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# Peroxisome Proliferation and its role in Carcinogenesis

Views and expert opinions of an IARC Working Group
Lyon, 7–11 December 1994

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## Lyon, 7–9 December 1994

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# 1. CONSENSUS REPORT

## 1.1 Introduction

A large number of chemicals have been shown to induce peroxisome proliferation in the livers of mice and rats. Such chemicals include hypolipidaemic and other drugs, some herbicides, plasticizers, solvents, food flavours and natural products. Since humans are exposed to peroxisome proliferators to a significant extent, assessment of the adverse biological effects of this group of compounds, and particularly their potential carcinogenicity, has become an important issue.

At a meeting in December 1993 (IARC, 1993) to identify priorities for *IARC Monographs* in 1995–2000, several groups of agents were identified that may have specific mechanisms of action. One such group was chemicals that induce proliferation of peroxisomes, particularly in hepatocytes of rats and mice. The present meeting on peroxisome proliferation and its relationship to carcinogenesis was convened as a result of a recommendation of that meeting to discuss generic mechanisms of carcinogenicity before certain groups of chemicals were evaluated.

The *IARC Monographs* programme aims to identify chemicals and other agents and mixtures that are carcinogenic to humans; the evaluations are agreed upon by groups of invited experts in relevant fields. Most of the information considered during this process is derived from studies of human epidemiology and experimental carcinogenicity; information that may be relevant to the mechanism by which the putative carcinogen acts is also considered, as it may be helpful in making an overall evaluation. At a meeting on the mechanisms of carcinogenesis, held in Lyon in 1991 (Vainio *et al.*, 1992), information relevant to an evaluation of carcinogenic risk was considered to include evidence of: genotoxicity (i.e. structural changes at the level of the gene), effects on the expression of relevant genes (i.e. functional changes at the intracellular level), effects on cell or tissue morphology or behaviour and time and dose relationships of carcinogenic effects and interactions between agents. That advisory meeting concluded that the available data on mechanisms should be summarized and the strength of the evidence for the action of certain mechanisms and their relevance to carcinogenicity should be evaluated. Information on mechanism of action may show that similar effects occur in humans and experimental animals, or it could suggest species specificity. The ways in which such information might be used to modify an evaluation of carcinogenicity are described in the Preamble to each volume of *Monographs* since Volume 54 (IARC, 1992).

## 1.2 Characteristics of peroxisome proliferation

### *1.2.1 Peroxisomes*

Peroxisomes are single, membrane-limited, cytoplasmic organelles that are found in cells of animals, plants, fungi and protozoa. In rat hepatocytes, they are normally spherical or oval, about 0.5 μm in diameter and contain a finely granular matrix with a crystalline nucleoid core (Cohen & Grasso, 1981); it has been suggested that peroxisomes are not discrete organelles but actually exist as a continuous reticulum. They account for about 2% of the cytoplasmic volume and total cellular protein (Cohen & Grasso, 1981; Reddy & Lalwani, 1983; Mannaerts & Van Veldhoven, 1993). They are characterized by their content of catalase and a number of hydrogen peroxide-generating oxidases (Cohen & Grasso, 1981; Reddy & Lalwani, 1983), and, like mitochondria, they contain a fatty acid β-oxidation enzyme system. The half-life of peroxisomal enzymes is generally about 36 h. Peroxisomal disorders can have serious consequences for the organism (Mannaerts & Van Veldhoven, 1993; Reddy & Mannaerts, 1994).

### *1.2.2 Peroxisome proliferation in rats and mice*

**Peroxisome proliferation** can be defined as a cellular process characterized by increases in the volume density of peroxisomes and of peroxisomal fatty acid β-oxidation activity. Determination of peroxisome proliferation may be based on either but preferably both of these end-points. A **peroxisome proliferator** can be defined as a chemical that increases peroxisome proliferation. In judging whether a chemical is a peroxisome proliferator, due attention should be given to adequacy of experimental design, conduct and analysis.

A characteristic structural feature of many, but not all, peroxisome proliferators is the presence of an acidic function (Lake & Lewis, 1993), which is normally a carboxyl group and either occurs free in the parent structure or is generated by metabolism. Marked differences in the potencies of peroxisome proliferators have been demonstrated in several studies. For example, the hypolipidaemic agent ciprofibrate is orders of magnitude more potent than the plasticizer di(2-ethylhexyl)phthalate (Reddy *et al.*, 1986). Potent compounds can increase peroxisome volume density (peroxisome volume as a proportion of cytoplasmic volume) in liver of rats and mice from around 2% to 20–25%, whereas smaller changes may be produced by weaker compounds and by physiological factors, including certain high-fat diets and vitamin E deficiency.

Liver enlargement induced by peroxisome proliferators is due to both hepatocyte hyperplasia (increased replicative DNA synthesis and cell division) and hypertrophy. Morphological examination reveals increased peroxisome volume density, which results from an increase primarily in the number of peroxisomes, although size may also be increased. The major biochemical alteration is induction of the activities of peroxisomal enzymes of the fatty acid β-oxidation system and of CYP4A subfamily isoenzymes. The activity of the peroxisomal fatty acid β-oxidation cycle is normally determined by measuring overall activity (e.g. as cyanide-insensitive palmitoyl-coenzyme A oxidation) or by assaying the first rate-limiting enzyme of the cycle, namely acyl-coenzyme A oxidase (Mannaerts & Van Veldhoven, 1993). There is differential induction of peroxisomal enzyme activities, in that while that of the β-oxidation cycle enzymes can be markedly induced, smaller increases are observed in the activities of other peroxisomal enzymes, such as catalase. The stimulation of microsomal fatty acid-oxidizing enzymes (normally measured as lauric acid 12-hydroxylase) is due to induction of cytochrome P450 isoenzymes in the CYP4A subfamily (Gibson, 1989). The activity of carnitine acetyltransferase can also be markedly induced by peroxisome proliferators (Cohen & Grasso, 1981; Reddy & Lalwani, 1983); however, as this enzyme is found in peroxisomal, mitochondrial and microsomal fractions, enzyme induction may reflect stimulation of activity in more than one subcellular compartment (Cohen & Grasso, 1981; Moody *et al.*, 1991). The morphological and biochemical changes described above persist at the steady-state level for as long as the peroxisome proliferator is administered; reversal depends on the half-lives of both the induced enzymes and the rate of elimination of the chemical.

Other reported effects of peroxisome proliferators in hepatocytes of rats and mice include mitochondrial proliferation (with changes in enzyme activities), increase in the number of lysosomal bodies (with changes in enzyme activities and lipofuscin deposition) and effects on UDPglucuronosyltransferase activities. Peroxisome proliferators have also been reported to induce microsomal and cytosolic epoxide hydrolase activities, to modulate intracellular calcium concentrations, to stimulate protein kinase C and to reduce the activities of glutathione peroxidase, glutathione *S*-transferase and superoxide dismutase (Reddy & Lalwani, 1983; Bentley *et al.*, 1993; Grasso, 1993; Lake, 1993, 1995).

While marked effects may be observed in hepatocytes, only small increases in mRNA levels of peroxisomal fatty acid β-oxidation enzymes have been observed in certain other tissues, such as the kidney, intestine and heart.

### *1.2.3 Peroxisome proliferation in vitro*

Peroxisome proliferation has been demonstrated *in vitro* in primary rat and mouse hepatocyte cultures in a number of different laboratories, with standard hepatocyte media and culture conditions (Lock *et al.*, 1989; Moody *et al.*, 1991; Lake & Lewis, 1993; Foxworthy & Eacho, 1994). The factors responsible for the induction of peroxisome proliferation have been shown in these studies to be intrahepatic, and they are retained in cell culture. The characteristics of peroxisome proliferation *in vivo*, including stimulation of DNA synthesis, increased peroxisome numbers, changes in morphology and differential induction of enzyme activities, have also generally been observed in cultured hepatocytes.

In most studies, the effects of chemicals on the activities of peroxisomal and microsomal fatty acid-oxidizing enzymes have been examined in hepatocytes cultured for 3–4 days; however, peroxisome proliferation has also been reported in long-term (> 7 days) hepatocyte cultures and in other systems, including certain liver-cell lines, hepatocyte spheroids and liver slices (Lake, 1995). Peroxisome proliferation has also been demonstrated in hepatocytes transplanted into subcutaneous fat or the anterior chamber of the eye of rats and in hepatocytes induced in rat pancreas (Reddy & Mannaerts, 1994).

### *1.2.4 Mechanisms of induction of peroxisome proliferation*

Hypotheses to explain the initiation of peroxisome proliferation in hepatocytes include: the involvement of a receptor, substrate overload and peroxisome proliferators serving as substrates for peroxisomal enzymes (Reddy & Lalwani, 1983; Bentley *et al.*, 1993). Attention has focused mainly on identifying receptors and on the effect of peroxisome proliferators on lipid metabolism. These two hypotheses are not mutually exclusive (Bentley *et al.*, 1993). In the hypothesis of substrate overload, peroxisome proliferation is considered to be an adaptive response to perturbation of lipid metabolism, and induction of CYP4A isoenzymes is involved. Certain peroxisome proliferators inhibit fatty acid oxidation, form coenzyme A esters, increase levels of fatty acids and displace fatty acids from the cytosolic fatty acid-binding protein (Lock *et al.*, 1989; Bentley *et al.*, 1993).

Green and coworkers (Issemann & Green, 1990) have cloned a peroxisome proliferator-activated receptor (PPAR) from mouse liver. This protein is a member of the steroid hormone receptor superfamily and, after activation, acts as a transcription factor. When a chimaeric receptor expression vector containing regions that encode the putative ligand-binding domain of this mouse PPAR and the DNA-binding domain of the human oestrogen receptor was transfected into COS 1 cells, it could be activated by peroxisome proliferators, leading to transcriptional activation of a gene containing an oestrogen response element. Several PPARs have now been described in the mouse, rat, frog and human beings. They may also be activated by certain fatty acids, and their target genes encompass those for peroxisomal, microsomal, mitochondrial and cytosolic enzymes, all of which are involved in fatty acid metabolism (Desvergne & Wahli, 1994; Reddy & Mannaerts, 1994; Lake, 1995), underlining the important physiological role of these PPARs in lipid metabolism. At the molecular level, PPARs form a heterodimer with RXR that binds to a specific response element in the target gene. The transcriptional effect is further modulated through interaction of PPAR or PPAR-RXR with other transcription factors, which act either positively, like Sp1 (Krey *et al.*, 1995), or are repressive, such as COUP-TF (Reddy & Mannaerts, 1994; Lake, 1995). Some PPARs may be dominant repressors of other forms (Kliewer *et al.*, 1994).

