Mitogenic and Carcinogenic Effects of a Hypolipidemic Peroxisome Proliferator, [4-Chloro-6-(2,3-xylidino)-2-pyrimidinylthio]acetic Acid (Wy-14,643), in Rat and Mouse Liver


Department of Pathology, The Northwestern University Medical School, Chicago, Illinois 60611; The Clinical Pharmacology and Toxicology Center, The University of Kansas Medical Center, Kansas City, Kansas 66163; and Department of Pathology, The University of California School of Medicine, San Diego, California 92037.

Abstract

Long-term effects of Wy-14,643 ([(4-chloro-6-(2,3-xylidino)-2-pyrimidinylthio]acetic acid), a potent hepatic peroxisome proliferator structurally unrelated to the clinically used drug clofibrate, were investigated in male acatalasemic CS\(^2\) mice and F344 rats. Acatalasemic mice were fed Wy-14,643 at a dietary concentration of 0.1% (w/w) for 6 months and then at 0.05% (w/w) until the termination of the experiment at 14.5 months. F344 rats were fed this compound at a 0.1% (w/w) level in the diet for 16 months. Hepatocellular carcinomas developed in 18 of 18 acatalasemic mice and 15 of 15 F344 rats that survived chronic Wy-14,643 treatment. Metastases to lungs were observed in 5 of 18 mice and 6 of 15 rats with Wy-14,643-induced hepatocellular carcinomas. The primary liver tumors in rats contained numerous peroxisomes. The increase in the number of these organelles in tumor cells was associated with a significant elevation of carnitine acetyltransferase activity, suggesting that these intrahepatic tumors respond to the peroxisome proliferative effect of Wy-14,643. The catalase activity of these tumors, however, was not increased.

Short-term administration of Wy-14,643 induced DNA replication and cell division in the rat liver as determined by \(^{3}H\)thymidine incorporation into DNA, autoradiography, and colchicine-arrested metaphases in liver cells. The stimulation of liver cell proliferation was not associated with hepatocellular necrosis. The elevations of serum \(\alpha\)-fetoprotein concentration were associated with and proportional to liver cell proliferation.

The observation that nafenopin and Wy-14,643, 2 structurally unrelated hypolipidemic agents, induce "primary" liver cell proliferation and hepatocellular carcinomas prompts a concern over the potential carcinogenicity of hepatic peroxisome proliferators as a class.

Introduction

Wy-14,643 ([(4-chloro-6-(2,3-xylidino)-2-pyrimidinylthio]acetic acid), which was originally synthesized by Santilli et al. (39), has been shown to possess both hypolipidemic and antiatherogenic properties (15). This compound was found to be 60 times more potent than the clinically used hypolipidemic drug clofibrate (ethyl-\(\alpha\)-p-chlorophenoxyisobutyrate) in lowering serum lipid levels in experimental animals (49) but it did not enter into clinical trials, possibly due to suspected hepatotoxicity in experimental animals. Previous work by Reddy and Krishnakanta (31) showed that Wy-14,643 caused a significant hepatomegaly and produced a marked increase in the hepatic peroxisome population in rats and mice. Subsequently, we have demonstrated that structurally related analogs of Wy-14,643 that lacked hypolipidemic effect failed to induce hepatic peroxisome proliferation and peroxisome-associated enzymes (28, 34).

During the past 5 years, several chemicals with a hypolipidemic property have been identified as hepatic peroxisome (microbody) proliferators in rats and mice (29, 31, 33). Because of this association, a relationship between hepatic peroxisome proliferation and lipid metabolism was suggested (31). Recently, Lazarow and DeDuve (18) and Lazarow (17) presented evidence to indicate that peroxisomes catalyze the \(\beta\)-oxidation of long-chain fatty acids. The presence in peroxisomes of a fatty acyl-CoA-oxidizing system (17, 18) and carnitine acetyltransferase (20) and their increase in the livers of animals treated with peroxisome proliferators (11, 12, 18, 22, 23, 26, 48) appear to substantiate the relationship between peroxisome proliferation and lipid metabolism.

