Comparison of the Hepatic Effects of Nafenopin and WY-14, 643 on Peroxisome Proliferation and Cell Replication in the Rat and Syrian Hamster

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Male Sprague-Dawley rats were fed control diet or diet containing 0.05% nafenopin (NAF) or 0.025% WY-14,643 (WY) and male Syrian hamsters were fed control diet or diet containing 0.25% NAF or 0.025% WY for periods of 1, 15, 40, and 60 weeks. Both NAF and WY produced a sustained increase in liver weight and induction of peroxisomal fatty acid β-oxidation in the rat and Syrian hamster. Replicative DNA synthesis was studied by implanting osmotic pumps containing [³H] thymidine during weeks 0-1, 14-15, 39-40, and 59-60. Cell replication, determined either as the hepatocyte labelling index or by incorporation of radioactivity into liver whole homogenate DNA, was increased in rats given NAF and WY for 1 week. However, only WY produced a sustained increased in cell replication after 15-60 weeks. After 40 weeks, liver nodules and tumors were present in WY-treated rats, and these lesions were observed in all WY-treated and some NAF-treated rats after 60 weeks. In contrast to the rat, no marked effect on replicative DNA synthesis and no liver nodules and tumors were observed in Syrian hamsters given NAF and WY for up to 60 weeks. The rat study demonstrates that liver tumors are produced more rapidly by doses of peroxisome proliferators that produce a sustained stimulation of cell replication, whereas the hamster study suggests that species differences may exist in both peroxisome proliferator-induced cell replication and liver tumor formation.

Introduction

A wide variety of compounds including herbicides, plasticizers, industrial chemicals, natural products, food flavors, and hypolipidemic and other types of therapeutic agents have been found to enlarge the liver, cause hepatic peroxisome proliferation, and induce peroxisomal and microsomal fatty acid oxidizing enzyme activities in rats and mice (1–5). Although peroxisome proliferators are not mutagenic in various short-term tests and do not appear to bind covalently to hepatic DNA after *in vivo* administration (6–8), sev-

eral of these compounds have been shown to increase the incidence of liver tumors in rats and mice (1,2,9,10). Indeed, Reddy and co-workers have suggested that peroxisome proliferators constitute a novel class of chemical carcinogens (10).

Because peroxisome proliferators appear to be nongenotoxic carcinogens, it has been suggested that liver tumor formation arises from a sustained "oxidative stress" to the hepatocytes due to an imbalance in the production and degradation of peroxisomal hydrogen peroxide (2,6–9). This imbalance is due to the fact that peroxisome proliferators markedly stimulate enzymes of the peroxisomal fatty acid β-oxidation cycle (which generate hydrogen peroxide), whereas only a small increase is observed in catalase activity, and selenium-dependent glutathione peroxidase activity is normally inhibited by these chemicals (7,8,11,12). Although some evidence has been obtained for the oxidative stress hypothesis (7,8), other workers have suggested that peroxisome proliferator-induced hepatocarcinogenicity may be due to alternative mechanisms including the sustained stimulation of replicative

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DNA synthesis and the promotion of spontaneously formed preneoplastic liver lesions (13,14).

Peroxisome proliferator-induced liver enlargement in rodents is due to both hepatocyte hypertrophy and hyperplasia (2-4). In addition to the initial burst of hepatocyte replication observed in the first few days of treatment with peroxisome proliferators, in some studies a sustained stimulation of hepatocyte cell replication has been observed (13, 15-18).

The purpose of this study was to further investigate the relationships between hepatic peroxisome proliferation, cell replication, and liver tumor formation in the rat and Syrian hamster. The Syrian hamster was selected because it is less responsive than the rat to a number of peroxisome proliferators including clobuzarit (19), clofibrate (20), di-(2-ethylhexyl) phthalate (20), LY 171883 (21), and nafenopin (22). In addition, there is a paucity of information on the effects of peroxisome proliferators on hepatic cell replication and tumor formation in the Syrian hamster. The two peroxisome proliferators (for structures see Fig. 1) used in this study were nafenopin (NAF; 2-methyl-2-[p-(1,2,3,4-tetrahydro-1-naphthyl)phenoxy]propionic acid) and Wy-14,643 (WY; [4-chloro-6-(2,3-xylidino)-2-pyrimidinylthio]acetic acid. Both these compounds have been previously shown to produce liver tumors in the rat and mouse (2).