Binding of peroxisome proliferators to PPAR has not been demonstrated but cannot be excluded. Peroxisome proliferators have been shown to increase the level of certain PPARs in mouse and rat liver (Reddy & Mannaerts, 1994; Lake, 1995). Activation of PPARs by peroxisome proliferators may require metabolism (e.g. to a coenzyme A ester or other derivative) or may occur by displacement of fatty acids from their cytosolic binding protein (Desvergne & Wahli, 1994). Several mechanisms could account for tissue and species differences in response to peroxisome proliferators. These include differences in the metabolism of peroxisome proliferators, interaction of PPARs with different sets of transcription factors, and species differences in the regulatory element of a given target gene (see Reddy & Rao, this volume). Moreover, tissue differences in the distribution of PPARs have been documented (Reddy & Mannaerts, 1994; Lake, 1995; Zhu *et al.*, 1995).

### *1.2.5 Hepatocellular proliferation induced by peroxisome proliferation*

Not only peroxisome proliferation but also hepatocyte proliferation is an important response in the livers of rats and mice receiving peroxisome proliferators. Acute hepatocyte proliferation, which involves about 50% of hepatocytes, is seen to begin about 48 h after the beginning of administration of a peroxisome proliferator. This early proliferative response subsides after several days. Chronic hepatocyte proliferation has been seen in the livers of rats and mice after administration of some, but not all, peroxisome proliferators. This replicative response may continue for the duration of exposure to the chemical. While the replication rate is less than that seen in the acute phase, the total proliferative response is much greater. Preferential hepatocyte proliferation occurs in the tumours that develop in rats and mice after administration of peroxisome proliferators and is seen in lesions that are the direct progenitors of tumours.

As enhanced hepatocyte proliferation is associated with administration of peroxisome proliferators to rats and mice, and in view of the importance of cell proliferation in carcinogenesis, hepatocyte proliferation is included in any evaluation of peroxisome proliferators, such as an assessment of species differences.

### *1.2.6 Species differences*

Differences between species with regard to hepatic peroxisome proliferation have been investigated *in vitro* and *in vivo* (Cohen & Grasso, 1981; Stott, 1988; Lock *et al.*, 1989; Moody *et al.*, 1991;

Bentley *et al.*, 1993). Factors that must be taken into account in such studies include the metabolism, disposition and dose of the chemical, sex differences and intrahepatic differences in response; *in vitro*, the functional viability of the hepatocyte preparations must also be considered.

Rats and mice are clearly responsive to peroxisome proliferators; although differences among strains have been observed, these are minor in comparison with the magnitude of species differences in response. On the basis of the activities of marker enzymes (e.g. cyanide-insensitive palmitoyl-coenzyme A oxidation, lauric acid 12-hydroxylase, carnitine acetyltransferase) and ultrastructural examination, Syrian hamsters appear to exhibit an intermediate response, whereas in most studies guinea pigs are either unresponsive or refractory. There is no evidence of significant peroxisome proliferation in either New (e.g. marmoset) or Old (e.g. rhesus) World monkeys (Lock *et al.*, 1989; Bentley *et al.*, 1993; Lake, 1995) *in vivo*, although high doses of ciprofibrate and DL-040 were reported to induce hepatic peroxisome proliferation in cynomolgus and rhesus monkeys (Reddy *et al.*, 1984; Lalwani *et al.*, 1985).

The results of studies with cultured primary hepatocytes from rats, mice, Syrian hamsters, guinea pigs and primates *in vitro* mirror those obtained *in vivo* in the same species (Bentley *et al.*, 1993; Lake, 1995): less effect is observed in Syrian hamster than in rat or mouse hepatocytes, and little or no effect is seen in guinea pig and primate hepatocytes, even though such preparations responded to other chemical challenges.

Comparatively few investigations have been conducted to evaluate species differences in cell replication. Although both nafenopin and Wy-14,643 are potent mitogens in rat liver, they do not appear to produce any significant stimulation of replicative DNA synthesis in the hepatocytes of Syrian hamsters treated *in vivo* either acutely or chronically (Lake, 1995). Similarly, methylclofenapate increased replicative DNA synthesis in rat hepatocytes *in vitro*, but no effect was observed in guinea pig or marmoset hepatocytes (Elcombe & Styles, 1989).

For data on human hepatocytes and human volunteers, see section 1.5, Peroxisome proliferators, human response and hazard.

## 1.3 Hepatocarcinogenicity in experimental animals

### *1.3.1 Concordance with peroxisome proliferation*

Prolonged administration of members of the structurally diverse class of peroxisome proliferators has been shown in many studies to produce liver tumours in rats and mice (Cohen & Grasso, 1981; Reddy & Lalwani, 1983; Bentley *et al.*, 1993), but the long-term effects of these compounds in other species have been examined in only a few studies. Clobuzarit, which induces peroxisome proliferation in rat and mouse liver (Orton *et al.*, 1984), did not induce tumours in Syrian hamsters in a two-year study (Tucker & Orton, 1995). Di(2-ethylhexyl)-phthalate, administered either by inhalation or by intraperitoneal injection, also did not induce tumours in this species (Schmezer *et al.*, 1988), although the doses and treatment regimens may not have been appropriate for an assessment of hepatocarcinogenicity. Both nafenopin and Wy-14,643 induce liver enlargement and sustained peroxisome proliferation in Syrian hamsters, but neither induced liver tumours after 80 weeks (Lake, 1995). Although peroxisome proliferation is therefore not sufficient for tumour induction, it may be necessary in the sequence of events that leads to the carcinogenicity of these compounds.

Some long-term studies have been performed in primates with ciprofibrate, clobuzarit and clofibrate. Although none was of lifetime duration, a 6.5-year study of clofibrate in marmosets covered about half of the expected lifespan of that species (Tucker & Orton, 1993). Increased relative liver weight was observed in some studies, but there was no evidence for significant peroxisome proliferation or peroxisome proliferator-induced liver lesions (Tucker & Orton, 1993; Graham *et al.*, 1994). These results suggest that the peroxisome proliferator-induced altered hepatic foci typically observed in rats and mice are not induced in Syrian hamsters or primates.

A comparison of 39 paired sets of data on carcinogenicity and peroxisome proliferation for 18 agents that have been shown to possess an intrinsic ability to induce peroxisome proliferation in the livers of rats and mice indicates a strong concordance (80%) between peroxisome proliferation and hepatocarcinogenicity after long-term exposure to these chemicals (Table 1), providing further support for the validity of peroxisome proliferation as an early biomarker for carcino-

genesis in the liver (Reddy *et al.*, 1980; Reddy & Rao, 1992).

Peroxisome proliferators are generally inactive in a wide range of short-term tests for genotoxicity *in vitro* and *in vivo*, including mutagenicity in *Salmonella*, unscheduled DNA synthesis, DNA damage as measured by $^{32}$P-postlabelling, intrachromosomal deletion and recombination in yeast (Warren *et al*, 1980; Reddy & Rao, 1992; Ashby *et al*, 1994). A recent, critical evaluation of the results of various tests for the mutagenicity of peroxisome proliferators thus eliminated intrinsic genotoxicity as the unifying mechanism of action for this class of carcinogenic chemicals (Ashby *et al*, 1994). It should be noted, however, that the properties of peroxisome proliferation and genotoxicity are not necessarily mutually exclusive, and some of these chemicals have genotoxic activity *in vitro*. A few hepatocarcinogenic peroxisome proliferators induced weak cytogenetic and/or cell transforming effects in cultured cells (Table 2), although this is not a consistent property of peroxisome proliferators. Several peroxisome proliferators also inhibited intercellular communication in rat and mouse hepatocytes and in hamster cells in culture (Table 2).

As genotoxicity is not a primary biological effect of peroxisome proliferators, any genetic alteration that may be necessary for the carcinogenic action could occur indirectly during long-term exposure to these agents as a result of biological alterations; consequently, the process of initiation would be rather slow and not as drastic as observed with genotoxic carcinogens. Structurally diverse peroxisome proliferators induce characteristic responses in hepatocytes by interacting with members of the PPAR subfamily. Thus, the hepatocarcinogenicity of these chemicals is strongly associated with the induction of these predictable and highly characteristic responses. Caution should be exercised, however, in drawing a mechanistic inference from this receptor-mediated response, despite the concordance between peroxisome proliferation and hepatocarcinogenicity. Knowledge of the carcinogenic mechanisms of peroxisome proliferators is incomplete; however, the characteristic biochemical composition of peroxisomes and the fact that peroxisome proliferation is associated with disproportionate changes in the levels of peroxisomal enzymes, leading to production of excess hydrogen peroxide concentrations in liver, may provide clues to the involvement of this organelle in carcinogenesis.

### *1.3.2 Plausible mechanisms*

Several mechanisms have been proposed for the induction of hepatocellular tumours in rats and mice.

#### *1.3.2.1 Receptor-mediated responses and oxidative stress*

This hypothesis relies on experimental evidence that the biological effects of peroxisome proliferators are confined predominantly to hepatic cells, that peroxisome proliferation is associated with disproportionate increases in the activities of enzymes that generate and degrade hydrogen peroxide, perturbing the hydrogen peroxide balance in the liver, and that tumours develop in this organ, which is also the main organ that responds to peroxisome proliferation. Peroxisomes in liver contain at least five distinct oxidases, which use a variety of substrates to generate hydrogen peroxide. Of these, urate oxidase, a liver-specific peroxisomal oxidase, and peroxisomal fatty acyl-coenzyme A oxidase, the first enzyme in the peroxisomal oxidation system, are of interest. Urate oxidase is expressed in the livers of most mammals, including rats and mice, but not in humans or higher primates. It is a pivotal enzyme in the metabolism of uric acid, a naturally occurring, potent biological antioxidant. The metabolic degradation of uric acid to allantoin by urate oxidase leads to the generation of hydrogen peroxide and accounts for the low serum levels of uric acid in these animals (Ames *et al.*, 1981). The activity of urate oxidase is two to three times higher in livers with peroxisome proliferation than in normal livers, with a consequent reduction in the concentration of uric acid in serum.