Because of the long-term administration of drugs such as clofibrate for the control of hyperlipidemic states in man (7), it is essential to investigate various aspects of the persistent hepatomegaly and peroxisome proliferation induced by these agents. Long-term feeding of nafenopin (2-methyl-\(\alpha\)-[\(p\)-\(\{(1,2,3,4\text{-tetrahydro}-1-naphthyl)phenoxy\}]propionic acid; Su-13,437), a closely related analog of clofibrate, has been shown to induce hepatocellular carcinomas in rats and acatalasemic mice (35, 38). The availability of several compounds with diverse chemical structures that induce peroxisome proliferation in liver cells (29, 31, 33, 47) provides an opportunity to investigate the relationship, if any, between peroxisome proliferation, hepatomegaly, and hepatocarcinogenesis. We now report that Wy-14,643 (Chart 1), a potent hypolipidemic peroxisome proliferator, which is structurally different from clofibrate, is a mitogen for liver and induces hepatocellular carcinomas in rats and mice.

Materials and Methods

Long-Term Administration of Wy-14,643. Male F344 rats weighing 80 to 100 g were obtained from ARS Sprague-Dawley stock.
**Hepatocarcinogenicity of a Peroxisome Proliferator**

![Chemical Structures](image)

**Chart 1. Structure of nafenopin (2-methyl-2-p-1,2,3,4-tetrahydro-1-naphthylphenoxo)propionic acid; Su-13,437) and Wy-14,643 ((4-chloro-6-(2,3-xylylidino)-2-pyrimidylythio)acetic acid). Nafenopin is a structural analog of the clinically used hypolipidemic drug clofibrate, but Wy-14,643 is not. These 2 structurally unrelated peroxisome proliferators produce liver tumors in rats and mice.**

**RESULTS**

**Liver Tumor Induction.** The incidence of liver tumors in male acatalasemic mice and F344 rats fed Wy-14,643 is...
shown in Table 1. Because 2 acatalasemic mice fed 0.1% Wy-14,643 died near the end of the first 6 months of the experiment, the surviving mice were fed this compound at a dietary concentration of 0.5% until the termination of the experiment at 14.5 months. All 18 acatalasemic mice that survived long-term administration of Wy-14,643 developed multiple tumors involving several lobes of the liver. The tumors measured 3 to 28 mm in diameter and appeared gray to gray-brown. The livers were greatly enlarged and revealed no evidence of cirrhosis or scarring. Microscopically, the cellular morphology of these mouse liver tumors ranged from well-differentiated to anaplastic. Although the majority of these hepatocellular carcinomas were of the trabecular type (Fig. 1), several had a pleomorphic, poorly differentiated pattern. Vascular invasion was frequently encountered in these tumors. Pulmonary metastases were present in 5 of 18 mice with hepatocellular carcinomas.

Between 14 and 16 months, 15 of 15 male F344 rats fed 0.1% Wy-14,643 developed liver tumors. These tumors were multiple and measured 3 to 42 mm in diameter. The tumor-bearing livers were markedly enlarged. The tumors appeared gray, and sectioned larger tumors revealed areas of focal hemorrhage and softening. Histologically, all these tumors were hepatocellular carcinomas with trabecular patterns (Fig. 2). Some tumors were moderately to poorly differentiated and had broad trabeculae with central necrosis. The nucleoli, in general, were very prominent in these hepatocellular carcinomas. Mitoses were frequently encountered, and an occasional tumor with a trabecular pattern showed evidence of fatty metamorphosis (Fig. 3). Metastases to the lungs were found in 6 rats (40%) bearing primary hepatocellular carcinomas. These occurred as clusters of well-differentiated cells resembling the cells of trabecular hepatocellular carcinomas. Some of these metastases were very small, composed of few cells, and were detected only on careful microscopic examination (Fig. 4). The primary transplants derived from rat liver tumors were palpable within 4 weeks and appeared as well-differentiated tumors.

