,643 also induced a large increase in cell proliferation which declined over time, as has been observed in previous studies.

The mean BrdU labeling indexes of the 250 and 1,000 ppm methapyrilene hydrochloride groups were generally significantly greater than those of the untreated controls at all time points. In the negative control group, the BrdU labeling index was significantly less than that of the untreated control group on day 29. The BrdU labeling index in the positive control group was significantly greater than that of the untreated control group at all time points.

On day 43 and at week 14, the mean PCNA labeling indexes of the 1,000 ppm methapyrilene hydrochloride group were significantly greater than those of the untreated control group. The mean PCNA labeling indexes of the

negative control group were significantly less than those of the untreated control group on days 29 and 43. On day 29, the mean PCNA labeling index of the positive control group was significantly greater than that of the untreated control group.

The mitotic indexes of the 1,000 ppm methapyrilene hydrochloride group were significantly greater than those of the untreated control group at all time points. The mitotic indexes of the 250 ppm group were significantly greater than those of the untreated control group on day 43 and at week 14.

At least 32 proteins underwent significant abundance changes at the highest exposure concentration of methapyrilene hydrochloride, and 39 protein changes were observed in the positive control group. Many, but not all, of the protein changes in the methapyrilene hydrochloride-exposed animals also occurred in the positive control group. Treatment with pyrilamine maleate produced no significant quantitative protein changes, as judged by the same criteria used for methapyrilene hydrochloride and Wy-14,643. Methapyrilene hydrochloride produced covalent modification of mitochondrial proteins as measured by the charge modification index. PCNA abundance in liver samples from the 250 and 1,000 ppm methapyrilene hydrochloride exposure groups on day 43 was significantly greater than that of the untreated control group.

Results of tests for induction of mutagenicity by methapyrilene hydrochloride were negative in *Salmonella typhimurium* strains TA98, TA100, TA1535, and TA1537 and in L5178Y mouse lymphoma cells, with and without S9 metabolic activation. However, positive responses were obtained in cytogenetic tests with cultured Chinese hamster ovary cells, in which methapyrilene hydrochloride induced sister chromatid exchanges and chromosomal aberrations. The increases in sister chromosome exchanges were obtained with and without S9, but chromosomal aberrations were increased only in the presence of S9.

In summary, the significance of the increased hepatic cell proliferation and the protein alterations observed in this study is not definite, but may be of predictive value for assessing the toxicity and carcinogenicity of chemicals in preclinical assays. A chemical which does not produce an increase in cell proliferation or a large number of protein changes may be considered safer than a similar chemical that produces many such changes.

INTRODUCTION

PHYSICAL AND CHEMICAL PROPERTIES

Methapyrilene hydrochloride, a white crystalline powder with a slight odor and a melting point range of 161° to 165° C, is an antihistamine (an H_1-receptor antagonist). It is soluble in water, chloroform, and alcohol and is insoluble in ether and benzene. In solution, methapyrilene hydrochloride is acidic, having a pH of about 5.5 (*Remington's*, 1980).

PRODUCTION, USE, AND EXPOSURE

Methapyrilene hydrochloride is prepared by condensing 2-(2-thenyl)aminopyridine with 2-(dimethylamino)ethyl chloride in the presence of sodamide and then reacting the base with an equimolar quantity of hydrogen chloride (*Remington's*, 1980). Human exposure to methapyrilene hydrochloride during the 1970s was extensive because it was an active ingredient in a wide variety of over-the-counter cold and allergy preparations and sleep aids (e.g., Excedrin PM®, Nytol®, Sominex®, and Compoz®) and it was a component of prescription medications (e.g., Co-Pyronil®, Histadyl®) (Mirsalis, 1987). Between the years 1972 and 1975, production of methapyrilene hydrochloride in the United States was 450 kg per year. Additionally, 790 kg were imported into the United States in 1975 (USITC, 1976).

In response to results by Lijinsky *et al*. (1980) of hepatocarcinogenesis studies in methapyrilene hydrochloride-treated Sprague-Dawley rats, the United States Food and Drug Administration declared methapyrilene hydrochloride a potential carcinogen and announced a voluntary recall of products containing methapyrilene hydrochloride (Couri *et al.*, 1982). In addition to the removal of this chemical from all medications sold in the United States, it was also banned in Canada and Great Britain (Mirsalis, 1987). In most cases, products were reformulated with pyrilamine maleate, a structurally similar analogue (Lijinsky, 1984).

ABSORPTION, DISTRIBUTION, METABOLISM, AND EXCRETION

Methapyrilene hydrochloride is in the ethylenediamine class of histamine H_1-receptor antagonists. These antihistamines act by competitively antagonizing the effects of histamine at receptor sites but do not block the release of histamine. After oral administration, palliation of allergy and cold symptoms is apparent within 15 to 30 minutes, is maximal within 1 hour, and persists for 4 to 6 hours (*Remington's*, 1980). Binding of methapyrilene hydrochloride to H_1 receptors in the central nervous system generally results in diminished alertness, slowed reaction times, and somnolence. The tendency of the ethylenediamines to produce somnolence has led to their use as hypnotics in proprietary remedies for insomnia that are sold over the counter (*Goodman and Gilman's*, 1990).

In general, the H_1 antagonists are well absorbed from the gastrointestinal tract (*Goodman and Gilman's*, 1990). Peak plasma concentrations are usually achieved 2 to 3 hours after oral dosing, and effects last for up to 6 hours. H_1 antagonists are widely distributed in the body, including the central nervous system. The major site for metabolism of these compounds is the liver (*Remington's*, 1980). Only minute amounts of methapyrilene hydrochloride are excreted unchanged; most of an administered dose is eliminated as metabolites (*Goodman and Gilman's*, 1990).

Experimental Animals

Within 24 hours after intravenous doses of 0.7 or 3.5 mg/kg [^{14}C]-methapyrilene hydrochloride were administered to male F344/N rats, the 0.7 mg/kg group eliminated 40% of the radiolabel in the urine and 38% in the feces, and the 3.5 mg/kg group eliminated 35% in the urine and 44% in the feces; approximately 98% of all radioactivity was excreted within 7 days (Kelly *et al.*, 1990). The urinary and plasma metabolic profiles of methapyrilene hydrochloride in the rat were essentially the same; one major and five minor radiolabeled compounds were identified. The major metabolic pathway of methapyrilene hydrochloride is aliphatic *N*-oxidation to methapyrilene *N*-oxide. Minor metabolites, which include side-chain cleavage products and demethylated and glucuronic acid-conjugated metabolites, were also identified in 24-hour urine samples along with unmetabolized methapyrilene (less than 10%). In two rat metabolism studies of orally administered methapyrilene hydrochloride, an additional major metabolite, *N*-(*N′,N′*-dimethylaminoethyl)-2-aminopyridine, was identified in the urine, suggesting that the urinary metabolic profile of methapyrilene hydrochloride in F344/N rats is dependent on the route of administration (Kelly *et al.*, 1990).

The elimination kinetics of intravenously administered pyrilamine maleate in male F344/N rats (0.7 or 7 mg per kg body weight) were shown to be similar to those of methapyrilene hydrochloride. However, while *N*-oxidation is the major pathway and glucuronidation is a minor pathway for metabolism and elimination of methapyrilene

hydrochloride, pyrilamine maleate is metabolized primarily by *O*-demethylation and conjugation with glucuronic acid; 28% to 38% is excreted in the urine and 27% to 30% in the feces within 24 hours (Kelly and Slikker, 1987).

The metabolism of methapyrilene hydrochloride was also studied in suspension cultures of hepatocytes from F344/N rats and B6C3F_1 mice by Kelly *et al.* (1992), who regarded these cultures as good qualitative predictors of the metabolism of the compound *in vivo*. The hepatocytes of both species biotransformed methapyrilene hydrochloride via glucuronidation, *N*- and side-chain oxidation, and *N*-dealkylation. Although glucuronidation predominated in both species, rat hepatocytes metabolized methapyrilene hydrochloride more rapidly than did mouse hepatocytes. The rate of side-chain oxidation in rats was nearly eight times faster than that in mice. These results were cited as a possible explanation for why rats were more sensitive to methapyrilene hydrochloride than mice. Another study designed to document interspecies differences in the *in vitro* metabolism of methapyrilene hydrochloride demonstrated the formation of additional minor metabolites by liver microsomes of male ICR mice and male LVG hamsters; these metabolites were not formed by microsomal fractions from male Sprague-Dawley rats (Lampe and Kammerer, 1990).

Histamine H_1-receptor antagonists have been shown to induce hepatic microsomal enzymes. Consequently, they may facilitate their own metabolism (*Goodman and Gilman's*, 1990).

Humans

Systemic bioavailability of methapyrilene hydrochloride in humans is low and ranged from 4% to 46% in healthy human volunteers given 20 mg intravenous or 25 or 50 mg oral doses. Recovery of unchanged methapyrilene hydrochloride from the urine over a 24-hour period was less than 2% of the administered dose (Calandre *et al.*, 1981).

TOXICITY

Experimental Animals

Within 4 days after administration of a single gavage dose of 225 mg methapyrilene hydrochloride/kg body weight in corn oil, male F344/N rats developed severe hepatotoxicity characterized by periportal and focal necrosis and increased numbers of mitotic figures. Results from serum analyses correlated with these histopathologic results, indicating elevated serum bilirubin concentrations and activities of liver enzymes (sorbitol dehydrogenase, glutamic oxaloacetate transaminase, and glutamic pyruvate transaminase) (Steinmetz *et al.*, 1988).

In a study in male Syrian golden hamsters administered 15 mg methapyrilene hydrochloride by water gavage twice weekly for 58 weeks (for a total dose of approximately 15 g/kg body weight), only 13 of 20 animals survived an acute convulsant effect, and several others died during the study with indications of liver toxicity and pancreatic hyperplasia (Lijinsky *et al.*, 1983).

One of the few hepatic ultrastructural changes in the F344/N rat associated with a dose of 1,000 ppm methapyrilene hydrochloride/kg body weight in feed is a substantial increase in the number of mitochondria per hepatocyte in portal areas, with numerous peripheral mitotic hepatocytes. This change begins shortly after the start of chronic treatment and ultimately also occurs in the induced hepatocellular carcinomas. In addition, a concomitant decrease in smooth and rough endoplasmic reticulum occurs in the same cells (Reznik-Schüller and Lijinsky, 1982). None of these effects were induced by methapyrilene in the Syrian golden hamster or guinea pig, nor were these effects induced in rats administered 2,000 ppm of the analogue, pyrilamine maleate, in feed (Reznik-Schüller and Lijinsky, 1982).

In a chronic study of pyrilamine maleate exposure, mean body weight gains of male and female F344/N rats exposed to 1,500 or 3,000 ppm in feed for 13 weeks were significantly less than those of the control groups (Greenman *et al.*, 1995a). In rats exposed for 104 weeks, body weights of males exposed to 3,000 ppm and females exposed to 1,500 or 3,000 ppm were significantly less than those of the control groups. The survival of exposed males was greater than that of the controls, and the difference was significant in the 1,500 ppm group. After 65 weeks of exposure, absolute liver, kidney, and thymus weights of females decreased significantly with increasing exposure concentration. In females exposed for 104 weeks, incidences of chronic inflammation of the nasolacrimal duct increased significantly with increasing exposure concentration, and adrenal medullary hyperplasia occurred significantly earlier in exposed females than in the controls, although the incidences were not increased. In B6C3F_1 mice exposed to pyrilamine maleate in feed for 104 weeks, the final mean body weight of females exposed to 1,500 ppm was significantly less than that of the control group (Greenman *et al.*, 1995b). The survival of male mice increased with increasing exposure concentration. Incidences of chronic liver inflammation in female mice exhibited a significant, positive, exposure concentration-related trend. The incidence of chronic liver inflammation in the 750 ppm male mice was significantly greater than that in the control group.

Humans

In cases of overdosage or allergic reaction to methapyrilene hydrochloride, the following acute symptoms have been observed: dizziness, lethargy, ataxia, skin rash (including urticaria), spasms of the pylorus and/or colon, blurred vision, palpitations (e.g., tachycardia), swelling of the mammary glands, muscular twitching, medullary

convulsions, coma, respiratory and/or circulatory collapse, and death (Thienes and Haley, 1972). A case of fatal overdose of methapyrilene hydrochloride has been described by Ainsworth and Biggs (1977).