Livers of rats and mice with peroxisome proliferation show a 20- to 40-fold increase in the activity of peroxisomal fatty acyl-coenzyme A oxidase, owing to transcriptional activation of the responsible gene, whereas catalase activity is increased by less than twofold. These disproportionate increases in hydrogen peroxide generating and hydrogen peroxide degrading peroxisomal enzymes, together with reductions in the overall cellular capacity to detoxify hydrogen peroxide, provide a plausible biological basis for the role of peroxisome proliferation-associated oxidative stress in hepatocarcinogenesis. Livers of animals with massive peroxisome proliferation have a biochemical milieu that is consistent with the presence

**Table 1. Database for examining the concordance between hepatocellular peroxisome proliferation (PP) and hepatocarcinogenicity (HC) in rats and mice (M, male; F, female)**

| Compound | CAS No. | Rats | | | | | Mice | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Strain | PP | | HC | | Strain | PP | | HC | |
| | | | M | F | M | F | | M | F | M | F |
| Benzylbutyl phthalate | 85-68-7 | F344 | | – | | – | | | | | |
| Cinnamyl anthranilate | 87-29-6 | F344 | – | + | – | – | B6C3F1 | + | + | + | + |
| Ciprofibrate | 52214-84-3 | F344 | + | | + | | C57Bl | + | | + | |
| Clobuzarit | 22494-47-9 | Wistar | + | | – | | C57Bl | + | | + | |
| Clofibrate | 637-07-0 | SD | + | + | + | + | C57Bl | + | | – | |
| | | F344 | + | | + | | Swiss | + | | – | |
| | | Wistar | + | | – | | | | | | |
| Di(2-ethylhexyl)adipate | 103-23-1 | F344 | + | + | – | – | B6C3F1 | + | + | – | + |
| Di(2-ethylhexyl)phthalate | 117-81-7 | F344 | + | + | + | + | | | | | |
| Di-isononyl phthalate | 28553-12-0 | F344 | – | – | – | – | | | | | |
| Gemfibrozil | 25812-30-0 | SD | + | | + | | | | | | |
| Lactofen | 3513-60-4 | | | | | | CD–1 | + | + | + | + |
| LY 171883 | 88107-10-2 | | | | | | B6C3F1 | | + | | + |
| Methylclofenapate | 21340-68-1 | F344 | + | | + | | | | | | |
| Nafenopin | 3771-19-5 | F344 | + | | + | | | | | | |
| Tetrachloroethylene | 127-18-4 | F344 | – | – | – | – | B6C3F1 | + | + | + | + |
| Tibric acid | 37087-94-8 | F344 | + | | + | | | | | | |
| Trichloroacetic acid | 76-03-9 | | | | | | B6C3F1 | + | | + | |
| Trichloroethylene | 79–01–6 | F344 | – | | – | | B6C3F1 | + | | + | |
| | | Osborne-Mendel | – | | – | | | | | | |
| Wy-14,643 | 50892-23-4 | F344 | + | | + | | | | | | |

Adapted from Ashby *et al.* (1994)

**Table 2. Overall activity of hepatocarcinogenic peroxisome proliferators in assays for morphological cell transformation and gap-junctional intercellular communication**

| Compound | Cell transformation | Intercellular communication |
|---|---|---|
| Clofibrate | + | + |
| Di(2-ethylhexyl)adipate | – | |
| Di(2-ethylhexyl)phthalate | + | + |
| Methylclofenapate | | – |
| Nafenopin | | + |
| Trichloroethylene | + | + |
| Trichloroacetic acid | | + |
| Wy-14,643 | + | + |

+, Most assays with the compound gave a positive response (i.e. induced cell transformation or decreased intercellular communication).
–, Most assays with the compound gave a negative response (i.e. did not induce cell transformation or did not decrease intercellular communication).

of a sustained increase in the levels of hydrogen peroxide (Reddy & Rao, 1992). Increased amounts of lipofuscin and high levels of conjugated dienes have been reported in livers of rats treated for long periods with peroxisome proliferators. Furthermore, fatty acyl-coenzyme A oxidase-rich peroxisomes isolated from the livers of rats treated with a peroxisome proliferator induced DNA strand breaks *in vitro* in one study; this finding was not reproduced in other studies. Taken together with the observation that livers with chronic peroxisome proliferation show a two- to fourfold increase in the level of 8-hydroxydeoxyguanosine in DNA, these reports can be construed as evidence for the role of receptor-mediated transcriptional activation of hydrogen peroxide-generating peroxisomal fatty acyl-coenzyme A oxidase in the carcinogenesis associated with peroxisome proliferation. A recent study (Cattley & Glover, 1993), however, demonstrated that the increase in 8-hydroxydeoxyguanosine is dependent on the method of DNA isolation, suggesting that it occurs in the homogenate and thus reflects changes in mitochondrial rather than nuclear DNA.

In rats given long-term treatment with both ciprofibrate, a peroxisome proliferator, and ethoxyquin, an antioxidant, a marked reduction in hepatocellular tumour development is seen, despite the increases in liver enlargement, hepatocellular proliferation and peroxisome proliferation (Rao *et al.*, 1984). Conflicting results were obtained, however, in studies of the modulating effects of vitamin E deficiency and peroxisome proliferation-induced carcinogenesis (Glauert *et al.*, 1990; Lake *et al.*, 1991).

It is less likely that the level of oxidative stress caused by sustained induction of peroxisome proliferation would yield measurable DNA breakage similar to that resulting from exposure to genotoxic chemicals. Induction of DNA damage by free radicals occurs relatively commonly in mammalian DNA, and, although it is rapidly repaired, it is implicated in spontaneous initiation (Saul & Ames, 1986; Loeb, 1989). As mentioned above, several lines of evidence indicate that induction of peroxisome proliferation is not the sole factor involved in tumour development. The lack of a consistent quantitative association between induction of peroxisome proliferation, DNA oxidation and tumour response also indicates that additional factors must operate.

The availability of transgenic cells containing the rat peroxisomal fatty acyl-coenzyme A oxidase gene has provided an opportunity to examine the role of increased expression of this hydrogen peroxide-generating peroxisomal protein in cell transformation (Chu *et al.*, 1995). These and other transgenic cells and transgenic animals that overexpress fatty acyl-coenzyme A oxidase should serve as useful systems for further elucidating the relative roles of peroxisome proliferation and oxidative stress in hepatocarcinogenesis.

#### *1.3.2.2 Increased cell proliferation*

Cell proliferation has been linked experimentally and conceptually to carcinogenesis induced by both genotoxic and nongenotoxic carcinogens (Rajewsky, 1972; Ames & Gold, 1990; Cohen & Ellwein, 1990). In tumorigenesis induced by genotoxic carcinogens, cell proliferation is a crucial event in converting DNA damage to heritable mutations and causing clonal expansion of mutated cell populations (Grisham *et al.*, 1983). Cell proliferation induced by genotoxic carcinogens is compensatory and may be associated with cytotoxic cell injury; this type of cell proliferation tends to augment the process of carcinogenesis (Columbano *et al.*, 1987). Peroxisome proliferator-induced hepatocellular proliferation is not preceded by hepatocellular injury (Reddy *et al.*, 1979); and Columbano *et al.* (1987) reported that this primary mitogenic proliferation is not as effective as compensatory hyperplasia in the carcinogenic process, although the conditions of the experiments were different from those in long-term bioassays. The suggestion that peroxisome proliferator-induced carcinogenesis is mediated by hepatocellular proliferation is based in part on the observed mitogenic properties of peroxisome proliferators, such as nafenopin, Wy-14,643, ciprofibrate and clofibric acid (Moody *et al.*, 1977; Reddy *et al.*, 1979; Marsman *et al.*, 1988; Yeldandi *et al.*, 1989; Marsman *et al.*, 1992), and on the hypothesis that the chronic mitogenic response has an indirect carcinogenic effect in that it increases the probability that endogenous DNA damage will be converted into mutations (Ames & Gold, 1990; Cohen & Ellwein, 1991).

The initial, acute cell proliferation is unlikely to have an important effect on carcinogenesis, for several reasons. First, very little cell proliferation occurs during this phase in comparison with that which takes place during the two years of a typical bioassay in rats or mice. Second, the acute phase of cell proliferation is finished before induction of peroxisome proliferation has been completed, so that acute hepatocyte proliferation cannot play a

role in enhancing the DNA damage that may occur secondary to induction of peroxisome proliferation. The increased hepatocellular proliferation observed during continued treatment with peroxisome proliferators may, however, contribute to the carcinogenic process. Chronic enhancement of hepatocyte proliferation is correlated with the greatest tumour responses (Marsman *et* al., 1988, 1992), suggesting a role in carcinogenesis. Proliferation of nonpreneoplastic hepatocytes may not be sufficient to elicit carcinogenesis (Reddy & Rao, 1992; Ashby *et al.*, 1994; Reddy & Rao, this volume).

#### *1.3.2.3 Preferential growth of preneoplastic lesions*

It has been proposed that peroxisome proliferators induce preferential growth of altered hepatocytes in developing liver tumours (Schulte-Hermann *et al.*, 1981, 1983; Marsman & Popp, 1994), and to a much greater degree than other nongenotoxic carcinogens, such as phenobarbital. As this growth depends on continued administration of a peroxisomal proliferating agent, it is not an inherent property of the preneoplastic cells. Support for the conclusion that these agents act by inducing preferential growth is provided by the observation that more tumours develop in older than younger rats fed nafenopin or Wy-14,643, although tumours were found in both two-month- and 15-month-old rats fed these compounds (Cattley *et al.*, 1991; Kraupp-Grasl *et al.*, 1991). In studies with ciprofibrate, a potent peroxisome proliferator, however, no appreciable difference in tumour incidence was seen between six- and 12-month-old rats (Rao *et al.*, 1990). Peroxisome proliferators can induce liver tumours in both young and old rats, and, like any other carcinogen, genotoxic or nongenotoxic, they can act to some extent at any stage of the process of carcinogenesis. The observation of *ras* gene mutations in B6C3F1 mouse liver tumours (Hegi *et al.*, 1993) is not consistent with the notion that peroxisome proliferators act solely as promoters of spontaneous carcinogenesis. The spectrum and frequency of the activating mutations detected in the H-*ras* and K-*ras* genes differ significantly between ciprofibrate-induced and spontaneously occurring liver tumours. These observations further support the suggestion that ciprofibrate and other peroxisome proliferators are genotoxic by indirect means, such as oxidative stress.

Several other possible carcinogenic mechanisms of action of peroxisome proliferators have been proposed, including stimulation of protein kinase C, uncoupling of oxidative phosphorylation and interference with intercellular communication (Keller *et al.*, 1993; Bayly *et al.*, 1994; Bojes & Thurman, 1994; Krutovskikh *et al.*, 1995).