The ultrastructural appearance of rat and mouse hepatocellular carcinomas was similar to that observed in our earlier studies with nafenopin (35, 36). Peroxisomes were prominent in these tumors, and several of these organelles were encountered in primary tumors, as well as transplants derived from these tumors, contained prominent nucleoids (Figs. 5 and 6).

Peroxisome-associated Enzymes in Rats Bearing Primary Hepatocellular Carcinomas. Since the animals were fed the peroxisome proliferator Wy-14,643 until sacrifice, it appeared important to compare the levels of catalase and carnitine acetyltransferase activities in primary liver tumors with the levels in uninvolved portions of livers. The data on these 2 peroxisome-associated enzymes are presented in Table 2. Although peroxisomes in these primary hepatocellular carcinomas appeared abundant (Fig. 5), the catalase activity in these tumors was not inducible. The activity of this enzyme in uninvolved portions of liver was slightly elevated. In contrast, the carnitine acetyltransferase activity in Wy-14,643-induced primary hepatocellular carcinomas was markedly increased (Table 2).

Fig. 7 illustrates the appearance of SDS-polyacrylamide gels of postnuclear pellets obtained from normal rat liver and from the tumorous as well as nontumor portions of livers of Wy-14,643-fed rats. A band with a molecular weight of approximately 80,000, which has been shown to be associated with peroxisome proliferation, is increased in both tumors and nontumorous portions of the livers of Wy-14,643-treated animals.

Mitogenic Effect of Wy-14,643. The liver weights of both rats and acatalasemic mice increased significantly within a few days of Wy-14,643 administration in the diet. To analyze the early events, the extent of continuous administration of Wy-14,643 for 5 days on the liver weight, liver DNA, and uptake of [3H]thymidine by rat liver was investigated. Table 3 presents the data obtained from this experiment. A signif-

### Table 1

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of animals</th>
<th>No. with liver tumors</th>
<th>% of No. with tumors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acatalasemic mice</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>10</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Wy-14,643 (0.1% for 6 mos.; 0.05% for 8.5 mos.)</td>
<td>20</td>
<td>18</td>
<td>18d 100c</td>
</tr>
<tr>
<td>F344 rats</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>10</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Wy-14,643 (0.1% for 16 mos.)</td>
<td>15</td>
<td>15</td>
<td>15d 100c</td>
</tr>
</tbody>
</table>

a Number of animals alive at appearance of first liver tumor.

b In relation to the effective number.

c Wy-14,643 was added to powdered chow at the concentrations (w/w) shown and fed ad libitum.

d Pulmonary metastases were found in 5 mice and 6 rats with liver tumors.

e Significantly different (p < 0.001) from animals fed control diet, as determined by Fisher's exact test.

### Table 2

<table>
<thead>
<tr>
<th>Group</th>
<th>Catalase Activity (units/mg protein)</th>
<th>Carnitine acetyltransferase Activity (units/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumor-bearing rats</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hepatocellular carcino- mas</td>
<td>31 ± 11 (9)</td>
<td>160 ± 46 (6)</td>
</tr>
<tr>
<td>Uninvolved liver</td>
<td>56 ± 7 (9)</td>
<td>193 ± 63 (8)</td>
</tr>
<tr>
<td>Normal rats</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control (untreated)</td>
<td>45 ± 3 (9)</td>
<td>2.9 ± 1.5 (4)</td>
</tr>
<tr>
<td>Wy-14,643 (0.05%) for 3 wk</td>
<td>86 ± 6c (5)</td>
<td>206 ± 14c (5)</td>
</tr>
</tbody>
</table>

a Mean ± S.D.
b Numbers in parentheses, number of animals studied.
c Significantly different from controls (p < 0.001).
significant increase in liver weight, liver DNA, and specific radioactivity of the DNA occurred in animals fed Wy-14,643 for 5 days. The nuclear labeling of liver cells, as analyzed by light microscopic autoradiography, was markedly increased (Fig. 8) in rats fed Wy-14,643 for 5 days.