Materials and Methods

Chemicals

Enzyme cofactors, etc., were purchased from Sigma Chemical Co. Ltd. (Poole, Dorset, UK) and Wy-14,643 was purchased from Chemsyn Science Laboratories (Lenexa, KS). Nafenopin (Su 13,437) was the generous gift of Ciba-Geigy Ltd. (Basel, Switzerland). Alzet osmotic pumps were obtained from Charles River UK Ltd. (Margate, Kent, UK) and [methyl-^{3}H]thymidine (79-85 mCi/mmole, 1.0 and 3.0 mCi/mL) was obtained from Amersham International, plc (Little Chalfont, Bucks, UK).

Animals and Treatment

Male Sprague-Dawley rats (4 weeks old) were purchased from Harlan Olac Ltd. (Bicester, Oxon, UK) and male MB strain Syrian hamsters (4 weeks old) from Consort Ltd. (Hovewood End, Hereford, UK). Animals were allowed free access to R and M No. 1 (rats diets) and R and M No. 3 (hamsters) diets (Special Diet Services, Witham, Essex, UK) and water. The animals were housed in mesh -floored cages in rooms maintained at 22 ± 3°C with a relative humidity of 40-70%. After acclimatizing to these conditions for 2 weeks, rats were fed control diet or diet containing 0.05% NAF or 0.025% WY, and hamsters were fed control diet or diet containing 0.025% NAF or 0.075% WY. The compounds were administered for periods of 1,15,40, and 60 weeks. Animals were killed by exsan-
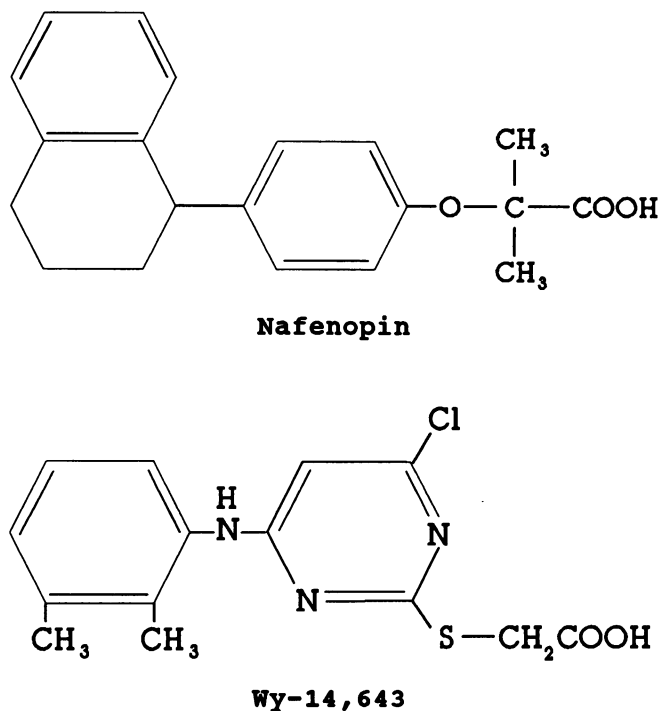

FIGURE 1. Structures of nafenopin and Wy-14,643.

guination under diethyl ether anesthesia, and the livers were immediately excised for biochemical and morphological studies.

Replicative DNA Synthesis

Animals were anesthetised with sodium pentobarbitone (60 mg/kg, ip) before subcutaneous implantation of 7-day Alzet osmotic pumps containing [^{3}H] thymidine. Osmotic pumps for rats (model 2ML1) and hamstes (model 2001) contained 2.0 and 0.6 mCi of [^{3}H] thymidine, respectively. Sections of liver and upper small intestine (to confirm pump efficiency) were dipped in liquid photographic emulsion and exposed in the dark at 4°C for 10 weeks. After processing, the hepatocyte labelling index (i.e., percentage of the nuclei undergoing replicative DNA synthesis) was assessed by microscopic examination of at least 1000 nuclei in random fields from the left lobe of the liver. Hepatic whole homogenate DNA content was determined in liver homogenates (see below) by the method of Setaro and Morley (23), and radioactivity incorporated into DNA was determined by scintillation counting.