CARCINOGENICITY

Experimental Animals

Following the demonstration by Lijinsky (1974) that methapyrilene hydrochloride reacts with nitrite in mildly acidic solutions to form the carcinogen dimethylnitrosamine, Lijinsky and Taylor (1977) observed a significant number of liver neoplasms (9/30; 30%) in Sprague-Dawley rats exposed to 1,000 ppm methapyrilene hydrochloride and 2,000 ppm sodium nitrite in drinking water for 90 weeks. This led them to conclude that the formation of carcinogenic nitrosamines by reaction of methapyrilene hydrochloride with nitrite in the stomach may pose an increased carcinogenic risk to humans. However, in a subsequent carcinogenesis bioassay in which methapyrilene hydrochloride was administered to F344/N rats with or without sodium nitrite for up to 64 weeks, the incidence and type of methapyriline hydrochloride-induced neoplasms at 83 weeks were unaffected by the simultaneous administration of sodium nitrite. Almost all (48/50) of F344/N rats exposed to 1,000 ppm methapyrilene hydrochloride in feed developed liver neoplasms, mainly hepatocellular carcinomas and cholangiocarcinomas. The spread of neoplasms to the stomach, spleen, and pancreas and metastases to the lung, omentum and peritoneum, brain, ovary, kidney, thymus, spleen, and lymph nodes (portahepatic, parapancreatic, and parathymic) were common (Lijinsky *et al.*, 1980).

In a later study designed to evaluate the chronic toxicity and carcinogenicity of lower concentrations of methapyrilene hydrochloride in feed, almost all F344/N rats exposed to 250 ppm had either hepatic carcinomas or neoplastic nodules (18/20 males and 20/20 females), whereas 40% of rats exposed to 125 ppm developed neoplastic nodules (Lijinsky, 1984). In the same study, other groups of rats were exposed to 2,000 ppm pyrilamine maleate either in the diet or in drinking water. The incidences of liver neoplasms in rats given pyrilamine maleate in drinking water were similar to those in the control group; however, in female rats that received pyrilamine maleate in the diet, the incidences of hepatocellular carcinoma and neoplastic nodules were significantly greater than those in the controls. In more recent studies, F344/N rats and B6C3F_1 mice given pyrilamine maleate in feed for 2 years at concentrations up to 3,000 ppm (rats) or 1,500 ppm (mice) exhibited no clear carcinogenic response (Greenman *et al.*, 1995a,b).

Results of studies conducted in other rodent species have indicated that the carcinogenicity of methapyrilene hydrochloride is species specific. No increased incidence of hepatic neoplasms occurred at 132 weeks in male or

female guinea pigs administered gavage doses of 200 mg methapyrilene hydrochloride/kg body weight twice weekly for 78 weeks, and none occurred at 61 weeks in male Syrian golden hamsters administered 15 mg/kg twice weekly for 58 weeks (Lijinsky *et al.*, 1983).

The promoting ability of methapyrilene hydrochloride has been demonstrated by Couri *et al.* (1982), who reported that although methapyrilene hydrochloride was not an initiator in rat liver, it was a potent promoter of γ-glutamyl-transpeptidase-positive foci in partially hepatectomized Sprague-Dawley rats following initiation with nitrosodiethylamine. A similar result was reported by Furuya *et al*. (1983), who demonstrated enhancement of methapyrilene-induced hepatocarcinogenicity in F344 rats previously administered *N*-2-fluorenylacetamide. Additional studies by Furuya and Williams (1984) indicated that administration of methapyrilene hydrochloride prior to diethylnitrosamine also enhanced hepatocarcinogenesis, suggesting that methapyrilene produces a neoplastic conversion of liver cells which, combined with the genotoxic effect of *N*-2-fluorenylacetamide, can produce syncarcinogenesis. More recently, Horn *et al.* (1996) demonstrated exposure concentration- and site (lobe)-dependent increases in the incidences of liver foci staining positive for glutathione S-transferase (placental form) in partially hepatectomized male F344 rats fed 62.5 to 1000 ppm methapyrilene hydrochloride (following initiation with diethylnitrosamine). In species that have relatively high spontaneous liver neoplasm rates, treatment with a tumor promoter may increase the number of neoplasms or decrease the time required for the appearance of neoplasms. The significant increases in hepatic cell proliferation induced by methapyrilene hydrochloride may lead to an increased liver neoplasm incidence, which may be sufficient to explain the carcinogenicity of this compound (Cunningham *et al*., 1995).

Humans

No epidemiology studies or information on the carcinogenicity of methapyrilene hydrochloride in humans were found in the literature.

GENETIC TOXICITY

Methapyrilene and its hydrochloride salt have been tested in a variety of mutagenicity assays. Weak responses have been noted in some *in vitro* assays, but no evidence of mutagenicity has been detected *in vivo*. An earlier review of the genotoxicity of methapyrilene and consideration of its mechanism of action were provided by Mirsalis (1987). Methapyrilene hydrochloride, tested in a standard plate incorporation assay with and without S9 metabolic activation enzymes, did not induce mutations in *Escherichia coli* (Oberly *et al.*, 1993), and no increased

mutation frequencies were noted in a variety of *Salmonella typhimurium* strains tested via either plate incorporation or liquid preincubation protocols (Andrews *et al.*, 1980; Kammerer *et al.*, 1986; Mortelmans *et al.*, 1986). However, Ashby *et al.* (1988) reported weak but reproducible positive results with pure methapyrilene, the hydrochloride salt, and methapyrilene fumarate in *S. typhimurium* strain TA1535 in the absence of S9; mutation frequencies following treatment were approximately twice the control values with all three compounds. A similar low-level response was noted by Oberly *et al.* (1993) in *S. typhimurium* strain TA1535 treated with methapyrilene hydrochloride in the absence of S9. Induction of mitotic gene conversion and mitotic crossing-over was observed in *Saccharomyces cerevisiae* strain D7 treated with methapyrilene hydrochloride with and without S9 (Mehta and von Borstel, 1984). Increased frequencies of mutations were detected at the thymidine kinase locus in L5178Y mouse lymphoma cells treated with 200 to 500 µg/mL methapyrilene or its hydrochloride salt in the presence of Sprague-Dawley rat liver S9 (Blazak *et al.*, 1986; Mirsalis, 1987; Turner *et al.*, 1987; Clive *et al.*, 1990; Casciano *et al.*, 1991). Mutagenicity in these cells was generally accompanied by marked cytotoxicity (cell growth in treated cultures less than 10% of control values) and the predominant size of mutant colonies was small, suggesting that chromosomal mutations, rather than point mutations, were induced. Other studies with L5178Y mouse lymphoma cells that employed similar doses of methapyrilene hydrochloride, with and without S9, reported no induction of mutations (Oberly *et al.*, 1984; McGregor *et al.*, 1991). Turner *et al.* (1987) determined that the use of freshly prepared S9 in the mouse lymphoma cell assay resulted in lower frequencies of methapyrilene-induced mutations than the use of aged S9, and the authors speculated that interlaboratory variations in response were related to the preparation and storage of S9.

No increases in frequencies of 6-thioguanine-resistant mutants were noted in Chinese hamster ovary cell cultures exposed to methapyrilene, with or without S9 (Casciano and Schol, 1984; Oberly *et al.*, 1990), and no increase in the frequency of sister chromatid exchanges was observed in Chinese hamster ovary cells cocultured with rat liver epithelial cells and treated with methapyrilene (Iype *et al.*, 1982). Finally, no induction of chromosomal aberrations was noted in either Chinese hamster ovary cells (Brunny *et al.*, 1989) or human lymphocytes (Thompson and Albanese, 1986) treated with methapyrilene hydrochloride, with or without S9.

DNA adduct formation was noted *in vitro* in isolated calf thymus DNA preparations (Lampe and Kammerer, 1987, 1990) but not in DNA of L5178Y mouse lymphoma cells (Casciano *et al.*, 1991) treated with methapyrilene or its hydrochloride salt in the presence of S9. Autoradiographic analyses revealed no induction of unscheduled DNA synthesis in Fischer 344 rat hepatocytes exposed to methapyrilene or its hydrochloride salt (Probst and Neal, 1980; McQueen and Williams, 1981; Probst *et al.*, 1981; Budroe *et al.*, 1984; Steinmetz *et al.*, 1988), but positive results were obtained by liquid scintillation counting of DNA preparations from hepatocytes of Holtzman rats treated with higher concentrations of methapyrilene hydrochloride than were used in the F344 rat studies (Althaus *et al.*,

1982a,b). A single unscheduled DNA synthesis study conducted in human hepatocytes detected no evidence of an effect after *in vitro* exposure to 5 to 500 µg/mL methapyrilene (Steinmetz *et al.*, 1988).

No evidence of methapyrilene-induced genotoxicity has been reported in *in vivo* tests. No formation of DNA adducts was detected in any of various tissues of F344 male rats administered methapyrilene via gavage (290 µmol/kg; Lijinsky and Muschik, 1982), drinking water (470 µmol/kg; Lijinsky and Yamashita, 1988), or intraperitoneal injection (500 µmol/kg; Casciano *et al.*, 1988). Cytogenetic studies revealed no increases in the frequency of sister chromatid exchanges in bone marrow cells of mice administered 40 or 80 mg/kg methapyrilene hydrochloride by gavage (Brunny *et al.*, 1989) or rats given 2.5 to 20 mg/kg intravenously (Iype *et al.*, 1982) and no induction of micronuclei or chromosomal aberrations in rat bone marrow cells after intraperitoneal injection of 300 mg/kg (Thompson and Albanese, 1986).

In conclusion, independently verified positive responses in mutagenicity assays have been obtained for methapyrilene and its salts only under limited conditions (i.e., *S. typhimurium* strain TA1535 without S9 or L5178Y mouse lymphoma cells with aged S9). Because of the lack of activity by methapyrilene in a broad spectrum of other *in vitro* and *in vivo* genotoxicity assays, the biological relevance of these positive findings to the hepatic carcinogenicity of methapyrilene remains unclear. It has been suggested that the ability of methapyrilene to induce S-phase DNA synthesis (a marker for increased cell proliferation) and significantly increased numbers of mitochondria in liver cells might be one possible nongenotoxic mechanism for its carcinogenicity (Steinmetz *et al.*, 1988).

STUDY RATIONALE AND DESIGN

Although methapyrilene hydrochloride is no longer in use, widespread exposure when it was available as a component of numerous pharmaceutical preparations presents a possible significant cancer risk to humans. This study was conducted to provide mechanistic information to assist in the determination of the carcinogenic risk associated with human exposure to methapyriline hydrochloride. Methapyriline hydrochloride was selected as part of an ongoing research effort at the National Toxicology Program (NTP) to provide mechanistic understanding of the class of compounds termed nongenotoxic carcinogens. Such data will be invaluable for understanding the mechanism of carcinogenicity of compounds that produce weak or no effects in standard *in vitro* tests for genetic damage, yet produce carcinogenic effects in chronic bioassays. Because the liver has been shown to be a target organ for methapyrilene hydrochloride toxicity, the current study was designed to more fully evaluate

hepatocellular effects and to determine the relationship between the hepatocarcinogenicity of methapyrilene hydrochloride and the production of chronic hepatotoxicity in F344/N rats.

The 14-week study, with 15-, 29-, and 43-day interim evaluations, was limited to histopathology of the liver, alteration in hepatic protein profiles, and cell proliferation assays. In addition to groups that received methapyrilene hydrochloride, a negative control group received pyrilamine maleate, a currently marketed, structural analogue of methapyrilene hydrochloride that is regarded as marginally carcinogenic or noncarcinogenic, and a positive control group received the known hepatocarcinogen Wy-14,643. The mutagenicity of methapyrilene hydrochloride was also evaluated in *S. typhimurium*, L5178Y mouse lymphoma cells, and cultured Chinese hamster ovary cells.

Data in the literature indicate a positive association between increased cell proliferation and carcinogenesis and illustrate the value of performing mechanistic studies such as cell proliferation assays in conjunction with short-term tests to further investigate the results of NTP bioassays. Mechanistic studies such as cell proliferation assays may also aid in the selection of doses for future bioassays and support assessments of human risk. Though some nonmutagenic chemicals cause increased neoplasm incidences that are associated with increased cell proliferation, increased cell proliferation alone appears to be insufficient to cause an increased incidence of neoplasms. Numerous chemicals have been shown to induce sustained cell proliferation in the absence of an increased neoplasm incidence. The hypothesis tested in the present study is that although cell proliferation may not be sufficient to induce carcinogenesis, it does create a favorable environment for neoplasm development. This favorable environment is thought to occur as a result of an increased number of replicating cells passing through the more vulnerable stages of mitosis. It is this favorable environment which permits the manifestations of the mutagenic activity of any chemicals that may be present, including the administered chemical, one or more of its metabolites, or other naturally occurring or foreign chemicals (Ames and Gold, 1998). The endpoints of BrdU incorporation and proliferating cell nuclear antigen (PCNA) labeling, indexes of cell proliferation, were used in the present study to determine their value in such investigations.

An additional marker of toxicity that may be associated with chemical exposure is the occurrence of protein alterations. Two-dimensional gel electrophoresis of proteins can be used to detect and quantify hundreds of proteins simultaneously from a single sample. Advances in computerized data acquisition, storage, and retrieval have permitted the establishment of a large database of two-dimensional patterns from several species under many treatment conditions. It is possible that such data would be of value for the understanding of chemical toxicity and for uncovering mechanisms of cellular response to DNA damage, cell membrane damage, and cellular mechanisms of adaptation to chemically induced stress or injury. Therefore, protein alterations were examined in the present

study in an attempt to obtain mechanistic information on the toxicity produced by hazardous chemicals as well as safety information for a less hazardous agent. Protein alterations were quantified in two ways: 1) by studying increases or decreases in abundance; and 2) by studying posttranslational modification resulting in changes in the overall charge of the protein. To quantify the extent of charge modification of individual proteins, the charge modification index was developed (Anderson *et al.*, 1992). This index is the sum of abundances of various charge-modified forms weighted by the number of charges added, divided by the unweighted sum of abundances. The index yields the overall average number of charges added per protein molecule and provides a useful measure of the level of covalent chemical modification of a target protein by a reactive drug metabolite or other reactive species.