Table 4 presents the effects of time course on the change in liver weight and the incorporation of [3H]thymidine into liver DNA of rats after a single dose of Wy-14,643 by stomach tube. An increase in the specific radioactivity of DNA was evident at 18 hr and appeared maximal at 24 hr after the administration of Wy-14,643.

The number of cells entering mitosis between 18 and 72 hr during the administration of Wy-14,643 was determined by recording the colchicine-arrested metaphases (Fig. 9) over a 6-hr period (Table 5). The total number of hepatocytes entering mitosis during 36 to 42 hr was approximately 11%. No evidence of hepatocellular necrosis was seen at any time interval during the first week of Wy-14,643 feeding, suggesting that the wave of DNA replication and mitosis does not represent reparative hyperplasia.

**Serum AFP.** The serum level of AFP was determined in rats fed 0.2% Wy-14,643 (w/w) for up to 16 weeks. The AFP concentration was significantly elevated at 5 and 7 days and declined thereafter (Chart 2). The serum AFP levels did not increase in 7 rats bearing Wy-14,643-induced primary hepatocellular carcinomas.

### DISCUSSION

The present study demonstrates that long-term administration of the hypolipidemic peroxisome proliferator Wy-14,643 in the diet of male F344 rats and acatalasemic C57BL mice results in the development of hepatocellular carcinomas, with a 100% incidence in both species. No tumors were observed in control F344 rats and in acatalasemic mice fed an identical diet minus Wy-14,643. Although the number of animals used in these experiments is small, the tumor incidence in Wy-14,643-fed animals, according to Fisher's exact test or χ² analysis, is significantly different from the appropriate controls (p < 0.001). Pulmonary metastases were found in 28% of acatalasemic mice and 40% of F344 rats bearing hepatocellular carcinomas. The incidence of lung metastases in acatalasemic mice observed in this study is comparable to that reported recently by Vesselinovitch et al. (52) in C57BL × C3H F1 mice, and by Reddy et al. (38) in nafenopin-treated mice with malignant liver cell tumors.

The primary hepatocellular carcinomas induced by Wy-14,643 in F344 rats in the present study provided an opportunity to delineate differences in the inducibility, between
normal and neoplastic cells, of the peroxisome-associated enzymes catalase and carnitine acetyltransferase. As expected (14, 51), the catalase activity in Wy-14,643-induced rat liver tumors was decreased, whereas the carnitine acetyltransferase activity increased significantly in response to dietary Wy-14,643. It is now clearly established that Wy-14,643 and all other peroxisome proliferators increase the activity of carnitine acetyltransferase in rat and mouse liver (22, 28, 31, 32, 45, 48) and that this increase parallels the increase in peroxisome population. Electron microscopic examination of Wy-14,643-induced primary hepatocellular carcinomas in the present study revealed the presence of several peroxisomes in the cytoplasm, and these organelles stained positively for catalase when incubated in the alkaline 3,3′-diaminobenzidine medium (25). Accordingly, the increase in carnitine acetyltransferase activity in these liver tumors may, in part, be related to the number of peroxisomes, and it suggests that the intrahepatic primary tumors respond to the peroxisome-proliferative effect of Wy-14,643. An alternative explanation for the increased numbers of peroxisomes in primary tumor cells may be that peroxisome proliferation has already occurred in the hepatocytes from which the tumor cells arise, and the tumor cells merely retain these organelles. This, however, is unlikely since the initiated cells divide several times prior to becoming grossly visible. It is of particular interest, however, to note that the increase in peroxisome population in these primary hepatocellular carcinomas was not associated with an increase in catalase activity, which is the marker enzyme for this organelle (4). The increase in carnitine acetyltransferase but not of catalase activity in these Wy-14,643-induced tumors suggests that the cellular mechanisms regulating these peroxisomal enzymes differ considerably. It should be noted that the catalase activity is very low in many liver tumors and in the livers of tumor-bearing animals (14, 51). The lowering of liver catalase in tumor-bearing animals has been attributed to the presence of a tumor component, called toxohormone, that inhibits the synthesis or enhances the degradation of catalase (14). The presence of peroxisomes in primary as well as transplantable hepatocellular carcinomas in rats induced by Wy-14,643 in these studies (Fig. 6), however, suggests that these tumors are better differentiated (13, 51).