Biochemical and Morphological Investigations

Whole liver homogenates (rat 0.25g and hamster 0.1g fresh tissue/mL) were prepared in 0.154 M KCl containing 50 mM Tris-HCl, pH 7.4, using a Potter-type, Teflon-glass, motor-driven homogenizer (A. H.

Thomas Co., Philadelphia, PA). When animals had large liver nodules or tumors, samples of the surrounding host tissue were selected for homogenization. Liver whole homogenates were assayed for cyanide-insensitive palmitoyl-CoA oxidation (22) and protein content (24).

Liver slices were fixed in neutral buffered formalin. Paraffin sections were cut at 5 μm, stained with hematoxylin and eosin, and examined by light microscopy.

Statistical Analysis

Statistical evaluation of data was performed by one-way analysis of variance. Comparisons between means were made using the least significant difference test.

Results

Effect on Liver Weight and Peroxisomal Fatty Acid β-Oxidation

Treatment of rats with diets containing 0.05% NAF or 0.025% WY markedly increased relative liver weight after 1, 15, 40, and 60 weeks of treatment (Fig. 2A). Relative liver weight was also significantly increased in Syrian hamsters fed diets containing either 0.25% NAF or 0.025% WY (Fig. 2B). In both species, WY produced a greater increase in relative liver weight than NAF after 15, 40 and 60 weeks of treatment.

Peroxisomal (whole homogenate cyanide-insensitive palmitoyl-CoA oxidation) fatty acid β-oxidation was markedly induced by both NAF and WY administration to the rat (Fig. 3A) and Syrian hamster (Fig. 3B) at all time points. Although enzyme activity (nmole/min/mg protein) was induced to similar levels in both species (Fig. 3A,B), the magnitude of induction was greater in the rat than in the Syrian hamster owing to a 2.3-fold higher basal level of enzyme activity in the hamster. Mean values for induction of palmitoyl-CoA oxidation over the 1-60 week treatment period by NAF and WY were 1180 and 1110% of control for the rat and 415 and 590% of control for the Syrian hamster, respectively.

Effect on Replicative DNA Synthesis

Replicative DNA synthesis was determined over study weeks 0-1, 14-15, 39-40 and 59-60 with 7-day Alzet osmotic pumps containing [³H] thymidine. After 1 week, replicative DNA synthesis as assessed by [³H] thymidine incorporation into hepatic DNA, was significantly increased in rats given both NAF and WY (Fig. 4A). However, after 15, 40, and 60 weeks of treatment, hepatic DNA radioactivity levels were still significantly increased by WY, whereas NAF had no effect. In contrast, in the Syrian hamster, neither compound produced a significant increase in hepatic DNA radioactivity levels at any time point (Fig 4B).