MATERIALS AND METHODS

PROCUREMENT AND CHARACTERIZATION

Methapyrilene Hydrochloride

Methapyrilene hydrochloride (lot 37F-0929), a crystalline solid, was obtained from Sigma Chemical Company (St. Louis, MO). The manufacturer estimated the purity to be greater than 99% by perchloric acid titration. The bulk chemical was stored protected from light at room temperature.

Pyrilamine Maleate

Pyrilamine maleate (lot 90H0501), a crystalline solid, was obtained from Sigma Chemical Company. The manufacturer estimated the purity to be 100% by perchloric acid titration. The bulk chemical was stored at room temperature.

Wy-14,643

Wy-14,643 (lots CSL-85-029-82-30 and CSL-91-315-72), a white powder, was obtained from Chemsyn Science Laboratories (Lenexa, KA). The manufacturer estimated the purity to be greater than 98% with high-performance liquid chromatography. The bulk chemical was stored at room temperature.

Bromodeoxyuridene

Bromodeoxyuridene (BrdU; lots 21H0066 and 10H0652), a white powder, was obtained from Sigma Chemical Company. Stability and purity were determined by the manufacturer. The bulk chemical was stored at -20° C and protected from light.

PREPARATION OF DOSE FORMULATIONS

All dosed feed formulations of methapyrilene hydrochloride, pyrilamine maleate, and Wy-14,643 were prepared by mixing the appropriate amount of the chemical with NIH-07 open formula diet (Ziegler Brothers, Inc., Gardners, PA). Dose formulations were prepared 7 days before the study began and at 2-week intervals thereafter.

All dose formulations were stored protected from light at room temperature and were used within 3 weeks of preparation.

BrdU dose formulations (30 mg/mL; 2 mL per pump; 10 μL/hour flow rate), administered to assess hepatocyte proliferation, were prepared by diluting the appropriate amount of the chemical with 0.01 N sodium hydroxide. The mixture was then vortexed, stirred, and sonicated at 37° to 40° C. Samples were then passed through sterile filters into sterile foil-wrapped vials.

14-WEEK STUDY

Male F344/N rats were obtained from Harlan Sprague-Dawley (Indianapolis, IN). Rats were 4 weeks old at receipt and were quarantined for 13 to 14 days before the study began. At the end of the quarantine, blood samples were collected from five animals. The sera were analyzed for antibody titers to rodent viruses (Boorman *et al.*, 1986; Rao *et al.*, 1989); all results were negative.

Groups of 40 male rats were exposed to methapyrilene hydrochloride in feed at concentrations of 0, 50, 100, 250, or 1,000 ppm. Additional groups of 40 male rats were exposed to 50 ppm Wy-14,643 (positive control) or 1,000 ppm pyrilamine maleate (negative control) in feed. Details of the study design and animal maintenance are listed in Table 1.

To assess the effects of methapyrilene hydrochloride exposure on hepatocyte proliferation, each group was divided into four groups of 10 rats to be evaluated on days 15, 29, and 43 and at week 14. Seven days before each evaluation period, rats in the scheduled group were anesthetized with Ketamine®:Rompun® (60:40); a 7-day osmotic minipump (Alzet® Model 2001, Alza Corp., Palo Alto, CA) containing BrdU (30 mg/mL) was then surgically implanted under the interscapular skin of each rat.

Necropsies were performed on all animals that survived to the end of the study, and the liver and duodenum were examined. Livers were removed and weighed. A 0.5- to 1.0-g sample of the liver was removed for proliferating cell nuclear antigen (PCNA) analysis by immunohistochemical analysis (Foley *et al*., 1991) and separately for protein analysis by two-dimensional gel electrophoresis at Large Scale Biology Corporation (Rockville, MD). The remaining liver tissue was fixed in 10% neutral buffered formalin for 24 hours and then transferred to 70% ethanol. Livers were trimmed and embedded in paraffin. Triplicate sections were made from each liver and duodenum; one section was stained with hematoxylin and eosin, a second section was stained to determine the BrdU labeling index, and a third section was stained to determine the PCNA labeling index.

Upon completion of the laboratory pathologist's histologic 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. Evaluation of hepatocyte hypertrophy was performed empirically by side-by-side comparison with the untreated controls. No attempt was made to quantitate hypertrophy. Details of these review procedures have been described by Maronpot and Boorman (1982) and Boorman *et al.* (1985).

BrdU- and PCNA-labeled tissues were mounted on Superfrost Plus® coated slides (Scientific Products, McGaw Park, IL). Tissue sections were deparaffinized in xylene and then passed twice (5 minutes per pass) through automation buffer (BrdU-labeled tissues) or phosphate-buffered saline plus 0.05% Tween 20 (PCNA-labeled tissues). The tissue sections were then treated with 2 N hydrochloric acid for 30 minutes at 37° C to denature double-stranded DNA. The BrdU-labeled tissue slides were rinsed with borate buffer (pH 7.6) for 3 minutes followed by the application of a trypsin solution for 3 minutes at 37° C to expose antigenic sites and were then rinsed with distilled water for 1 minute and then with automation buffer (two changes at 5 minutes each). A 3% hydrogen peroxide block was applied to BrdU- and PCNA-labeled tissues for 10 minutes and was followed by two buffer rinses (5 minutes each). The blocking serum was then applied and the slides were incubated for 20 minutes at room temperature. Excess fluid was shaken from the tissue sections, the anti-BrdU or anti-PCNA primary antibody was applied, and the slides were incubated at room temperature for 60 minutes. The slides were again rinsed with buffer (two changes at 5 minutes each), and the biotinylated secondary antibody was applied for 30 minutes at room temperature, followed by two automation buffer rinses (5 minutes each). An avidin-biotin complex was applied to the tissue sections, which were then incubated for 30 minutes at room temperature. After two rinses with buffer (5 minutes for each rinse), 3,3'-diaminobenzidine tetrahydrochloride substrate was applied and incubated for 6 minutes at room temperature. The tissue sections were rinsed thoroughly in tap water prior to being stained with Mayer's hematoxylin for 1 minute (BrdU-labeled tissues) or 2 minutes (PCNA-labeled tissues). The tissue sections were rinsed in bluing solution, washed, and dehydrated through graded alcohols to xylene; a coverslip was applied with mounting media.

Cell proliferation based on BrdU staining was evaluated for the liver of each animal that demonstrated immunodetectable proliferation in the duodenum. The number of BrdU-labeled and unlabeled hepatocyte nuclei per eight randomly selected high-power fields ($\geq$ 3,000 cells) was counted for each animal. The BrdU labeling index was calculated by dividing the number of BrdU-labeled hepatocyte nuclei by the total number of nuclei counted (expressed as a percentage). The number of mitotic figures present in the nuclei of the hepatocytes from

eight high-power fields was recorded from the BrdU-stained slides. The mitotic index was calculated by dividing the number of cells in mitosis by the total number of cells counted (expressed as a percentage).

Cell proliferation based on PCNA labeling was evaluated in all animals by two methods, immunohistochemically on paraffin-embedded sections and by 2-dimensional electrophoresis. The number of PCNA-labeled hepatocyte nuclei in S-phase and all other nuclei per eight randomly selected high-power fields was counted for each animal. The PCNA labeling index was calculated by dividing the number of PCNA-labeled hepatocyte nuclei by the total number of nuclei counted (expressed as a percentage).

At the 43-day interim evaluation, samples from the left lobes of livers of five rats per exposure group were collected for two-dimensional gel electrophoresis for total protein analysis and quantification of PCNA. The samples were solubilized with 9 M urea, 2% NP-40 detergent, 0.5% dithiothreitol, and 2% ampholytes (pH 9-11) (Cunningham *et al.*, 1995). Samples were then centrifuged and the supernatant was removed. Acidic and neutral soluble proteins were then separated on sodium dodecyl sulfate-denaturing polyacrylamide gels based on molecular weight and isoelectric point (Anderson *et al.*, 1992) using a 20 to 25 cm ISO-DALT® system (Large Scale Biology Corp., Rockville, MD). Following electrophoresis, proteins were fixed and stained with Coomassie Blue G-250, and the resulting protein spots were digitized. Protein abundance was measured as pixel density on the digitized image.

The charge modification index, which describes the level of charge modification of each protein, was calculated by first weighting the abundance of charge-modified forms of a protein based on the number of charges added and then dividing the sum of the weighted protein abundances by the sum of unweighted protein abundances. The index yields the overall average number of charges added per protein molecule and gives a measurement of the covalent chemical modification of a target protein by a reactive drug metabolite or by other reactive species.

The charge modification index was compared between exposure groups for three mitochondrial proteins: Mitcon:1, the β subunit of the mitochondrial F_1 ATPase located on the inner membrane; Mitcon:2, a mitochondrial matrix protein inducible by heat shock and apparently analogous to the *Escherichia coli* groEL gene product, which is involved in the assembly of multimeric protein structures; and Mitcon:3, a mitochondrial matrix protein in the family of mammalian stress-inducible proteins, which binds ATP and is apparently analogous to the *E. coli dna*K and yeast SSC1 proteins. These *E. coli*- and yeast-derived proteins appear to be involved in the ATP-dependent stabilization of proteins being incorporated into larger structures. Previous studies have demonstrated that these mitochondrial proteins are altered following methapyrilene hydrochloride exposure, but no data were available for comparison to exposure to pyrilamine maleate.

The region surrounding the known location of PCNA on the two-dimensional gels (20 cm × 25 cm) was transferred onto polyvinylidene fluoride membranes (Immobilon-P, Millipore Corp., Bedford, MA) by Western blot in a DALT electrophoresis tank (Hoefer Scientific Instruments, San Francisco, CA) with Tris/glycine/SDS tank buffer at 10° C and 300 V for 30 minutes. PCNA was detected by reaction with a specific monoclonal antibody (clone PC10, Boehringer Mannheim, Indianapolis, IN) followed by an alkaline phosphatase-conjugated second antibody (goat anti-mouse Ig, KPL Laboratories, Rockville, MD). A monoclonal antibody directed against α-tropomyosin (clone TM311, Sigma Chemical Company) was included with the first antibody to allow measurement of this protein. The resulting spots were digitized. PCNA abundance was measured as pixel density on the digitized image.

TABLE 1
Experimental Design and Materials and Methods in the 14-Week Feed Study of Methapyrilene Hydrochloride

Study Laboratory
Microbiological Associates, Inc. (Bethesda, MD)

Strain and Species
Male F344/N rats

Animal Source
Harlan Sprague-Dawley (Indianapolis, IN)

Time Held Before Study
13 or 14 days

Average Age When Study Began
6 weeks

Date of First Exposure
18 or 19 November 1991

Duration of Exposure
15, 29, or 43 days, or 14 weeks

Date of Last Exposure and Necropsy
17 or 18 February 1992

Average Age at Necropsy
8, 10, 12, or 19 weeks

Size of Study Groups
40 males

Method of Distribution
Animals were distributed randomly into groups of approximately equal initial mean body weights.

Animals per Cage
5

Method of Animal Identification
Tail tattoo

Diet
NIH-07 open formula diet (Ziegler Bros., Inc., Gardners, PA) in meal form, available *ad libitum*

Water
Washington Suburban Sanitary Commission, Potomac Plant, available *ad libitum*

Cages
Polycarbonate (Laboratory Products, Inc., Maywood, NJ)

Bedding
Sani-Chip (P.J. Murphy Forest Products, Montville, NJ)

Animal Room Environment
Temperature: 22° ± 3° C
Relative humidity: 50% ± 15%
Fluorescent light: 12 hours/day
Room air: at least 10 air changes per hour

TABLE 1
Experimental Design and Materials and Methods in the 14-Week Feed Study of Methapyrilene Hydrochloride

Exposure Concentrations
Methapyrilene hydrochloride: 0, 50, 100, 250, or 1,000 ppm in feed
Wy-14,643 (positive control): 50 ppm in feed
Pyrilamine maleate (negative control): 1,000 ppm in feed

Type and Frequency of Observation
Animals were observed twice daily. Body weights and feed consumption were recorded at the start of the study and weekly thereafter.

Method of Sacrifice
Metofane® anesthetization and exsanguination

Necropsy
All surviving animals were necropsied; the liver and duodenum were examined. Liver weights were recorded.

Histopathology
None

Supplemental Evaluations
Liver samples were collected from all core study animals that survived to the end of the study. The following hepatocyte proliferation measurements were recorded:

BrdU Labeling Index
The number of BrdU-labeled and unlabeled hepatocyte nuclei per eight randomly selected high-power fields was counted; the BrdU labeling index was calculated by dividing the number of BrdU-labeled hepatocyte nuclei by the total number of nuclei counted (expressed as a percentage).