The mechanism by which Wy-14,643, a peroxisome proliferator, exerts its carcinogenic effect is not known. Recently, Reddy et al. (35, 36, 38) reported the development of hepatocellular carcinomas in rats and mice fed the peroxisome proliferator, nafenopin. The observation that nafenopin and Wy-14,643, 2 structurally unrelated hypolipidemic agents, induce hepatocellular carcinomas prompts a concern over the potential carcinogenicity of hepatic peroxisome proliferators as a class. Wy-14,643 causes marked liver enlargement in rats and mice (31, 34). The liver cells are hypertrophic and reveal proliferation of peroxisomes and smooth endoplasmic reticulum. It is evident from this study that Wy-14,643 induces liver cells to proliferate, as judged by the incorporation of [3H]thymidine into rat liver DNA, [3H]thymidine autoradiography, and the analysis of colchicine-arrested metaphases during the 5-day treatment period. The increase in [3H]thymidine incorporation was also evident at 24 hr after a single dose of Wy-14,643 by stomach tube. The absence of any histological evidence for hepatocellular necrosis in these livers suggests that the mitogenic effect is a primary action of Wy-14,643. The fact that Wy-14,643 and nafenopin (21), 2 carcinogenic peroxisome proliferators, stimulate DNA replication [unlike most carcinogens, which strongly inhibit DNA replication both in vivo and in vitro (53)] appears to place the peroxisome proliferators in a different class of carcinogens. The fact that the dose of Wy-14,643 administered to rats and mice in these studies may be higher than the hypolipidemic dose used for humans should not detract from the potential implications of these findings.

The importance of the primary mitogenic effect of 2 carcinogenic peroxisome proliferators, Wy-14,643 and nafenopin (21), in the initiation or promotion of liver tumorigenesis (37) remains to be elucidated. It is conceivable that these agents may induce mitotic irregularities, as well as cause DNA damage. If these agents do cause DNA damage, their ability to induce DNA replication and cell proliferation may convert a transitory abnormality in DNA into an inheritable change, leading to liver tumorigenesis (2, 3, 50).

Wy-14,643 like nafenopin (29), is a potent inducer of peroxisomes. The liver growth, increase in peroxisome population, and increase of several peroxisomal enzymes in rats fed these agents appear analogous to adaptive changes occurring in livers of animals exposed to a variety of microsomal enzyme inducers, such as phenobarbital (40). Several of these xenobiotics, which induce drug-metabolizing enzymes in liver together with an increase of smooth endoplasmic reticulum, have been shown to possess hepatocarcinogenic activity, in addition to their ability to promote liver tumor induction by other chemical carcinogens (2, 24, 27, 40). The exact mechanism of these actions is not understood.

Recently, Feinstein et al. (6) presented evidence to suggest that catalase, a marker enzyme for peroxisomes, and H2O2, which is generated also by peroxisomal oxidases, may be relevant in carcinogenesis. They observed an increased incidence of liver tumors in acatalasemic mice fed aminotriazole, a potent inhibitor of catalase, and attributed this to a diminished rate of degradation of H2O2, which has been shown to be mutagenic (9). Although this hypothesis appears attractive in explaining the differences observed with aminotriazole (6) or nafenopin liver tumorigenesis (38) between substrains of mice differing only in the catalase-synthesizing gene locus, it would be difficult to explain the development of liver tumors in rats fed Wy-14,643 and nafenopin, in which catalase activity is markedly elevated. The increased catalase activity in these livers with peroxisome proliferation should lead to an enhanced rate of H2O2 degradation, thereby reducing its presumed mutagenicity and carcinogenicity. However, the possibility that the peroxisome proliferation-associated increase in catalase activity is not adequate to degrade the H2O2 produced by the oxidases present in numerous peroxisome profiles in these cells cannot be ruled out. It is not certain if the mutagenicity is due to H2O2 or to the nascent oxygen liberated as a result of H2O2 degradation.