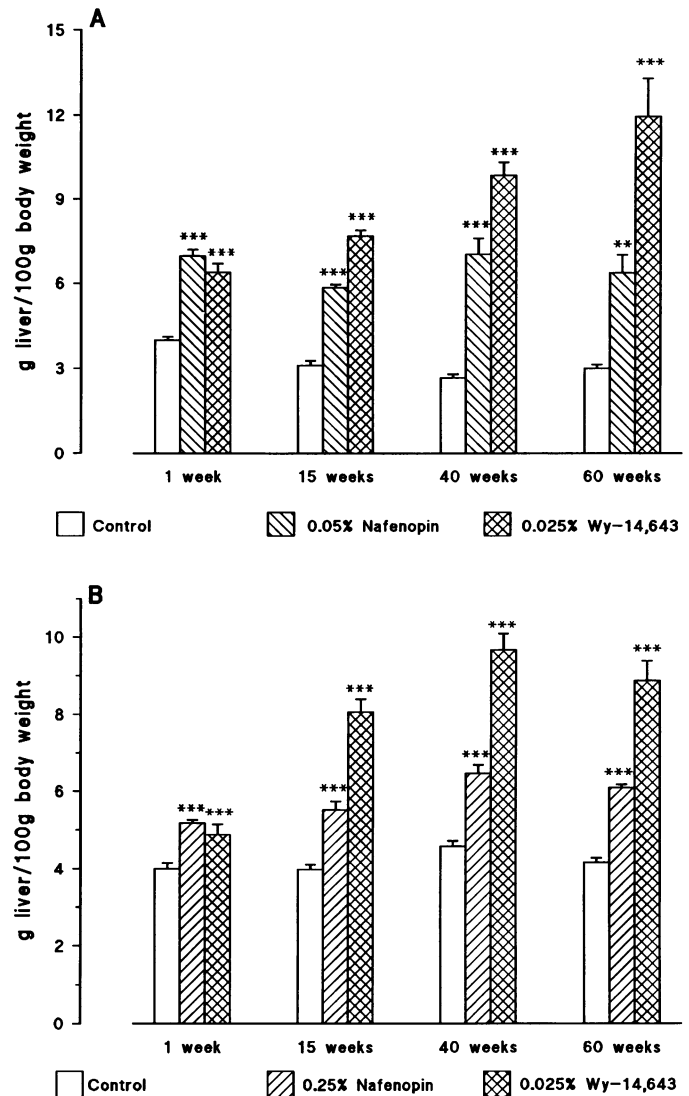Replicative DNA synthesis, as assessed by the hepa-

FIGURE 2. Effect of dietary administration of nafenopin and WY for 1, 15, 40, and 60 weeks on relative liver weight in the rat (A) and Syrian hamster (B). Results are presented as means ± SEM (n = 5-7). Values significantly different from controls (**) $p < 0.01$; (***) $p < 0.001$.

tocyte labelling index (LI), was significantly increased in the rat to 580 and 535% of control levels by treatment with NAF and WY, respectively, for 1 week (Fig 5A). However, after 15 and 40 weeks of treatment, only WY produced a significant increase in hepatocyte LI values to 395 and 855% of control, respectively. In contrast to the rat, neither compound produced a significant increase in hepatocyte LI values in the Syrian hamster at any time point examined (Fig. 5B).

Effect on Liver Morphology

No macroscopic liver lesions (i.e., liver nodules or tumors) were observed in either NAF- or WY- treated rats after 1 and 15 weeks and in control animals after
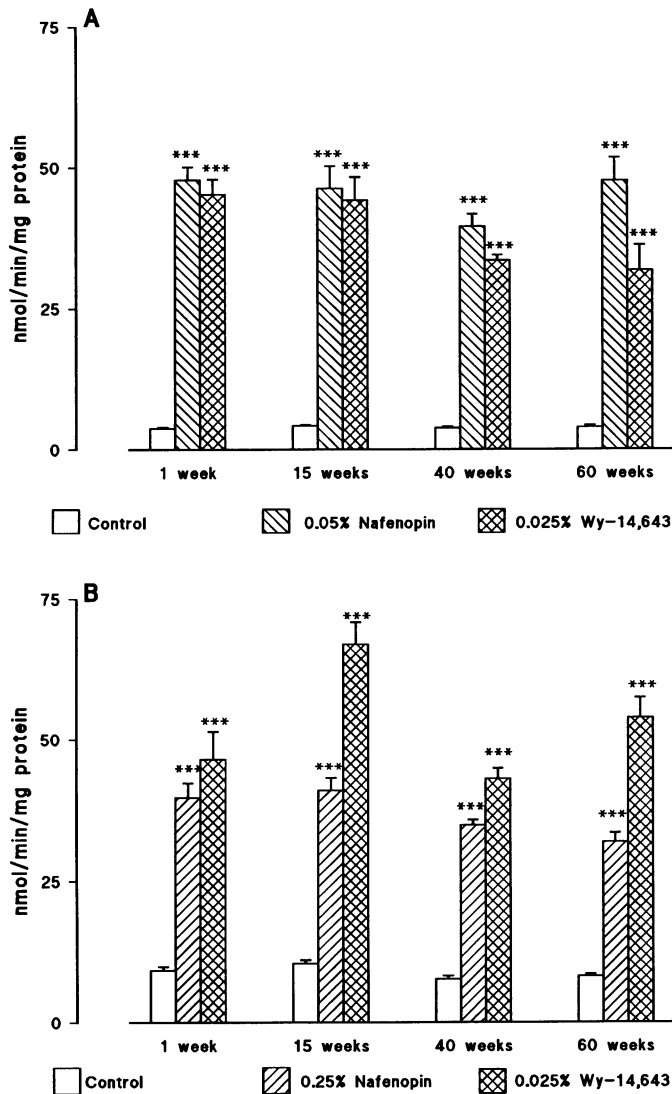
FIGURE 3. Effect of dietary administration of nafenopin and WY for 1, 15, 40, and 60 weeks on cyanide-insensitive palmitoyl-CoA oxidation in the rat (A) and Syrian hamster (B). Results are presented as means ± SEM (n = 5-7). Values significantly different from controls (***) $p < 0.001$.