PCNA Labeling Index
The number of PCNA-labeled hepatocyte nuclei in S-phase and all other nuclei per eight randomly selected high-power fields was counted for each animal; the PCNA labeling index was calculated by dividing the number of PCNA-labeled hepatocyte nuclei by the total number of nuclei counted (expressed as a percentage).

Mitotic Index
The number of mitotic figures present in the nuclei of the hepatocytes from eight high-power fields was recorded from the BrdU-stained slides; the mitotic index was calculated by dividing the number of cells in mitosis by the total number of cells counted (expressed as a percentage).

Two-Dimensional Gel Electrophoresis
Proteins from the livers of five rats per exposure group exposed for 43 days were separated based on molecular weight and isoelectric point using a two-dimensional electrophoretic gel. Proteins from the PCNA region of the gels were transferred by Western blotting to a membrane for PCNA analysis.

STATISTICAL METHODS

Calculation and Analysis of Lesion Incidences

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

Analysis of Continuous Variables

Two approaches were employed to assess the significance of pairwise comparisons between exposed and untreated control groups in the analysis of continuous variables. Organ and body weight data, which are approximately normally distributed, were analyzed with the parametric multiple comparisons procedure of Williams (1971, 1972) or Dunnett (1955). For animals exposed to pyrilamine maleate or Wy-14,643, body weight comparisons consisted of the untreated control group and one exposed group (either chemical). Therefore, these data were analyzed by a *t*-test. For animals exposed to methapyrilene hydrochloride, the BrdU labeling index, PCNA labeling index, and mitotic index data were analyzed with the nonparametric comparisons test of Shirley (1977). For animals exposed to pyrilamine maleate or Wy-14,643, the BrdU labeling index, PCNA labeling index, and mitotic index data were analyzed using Wilcoxon's rank sum test (Conover, 1971). 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.

Proteins showing meaningful treatment-related changes in abundance were defined as those that had a *t*-test P value less than 0.001, an average within-group coefficient of variation less than 20%, and a group average value at least 30% different from the untreated control average for at least one exposed group and that were absent on no more than five gels from any group.

QUALITY ASSURANCE METHODS

The animal study of methapyrilene hydrochloride was performed in compliance with United States FDA Good Laboratory Practices regulations (21 CFR, Part 58). The Quality Assurance Unit of Microbiological Associates, Inc., performed audits and inspections of protocols, procedures, data, and reports throughout the course of the study.

GENETIC TOXICOLOGY

Salmonella typhimurium Mutagenicity Test Protocol

Testing was performed as reported by Mortelmans *et al.* (1986). Methapyrilene hydrochloride was sent to the laboratory as a coded aliquot from Radian Corporation (Austin, TX). It was incubated with the *Salmonella typhimurium* tester strains TA98, TA100, TA1535, and TA1537 either in buffer or S9 mix (metabolic activation enzymes and cofactors from Aroclor 1254-induced male Sprague-Dawley rat or Syrian hamster liver) for 20 minutes at 37° C. Top agar supplemented with L-histidine and d-biotin was added, and the contents of the tubes

were mixed and poured onto the surfaces of minimal glucose agar plates. Histidine-independent mutant colonies arising on these plates were counted following incubation for 2 days at 37° C.

Each trial consisted of triplicate plates of concurrent positive and negative controls and five doses of methapyrilene hydrochloride. The high dose was limited to 10,000 µg/plate by experimental design. All trials were repeated.

In this assay, a positive response is defined as a reproducible, dose-related increase in histidine-independent (revertant) colonies in any one strain/activation combination. An equivocal response is defined as an increase in revertants that is not dose related, is not reproducible, or is not of sufficient magnitude to support a determination of mutagenicity. A negative response is obtained when no increase in revertant colonies is observed following chemical treatment. There is no minimum percentage or fold increase required for a chemical to be judged positive or weakly positive.

Mouse Lymphoma Mutagenicity Test Protocol

The experimental protocol is presented in detail by McGregor *et al.* (1991). Methapyrilene hydrochloride was supplied as a coded aliquot by Radian Corporation. The high dose of 550 µg/mL was determined by toxicity. L5178Y mouse lymphoma cells were maintained at 37° C as suspension cultures in supplemented Fischer's medium; normal cycling time was approximately 10 hours. To reduce the number of spontaneously occurring cells resistant to trifluorothymidine (TFT), subcultures were exposed to medium containing thymidine, hypoxanthine, methotrexate, and glycine for 1 day; to medium containing thymidine, hypoxanthine, and glycine for 1 day; and to normal medium for 3 to 5 days. For cloning, horse serum content was increased and Noble agar was added to the medium.

All treatment levels within an experiment, including concurrent positive and solvent controls, were replicated. Treated cultures contained 6×10^6 cells in 10 mL medium. This volume included the S9 fraction in those experiments performed with metabolic activation. Incubation with methapyrilene hydrochloride continued for 4 hours, at which time the medium plus methapyrilene hydrochloride was removed, and the cells were resuspended in fresh medium and incubated for an additional 2 days to express the mutant phenotype. Cell density was monitored so that log phase growth was maintained. After the 48-hour expression period, cells were plated in medium and soft agar supplemented with TFT for selection of TFT-resistant cells, and cells were plated in nonselective medium and soft agar to determine cloning efficiency. Plates were incubated at 37° C in 5% CO_2 for 10 to 12 days. The test was initially performed without S9. Because a clearly positive response was not obtained, the test was repeated using freshly prepared S9 from the livers of either Aroclor 1254-induced or noninduced male Fischer 344 rats.

Minimum criteria for accepting an experiment as valid and a detailed description of the statistical analysis and data evaluation are presented in Caspary *et al.* (1988). All data were evaluated statistically for trend and peak responses. Both responses had to be significant ($P \leq 0.05$) for methapyrilene hydrochloride to be considered positive, i.e., capable of inducing TFT resistance. A single significant response led to a call of "questionable," and the absence of both a trend and a peak response resulted in a "negative" call.

Chinese Hamster Ovary Cell Cytogenetics Protocols

Testing was performed as reported by Galloway *et al.* (1987). Methapyrilene hydrochloride was sent to the laboratory as a coded aliquot by Radian Corporation. It was tested in cultured Chinese hamster ovary (CHO) cells for induction of sister chromatid exchanges (SCEs) and chromosomal aberrations (Abs), both in the presence and absence of Aroclor 1254-induced male Sprague-Dawley rat liver S9 and cofactor mix. Cultures were handled under gold lights to prevent photolysis of BrdU-substituted DNA. Each test consisted of concurrent solvent and positive controls and of at least three doses of methapyrilene hydrochloride; the high dose was limited by toxicity. A single flask per dose was used.

Sister Chromatid Exchange Test: In the SCE test without S9, CHO cells were incubated for 26 hours with methapyrilene hydrochloride in supplemented McCoy's 5A medium. BrdU was added 2 hours after culture initiation. After 26 hours, the medium containing methapyrilene hydrochloride was removed and replaced with fresh medium plus BrdU and Colcemid, and incubation was continued for 1.8 hours. Cells were then harvested by mitotic shake-off, fixed, and stained with Hoechst 33258 and Giemsa. In the SCE test with S9, cells were incubated with methapyrilene hydrochloride, serum-free medium, and S9 for 2 hours. The medium was then removed and replaced with medium containing serum and BrdU and no methapyrilene hydrochloride. Incubation proceeded for an additional 25.8 to 26 hours, with Colcemid present for the final 2 hours. Harvesting and staining were the same as for cells treated without S9. All slides were scored blind and those from a single test were read by the same person. Fifty second-division metaphase cells were scored for frequency of SCEs/cell from each dose level. Because significant chemical-induced cell cycle delay was seen at higher concentrations of methapyrilene hydrochloride, incubation time was lengthened to ensure a sufficient number of scorable (second-division metaphase) cells.

Statistical analyses were conducted on the slopes of the dose-response curves and the individual dose points (Galloway *et al.*, 1987). An SCE frequency 20% above the concurrent solvent control value was chosen as a statistically conservative positive response. The probability of this level of difference occurring by chance at one dose point is less than 0.01; the probability for such a chance occurrence at two dose points is less than 0.001. An increase of 20% or greater at any single dose was considered weak evidence of activity; increases at two or more

doses resulted in a determination that the trial was positive. A statistically significant trend ($P<0.005$) in the absence of any responses reaching 20% above background led to a call of equivocal.

Chromosomal Aberrations Test: In the Abs test without S9, cells were incubated in McCoy's 5A medium with methapyrilene hydrochloride for 22.2 hours; Colcemid was added and incubation continued for 2 hours. The cells were then harvested by mitotic shake-off, fixed, and stained with Giemsa. For the Abs test with S9, cells were treated with methapyrilene hydrochloride and S9 for 2 hours, after which the treatment medium was removed and the cells were incubated for 8.5 hours in fresh medium, with Colcemid present for the final 2 hours. Cells were harvested in the same manner as for the treatment without S9. The harvest time for the Abs test was based on the cell cycle information obtained in the SCE test: because cell cycle delay was anticipated in the absence of S9, the incubation period was extended.

Cells were selected for scoring on the basis of good morphology and completeness of karyotype (21 ± 2 chromosomes). All slides were scored blind and those from a single test were read by the same person. One hundred first-division metaphase cells were scored at each dose concentration. Classes of aberrations included simple (breaks and terminal deletions), complex (rearrangements and translocations), and other (pulverized cells, despiralized chromosomes, and cells containing 10 or more aberrations).

Chromosomal aberration data are presented as percentages of cells with aberrations. To arrive at a statistical call for a trial, analyses were conducted on both the dose response curve and individual dose points. For a single trial, a statistically significant ($P \leq 0.05$) difference for one dose point and a significant trend ($P \leq 0.015$) were considered weak evidence for a positive response; significant differences for two or more doses indicated the trial was positive. A positive trend in the absence of a statistically significant increase at any one dose resulted in an equivocal call (Galloway *et al*., 1987). Ultimately, the trial calls were based on a consideration of the statistical analyses as well as the biological information available to the reviewers.

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 protocols. There have been instances, however, in which multiple aliquots of a chemical were tested in the same assay, and differing 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

Four rats scheduled for evaluation at 15 days died on day 8 due to anesthesia complications during the bromodeoxyuridine (BrdU) pump implantation procedure (Table 2a). These rats included one rat each from the untreated control and 100 ppm groups and two rats from the 50 ppm Wy-14,643 (positive control) group. No other deaths occurred during the study (Tables 2b, 2c, and 2d).

Low mean body weights were generally observed in the 1,000 ppm methapyriline hydrochloride group and the positive control group. Final mean body weights and mean body gains of rats exposed to 1,000 ppm methapyriline hydrochloride were significantly less than those of the untreated control group on days 15, 29, and 43 and at week 14 (Tables 2a, 2b, 2c, and 2d and Figure 1). The 1,000 ppm concentration exceeded the maximum tolerated dose, as demonstrated by the reduced body weight gain in this group (Figure 1).

The average daily doses delivered to rats in the 14-week study by dietary concentrations of 50, 100, 250, and 1,000 ppm methapyrilene hydrochloride, 1,000 ppm pyrilamine maleate (negative control), and 50 ppm Wy-14,643 are provided in Tables 2a, 2b, 2c, and 2d. Feed consumption by rats exposed to 1,000 ppm was significantly less than that by the untreated control group throughout the study, likely due to unpalatability of the dosed feed. The predominant clinical finding related to methapyrilene hydrochloride exposure was thinness in rats exposed to 1,000 ppm; this finding was first observed on day 29.

TABLE 2a
Survival, Body Weights, and Feed Consumption of Male Rats in the 14-Week Feed Study of Methapyrilene Hydrochloride: 15-Day Interim Evaluation

Exposure Concentration (ppm)	Survival[a]	Mean Body Weight[b] (g)			Day 15 Weight Relative to Untreated Controls (%)	Average Feed Consumption[c] (g/day)	Average Dose[d] (mg/kg/day)
		Initial	Day 15	Change			
Methapyrilene Hydrochloride							
0	9/10[e]	134 ± 2	202 ± 3	67 ± 3		15.4	
50	10/10	130 ± 2	200 ± 4	63 ± 4	99	15.5	5
100	9/10[e]	130 ± 4	207 ± 5	66 ± 3	102	15.5	9
250	10/10	129 ± 3	198 ± 5	62 ± 5	98	15.6	24
1,000	10/10	130 ± 3	168 ± 3**	37 ± 3**	83	13.9	93
Pyrilamine Maleate (– control)							
1,000	10/10	124 ± 4*	191 ± 5	62 ± 4	94	15.2	97
Wy-14,643 (+ control)							
50	8/10[e]	126 ± 4	191 ± 5	63 ± 7	94	16.3	5

* Significantly different ($P \leq 0.05$) from the untreated control group by a *t*-test
** Significantly different ($P \leq 0.01$) from the untreated control group by Williams' test
[a] Number of animals surviving on day 15/number initially in group
[b] Weights and weight changes are given as mean ± standard error. Subsequent calculations are based on animals surviving to the end of the study.
[c] Feed consumption is expressed as grams of feed consumed per animal per day.
[d] Compound consumption is expressed as mg chemical/kg body weight per day.
[e] Day of death: 8. Deaths were due to anesthesia complications during bromodeoxyuridine pump implantation.