The elevations of serum AFP concentration are most probably related to the proliferative events induced by Wy-14,643. AFP production follows proliferation induced in...
vivo by partial heptectomy or chemically induced liver injury (42, 44). AFP production is also associated with proliferation of hepatocytes in vitro (42). Of further interest is the fact that Wy-14,643 does not induce liver cell necrosis, but only proliferation (hyperplasia). Thus, AFP production has been shown to be an early event following proliferation of hepatocytes in vitro (42). Of further interest is the fact that Wy-i4,643 does not induce liver cell necrosis, but the relationship of this to proliferation or to carcinogenic events remains uncertain (42). In the case of N-2-fluorenylacetamide or ethionine, AFP is found in small transitional "oval" cells early after carcino gen exposure (41, 45). These cell types are not seen during exposure to Wy-14,643. On the other hand, neoplastic nodules do develop after prolonged exposure to Wy-14,643. The neoplastic nodules produced by N-2-fluorenylacetamide do not contain AFP (41, 50), and the nodules produced by Wy-14,643 exposure appear at a time when serum AFP concentrations are almost normal. We have obtained preliminary evidence that none of the hepatocellular carcinomas arising from Wy-14,643 produce AFP. These findings are consistent with the hypothesis that non-AFP-producing hepatocellular carcinomas may arise from a different precursor lesion (neoplastic nodules) than do AFP-producing tumors, which may be derived from oval cells or zones of atypical hyperplasia (41).

ACKNOWLEDGMENTS


40. Selig, S. Distribution of α-Fetoprotein and Albumin-containing Cells in...
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Fig. 1. Hepatocellular carcinoma with trabecular pattern from a male acatalasemic mouse fed Wy-14,643 for 14 months. × 70.

Fig. 2. Hepatocellular carcinoma showing a well-differentiated trabecular pattern from a rat fed 0.1% Wy-14,643 for 15 months. × 100.

Fig. 3. Hepatocellular carcinoma with trabecular pattern reveals fatty metamorphosis. Rat fed Wy-14,643 for 16 months. × 125.

Fig. 4. Multiple metastases in the lungs from a trabecular hepatocellular carcinoma of a rat fed Wy-14,643 for 14 months. × 100.

Fig. 5. Primary hepatocellular carcinoma of the rat. Several peroxisomes (p) are present in the tumor cells, suggesting that these primary liver tumors respond to Wy-14,643-induced peroxisome proliferation. × 18,000.

Fig. 6. Hepatocellular carcinoma transplant in F344 rat. These transplantable tumors are well differentiated and contain peroxisomes (p) with prominent nucleoids. × 12,500.

Fig. 7. SDS-polyacrylamide gel electrophoretic profile of postnuclear pellet (100-μg protein samples) of normal rat liver (B and 1), liver tumor (B and 2), and an uninvolved portion of the liver (Band 3) of a Wy-14,643-fed rat. Arrows, position of 80,000-molecular-weight polypeptide.

Fig. 8. Autoradiograph of liver from rat fed Wy-14,643 for 5 days and given [3H]thymidine injections at 24, 48, 72, and 96 hr. Note dense labeling of several hepatocyte nuclei (arrows). × 200.

Fig. 9. Colchicine-arrested metaphases (arrows) between 36 and 42 hr in the liver of a rat treated with Wy-14,643. Colchicine (0.1 mg/kg body weight) was given i.p. 36 hr after the start of Wy-14,643. The animal was killed 6 hr after colchicine injection. × 200.
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