FIGURE 4. Effect of dietary administration of nafenopin and WY for 1, 15, 40, and 60 weeks on [³H] thymidine incorporation into hepatic DNA in the rat (A) and Syrian hamster (B). Results are presented as means ± SEM (n = 5-7). Values significantly different from controls (**) $p < 0.01$; (***) $p < 0.001$.

1, 15, 40, and 60 weeks of treatment (Table 1). However, macroscopic liver lesions were present in all WY-treated rats after 40 and 60 weeks and in 73% of NAF-treated rats after 60 weeks. In contrast to the rat, no macroscopic liver lesions were observed in control, NAF-, and WY-treated Syrian hamsters at any time point (Table 1).

Histological examination of liver sections from control and NAF-treated rats after 40 weeks revealed no abnormalities, whereas basophilic foci and nodules were observed in liver sections from all WY-treated rats. In addition, areas of adenoma and carcinoma were identified in liver sections from some WY-treated rats. No abnormalities were observed in liver sections from control rats after 60 weeks, whereas liver sections
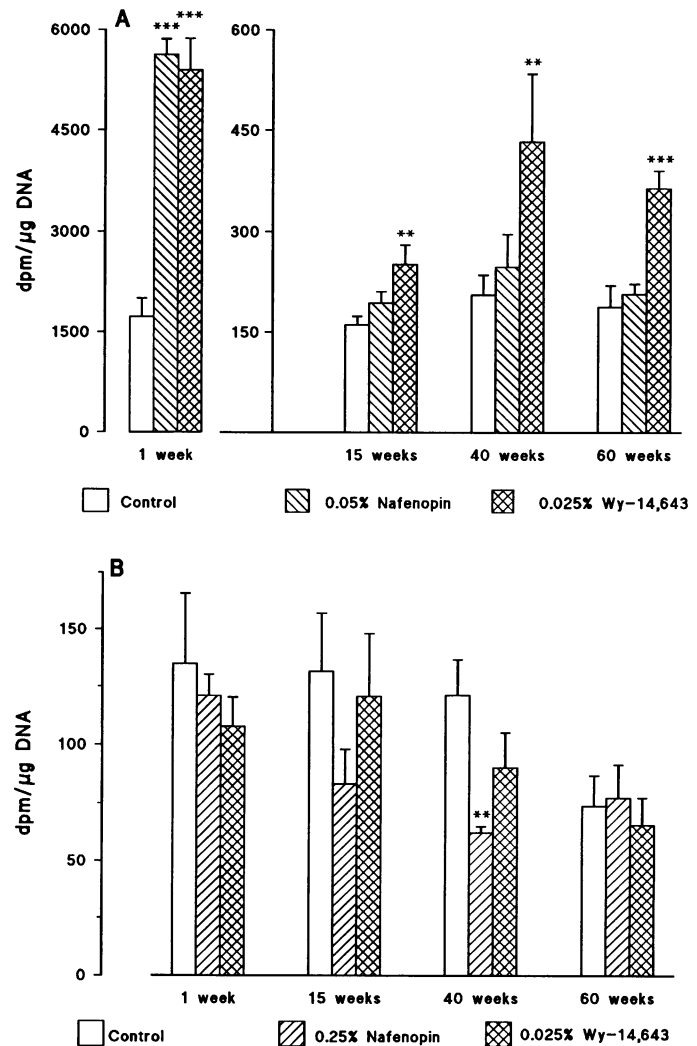
from all WY-treated and most NAF- treated rats contained basophilic foci and nodules together with areas of adenoma and carcinoma.