#### *1.3.3 Neoplasms in organs other than the liver*

Some hepatocarcinogenic peroxisome proliferators have been found also to induce tumours in organs other than the liver. Of the 18 peroxisome proliferators listed in Table 1, benzylbutyl phthalate and tetrachloroethylene have been reported to induce mononuclear-cell leukaemia in Fischer 344 rats; cinnamyl anthranilate, clofibrate and gemfibrozil to induce tumours in rat pancreas; and cinnamyl anthranilate, tetrachloroethylene and trichloroethylene to induce renal tumours in male rats. A number of studies have also been carried out to investigate possible promoting activity in two-stage carcinogenesis models. Di(2-ethylhexyl)phthalate was reported to promote renal tumours (Kurokawa *et al.*, 1988) and to act as a second-stage promoter in skin carcinogenesis in SENCAR mice (Diwan *et al.*, 1985). Clofibrate has a promoting effect in urinary bladder carcinogenesis in Fischer 344 rats (Hagiwara *et al.*, 1990). These effects appear not to be related to the peroxisome proliferating properties of the chemicals, but may represent independent properties. (See also the paper by Dybing *et al.*, this volume.)

### 1.4 Peroxisome proliferation as a biological marker for hepatocarcinogenesis

It has been suggested that the morphological and biochemical phenomenon of peroxisome proliferation in liver cells could serve as a useful biological marker for identifying the potential carcinogenicity of this class of generally nonmutagenic chemicals (Reddy *et al.*, 1980; Reddy & Lalwani, 1983). Short-term biological effects *in vivo* can be evaluated easily by ascertaining the alterations in peroxisome number and volume density in the livers of rats and mice exposed to several doses of the compound and by determining changes in peroxisomal β-oxidation enzyme activity and in specific mRNA levels (Reddy & Rao, 1992). The ability of chemicals to induce peroxisome proliferation in primary cultures of liver cells could also be used to identify peroxisome proliferators *in vitro* and to evaluate possible species differences in response.

The purpose of a short-term biological marker is to identify the potential carcinogenic nature of an agent and not necessarily to serve as a quantitative indicator of carcinogenicity. Nonetheless, if a

chemical can induce maximal peroxisome proliferation (i.e. increase the peroxisome volume to 15–25% of the cytoplasmic volume), it is reasonable to anticipate a high incidence of hepatocellular tumours in rats and mice exposed for long periods.

### 1.5 Peroxisome proliferators, human response and hazard

The potential human response to agents that induce peroxisome proliferation in rats or mice has been examined both *in vitro*, in cultured human hepatocytes, and *in vivo,* in subjects receiving hypolipidaemic agents.

As in cultured nonhuman primate hepatocytes, peroxisome proliferators do not have significant effects on marker enzyme activities or the number of peroxisomes in cultured human hepatocytes (for reviews, see Bentley *et al.*, 1993; Ashby *et al*, 1994; Foxworthy & Eacho, 1994). The compounds examined in cultured human hepatocytes include beclobric acid, benzbromarone, ciprofibrate, clofibric acid, fomesafen, monoethylhexylphthalate, methylclofenapate and trichloroacetic acid. In many of these studies, the functional viability of the human hepatocyte preparations was confirmed in parallel experiments in which other end-points were determined. Additionally, although replicative DNA synthesis could be induced in these hepatocytes by epidermal growth factor, no significant changes were induced by methylclofenapate or nafenopin (reviewed by Lake, 1995).

The effects of several hypolipidaemic agents, including ciprofibrate, clofibrate, fenofibrate and gemfibrozil (all well documented peroxisome proliferators in mouse and rat liver), have been studied in human volunteers (for reviews, see Bentley *et al.*, 1993; Ashby *et al.*, 1994). While no significant change was seen in most investigations, one study of clofibrate resulted in a 50% statistically significant increase in the mean number of peroxisomes but a nonsignificant, 23% increase in peroxisome volume density (Hanefeld *et al.*, 1983). Since the measurement of volume density rather than the number of peroxisomes is indicative of peroxisome proliferation, the results of this study indicate a negative human response.

The results of studies of human hepatocytes *in vivo* and *in vitro*, together with the data on effects in experimental animals, suggest that there are marked species differences in response to peroxisome proliferators. Although further studies are desirable, the current literature suggests that compounds that are peroxisome proliferators in rats and mice have little, if any, effect on human liver. In a study by Sher *et al.* (1993), however, a human liver PPARα was as effective as mouse PPARα in a trans-activation assay system *in vitro*. The apparent disparity between the effect of peroxisome proliferators in rodent and human hepatocyte cultures may therefore be due to a number of modulating or confounding factors (Ashby *et al.*, 1994; Reddy & Mannaerts, 1994; Lake, 1995).

Several clinical trials have addressed the potential carcinogenicity of therapeutic hypolipidaemic agents in the human population. A meta-analysis of all randomized clinical trials of cholesterol lowering did not reveal excess mortality from cancer at all sites combined among the actively treated subjects (Law *et al.*, 1994). Two randomized studies of the effect of fibrates in preventing coronary heart disease, however, raised concern, as an excess of deaths from cancer was seen in the fibrate-treated subjects. In the five-year WHO study of clofibrate (WHO European Collaborative Group, 1986; Law *et al.*, 1994), 72 deaths from cancer were observed in the group treated with clofibrate and 54 in the group receiving a placebo ($p = 0.12$). The difference disappeared during the follow-up after the intervention. Data on cancer incidence were not available. No difference was seen in the incidence of or mortality from cancer in the Helsinki Heart Study of gemfibrozil (Huttunen *et al.*, 1994) during the five-year intervention period, but an excess of deaths from cancer (20 *versus* 7) was observed in the original gemfibrozil group during the 3.5-year post-trial follow-up, when about 60% of the participants in both trial groups were taking gemfibrozil. The differences disappeared again when the follow-up was extended to 10 years. Cancer incidence did not differ between the two groups during the observation period. It should be noted that the studies of users of cholesterol-lowering drugs have insufficient statistical power to evaluate the risk for hepatocellular cancer.

Increased mortality from cancer has been associated with a low serum cholesterol level in about half of the longitudinal epidemiological studies published so far (Epstein, 1990, 1992; Jacobs *et al.*, 1992; Law *et al.*, 1994). The excess mortality from cancer seen in several studies was confined to deaths occurring within a few years of cholesterol measurements and was attributed to preclinical cancer. In an analysis of all published cohort studies, the association was present on a long-term basis only for cancers of the lung and of

the lymphatic and haematopoietic system (Law & Thompson, 1991). The finding that the association between low cholesterol and lung cancer was restricted to community cohorts and to certain groups within the cohorts was interpreted as suggesting that the relationship is a result of confounding by a factor linked to both low serum cholesterol level and a high risk for lung cancer.

### 1.6 Conclusions

The responses to the following questions are based on the interpretation of hepatocellular tumour induction in rats and mice, since the mechanisms of carcinogenesis have been evaluated in detail only in liver. The available information on the mechanisms of tumour response elicited by some peroxisome proliferators in rats and mice at sites other than the liver suggests that peroxisome proliferation does not play a role in the formation of tumours at those sites.

1. What mechanisms are critical to peroxisome proliferation?

The evidence suggests that peroxisome proliferation in mouse and rat liver is mediated by activation of peroxisome proliferator-activated receptors, which are members of the steroid hormone receptor superfamily. Receptor activation may be a direct effect of the peroxisome proliferator or may be mediated through perturbation of lipid metabolism. Such receptors have also been identified in humans.

2. Is peroxisome proliferation an indicator of cancer risk in rats and mice?

There is a strong concordance between peroxisome proliferation and hepatocellular carcinogenesis in rats and mice. On the basis of a more limited database, a similar concordance is seen between hepatocellular proliferation induced by peroxisome proliferators and hepatocellular tumour induction.

3. What are the mechanisms of carcinogenesis mediated by chemically induced peroxisome proliferation?

Two major biological responses to peroxisome proliferators are associated with increased cancer induction in rats and mice. One is peroxisome proliferation, and the other is increased hepatocellular proliferation. The proposed mechanisms of peroxisome proliferator-induced hepatocellular carcinogenesis include oxidative stress, increased hepatocellular proliferation and preferential growth of preneoplastic lesions. These mechanisms may not be mutually exclusive.

Hepatocellular carcinogenic peroxisome proliferators are generally inactive in assays for genotoxicity. Some such agents can cause morphological cell transformation and inhibit gap-junctional intercellular communication. These cellular effects appear to be independent of the process of peroxisome proliferation. Chemicals that induce peroxisome proliferation may have additional carcinogenic effects unrelated to that phenomenon.

4. Does peroxisome proliferation also occur in humans, and do the mechanisms of carcinogenesis mediated by peroxisome proliferation in rats and mice also operate in humans?

Data on the effects in humans of peroxisome proliferators are derived from studies of subjects receiving hypolipidaemic drugs and from studies of cultured human hepatocytes. The limited data *in vivo* suggest that therapeutic doses of hypolipidaemic agents produce little if any peroxisome proliferation in human liver. Hypolipidaemic fibrates and other chemicals that induce peroxisome proliferation in rat and mouse hepatocytes when given at high concentrations do not do so in cultured human hepatocytes.

Marginal, statistically nonsignificant increases in hepatocellular peroxisome proliferation in human liver have been reported after exposure to clofibrate, but a comparable increase in peroxisome proliferation was not associated with hepatocellular carcinogenesis in rats or mice.

5. How can data on peroxisome proliferation be used in making overall evaluations of carcinogenicity to humans?

Chemicals that show evidence of inducing peroxisome proliferation should be evaluated on a case-by-case basis. The evaluation of agents by independent expert groups is a matter of scientific judgement.

When the database supports the conclusion that a tumour response in mice or rats is secondary only to peroxisome proliferation, consideration could be given to modifying the overall evaluation, as described in the Preamble to the *IARC Monographs*, taking into account the following evidence:

(a) Information is available to exclude mechanisms of carcinogenesis other than those related to peroxisome proliferation.

(b) Peroxisome proliferation (increases in peroxisome volume density or fatty acid β-oxidation activity) and hepatocellular

proliferation have been demonstrated under the conditions of the bioassay.

(c) Such effects have not been found in adequately designed and conducted investigations of human groups and systems.

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# 2. AUTHORED PAPERS

# A. THE BIOLOGY AND MOLECULAR CONSEQUENCES OF PEROXISOME PROLIFERATION IN EXPERIMENTAL ANIMALS AND HUMANS

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## Introduction

The purpose of this paper is to consider briefly the following aspects of hepatic peroxisome proliferation

- characteristics of peroxisome proliferation in rodent liver;
- rodent liver peroxisome proliferators, including compound potency differences and structure–activity relationships;
- peroxisome proliferation *in vitro*, in primary hepatocyte cultures and other systems;
- mechanisms of induction of peroxisome proliferation in rodent hepatocytes, including lipid hypothesis and peroxisome proliferator-activated receptors; and
- species differences in response, in studies on organelle proliferation, associated enzyme activities and other end-points *in vivo* and *in vitro*.