TABLE 2b
Survival, Body Weights, and Feed Consumption of Male Rats in the 14-Week Feed Study of Methapyrilene Hydrochloride: 29-Day Interim Evaluation

Exposure Concentration (ppm)	Survival[a]	Mean Body Weight[b] (g)			Day 29 Weight Relative to Untreated Controls (%)	Average Feed Consumption[c] (g/day)	Average Dose[d] (mg/kg/day)
		Initial	Day 29	Change			
Methapyrilene Hydrochloride							
0	10/10	129 ± 3	251 ± 4	125 ± 5		16.2	
50	10/10	128 ± 5	249 ± 6	131 ± 6	99	16.2	4
100	10/10	130 ± 3	250 ± 3	121 ± 3	100	16.2	9
250	10/10	125 ± 3	242 ± 3	117 ± 5	97	16.5	22
1,000	10/10	125 ± 3	192 ± 4**	63 ± 3**	77	14.0	88
Pyrilamine Maleate (– control)							
1,000	10/10	127 ± 4	249 ± 3	122 ± 6	99	16.1	86
Wy-14,643 (+ control)							
50	10/10	126 ± 3	236 ± 5*	107 ± 5*	94	17.9	5

* Significantly different ($P \leq 0.05$) from the untreated control group by a *t*-test
** Significantly different ($P \leq 0.01$) from the untreated control group by Williams' test
[a] Number of animals surviving on day 29/number initially in group
[b] Weights and weight changes are given as mean ± standard error.
[c] Feed consumption is expressed as grams of feed consumed per animal per day.
[d] Compound consumption is expressed as mg chemical/kg body weight per day.

TABLE 2c
Survival, Body Weights, and Feed Consumption of Male Rats in the 14-Week Feed Study of Methapyrilene Hydrochloride: 43-Day Interim Evaluation

Exposure Concentration (ppm)	Survival[a]	Mean Body Weight[b] (g)			Day 43 Weight Relative to Untreated Controls (%)	Average Feed Consumption[c] (g/day)	Average Dose[d] (mg/kg/day)
		Initial	Day 43	Change			
Methapyrilene Hydrochloride							
0	10/10	125 ± 4	281 ± 5	149 ± 5		16.3	
50	10/10	127 ± 3	284 ± 5	162 ± 5	101	16.3	4
100	10/10	121 ± 4	271 ± 3	151 ± 5	96	16.4	8
250	10/10	129 ± 2	281 ± 4	144 ± 4	100	16.7	20
1,000	10/10	126 ± 4	202 ± 4**	72 ± 4**	72	13.7	84
Pyrilamine Maleate (– control)							
1,000	10/10	125 ± 3	283 ± 6	159 ± 6	101	16.4	80
Wy-14,643 (+ control)							
50	10/10	128 ± 3	265 ± 4*	143 ± 4	94	18.5	5

* Significantly different ($P \leq 0.05$) from the untreated control group by a *t*-test
** Significantly different ($P \leq 0.01$) from the untreated control group by Williams' test
[a] Number of animals surviving on day 43/number initially in group
[b] Weights and weight changes are given as mean ± standard error.
[c] Feed consumption is expressed as grams of feed consumed per animal per day.
[d] Compound consumption is expressed as mg chemical/kg body weight per day.

TABLE 2d
Survival, Body Weights, and Feed Consumption of Male Rats in the 14-Week Feed Study of Methapyrilene Hydrochloride: Study Termination

Exposure Concentration (ppm)	Survival[a]	Mean Body Weight[b] (g)			Final Weight Relative to Untreated Controls (%)	Average Feed Consumption[c] (g/day)	Average Dose[d] (mg/kg/day)
		Initial	Final	Change			
Methapyrilene Hydrochloride							
0	10/10	125 ± 3	355 ± 5	228 ± 6		16.2	
50	10/10	132 ± 2	363 ± 5	239 ± 5	102	16.5	3
100	10/10	129 ± 2	359 ± 6	228 ± 4	101	16.5	7
250	10/10	129 ± 4	357 ± 5	237 ± 7	100	16.8	17
1,000	10/10	129 ± 3	254 ± 6**	128 ± 7**	71	13.3	69
Pyrilamine Maleate (– control)							
1,000	10/10	129 ± 3	350 ± 5	221 ± 8	99	16.1	67
Wy-14,643 (+ control)							
50	10/10	130 ± 3	316 ± 7**	180 ± 6**	89	19.1	4

** Significantly different ($P \leq 0.01$) from the untreated control group by Williams' test (methapyrilene hydrochloride) or by a *t*-test (Wy-14,643)
[a] Number of animals surviving at 13 weeks/number initially in group
[b] Weights and weight changes are given as mean ± standard error.
[c] Feed consumption is expressed as grams of feed consumed per animal per day.
[d] Compound consumption is expressed as mg chemical/kg body weight per day.

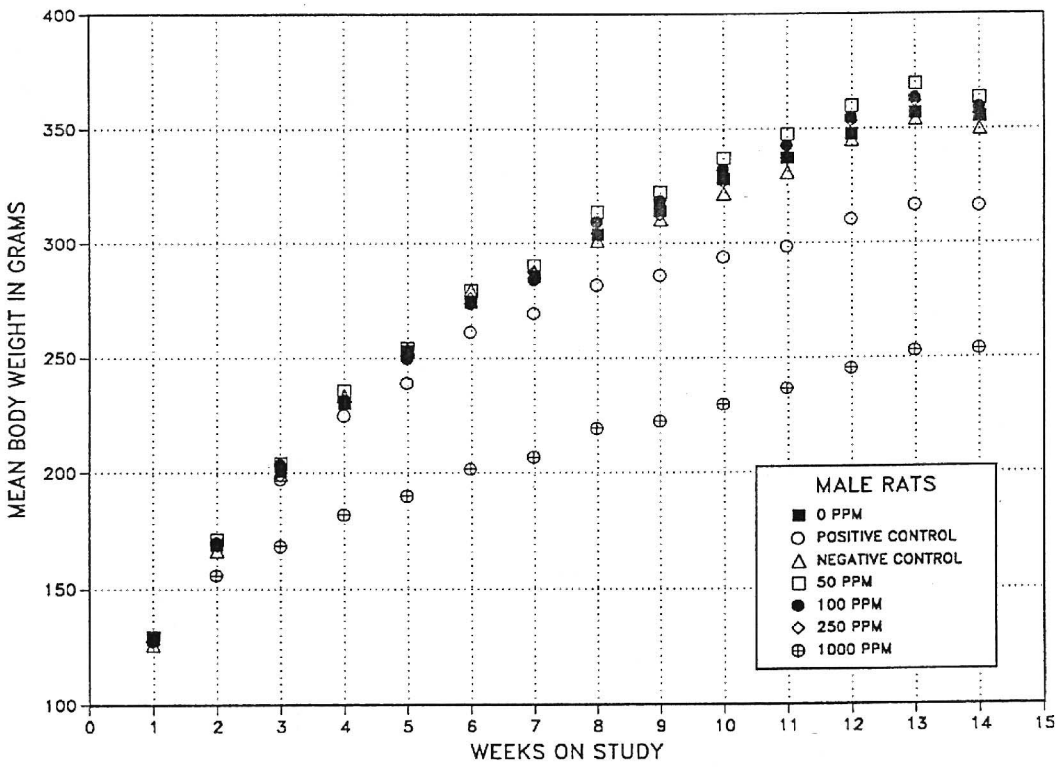


FIGURE 1
Body Weights for Male Rats Exposed to Methapyrilene Hydrochloride in Feed for 14 Weeks

On days 29 and 43 and at 14 weeks, the absolute liver weights of rats exposed to 1,000 ppm methapyriline hydrochloride were significantly less than those of the untreated control group (Table A). At all time points, the relative liver weights of rats exposed to 1,000 ppm and the absolute and relative liver weights of positive control rats were significantly greater than those of the untreated control group. No significant differences in absolute or relative liver weights were observed between the negative control and untreated control groups at any time point.

Hepatic lesions were observed predominantly in the 250 and 1,000 ppm methapyrilene hydrochloride groups and in the positive control group. The incidences of bile duct hyperplasia, hepatocyte necrosis, hepatocyte mitosis, and hepatocyte hypertrophy in rats in the 1,000 ppm group were significantly greater than those in the untreated control group at all time points (Table 3). The severities of hepatocyte mitosis and hepatocyte hypertrophy in 1,000 ppm rats were generally mild to moderate; the lesions occurring in the 250 ppm group were less severe. At each time

TABLE 3
Incidences of Selected Nonneoplastic Lesions of the Liver in Male Rats in the 14-Week Feed Study of Methapyrilene Hydrochloride

	Methapyrilene Hydrochloride										1,000 ppm Pyrilamine Maleate (– control)		50 ppm Wy-14,643 (+ control)	
	0 ppm		50 ppm		100 ppm		250 ppm		1,000 ppm					
Day 15														
Number Examined Microscopically	9		10		9		10		10		10		8	
Bile Duct Hyperplasia[a]	0		0		0		10**	(1.0)[b]	10**	(1.9)	0		1	(2.0)
Hepatocyte Necrosis	0		0		0		3	(1.0)	10**	(1.2)	0		0	
Hepatocyte Mitosis	0		1	(1.0)	1	(2.0)	10**	(2.4)	10**	(3.1)	5*	(1.4)	8**	(1.3)
Hepatocyte Hypertrophy	0		0		0		0		10**	(2.0)	0		7**	(2.0)
Granulomatous Inflammation	0		1	(3.0)	0		0		0		0		7**	(1.4)
Day 29														
Number Examined Microscopically	10		10		10		10		10		10		10	
Bile Duct Hyperplasia	0		0		0		10**	(1.4)	10**	(2.7)	0		1	(1.0)
Hepatocyte Necrosis	0		0		0		10**	(1.0)	10**	(1.2)	0		0	
Hepatocyte Mitosis	0		0		0		6**	(1.2)	10**	(2.2)	0		6**	(1.0)
Hepatocyte Hypertrophy	0		0		0		0		10**	(3.0)	0		10**	(2.1)
Pigmentation	0		0		0		7**	(1.0)	10**	(1.0)	0		0	
Granulomatous Inflammation	0		0		0		1	(1.0)	1	(1.0)	0		8**	(1.8)
Day 43														
Number Examined Microscopically	10		10		10		10		10		10		10	
Bile Duct Hyperplasia	0		0		0		10**	(1.7)	10**	(3.1)	0		0	
Hepatocyte Necrosis	0		0		0		8**	(1.0)	10**	(1.1)	0		0	
Hepatocyte Mitosis	0		0		0		3	(1.3)	10**	(2.2)	0		5*	(1.0)
Hepatocyte Hypertrophy	0		0		0		0		10**	(2.6)	0		10**	(2.0)
Pigmentation	0		0		0		10**	(1.0)	9**	(1.9)	0		0	
Eosinophilic Focus	0		0		0		0		1	(1.0)	0		0	
Granulomatous Inflammation	0		0		0		0		0		0		6**	(1.3)
Week 14														
Number Examined Microscopically	10		10		10		10		10		10		10	
Bile Duct Hyperplasia	1	(1.0)	1	(1.0)	7**	(1.0)	10**	(1.9)	10**	(4.0)	0		2	(1.0)
Hepatocyte Necrosis	0		0		0		2	(1.0)	10**	(1.0)	0		0	
Hepatocyte Mitosis	0		0		0		0		10**	(1.8)	0		0	
Hepatocyte Hypertrophy	0		0		0		0		10**	(3.3)	0		10**	(2.0)
Pigmentation	0		0		0		10**	(1.9)	10**	(3.0)	0		3	(1.0)
Eosinophilic Focus	0		0		0		0		8**		0		0	
Cholangiofibrosis	0		0		0		0		8**	(2.9)	0		0	
Granulomatous Inflammation	0		0		0		0		0		0		1	(1.0)

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

point, the incidence of bile duct hyperplasia in the 250 ppm rats was significantly greater than that in the untreated control group. The incidences of hepatocyte mitosis on days 15 and 29 and the incidences of hepatocyte necrosis on days 29 and 43 in the 250 ppm rats were significantly greater than those in the untreated control group. With the exception of bile duct hyperplasia at 14 weeks in the 100 ppm group, incidences of nonneoplastic lesions in the 50 and 100 ppm groups were not significantly different from those in the untreated controls group. Incidences of pigmentation in the 250 and 1,000 ppm groups were significantly greater than those in the untreated control group on days 29 and 43 and at 14 weeks. The severity of hepatocyte hypertrophy in the 1,000 ppm group was generally greater than that in the positive control group at each time point. No rats developed liver neoplasms during the 14-week study.