Histological examination of liver sections from control hamsters after 40 and 60 weeks revealed no abnormalities, whereas slight cellular hypertrophy of periportal hepatocytes was observed in liver sections from NAF- and WY-treated hamsters. No peroxisome proliferator-induced liver foci, nodules, adenomas or carcinomas were observed in this species. However, after 60 weeks in WY-treated but not in NAF-treated hamsters, some fatty vacuolation of periportal hepatocytes and slight bile duct proliferation was observed.

Discussion

These results demonstrate that the chronic administration of both NAF and WY results in a sustained
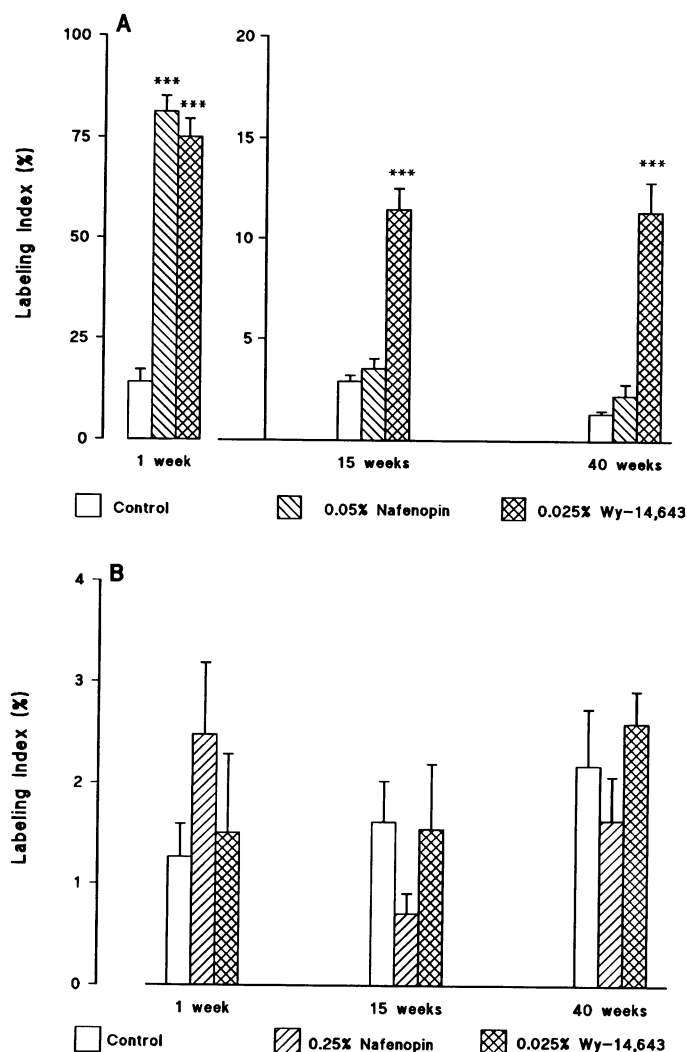
FIGURE 5. Effect of dietary administration of nafenopin and WY for 1, 15, and 40 weeks on hepatocyte labelling index in the rat (A) and Syrian hamster (B). Results are presented as means ± SEM (n = 5-7). Values significantly different from controls (***) p < 0.001.

were administered for much shorter periods ranging from 2 to 6 weeks.

In the rat, both peroxisome proliferators produced a similar magnitude of induction of hepatic peroxisome proliferation, but had different effects on hepatic replicative DNA synthesis. Although both compounds stimulated hepatic replicative DNA synthesis (assessed either as the hepatocyte labelling index or as incorporation of [³H] thymidine into whole homogenate DNA) during the first week of treatment, only WY produced a sustained stimulation throughout the remainder of the 60 weeks of administration. In addition, WY produced liver nodules, adenomas, and carcinomas within 40 weeks, whereas these liver lesions were only observed after 60 weeks of NAF administration. As such, these results are in agreement with those of a previous 1 year study conducted in F344 rats with di-(2-ethylhexyl)phthalate and WY (13). Although both compounds produced a similar magnitude of induction of peroxisome proliferation, only WY produce a sustained stimulation of cell replication and liver tumors after 1 year of treatment. Thus, from the results of the 1-year study and the present study, a similar magnitude of induction of hepatic peroxisome proliferation in the rat does not necessarily result in a similar time course of liver tumor formation. Rather, liver tumors appear to be produced more rapidly by doses of peroxisome proliferators that produce a sustained stimulation of cell replication in this species.