## Characteristics of peroxisome proliferation in rodent liver

Peroxisomes (or 'microbodies') are single-membrane-limited cytoplasmic organelles present in cells of animals, plants, fungi and protozoa. They are characterized by their content of catalase and a number of hydrogen peroxide-generating oxidase enzymes (Cohen & Grasso, 1981; Reddy & Lalwani, 1983). Like mitochondria, peroxisomes contain a complete fatty acid β-oxidation cycle (Lazarow & deDuve, 1976). In rat hepatocytes, peroxisomes are normally spherical or oval and approximately 0.5 μm in diameter and contain a finely granular matrix with a crystalline nucleoid core (Cohen & Grasso, 1981). They account for about 2% of the cytoplasmic volume and total cellular protein (Cohen & Grasso, 1981; Reddy & Lalwani, 1983; Mannaerts & Van Veldhoven, 1993;). Peroxisomes have a number of important functions in intermediary metabolism (Mannaerts & Van Veldhoven, 1993), and peroxisomal disorders can have serious consequences for the organism (Wanders *et al.*, 1993).

A wide variety of chemicals have been shown to produce liver enlargement and peroxisome proliferation and to induce peroxisomal and microsomal fatty acid oxidizing enzyme activities in rodents (Cohen & Grasso, 1981; Reddy & Lalwani, 1983; Stott, 1988; Lock *et al.*, 1989; Moody *et al.*, 1991; Bentley *et al.*, 1993; Lake & Lewis, 1993). Some characteristics of peroxisome proliferation in rat and mouse hepatocytes are shown in Table 1. Liver enlargement is due to both hepatocyte hyperplasia and hypertrophy. Morphological examination reveals an increase in both the size and number of peroxisomes and in the amount of smooth endoplasmic reticulum. Unlike the livers of untreated animals, livers treated with peroxisome proliferators have many 'coreless' peroxisomes (which lack the characteristic crystalline nucleoid containing urate oxidase). The major

biochemical alterations consist of induction of both peroxisomal and microsomal fatty acid oxidizing enzyme activities. The activity of the peroxisomal fatty acid β-oxidation cycle is normally determined by measuring either overall activity (e.g. as cyanide-insensitive palmitoyl-coenzyme A (CoA) oxidation) or by assaying the first-rate limiting enzyme of the cycle, namely acyl-CoA oxidase (Lazarow & deDuve, 1976; Mannaerts & Van Veldhoven, 1993). It should be noted that there is differential induction of peroxisomal enzyme activities, in that while the β-oxidation cycle enzymes can be markedly induced, much smaller increases are observed in other peroxisomal enzymes such as D-amino acid oxidase and catalase. The stimulation of microsomal fatty acid oxidizing enzymes (normally measured as lauric acid 12-hydroxylase) is due to induction of cytochrome P450 isoenzymes in the CYP4A subfamily (Sharma et al., 1988a,b; Gibson, 1989). Carnitine acetyltransferase activity can also be markedly induced by rodent peroxisome proliferators (Cohen & Grasso, 1981; Reddy & Lalwani, 1983). However, as this enzyme is found in peroxisomal, mitochondrial and microsomal fractions, enzyme induction may reflect stimulation of activity in more than one subcellular compartment (Ishii *et al.*, 1980; Bieber *et al.*, 1981; Cohen & Grasso, 1981; Moody *et al.* 1991).

**Table 1. Some characteristics of peroxisome proliferation in rat and mouse liver**

| |
|---|
| ***Liver weight*** |
| – Enlargement due to both hepatocyte hyperplasia and hypertrophy |
| – Increased replicative DNA synthesis (either transient or sustained)[a] |
| ***Morphological changes*** |
| – Increased number and size of peroxisomes |
| – Many 'coreless' peroxisomes[b] |
| – Increased smooth endoplasmic reticulum |
| – Lysosomal changes and lipofuscin deposition[a] |
| – Liver nodules and hepatocellular carcinoma[a] |
| ***Biochemical changes*** |
| – Selective induction of peroxisomal enzymes (e.g. marked induction of peroxisomal fatty acid β-oxidation enzymes but only a small increase in catalase activity) |
| – Induction of microsomal fatty acid (ω-1)- and particularly ω-oxidizing enzyme activities (due to induction of cytochrome P450 isoenzymes in the CYP4A subfamily) |
| – Induction of carnitine acetyltransferase activity |
| – Increase in an 80-kDa relative molecular mass polypeptide (due to induction of component enzymes of the peroxisomal fatty acid β-oxidation cycle) |
| – Induction of microsomal and cytosolic epoxide hydrolases |
| – Induction of microsomal UDPglucuronosyltransferase |
| – Inhibition of glutathione peroxidase, glutathione S-transferase and superoxide dismutase activities |

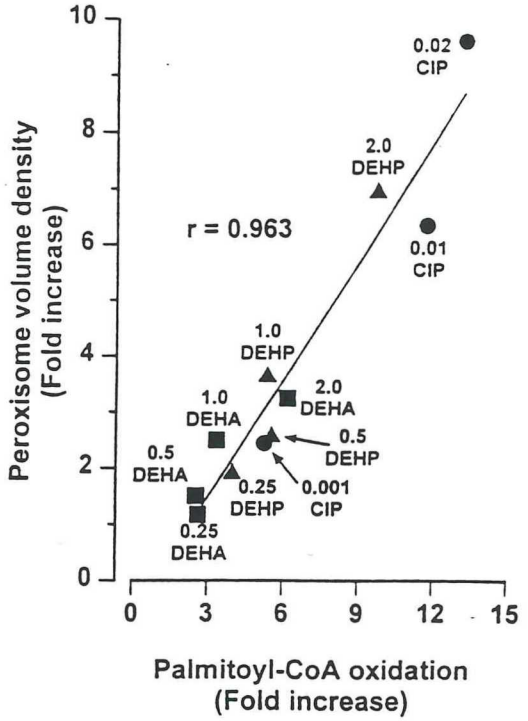
[a] Depends on test compound, dose and duration of treatment

[b] Normal rat and mouse liver peroxisomes contain a crystalline nucleoid core consisting of insoluble urate oxidase

For further details see Cohen and Grasso (1981), Reddy and Lalwani (1983), Stott (1988), Conway *et al.* (1989), Lock *et al.* (1989), Moody *et al.* (1991), Bentley *et al.* (1993), Lake (1993) and Lake (1995).

Lobular differences in the effects of peroxisome proliferators in rodent liver have also been observed. For example, in rat liver peroxisome proliferation is more marked in centrilobular than in periportal hepatocytes, in contrast to cell replication (Eldridge *et al.*, 1990; Bell *et al.*, 1991; Eacho *et al.*, 1991; Barrass *et al.*, 1993). As seen in Figure 1, correlations have been reported in rat liver between the induction of peroxisomal fatty acid β-oxidation (palmitoyl-CoA oxidation) and organelle proliferation (peroxisome volume density) (Reddy *et al.*, 1986; Lin, 1987; Sharma *et al.*, 1988a,b). Correlations have also been reported between the induction of peroxisomal and microsomal fatty acid oxidizing enzyme activities (Sharma *et al.*, 1988a,b; Dirven *et al.*, 1992).

**Figure 1. Correlation between peroxisomal proliferation and peroxisomal fatty acid β oxidation**




Comparisons with ciprofibrate (CIP, ●), di(2-ethylhexyl)phthalate (DEHP, ▲) and di(2-ethylhexyl)adipate (DEHA, ■)
From Reddy *et al.* (1986)

Some other aspects of the effects of acute and chronic administration of peroxisome proliferators have been described elsewhere (Cohen & Grasso, 1981; Reddy & Lalwani, 1983; Hawkins *et al.*, 1987; Stott, 1988; Lock *et al.*, 1989; Moody *et al.*, 1991; Rao & Reddy, 1991; Bentley *et al.*, 1993; Grasso, 1993; Ashby *et al.*, 1994). Peroxisome proliferators have also been reported to produce mitochondrial proliferation and changes in mitochondrial enzyme activities (Hawkins *et al.*, 1987; Lake *et al.*, 1987a; Eacho & Feller, 1991; Berge et al., 1993; Grasso, 1993). Other investigators have reported the induction of microsomal UDPglucuronosyltransferase towards bilirubin and certain other substrates and of both microsomal and cytosolic epoxide hydrolase activities (Fournel *et al.*, 1987; Moody *et al.*, 1992).

Chemicals may be screened for their ability to produce peroxisome proliferation in rodent hepatocytes, after administration either *in vivo* or *in vitro* (see below), by both biochemical and morphological markers. The most sensitive marker enzyme activities include palmitoyl-CoA oxidation, acyl-CoA oxidase, lauric acid 12-hydroxylase and carnitine acetyltransferase; catalase is not to be recommended, as it shows only a relatively small increase in activity. Peroxisome proliferation may also be assessed by ultrastructural examination of liver sections, and in some

studies staining with 3,3′-diaminobenzidine has been used to distinguish peroxisomes from other intracellular organelles. For small increases in peroxisome numbers, a quantitative morphometric, rather than a qualitative subjective, procedure should be employed, and care should be taken to compare effects in the same area of the liver lobule. A reduction in plasma or serum cholesterol, and particularly triglyceride levels, may indicate that a compound is a peroxisome proliferator, but it should be noted that several studies have demonstrated that a hypolipidaemic effect is not necessarily associated with peroxisome proliferation in rodent liver (Eacho *et al.*, 1993; Lake & Lewis, 1993).

Peroxisome proliferators have also been reported to produce effects in extrahepatic rodent tissues (Nemali *et al.*, 1988; Hinton & Price, 1993). Organelle proliferation and/or changes in peroxisomal mRNA levels or enzyme activities have been demonstrated in the kidney, intestine and heart.