Hepatic lesions were also observed in the positive control group (Table 3). The incidences of granulomatous inflammation were significantly greater than those in the untreated control group on days 15, 29, and 43; significantly increased incidences of granulomatous inflammation were not observed in rats exposed to methapyrilene hydrochloride. The incidences of hepatocyte hypertrophy and hepatocyte mitosis in the positive control group were significantly greater than those in the untreated group on days 15, 29, and 43. The incidence of hepatocyte hypertrophy, but not hepatocyte mitosis, was also significantly increased in the positive control group at 14 weeks. Except for an increased incidence of hepatocyte mitosis on day 15, no lesions occurred in the livers of negative control rats (Table 3).

Due to a problem with the BrdU-containing infusion pumps, no animals examined on day 15 and only half of those examined on day 29 were evaluated for hepatocyte proliferation by BrdU staining. With the exception of the 250 ppm group on day 29, the mean BrdU labeling indexes of the 250 and 1,000 ppm groups were significantly greater than those of the untreated controls at all time points (Table B). The BrdU labeling index of the positive control group was significantly greater than that of the untreated controls at all time points. The BrdU labeling index of the negative control group was significantly less than that of the untreated controls on day 29. The indexes of the 50 and 100 ppm groups were also less than that of the negative controls on day 29.

On day 43 and at week 14, the mean proliferating cell nuclear antigen (PCNA) labeling indexes of the 1,000 ppm group were significantly greater than those of the untreated control group (Table B). On day 29, the mean PCNA labeling index of the positive control group was significantly greater than that of the untreated control group. The mean PCNA labeling indexes of the negative control group were significantly less than those of the untreated control group on days 29 and 43.

The mitotic indexes of the 1,000 ppm group were significantly greater than those of the untreated control group at all time points (Table B). The mitotic indexes of the 250 ppm group were significantly greater than those of the untreated control group on day 43 and at week 14. It appears that the increased liver weights and increased liver-weight-to-body-weight ratios were a combination of increased hepatocyte volume due to hypertrophy (Table 3) and an increased rate of cell division demonstrated by the increased mitotic indexes and BrDU incorporation (Table B).

Alterations in the number of protein abundance changes were observed following methapyrilene hydrochloride exposure. Abundance changes of at least 32 proteins were observed after 43 days of exposure to 1,000 ppm (Table 4). At least 39 protein abundance changes were observed in samples from the positive control group after 43 days of exposure. Many of the protein abundance changes were observed in both the methapyrilene hydrochloride-exposed groups and the positive control group. Methapyrilene hydrochloride produced covalent modification of mitochondrial proteins as measured by the charge modification index; however, there was no covalent modification of cytosolic proteins (Table 4). No covalent modification of mitochondrial or cytosolic proteins was observed in the positive control group. No protein abundance changes or covalent modification of mitochondrial or cytosolic proteins were observed in the negative control group.

PCNA abundance values in liver samples from the 250 and 1,000 ppm groups were significantly greater than the value for the untreated control group as measured by 2-dimensional gel electrophoresis (Table 5). The PCNA abundance in the liver of 1,000 ppm rats was approximately fourfold greater than that of the untreated control group. These data are in agreement with data obtained immunohistochemically which indicated an increase in PCNA expression following exposure to 1,000 methapyrilene hydrochloride (Table B).

TABLE 4
Protein Abundance Changes and Charge Modification Indexes of Three Mitochondrial Proteins in the Liver of Male Rats on Day 43 in the 14-Week Feed Study of Methapyrilene Hydrochloride[a]

Exposure Concentration (ppm)	Number of Protein Abundance Changes	Charge Modification Index		
		Mitcon:1	Mitcon:2	Mitcon:3
Methapyrilene Hydrochloride				
0	–	−0.30	−0.34	−0.36
50	0	−0.34	−0.34	−0.35
100	2	−0.37	−0.39	−0.37
250	6	−0.59	−0.61	−0.66
1,000	32	−1.45	−1.45	−1.32
Pyrilamine Maleate (– control)				
1,000	0	−0.24	−0.31	−0.31
Wy-14,643 (+ control)				
50	39	−0.39	−0.40	−0.33

[a] Cunningham *et al*. (1995)

TABLE 5
PCNA Abundance in Liver Protein Samples from Male Rats on Day 43 in the 14-Week Feed Study of Methapyrilene Hydrochloride[a]

	Methapyrilene Hydrochloride					1,000 ppm Pyrilamine Maleate (– control)	50 ppm Wy-14,643 (+ control)
	0 ppm	50 ppm	100 ppm	250 ppm	1,000 ppm		
Number of Rats	5	5	5	5	5	5	5
Number of Samples	18	22	15	16	23	21	19
PCNA Abundance × 10^3	7.08 ± 3.15	7.65 ± 3.73	6.75 ± 3.75	11.58 ± 4.09*	26.90 ± 10.64*	7.94 ± 3.68	10.16 ± 3.75

* Significantly different ($P \leq 0.05$) from the untreated control group by a *t*-test
[a] Abundance is a pixel density of spots digitized from two-dimensional electrophoretic gels; PCNA abundance data are presented as mean ± standard deviation.

GENETIC TOXICOLOGY

Results of tests for induction of mutations by methapyrilene hydrochloride (33 to 10,000 µg/plate) were negative in *Salmonella typhimurium* strains TA98, TA100, TA1535, and TA1537 when tested in a preincubation protocol with or without induced hamster or rat liver S9 metabolic activation enzymes (Mortelmans *et al.*, 1986; Table C1). No significant, reproducible increases in mutations were observed in L5178Y mouse lymphoma cells treated with methapyrilene hydrochloride, with or without S9 (McGregor *et al.*, 1991; Table C2). Viable doses tested in the absence of S9 ranged from 50 to 300 µg/mL; with S9, concentrations up to 550 µg/mL were tested. In contrast to the negative results obtained in bacterial and mammalian cell mutagenicity tests, results of tests for induction of chromosomal damage in cultured Chinese hamster ovary cells were positive. Methapyrilene hydrochloride induced sister chromosome exchanges, with and without S9, at doses that induced significant cell cycle delay (Table C3). Without S9, only the highest dose tested in each of two trials yielded a weakly positive response. With S9, positive responses were recorded at doses of 696.4 µg/mL and greater. The doses of methapyrilene hydrochloride that were tested with S9 (for example, 850 and 900 µg/mL, Trial 3) were not widely separated, however, and the positive responses observed represented only about a 20% increase over background. Chromosomal aberrations were induced by methapyrilene hydrochloride in cultured Chinese hamster ovary cells, but only in the presence of S9 (Table C4). In this test, strongly positive, dose-related responses were noted at all three concentrations tested.

DISCUSSION

The biochemical effects of carcinogenic chemicals that exert little or no genotoxicity in *in vitro* assays are a subject of intense research interest. The fact that a nongenotoxic compound such as methapyrilene hydrochloride produces such a dramatic carcinogenic effect in rats limits the usefulness of commonly used *in vitro* assays of genotoxicity for the prediction of carcinogenicity. Therefore, research into such nongenotoxic mechanisms of carcinogenesis provides information about the process of carcinogenesis as well as information about how a specific chemical induces carcinogenesis.

In the current study, the predominant clinical manifestation of exposure to methapyrilene hydrochloride was decreased body weight gain secondary to reduced feed consumption. This was likely due to unpalatibility of the dosed feed. In spite of the reduced feed intake of the exposed rats, the average dose of methapyrilene hydrochloride (69 to 93 mg/kg body weight per day) for the 1,000 ppm group was quite similar to the average dose (69 to 97 mg/kg per day) for the 1,000 ppm pyrilamine maleate (negative control) group at all time points. Although the 1,000 ppm group consumed less feed, methapyrilene hydrochloride intake was increased over the lower exposure groups when corrected for body weight. This also explains the observation that while 1,000 ppm methapyrilene hydrochloride caused reductions in absolute liver weights compared to the untreated controls on days 29 and 43 and at the end of the study, the relative liver weights were greater at all time points.

Histopathologic examination of liver sections showed that methapyrilene hydrochloride produced extensive hepatotoxicity, with hepatic lesions occurring in the 250 and 1,000 ppm groups at all time points. Bile duct hyperplasia was observed on day 15 in the 250 and 1,000 ppm groups and was sustained throughout the study. By 14 weeks, bile duct hyperplasia occurred in the 100 ppm group, and the severity of this lesion in the 1,000 ppm group was marked. Hepatocyte necrosis occurred in all animals exposed to 1,000 ppm methapyrilene hydrochloride at all time points and in most animals exposed to 250 ppm for 29 or 43 days. Minimal hepatocyte necrosis was observed on day 15 in the 250 ppm group. Hepatocyte hypertrophy was observed on day 15 in the 1,000 ppm group only and increased in severity over time. Pigmentation was observed in the 250 and 1,000 ppm groups beginning on day 29 and increased in severity with time; pigmentation in the 1,000 ppm group was more severe than that in the 250 ppm group. In carcinogenicity studies of methapyrilene hydrochloride in feed, almost

all F344/N rats exposed to 250 ppm had either hepatic carcinomas or neoplastic nodules (18/20 males and 20/20 females), whereas 40% of rats exposed to 125 ppm developed neoplastic nodules (Lijinsky, 1984).

Hepatocyte hypertrophy was the primary histopathologic effect observed in the animals treated with the positive control, the hepatocarcinogen Wy-14,643. Granulomatous inflammation was also observed on day 15 in the positive controls and increased in severity by day 29, but cleared by 14 weeks. This lesion was uncommon in the methapyrilene hydrochloride-exposed groups throughout the study. Incidences of hepatocyte hypertrophy and mitosis were significantly greater in positive control animals than in the untreated controls on days 15, 29, and 43. Incidences of hepatocyte mitosis, but not hypertrophy, in the positive controls returned to untreated control levels by 14 weeks. Pyrilamine maleate has been determined to be noncarcinogenic or equivocal for neoplasm induction in rats (Lijinsky, 1984). In the current study, the only nonneoplastic lesion occurring with a significantly increased incidence in the negative control group was hepatocyte mitosis on day 15. This lesion disappeared by day 29.

All three indexes of cell division, namely, bromodeoxyuridine (BrdU) incorporation, proliferating cell nuclear antigen (PCNA) expression, and mitotic index, were increased by methapyrilene hydrochloride exposure, although with slightly different time and dose responses. These differences are likely due to the different endpoints assessed by these three methods. Thus, while the BrdU labeling index is a cumulative measure of total cell division over the course of the osmotic minipump implantation, the PCNA labeling index quantifies the amount of PCNA protein expressed in S-phase cells at the moment of death, and the mitotic index measures the number of cells actually dividing at the time of death. Because full necropsies were not performed, animals were sacrificed close together to avoid diurnal effects on cell replication.

PCNA staining for hepatocyte cell division was increased on day 43 and at week 14 in the 1,000 ppm methapyrilene hydrochloride group. Significant BrdU labeling was observed in rats exposed to 1,000 ppm at all time points and in rats exposed to 250 ppm on day 43 and at week 14. Significantly, the magnitude of the response was maintained throughout the exposure period, indicating that cell proliferation was of a regenerative nature secondary to cytotoxicity and necrosis.

The positive control produced a transient significant increase in PCNA labeling on day 29. It also increased the BrdU labeling index at all time points measured, although this effect decreased in magnitude over time, reflecting the mitogenic nature of this compound. The negative control produced a significant decrease in PCNA labeling on days 29 and 43. Pyrilamine maleate did not increase the BrdU labeling index at any time and may have actually decreased it on day 29.

The results for Wy-14,643 are consistent with results reported previously (Marsman *et al*., 1988). The mitotic index evaluations failed to identify this chemical as increasing the rate of cell proliferation. Measurement of cell proliferation by BrdU incorporation or PCNA analysis produced qualitatively similar results. The BrdU labeling index appears to be a much more sensitive indicator of cell proliferation than either the PCNA labeling index or the mitotic index. Like the PCNA labeling index, the mitotic index offers only a snapshot of those cells in mitosis at the time of sacrifice, whereas the BrdU labeling index reflects all cells that had incorporated BrdU into DNA since the time of implantation of the osmotic minipump.

Two-dimensional gel electrophoresis was used to quantify PCNA abundance on day 43. PCNA abundance in liver samples from the rats exposed to 250 or 1,000 ppm methapyrilene hydrochloride was significantly greater than untreated control values and resembled the results obtained from the immunohistochemical analysis. Wy-14,643 produced protein abundance changes that were consistent with its effects as a cell and peroxisome proliferator. Of interest is the identity of the proteins changed by chemical treatment. Twenty of the 32 protein changes in the livers of animals exposed to 1,000 ppm methapyrilene hydrochloride also occurred in the livers of the positive controls. These 20 may represent proteins involved in the mechanics of cell replication, because both methapyrilene hydrochloride and Wy-14,643 produced this effect. The remaining protein abundance changes are less well characterized and may represent the unique toxic biochemical effects of these chemicals.