In agreement with previous studies on species differences in hepatic peroxisome proliferation (19–22), the magnitude of induction of palmitoyl-CoA oxidation was greater in the rat than in the Syrian hamster. However, although hepatic peroxisome proliferation was observed in both the rat and Syrian hamster, species differences in the hepatic effects of NAF and WY were also apparent. For example, neither compound significantly increased replicative DNA synthesis in hamster liver at any of the time points examined. In a comparative study of acute hyperplasia induced by methylclofenapate, a potent peroxisome proliferator and hepatocarcinogen (2,3), Styles and co-workers (27) observed a marked effect in rat and mouse liver, but only a small effect in Syrian hamster hepatocytes. Clearly, the Syrian hamster is much less susceptible than the rat to peroxisome proliferator-induced hyperplasia, and in the present study morpholocial examination of liver sections indicated that the increase in liver

increase in liver weight and induction of hepatic peroxisome proliferation (as assessed by palmitoyl-CoA oxidation) in the rat and Syrian hamster. Although these data confirm the results of previous rat studies (1–4, 12,13) they extend the findings of previous hamster studies (19–22,25,26) where peroxisome proliferators

Table 1. Macroscopic lesions in the livers of rats and Syrian hamsters treated with nafenopin (NAF) and Wy-14,643 (WY).

Weeks of treatment	Rat[a]			Syrian hamster		
	Control	0.05% NAF	0.025% WY	Control	0.25% NAF	0.025% WY
1 and 15	0(6)[b]	0(6)	0(6)	0(6)	0(6)	0(6)
40	0(6)	0(6)	6(6)	0(6)	0(6)	0(6)
60	0(8)	8(11)	12(12)	0(9)	0(8)	0(8)

[a]Lesions include small nodules and liver tumors.
[b]Figures in parentheses are the numbers of animals examined.

weight was primarily due to hepatocyte hypertrophy.

A second important species difference observed in this study is that, although both NAF and WY produced liver nodules, adenomas and carcinomas in the rat, no such peroxisome proliferator-induced liver lesions were observed after 60 weeks of treatment in the Syrian hamster. This observation suggests that the Syrian hamster is more resistant than the rat to peroxisome proliferator-induced hepatocarcinogenicity and, indeed, that species differences may exist in both hepatic peroxisome proliferation and liver tumor formation.

Several hypotheses have been proposed to account for the formation of liver tumors by peroxisome proliferators in rats and mice (2,6–9,13–14). If these hypotheses are combined, a role for increased cell replication in peroxisome proliferator-induced hepatocarcinogenicity may be identified. For example, if hepatocytes are transformed either by oxidative stress-induced damage (2,6–9) or spontaneously (14), such initiated cells could be promoted into liver tumors by enhanced cell replication (13). Certainly the present rat study with NAF and WY supports the concept that liver tumors are produced more rapidly by doses of peroxisome proliferators that produce a sustained stimulation of cell replication. In contrast, no marked induction of replicative DNA synthesis and no liver tumors were observed in Syrian hamsters given five times and the same dietary level of NAF and WY, respectively. Because hepatic peroxisome proliferation but not cell replication may be observed in the Syrian hamster, this species may be suitable for further studies aimed at establishing the respective roles of peroxisome proliferator-induced oxidative stress and enhanced cell replication in the hepatocarcinogenicity of peroxisome proliferators. Finally, in keeping with our previous suggestions (20,22), because the Syrian hamster is less responsive than the rat to such chemicals, appropriate bioassays conducted in the Syrian hamster may provide valuable data to assist in the assessment of the hazard, if any, of hepatocarcinogenic rodent peroxisome proliferators to man.

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