## Rodent liver peroxisome proliferators

A large number of chemicals have been shown to produce peroxisome proliferation in rodent hepatocytes (Cohen & Grasso, 1981; Reddy & Lalwani, 1983; Stott, 1988; Lock *et al.*, 1989; Moody *et al.*, 1991; Bentley *et al.*, 1993; Lake & Lewis, 1993; Lake, 1995). Because of the wide range of classes of chemicals that produce organelle proliferation, peroxisome proliferators have been referred to as 'chemically unrelated' (Cohen & Grasso, 1981) or 'structurally dissimilar' (Reddy & Lalwani, 1983) compounds. Classes of chemicals that produce this effect include hypolipidaemic drugs and other types of therapeutic agents, herbicides, plasticizers, industrial solvents, natural products and food flavours (Table 2). The structures of some representative rodent peroxisome proliferators are shown in Figure 2.

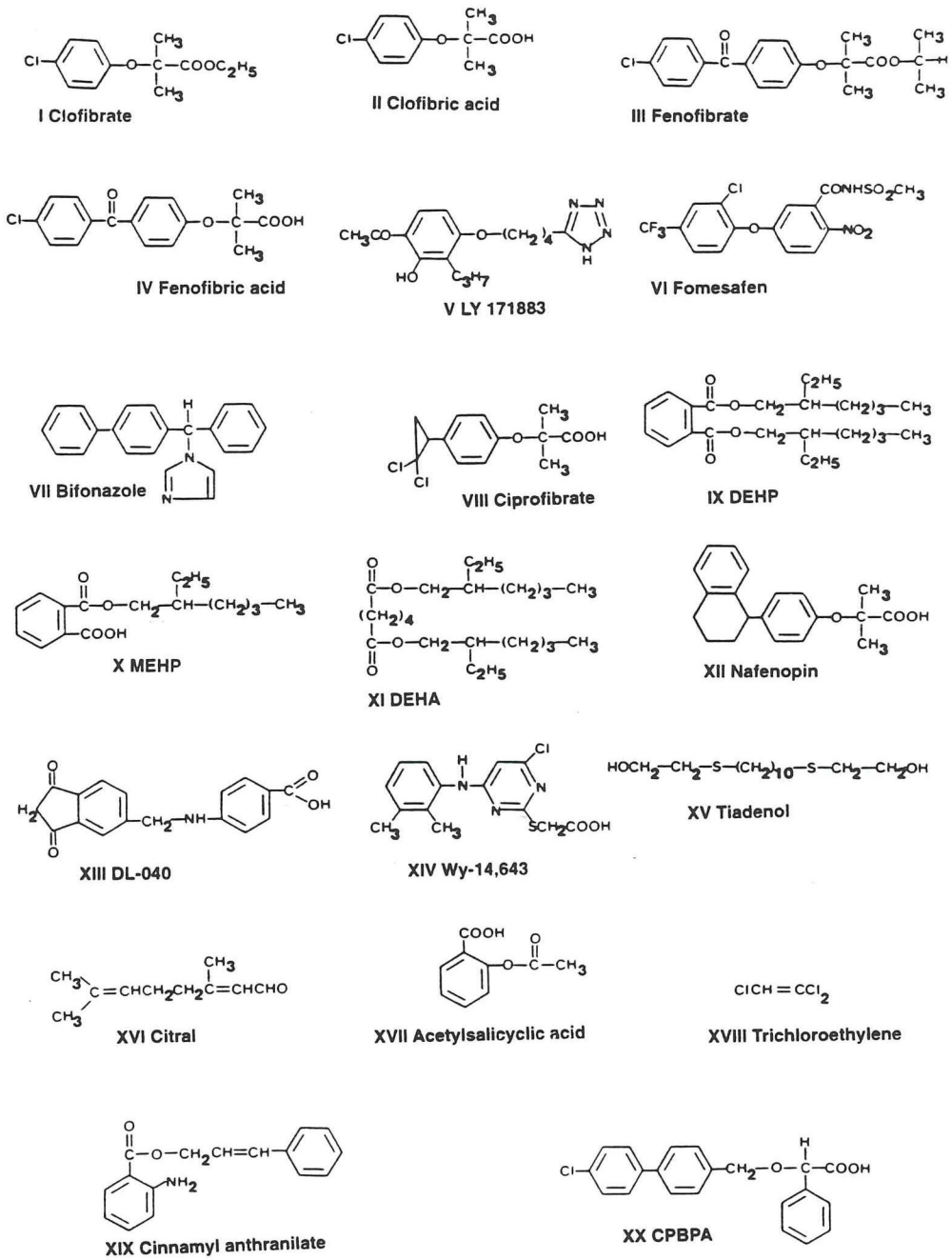
**Table 2. Examples of classes of chemicals which produce peroxisome proliferation in rodent liver**

| Chemical class | Examples |
|---|---|
| Therapeutic agents[a] | Acetylsalicylic acid, bezafibrate, bifonazole, ciprofibrate, clobuzarit, clofibrate, DL-040, fenofibrate, gemfibrozil, LY 171883, methylclofenapate, nafenopin, tiadenol, Wy-14,643 |
| Steroids | Dehydroepiandrosterone |
| Herbicides | 2,4-Dichlorophenoxyacetic acid, fomesafen, lactofen, 2,4,5-trichlorophenoxyacetic acid |
| Plasticizers | Di(2-ethylhexyl)adipate (DEHA), di(2-ethylhexyl)phthalate (DEHP), di(2-ethylhexyl)terephthalate, di(isodecyl)phthalate, di(isononyl)phthalate, tri(2-ethylhexyl)trimellitate |
| Solvents and industrial chemicals | Chlorinated paraffins, perfluoro-*n*-octanoic acid, tetrachloroethylene, trichloroethylene |
| Food flavours and natural products | Cinnamyl anthranilate, citral, linalool |

[a] Examples include compounds that were either developed and not marketed or have been withdrawn.

For further details see Cohen and Grasso (1981), Reddy and Lalwani (1983), Stott (1988), Lock *et al.* (1989), Moody *et al.* (1991), Bentley *et al.* (1993) and Lake and Lewis (1993).

**Figure 2. Structures of some compounds known to produce peroxisome proliferation**




Clofibrate (I) and fenofibrate (III) are metabolized to clofibric acid (II) and fenofibric acid (IV), respectively. Di(2-ethylhexyl)phthalate (DEHP, IX) is metabolised to mono(2-ethylhexyl)phthalate (MEHP, X) and 2-ethylhexanol, which are both further metabolized to more active peroxisome proliferators. Di(2-ethylhexyl)adipate (DEHA, XI) is hydrolysed to give two molecules of 2-ethylhexanol, which are subsequently converted to 2-ethylhexanoic acid. Note that trichloroethylene (XVIII) and cinnamyl anthranilate (XIX) exhibit species differences, in that they produce peroxisome proliferation in mouse, but not rat, liver. In the case of trichloroethylene, the active metabolite is trichloroacetic acid, whereas for cinnamyl anthranilate the parent compound is considered to be the active agent. 2-[4(4-Chlorophenyl)benzyloxy]-2-phenylacetic acid (CPBPA, XX), like some other peroxisome proliferators, has a chiral centre and the two enantiomers have differing potencies. For further details, see Cohen & Grasso (1981), Reddy & Lalwani (1983), Stott (1988), Lock *et al.* (1989), Moody *et al.* (1991), Bentley *et al.* (1993), Lake & Lewis (1993) and Ashby *et al.* (1994)

A characteristic feature of many, but not all, peroxisome proliferators is the presence of an acidic function (Lake et al., 1988; Lock et al., 1989; Lake & Lewis, 1993). This function is normally a carboxyl group, present as a free carboxyl group in the parent structure or one that is unmasked by metabolism. For example, after administration in vivo, clofibrate and fenofibrate are metabolized to clofibric acid and fenofibric acid, respectively (see Figure 2). Alternatively, the compound may contain a chemical grouping that is a bioisostere (Thornber, 1979) of a carboxyl group, such as the respective tetrazole and sulfonamide moieties (Figure 2) of LY 171883 (Eacho et al., 1986) and fomesafen (Lock et al., 1989). In the case of bifonazole, which produces peroxisome proliferation in rat liver (Horie et al., 1991), the imidazole moiety may be sufficiently similar to other chemical groups that are known bioisosteres of a carboxyl group (Thornber, 1979).For many compounds, the 'proximate' peroxisome proliferator may be the parent structure or a metabolite containing an unmasked carboxyl group. Compounds such as clofibric acid, ciprofibrate and fenofibric acid (see Figure 2) do not appear to be subjected to extensive phase I metabolism and tend to be excreted after phase II metabolism as their respective glucuronide conjugates (Lake & Lewis, 1993). It is assumed, but not experimentally proven, that such glucuronides or other phase II conjugates would not be active peroxisome proliferators in rodent liver.

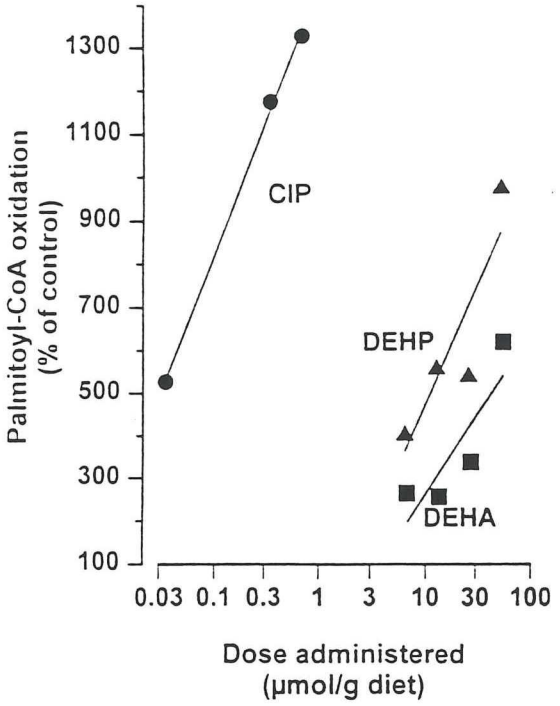
Certain other compounds require more extensive metabolism to produce the 'proximate' peroxisome proliferators. For example, both the primary metabolites of di(2-ethylhexyl)phthalate (DEHP), namely mono(2-ethylhexyl)phthalate (MEHP) and 2-ethylhexanol, are known to produce peroxisome proliferation in rodent hepatocytes (Lake *et al.* 1975; Rhodes *et al.*, 1984; Lhuguenot & Cornu, 1993); however, both compounds are known to be further metabolized (Albro, 1975; Albro & Lavenhar, 1989; Lhuguenot & Cornu, 1993). Additional studies with MEHP have demonstrated that the 'proximate' peroxisome proliferators are (ω-1)-hydroxy and keto metabolites (Mitchell *et al.*, 1985; Elcombe & Mitchell, 1986), whereas the 'proximate' peroxisome proliferator of 2-ethylhexanol appears to be 2-ethylhexanoic acid (Lundgren *et al.*, 1987; Lhuguenot & Cornu, 1993). Studies with di(2-ethylhexyl)adipate (DEHA) have indicated that the 'proximate' peroxisome proliferator is 2-ethylhexanoic acid (Cornu *et al.*, 1992; Lhuguenot & Cornu, 1993).