Protein analysis by 2-dimensional electrophoresis has been shown to be a useful tool for investigational toxicology studies. In the present study, protein changes that were different from the control were identified in methapyrilene hydrochloride groups exposed to concentrations as low as 100 ppm. These changes occurred in an exposure concentration-dependent manner, increasing with increasing methapyrilene hydrochloride exposure. These results confirm the protein changes observed by other groups (Anderson *et al.*, 1992; Richardson *et al.*, 1993). Two-dimensional gel electrophoresis is being increasingly used for the estimation of protein alterations induced by xenobiotics. This type of analysis complements and extends gene expression assays such as differential display because it measures expressed protein and not just mRNA. Although the identification of each altered protein is not without some difficulty, the simultaneous measurement of a large number of proteins allows for the possibility of identifying individual proteins expressed as a result of various chemical treatments, thus leading to a greater understanding of toxicological mechanisms. The significance of the protein alterations is not known, but they may be of predictive value for assessing the carcinogenicity and toxicity of chemicals in preclinical assays.

In summary, both methapyrilene hydrochloride and Wy-14,643, a nonmutagenic but potent hepatocarcinogen, induced hepatic cell proliferation in rats, further supporting the link between induced cell proliferation and carcinogenesis. Data in support of this conclusion were also presented by other investigators. Richardson *et al*.

(1992) demonstrated that 1,000 ppm methapyrilene produced hepatocellular labeling indexes of up to 67.8% in rats treated for 12 weeks. This strong degree of cell proliferation was not observed in the mouse, a species resistant to the hepatocarcinogenicity of this chemical, following 12 weeks of exposure to 1,000 ppm methapyrilene. These authors concluded that chronic toxicity was related to the carcinogenicity of methapyrilene in the rat and that neither toxicity nor carcinogenicity were induced in the mouse.

The increased cell proliferation following exposure to methapyrilene hydrochloride observed in the present study may be considered compensatory hyperplasia to replace necrotic cells. In contrast, the cell proliferation induced by Wy-14,643 is not associated with toxicity and may be considered to be due to a mitogen-type stimulation. Both types of induced cell proliferation are associated with high incidences of hepatocarcinogenesis. It has been speculated that increased cell proliferation *per se* may result in DNA lesions as a consequence of unfaithful DNA replication, mitotic recombination, or gene conversion (Ames *et al*., 1993). Mutagenesis increased by mitogenesis is an appealing theory to explain the effects of nongenotoxic carcinogens that are negative in *in vitro* assays for mutagenesis but are positive carcinogens *in vivo*. Mutagenic by-products of cytotoxicity and cell division, such as active oxygen species and lipid peroxidation metabolites, may induce mutagenesis *in vivo* yet remain undetected in the commonly used mutagenesis assays (Ames *et al.,* 1993). Rapidly proliferating cells may not have sufficient time to repair DNA damage from various sources. Enhanced cell replication may also result in oncogene activation due to hypomethylation of chromatin (Vorce and Goodman, 1989; Ray *et al.,* 1994). Chemical-induced cell proliferation as a result of chronic treatment may also promote the clonal expansion of preneoplastic cells during the promotion and progression stages of multistage carcinogenesis. Additionally, it has become clear that for the proper interpretation of studies on chemically induced cell proliferation, the proliferation must occur in the pluripotent cell population (stem cells) for the tissue at risk. These cells have the capacity to become neoplastic under the right circumstances, and increasing cell proliferation in this population increases cancer risk. Increasing cell proliferation rates in nonpluripotent cells (tetraploid or higher ploidy) are not associated with a higher cancer risk (Cohen, 1998).

These results provide support for the hypothesis that chemically induced cell proliferation is associated with carcinogenesis. Similar conclusions have been drawn by other investigators (Cohen and Ellwein, 1990; Preston-Martin *et al.,* 1990). Other investigators (Tennant *et al.,* 1987; Zeiger, 1987) have demonstrated that *in vitro* mutagenicity data for a chemical is not sufficient to predict its carcinogenicity in 2-year bioassays as conducted by the National Toxicology Program. The results presented here represent a continuing effort to determine the sources of these apparently discordant results and are of value in understanding the differences between *in vitro* and *in vivo* test systems for the evaluation of results of animal bioassays for chemical carcinogenesis.

Based on the data presented in this report, it is concluded that the ability of methapyrilene hydrochloride to produce hepatic carcinoma in almost 100% of treated animals in a carcinogenesis bioassay is related to its ability to produce a massive and sustained increase in cell replication. This conclusion is supported by the inability of the structurally similar yet nonhepatocarcinogenic chemical, pyrilamine maleate, to produce an increase in cell proliferation at the exposure concentration (1,000 ppm) at which methapyrilene hydrochloride induced a 20- to 40-fold increase in the hepatocyte labeling indexes above those seen in the untreated controls.

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APPENDIX A
LIVER WEIGHTS AND LIVER-WEIGHT-TO-BODY-WEIGHT RATIOS

TABLE A
Liver Weights and Liver-Weight-to-Body-Weight Ratios for Male Rats in the 13-Week Feed Study of Methapyrilene Hydrochloride[a]

	Methapyrilene Hydrochloride					1,000 ppm Pyrilamine Maleate (– control)	50 ppm Wy-14,643 (+ control)
	0 ppm	50 ppm	100 ppm	250 ppm	1,000 ppm		
Day 15							
n	9	10	8	10	10	10	8
Necropsy body wt	202 ± 3	200 ± 4	207 ± 5	198 ± 5	168 ± 3**	191 ± 5	191 ± 5
Absolute	9.416 ± 0.302	9.293 ± 0.276	9.665 ± 0.357	9.207 ± 0.322	8.855 ± 0.226	8.652 ± 0.232	17.715 ± 0.632**
Relative	46.44 ± 0.82	46.35 ± 1.03	46.75 ± 0.83	46.54 ± 0.67	52.64 ± 1.11**	45.36 ± 0.49	92.71 ± 2.58**
Day 29							
n	10	10	10	10	10	10	10
Necropsy body wt	251 ± 4	249 ± 6	250 ± 3	242 ± 3	192 ± 4**	249 ± 3	236 ± 5*
Absolute	10.588 ± 0.222	10.643 ± 0.309	10.571 ± 0.160	10.503 ± 0.231	9.102 ± 0.216**	10.534 ± 0.298	21.913 ± 0.582**
Relative	42.23 ± 0.59	42.74 ± 0.67	42.23 ± 0.33	43.33 ± 0.50	47.41 ± 0.72**	42.27 ± 0.86	92.65 ± 1.19**
Day 43							
n	10	10	10	10	10	10	10
Necropsy body wt	281 ± 5	284 ± 5	271 ± 3	281 ± 4	202 ± 4**	283 ± 6	265 ± 4*
Absolute	11.219 ± 0.273	11.143 ± 0.250	10.607 ± 0.173	11.838 ± 0.289	9.028 ± 0.244**	11.478 ± 0.358	23.028 ± 0.648**
Relative	39.89 ± 0.42	39.29 ± 0.51	39.14 ± 0.46	42.04 ± 0.51*	44.78 ± 0.85**	40.57 ± 1.19	86.92 ± 1.29**
Week 13							
n	10	10	10	10	10	10	10
Necropsy body wt	355 ± 5	363 ± 5	359 ± 6	357 ± 5	254 ± 6**	350 ± 5	316 ± 7**
Absolute	12.632 ± 0.234	12.356 ± 0.204	12.706 ± 0.309	12.745 ± 0.188	10.584 ± 0.220**	12.494 ± 0.302	27.571 ± 0.862**
Relative	35.58 ± 0.56	34.01 ± 0.21	35.33 ± 0.50	35.76 ± 0.27	41.75 ± 0.35**	35.68 ± 0.56	87.06 ± 0.95**

* Significantly different ($P \leq 0.05$) from the untreated control group by Williams' test (methapyrilene hydrochloride) or by a *t*-test (pyrilamine maleate and Wy-14,643)
** $P \leq 0.01$
[a] Liver weights (absolute weights) and body weights are given in grams; liver-weight-to-body-weight ratios (relative weights) are given as mg organ weight/g body weight (mean ± standard error).

APPENDIX B
CELL PROLIFERATION INDEXES

TABLE B
Indexes of Hepatocyte Proliferation for Male Rats in the 13-Week Feed Study of Methapyrilene Hydrochloride[a]

	Methapyrilene Hydrochloride					1,000 ppm Pyrilamine Maleate (– control)	50 ppm Wy-14,643 (+ control)
	0 ppm	50 ppm	100 ppm	250 ppm	1,000 ppm		
n							
Day 15	9	10	9	10	10	10	8
Day 29	5	5	5	5	5	5	5
Day 43	10	10	10	10	10	10	10
Week 13	10	10	10	10	10	10	10
BrdU labeling index (%)							
Day 29	3.76 ± 0.38	1.49 ± 0.38	1.61 ± 0.37	9.66 ± 3.67	56.43 ± 7.42*	2.33 ± 0.38*	14.87 ± 3.34*[b]
Day 43	2.46 ± 0.56	2.06 ± 0.31	2.07 ± 0.32	9.28 ± 1.14**	52.24 ± 4.56**	1.25 ± 0.20	9.98 ± 1.52**
Week 13	1.34 ± 0.22[c]	1.30 ± 0.33[d]	3.25 ± 1.49	7.58 ± 2.01**[d]	51.01 ± 4.56**[c]	0.81 ± 0.12[c]	6.20 ± 1.80**[e]
PCNA labeling index (%)							
Day 15[f]	1.985 ± 0.388	1.404 ± 0.335	1.813 ± 0.512	3.414 ± 0.759	5.431 ± 0.574	0.999 ± 0.278	2.036 ± 0.233
Day 29	1.402 ± 0.363	0.088 ± 0.055	0.234 ± 0.118	2.820 ± 0.767	9.568 ± 1.853	0.408 ± 0.184*	2.62 ± 0.54*
Day 43	0.764 ± 0.077	0.661 ± 0.163	0.561 ± 0.178	2.185 ± 0.415	14.474 ± 2.663**	0.397 ± 0.140*	1.270 ± 0.228
Week 13	0.396 ± 0.131	0.214 ± 0.085	0.180 ± 0.045	1.424 ± 0.433	8.909 ± 0.699**	0.612 ± 0.167	0.689 ± 0.122
Mitotic index (%)							
Day 29	0.012 ± 0.007	0.000 ± 0.000	0.000 ± 0.000	0.080 ± 0.049	0.512 ± 0.127**	0.026 ± 0.013	0.108 ± 0.071[b]
Day 43	0.010 ± 0.007	0.003 ± 0.003	0.009 ± 0.005	0.060 ± 0.020**	0.416 ± 0.093**	0.003 ± 0.003	0.043 ± 0.014
Week 13	0.000 ± 0.000[c]	0.008 ± 0.005[d]	0.007 ± 0.007	0.050 ± 0.013**[d]	0.333 ± 0.064**[c]	0.000 ± 0.000[c]	0.000 ± 0.000[e]

* Significantly different ($P \leq 0.05$) from the untreated control group by Shirley's test (methapyrilene hydrochloride) or by Wilcoxon's rank sum test (pyrilamine maleate and Wy-14,643)
** $P \leq 0.01$
[a] Mean ± standard error. BrdU=bromodeoxyuridine; PCNA=proliferating cell nuclear antigen
[b] n=4
[c] n=9
[d] n=8
[e] n=7
[f] Statistical analyses not performed

APPENDIX C
GENETIC TOXICOLOGY

TABLE C1
Mutagenicity of Methapyrilene Hydrochloride in Salmonella typhimurium[a]