Some peroxisome proliferators are known to form acyl-CoA thioesters (Bronfman, 1993), and it has been suggested that these are the active metabolites responsible for the various biological effects of peroxisome proliferators. As not all peroxisome proliferators possess a free carboxyl group, however, it is difficult to envisage peroxisome proliferator-coenzyme A thioester formation as a common mechanism of activation of these compounds (Lake & Lewis, 1993; Sohlenius *et al.*, 1993).

Although rodent peroxisome proliferators appear to be structurally diverse, similarities have been reported in the three-dimensional structures of some compounds (Lake *et al.*, 1988; Lake & Lewis, 1993; Lewis & Lake, 1993). Moreover, for various groups of peroxisome proliferators both structure–activity relationships and quantitative structure–activity relationships have been observed (Lake & Lewis, 1993). Certain peroxisome proliferators have chiral centres, which may result in the enantiomers having differing potencies (Lake & Lewis, 1993).

Several studies have demonstrated marked differences in compound potency. For example, the data shown in Figure 3 demonstrate that the hypolipidaemic agent ciprofibrate is orders of magnitude more potent than the plasticizer DEHP, which is somewhat more potent than another plasticizer, DEHA (Reddy *et al.*, 1986). Calculations from data in the literature suggest that DEHP and DEHA are 15 and 5 times more potent, respectively, than acetylsalicylic acid (Barber *et al.*, 1987).

**Figure 3. Potency to produce peroxisome proliferation (assessed as induction of palmitoyl-coenzyme A oxidation) in the livers of male Fischer 344 rats after 30 days' treatment**




Comparison with ciprofibrate (CIP, ●), di(2-ethylhexyl)phthalate (DEHP, ▲) and di(2-ethylhexyl)adipate (DEHA, ■) From Reddy *et al.* (1986)

## Peroxisome proliferation *in vitro*

Studies conducted in a number of laboratories have demonstrated that peroxisome proliferation may be readily demonstrated *in vitro* in primary rat and mouse hepatocyte cultures by employing standard hepatocyte media and culture conditions (Gray *et al.*, 1982, 1983a,b; Bieri *et al.*, 1984; Lake *et al.*, 1984a; Mitchell *et al.*, 1984; Elcombe, 1985; Elcombe & Mitchell, 1986; Foxworthy & Eacho, 1986; Lake *et al.*, 1986; Butler *et al.*, 1988; Lock *et al.*, 1989; Smith & Elcombe, 1989; Bieri *et al.*, 1990; Eacho & Feller, 1991; Moody *et al.*, 1991; Bentley *et al.*, 1993; Bieri, 1993; Eacho *et al.*, 1993; Lake & Lewis, 1993; Lake *et al.*, 1993a; Foxworthy & Eacho, 1994; Lake, 1995). These studies demonstrate that the factors responsible for the induction of peroxisome proliferation are intrahepatic and are retained in cell culture.

Generally, the characteristics of peroxisome proliferation *in vivo*, including the stimulation of DNA synthesis, increased peroxisome numbers and change in morphology, and differential induction of enzyme activities have also been observed in cultured hepatocytes. Most studies have examined the effects of peroxisome proliferators and certain fatty acids on peroxisomal and microsomal fatty acid oxidizing enzyme activities in hepatocytes cultured for periods of 3–4 days (Christiansen *et al.*, 1985; Eacho & Feller, 1991; Moody *et al.*, 1991; Bieri, 1993; Feller & Intrasuksri, 1993; Lake and Lewis, 1993; Foxworthy & Eacho, 1994). In keeping with studies *in vivo* (Sharma *et al.*, 1988a), a good correlation has been observed between the induction of palmitoyl-CoA oxidation and lauric acid hydroxylation in rat hepatocyte cultures (Lake *et al.*, 1984a), and significant increases in mRNA levels for β-oxidation cycle enzymes and CYP4A1 have also been detected (Thangada *et al.*, 1989; Bieri *et al.*, 1990; Bell & Elcombe, 1993; Bieri, 1993).

Other investigators have observed the effects of peroxisome proliferators on organelle proliferation by ultrastructural examination and in replicative DNA synthesis by incorporation of DNA precursors (Bieri *et al.*, 1984; Mitchell *et al.*, 1984; Elcombe & Styles 1989; Bieri *et al.*, 1990). Although enzyme activities (e.g. palmitoyl-CoA oxidation) may fall in cultured hepatocytes (Gray *et al.*, 1983b; Foxworthy & Eacho, 1986; Bieri, 1993), this does not preclude their use for studies of peroxisome proliferation. Indeed, peroxisome proliferation has also been reported in long-term (e.g. > 7-day) hepatocyte culture systems, such as media containing high levels of dimethyl sulfoxide and the collagen sandwich technique (Muakkassah-Kelly *et al.*, 1987; Beamand *et al.*, 1991). Peroxisome proliferation may be observed not only in primary hepatocyte cultures *in vitro* but also in certain cell lines (Osumi *et al.*, 1990; Bayly *et al.*, 1993), cultured rat hepatocyte spheroids (Roberts & Soames, 1993) and cultured precision-cut rat liver slices (Beamand *et al.*, 1993).

The bulk of the available literature suggests that primary hepatocyte cultures are a validated model system for studying various aspects of hepatic peroxisome proliferation *in vitro*. As with any system *in vitro*, hepatocyte cultures have both potential advantages and disadvantages. They permit the relatively rapid screening of large numbers of chemicals for their ability to produce peroxisome proliferation. Only small amounts of the test compounds are required and differences in compound pharmacokinetics seen *in vivo* are avoided. It has been suggested, however, that if a weak peroxisome proliferator is toxic to hepatocyte cultures a false negative result may be obtained, whereas a positive result *in vitro* will perhaps not be observed *in vivo* if the pharmacological effects of the chemical result in systemic toxicity at a lower concentration than that required to produce peroxisome proliferation (Bieri *et al.*, 1990; Bieri, 1993). The metabolism of the test chemical may also be an important factor. For example, like other phthalate esters, DEHP is hydrolysed by pancreatic and intestinal lipases, and, while the parent compound has little effect in cultured rat hepatocytes, the primary monoester and alcohol products and their further metabolites are much more potent (Gray *et al.*, 1983b; Rhodes *et al.*, 1984; Mitchell *et al.*, 1985; Bieri *et al.*, 1990; Bieri, 1993). As described above, primary rat hepatocyte cultures have been used to identify the active metabolites (i.e. 'proximate' peroxisome proliferators) derived from compounds such as DEHP and DEHA (Mitchell *et al.*, 1985; Lhuguenot & Cornu, 1993) and mouse hepatocytes have been shown to metabolize trichloroethylene to the active peroxisome proliferator trichloroacetic acid (Elcombe, 1985). For many compounds, there appears to be a good correlation between potency *in vitro* and potency *in vivo* in the intact animal. Indeed primary hepatocyte cultures have been used to derive information on compound structure–activity and quantitative structure–activity relationships (Lake *et al.*, 1987b, 1988; Eacho *et al.*, 1993; Lake & Lewis, 1993; Lake *et al.*, 1993a). Examples in which good agreement has been obtained between findings *in vitro* and *in vivo* include clofibrate analogues (Esbenshade *et al.*, 1990), phthalate monoesters (Lake *et al.*, 1987b) and tetrazole-substituted acetophenones (Eacho *et al.* 1993). Apart from screening chemicals and investigations of structure–activity relationships, primary hepatocyte cultures have also been employed for mechanistic studies and to assess species differences in hepatic peroxisome proliferation (Moody *et al.*, 1991; Bieri, 1993). The data on the use of hepatocyte cultures to investigate species differences in response is reviewed below.

## Mechanisms of induction of peroxisome proliferation in rodent hepatocytes

Proposed mechanisms for the initiation of peroxisome proliferation in rodent hepatocytes include involvement of a receptor, substrate overload and use of peroxisome proliferators as substrates for peroxisomal enzymes (Reddy & Lalwani, 1983; Lock *et al.*, 1989; Bell & Elcombe, 1993; Bentley *et al.*, 1993). Most attention has focused on the identification of receptors and the effect of peroxisome proliferators on lipid metabolism, and it should be noted that these two hypotheses are not mutually exclusive (Lock *et al.*, 1989; Bell & Elcombe, 1993; Bentley *et al.*, 1993). In the substrate overload hypothesis, it is proposed that peroxisome proliferation is an

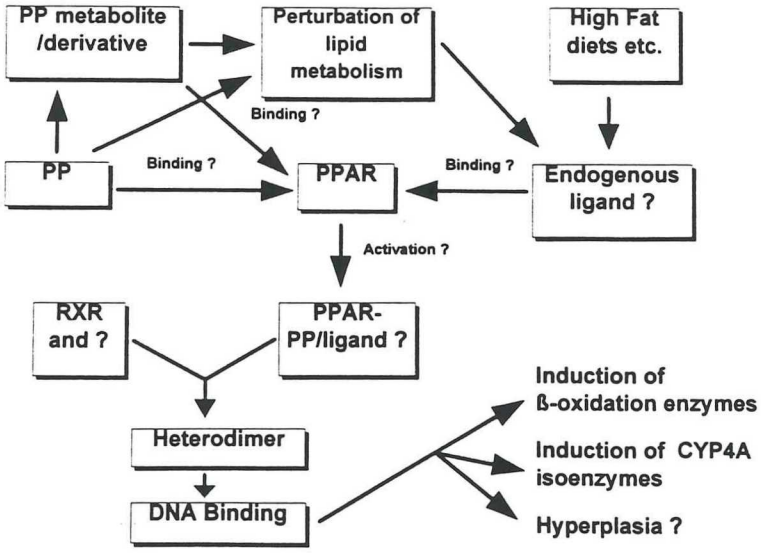
adaptive response to a perturbation of lipid metabolism and also involves induction of CYP4A isoenzymes (Elcombe & Mitchell, 1986; Sharma *et al.*, 1988a; Lock *et al.*, 1989; Bell & Elcombe, 1993; Bentley *et al.*, 1993). Certainly peroxisome proliferators inhibit fatty acid oxidation, form CoA esters, increase levels of fatty acids and displace fatty acids from the cytosolic fatty acid-binding protein (Elcombe & Mitchell, 1986; Lock *et al.*, 1989; Eacho & Feller, 1991; Bell & Elcombe, 1993; Bentley *et al.*, 1993; Bronfman, 1993; Eacho *et al.*, 1993; Lewis & Lake, 1993).