Strain	Dose (µg/plate)	Revertants/Plate[b] −S9 Trial 1	−S9 Trial 2	−S9 Trial 3	+ 10% hamster S9 Trial 1	+ 10% hamster S9 Trial 2	+ 10% rat S9 Trial 1	+ 10% rat S9 Trial 2
TA100	0	106 ± 8.1	96 ± 4.0	97 ± 2.5	201 ± 2.6	185 ± 7.1	178 ± 1.5	226 ± 13.0
	33		108 ± 5.8	127 ± 10.5				
	100	99 ± 12.5	95 ± 2.2	122 ± 6.7	183 ± 3.6	160 ± 22.5	172 ± 11.0	245 ± 8.5
	333	109 ± 6.6	89 ± 3.2	125 ± 4.7	163 ± 3.8	163 ± 6.9	162 ± 11.0	252 ± 13.1
	1,000	102 ± 8.8	97 ± 13.0	98 ± 1.5	165 ± 13.1	167 ± 7.6	163 ± 3.7	187 ± 3.8
	3,333	Toxic	66 ± 3.3	Toxic	137 ± 22.0	131 ± 8.4	168 ± 8.3	165 ± 7.4
	10,000	0 ± 0.0			134 ± 9.9	119 ± 25.1	126 ± 21.6	118 ± 4.0
Trial summary		Negative	Negative	Negative	Negative	Negative	Negative	Negative
Positive control[c]		932 ± 10.6	2,207 ± 35.7	1,032 ± 87.9	1,321 ± 136.1	962 ± 36.7	977 ± 24.4	2,125 ± 56.7
TA1535	0	14 ± 0.6	11 ± 0.3	19 ± 0.6	20 ± 1.5	12 ± 0.7	23 ± 3.0	16 ± 4.2
	33		11 ± 0.3	17 ± 3.0				
	100	15 ± 2.6	9 ± 0.6	16 ± 2.9	23 ± 1.7	13 ± 1.7	11 ± 2.3	17 ± 1.3
	333	8 ± 1.0	9 ± 0.6	17 ± 1.5	17 ± 3.2	11 ± 2.2	16 ± 4.2	12 ± 2.0
	1,000	11 ± 2.1	10 ± 1.2	15 ± 1.3	16 ± 1.0	9 ± 2.5	16 ± 1.0	10 ± 1.9
	3,333	Toxic	9 ± 1.2	Toxic	16 ± 3.2	4 ± 0.7	13 ± 3.3	6 ± 0.3
	10,000	0 ± 0.0			9 ± 2.5	4 ± 1.3	10 ± 2.0	2 ± 0.3
Trial summary		Negative	Negative	Negative	Negative	Negative	Negative	Negative
Positive control		1,158 ± 44.0	1,241 ± 33.2	1,113 ± 49.6	183 ± 10.4	847 ± 170.6	168 ± 24.0	1,040 ± 83.1
TA1537	0	10 ± 4.1	6 ± 1.2	11 ± 2.8	22 ± 1.8	13 ± 1.3	18 ± 0.3	13 ± 2.6
	33		6 ± 1.2	14 ± 1.2				
	100	14 ± 1.2	7 ± 0.6	11 ± 1.7	27 ± 1.5	16 ± 3.6	24 ± 3.2	14 ± 2.2
	333	9 ± 0.9	4 ± 1.5	11 ± 2.1	23 ± 4.2	12 ± 2.4	18 ± 1.5	8 ± 0.9
	1,000	6 ± 2.1	5 ± 0.6	5 ± 0.7	20 ± 3.1	5 ± 2.6	16 ± 3.8	6 ± 1.2
	3,333	Toxic	2 ± 0.3	Toxic	20 ± 1.7	9 ± 1.2	19 ± 2.5	7 ± 2.5
	10,000	0 ± 0.0			10 ± 2.7	7 ± 0.3	18 ± 2.5	1 ± 0.6
Trial summary		Negative	Negative	Negative	Negative	Negative	Negative	Negative
Positive control		678 ± 121.2	251 ± 26.3	935 ± 55.3	328 ± 6.9	133 ± 7.0	273 ± 42.7	234 ± 73.9
TA98	0	17 ± 1.8	20 ± 0.3	28 ± 1.5	43 ± 2.0	36 ± 4.0	43 ± 4.9	29 ± 2.6
	33		22 ± 1.2	30 ± 3.0				
	100	30 ± 4.5	20 ± 0.3	32 ± 1.5	44 ± 4.8	34 ± 3.6	35 ± 2.4	29 ± 1.9
	333	19 ± 3.9	19 ± 3.6	33 ± 1.5	40 ± 0.6	30 ± 5.5	41 ± 2.3	19 ± 3.2
	1,000	11 ± 0.7	13 ± 3.5	28 ± 1.9	38 ± 3.5	30 ± 3.5	28 ± 4.3	19 ± 2.0
	3,333	Toxic	15 ± 0.7	Toxic	34 ± 4.4	23 ± 3.7	28 ± 2.9	14 ± 0.3
	10,000	0 ± 0.0			32 ± 1.5	22 ± 0.6	32 ± 2.3	14 ± 1.5
Trial summary		Negative	Negative	Negative	Negative	Negative	Negative	Negative
Positive control		548 ± 26.5	374 ± 10.1	602 ± 13.3	736 ± 9.3	528 ± 52.4	363 ± 7.9	1,258 ± 66.5

[a] Study was performed at Case Western Reserve University. The detailed protocol and these data are presented in Mortelmans *et al.* (1986). 0 µg/plate was the solvent control.
[b] Revertants are presented as mean ± standard error from three plates.
[c] The positive controls in the absence of metabolic activation were sodium azide (TA100 and TA1535), 9-aminoacridine (TA1537), and 4-nitro-*o*-phenylenediamine (TA98). The positive control for metabolic activation with all strains was 2-aminoanthracene.

TABLE C2
Induction of Trifluorothymidine Resistance in L5178Y Mouse Lymphoma Cells by Methapyrilene Hydrochloride[a]

Compound	Concentration (μg/mL)	Cloning Efficiency (%)	Relative Total Growth (%)	Mutant Count	Mutant Fraction[b]	Average Mutant Fraction
−S9						
Trial 1						
Distilled water[c]		93	109	188	67	
		100	114	195	65	
		105	107	211	67	
		68	69	192	94	73
Methyl methanesulfonate[d]						
	15	47	27	368	262	
		26	16	438	551	406*
Methapyrilene hydrochloride						
	50	90	74	213	79	
		88	77	191	72	76
	100	78	62	229	98	
		64	59	195	102	100
	200	68	42	238	117	
		65	37	241	124	121*
	300	51	20	183	120	
		87	35	185	71	96
	400	Lethal				
Trial 2						
Distilled water		65	105	109	56	
		71	109	85	40	
		82	92	108	44	
		68	94	89	44	46
Methyl methanesulfonate						
	15	43	33	154	121	
		36	29	130	120	121*
Methapyrilene hydrochloride						
	100	65	79	84	43	
		56	64	106	63	53
	200	55	54	90	55	
		54	51	98	61	58
	300	49	36	92	62	
		54	39	98	60	61
	400	Lethal				

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Induction of Trifluorothymidine Resistance in L5178Y Mouse Lymphoma Cells by Methapyrilene Hydrochloride

Compound	Concentration (μg/mL)	Cloning Efficiency (%)	Relative Total Growth (%)	Mutant Count	Mutant Fraction	Average Mutant Fraction
+S9						
Trial 1						
Distilled water		77	105	52	23	
		79	98	68	29	
		76	97	46	20	
		69	100	48	23	24
Methylcholanthrene[d]						
	2.5	49	26	519	353	
		55	26	592	357	355*
Methapyrilene hydrochloride						
	100	93	97	90	32	
		68	98	54	27	30
	200	72	84	68	31	
		57	70	67	39	35
	300	74	67	97	44	
		80	84	82	34	39*
	400	88	65	90	34	
		97	67	88	30	32
	500	99	39	95	32	
		79	55	95	40	36
Trial 2						
Distilled water		74	88	103	46	
		79	109	98	41	
		75	99	105	47	
		83	105	89	36	42
Methylcholanthrene						
	2.5	72	53	463	213	
		71	66	381	180	197*
Methapyrilene hydrochloride						
	150	52	54	104	66	
		53	57	27	17	42
	250	67	64	44	22	
		63	45	40	21	22
	350	64	38	59	31	
		65	40	78	40	36
	450	69	33	48	23	
		63	28	57	30	27
	550	49	5	43	29	
		63	12	50	26	28
	650	Lethal				

* Positive response ($P \leq 0.05$) versus the solvent control
[a] Study was performed at Inveresk Research International. The detailed protocol and these data are presented in McGregor *et al.* (1991).
[b] Mutant fraction (MF) (frequency) is a ratio of the mutant count to the cloning efficiency, divided by 3 (to arrive at $MF/10^6$ cells treated).
[c] Solvent control
[d] Positive control

TABLE C3
Induction of Sister Chromatid Exchanges in Chinese Hamster Ovary Cells by Methapyrilene Hydrochloride[a]

Compound	Dose (µg/mL)	Total Cells Scored	No. of Chromo-somes	No. of SCEs	SCEs/ Chromo-some	SCEs/ Cell	Hrs in BrdU	Relative Change of SCEs/ Chromosome[b] (%)
−S9								
Trial 1								
Summary: Weak positive								
Medium[c]		50	1,032	442	0.42	8.8	25.8	
Mitomycin-C[d]	0.01	5	103	215	2.08	43.0	25.8	387.37
Methapyrilene hydrochloride								
	50.0	50	1,033	466	0.45	9.3	25.8	5.33
	166.7	50	1,031	595	0.57	11.9	35.5	34.75*
	500.0	0					35.5	
					P<0.001[e]			
Trial 2								
Summary: Weak positive								
Medium		50	1,046	483	0.46	9.7	25.8	
Mitomycin-C	0.001	50	1,032	627	0.60	12.5	25.8	31.58
	0.010	5	103	217	2.10	43.4	25.8	356.26
Methapyrilene hydrochloride								
	125.6	50	1,030	476	0.46	9.5	25.8	0.08
	150.8	50	1,024	567	0.55	11.3	25.8	19.92
	174.4	50	1,037	596	0.57	11.9	31.3[f]	24.47*
	201.0	0					31.3[f]	
					P<0.001			

TABLE C3
Induction of Sister Chromatid Exchanges in Chinese Hamster Ovary Cells by Methapyrilene Hydrochloride

Compound	Dose (µg/mL)	Total Cells Scored	No. of Chromo-somes	No. of SCEs	SCEs/ Chromo-some	SCEs/ Cell	Hrs in BrdU	Relative Change of SCEs/ Chromosome (%)
+S9								
Trial 1								
Summary: Negative								
Medium		50	1,036	451	0.43	9.0	25.8	
Cyclophosphamide[d]	2	5	102	217	2.12	43.4	25.8	388.71
Methapyrilene hydrochloride								
	166.7	50	1,029	481	0.46	9.6	25.8	7.38
	500.0	50	1,028	461	0.44	9.2	25.8	3.01
	1,700.0	0						
					P=0.328			
Trial 2								
Summary: Positive								
Medium		50	1,018	466	0.45	9.3	25.8	
Cyclophosphamide	0.3	50	1,030	697	0.67	13.9	25.8	47.83
	2.0	5	105	178	1.69	35.6	25.8	270.33
Methapyrilene hydrochloride								
	597.8	50	1,032	564	0.54	11.3	31.3[f]	19.39
	696.4	50	1,016	563	0.55	11.3	31.3[f]	21.05*
	797.0	50	1,027	586	0.57	11.7	31.3[f]	24.65*
	904.4	0						
					P<0.001			
Trial 3								
Summary: Positive								
Medium		50	1,042	517	0.49	10.3	26.0	
Cyclophosphamide	0.3	50	1,037	623	0.60	12.5	26.0	21.08
	2.0	5	104	172	1.65	34.4	26.0	233.33
Methapyrilene hydrochloride								
	800	50	1,028	553	0.53	11.1	31.0[f]	8.42
	850	50	1,025	617	0.60	12.3	31.0[f]	21.32*
	900	50	1,026	614	0.59	12.3	31.0[f]	20.61*
					P<0.001			

* Positive response (≥20% increase over solvent control)
[a] Study was performed at Litton Bionetics, Inc. A detailed description of the protocol is presented by Galloway *et al.* (1987). SCE=sister chromatid exchange; BrdU=bromodeoxyuridine
[b] SCEs/chromosome in treated cells versus SCEs/chromosome in solvent control cells
[c] Solvent control
[d] Positive control
[e] Significance of relative SCEs/chromosome tested by the linear regression trend test versus log of the dose
[f] Due to cell cycle delay, harvest time was extended to maximize the number of second-division metaphase cells available for analysis.

TABLE C4
Induction of Chromosomal Aberrations in Chinese Hamster Ovary Cells by Methapyrilene Hydrochloride[a]

−S9					+S9				
Dose (µg/mL)	**Total Cells Scored**	**No. of Abs**	**Abs/ Cell**	**Cells with Abs (%)**	**Dose (µg/mL)**	**Total Cells Scored**	**No. of Abs**	**Abs/ Cell**	**Cells with Abs (%)**
Harvest time: 24.2 hours[b]					Harvest time: 10.5 hours				
Summary: Negative					Summary: Positive				
Medium[c]					Medium				
	100	1	0.01	1.0		100	1	0.01	1.0
	100	0	0.00	0.0		100	0	0.00	0.0
Mitomycin-C[d]					Cyclophosphamide[d]				
0.05	50	26	0.52	36.0	25	50	8	0.16	14.0
				P=0.079[e]					P<0.001
Methapyrilene hydrochloride					Methapyrilene hydrochloride				
352	100	2	0.02	1.0	747	100	12	0.12	10.0*
376	100	2	0.02	2.0	798	100	23	0.23	16.0*
400	100	2	0.02	2.0	850	100	53	0.53	31.0*

* Positive response ($P \leq 0.05$) versus the solvent control
[a] Study was performed at Litton Bionetics, Inc. The detailed protocol is presented by Galloway *et al.* (1987). Abs=aberrations
[b] Due to cell cycle delay, harvest time was extended to maximize the number of first-division metaphase cells available for analysis.
[c] Solvent control
[d] Positive control
[e] Significance of percent cells with aberrations tested by the linear regression trend test versus log of the dose

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