Although the presence of cytosolic peroxisome proliferator binding proteins has been reported in some studies (Reddy & Lalwani, 1983; Milton *et al.*, 1988; Alvares *et al.*, 1990), more recently Green and coworkers cloned a peroxisome proliferator-activated receptor (PPAR) from mouse liver (Issemann & Green, 1990; Green *et al.*, 1993). This protein is a member of the steroid hormone receptor superfamily, has a relative molecular mass of about 52 kDa and possesses both putative ligand and DNA binding domains. When a chimaeric receptor expression vector containing regions encoding the putative ligand binding domain of this mouse PPAR and the DNA binding domain of the human oestrogen receptor was transfected into COS 1 cells, it could be activated by peroxisome proliferators (Issemann & Green, 1990; Green *et al.*, 1993). Other studies have demonstrated that the PPAR is involved in the activation of genes for both peroxisomal and microsomal (i.e. CYP4A subfamily) fatty acid oxidizing enzymes (Dreyer *et al.*, 1992; Muerhoff *et al.*, 1992; Tugwood *et al.*, 1992). Several different PPARs have now been described, in the mouse (Issemann & Green, 1990; Chen *et al.*, 1993; Zhu *et al.*, 1993; Kliewer *et al.*, 1994), rat (Gebel *et al.*, 1992; Göttlicher *et al.*, 1992), *Xenopus* (Dreyer *et al.*, 1992; Krey *et al.*, 1993) and human (Schmidt *et al.*, 1992; Sher *et al.*, 1993). The administration of peroxisome proliferators may increase the levels of certain PPARs in rodent liver (Gebel *et al.*, 1992; Zhu *et al.*, 1993).

Figure 4 summarizes some possible mechanisms of induction of peroxisome proliferation in rodent hepatocytes. Clearly, there is much scope for further studies in order to elucidate fully the precise mechanism(s) of peroxisome proliferation in rodent hepatocytes and to determine differences between rodent and human hepatocytes. Hepatic peroxisome proliferation in rodents can also be produced by various physiological factors, such as feeding high-fat diets, vitamin E deficiency and starvation, which may be related to xenobiotic-induced organelle proliferation (Reddy & Lalwani, 1983; Lock *et al.*, 1989; Bentley *et al.*, 1993). Although some evidence has been obtained for the formation of CoA esters of peroxisome proliferators, this could not be a universal mechanism: while most peroxisome proliferators contain an acidic function, this is not always a free carboxyl group (Lake *et al.*, 1988; Lock *et al.*, 1989; Bentley *et al.*, 1993; Bronfman, 1993; Eacho *et al.*, 1993; Lake & Lewis, 1993; Lewis & Lake, 1993). While molecular modelling has identified a putative peroxisome proliferator binding site in one PPAR, the binding of nafenopin has not been observed (Issemann & Green, 1990; Lewis & Lake, 1993; Morrison & Elcombe, 1995). In contrast, certain fatty acids, such as oleic and arachidonic acids, may act as endogenous ligands, as they have been shown both to bind to and to activate PPARs in expression systems *in vitro* (Göttlicher *et al.*, 1992; Schmidt *et al.*, 1992; Krey *et al.*, 1993; Göttlicher *et al.*, 1993; Morrison & Elcombe, 1995;). Assuming that peroxisome proliferators do interact directly with PPARs, the binding may be only transient. Steroid hormone receptors are associated with heat-shock proteins, and PPAR activation could conceivably involve displacement of the peroxisome proliferator and a heat-shock protein such as HSP 72 (Green & Chambon, 1988; Huang *et al.*, 1994). PPARs have been shown to bind to DNA as a heterodimer with retinoid X receptor (RXR); and another member of the steroid hormone receptor superfamily, namely the chicken ovalbumin upstream promoter transcription factor (COUP-TF), has been shown to act as a repressor (Kliewer *et al.*, 1992; Bardot *et al.*, 1993; Green, 1993; Miyata *et al.*, 1993). In addition, studies with three mouse liver PPARs (designated $\alpha$, $\delta$ and $\gamma$) have demonstrated differences in activation by some peroxisome proliferators and fatty acids (Kliewer *et al.*, 1994). Moreover, the PPAR $\delta$ and $\gamma$ forms interfere with the activation of the PPAR

α form by Wy-14,643, suggesting that some PPARs may be dominant repressors of other forms (Kliewer *et al.*, 1994).

**Figure 4. Some possible mechanisms of induction of peroxisome proliferation in rodent hepatocytes**




The peroxisome proliferator (PP) may either bind directly to or otherwise activate one or more peroxisome proliferator-activated receptors (PPARs) which bind to DNA as a heterodimer with retinoid X receptor (RXR) and possibly other receptors. Alternatively the PP may require metabolism (e.g. formation of an acidic function and/or a coenzyme A ester) which could interact with a PPAR either directly or via an endogenous ligand (a fatty acid?) formed as a consequence of a perturbation of lipid metabolism.

Multiple PPARs, together with activating receptors (e.g. RXR) and repressing receptors (e.g. COUP-TF and possibly truncated receptors), may account for known tissue and species differences in response to peroxisome proliferators (Cohen & Grasso, 1981; Nemali et al., 1988; Issemann & Green, 1990; Bentley et al., 1993; Ashby et al., 1994; Kliewer et al., 1994). It should be noted that one human PPAR has been shown to be activated by rodent peroxisome proliferators in an in-vitro expression system (Sher et al., 1993). As peroxisome proliferation does not appear to occur in man to any significant extent (see below), further work is required to assess the significance of this study. Modulating factors may include levels of expression of PPARs, activators and repressors in human hepatocytes and differences between the regulatory elements of human and rodent genes for enzymes such as acyl-CoA oxidase (Tugwood et al., 1992; Sher et al., 1993; Varanasi et al., 1994).

## Species differences in response

Many studies have investigated species differences in hepatic peroxisome proliferation (Cohen & Grasso, 1981; Rodricks & Turnbull, 1987; Stott, 1988; Lock *et al.*, 1989; Moody *et al.*, 1991; Bentley *et al.*, 1993; Lake, 1995). These studies were conducted either *in vivo* or *in vitro* in hepatocyte cultures and focused mainly on the measurement of organelle proliferation and induction of peroxisomal and microsomal enzyme activities. Table 3 lists some representative studies *in vivo*. Clearly, the rat and the mouse may be considered responsive to peroxisome proliferators, and, although strain differences have been observed, these are minor in comparison with the magnitude of the species differences in response. On the basis of marker enzyme activities (e.g. palmitoyl-CoA oxidation, lauric acid 12-hydroxylase, carnitine acetyltransferase) and ultrastructural examination,

the Syrian hamster appears to exhibit an intermediate response, whereas in most studies the guinea pig is either non-responsive or refractory (Table 3).

**Table 3. Some examples of species differences in hepatic peroxisome proliferation**

| Compound | Species examined[a] | | | References |
|---|---|---|---|---|
| | Responsive | Intermediate | Non responsive | |
| Bezafibrate | Rat, mouse | Syrian hamster, guinea pig | Dog, rabbit, rhesus monkey | Watanabe *et al.* (1989) |
| Ciprofibrate | Rat, mouse | Syrian hamster, rabbit | Guinea pig, marmoset | Graham *et al.* (1994) Makowska *et al.* (1992) |
| Clobuzarit | Rat, mouse | Syrian hamster | Dog, marmoset | Orton *et al.* (1984) |
| Clofibrate | Rat, mouse | Syrian hamster | Marmoset, rhesus monkey | Holloway *et al.* (1982) |
| Di(2-ethylhexyl)-phthalate | Rat, mouse | Syrian hamster | Guinea pig, cynomolgus monkey, marmoset | Lake *et al.* (1984b), Osumi & Hashimoto (1978), Rhodes *et al.* (1986), Short *et al.* (1987) |
| Dehydroepi-androsterone | Rat, mouse | Syrian hamster | Guinea pig | Sakuma *et al.* (1992) |
| LY 171883 | Rat, mouse | Syrian hamster | Guinea pig, dog, rhesus monkey | Eacho *et al.* (1986) |
| Nafenopin | Rat | Syrian hamster | Guinea pig, marmoset | Lake *et al.* (1989) |

[a] Peroxisome proliferation assessed by ultrastructural examination and/or measurement of marker enzyme activities. Intermediate species are less responsive than the rat and mouse, whereas non -responsive species are either refractory or exhibit only a small response at high doses. For further details, see individual references.

In assessing species differences in hepatic peroxisome proliferation, a number of factors should be considered. These include the metabolism, disposition and dose of the test compound, sex differences and intrahepatic differences in response. The importance of metabolism is illustrated by trichloroethylene, which produces hepatic peroxisome proliferation in the mouse but has little effect in the rat (Elcombe, 1985). Metabolic studies demonstrate that trichloroethylene is extensively metabolized to trichloroacetic acid in the mouse, whereas this is a minor saturable route of metabolism in the rat. That the difference in trichloroacetic acid formation is responsible for the observed species difference is demonstrated by the fact that this compound produces peroxisome proliferation in rat and mouse hepatocytes both *in vivo* and *in vitro* (Elcombe, 1985). With respect to compound disposition, DEHP is known to be more extensively absorbed after oral administration in the rat than in the marmoset (Rhodes *et al.*, 1986); however, the observed species differences in response *in vivo* (Table 3) are supported by the observation that metabolites of DEHP that produce peroxisome proliferation in rat hepatocytes *in vitro* have no significant effect in cultured marmoset hepatocytes (Elcombe & Mitchell, 1986).

Generally, the results of studies with primary hepatocyte cultures from the rat, mouse, Syrian hamster and guinea pig support the results of studies in these species *in vivo* (Elcombe, 1985; Elcombe & Mitchell, 1986; Lake *et al.*, 1986; Bieri *et al.*, 1988; Bentley *et al.*, 1993; Bieri, 1993; Lake, 1995). Although a large number of compounds have been shown to produce peroxisome proliferation in rat and mouse hepatocytes, less effect is observed in Syrian hamster hepatocytes and little or no effect in guinea pig hepatocytes. The failure of peroxisome proliferators to produce effects in

non responsive species is considered not to be due to lack of functional viability of the preparations, as they have been shown to respond to other chemicals (e.g. induction of cytochrome P450-dependent enzyme activities).

A number of studies in primates *in vivo* have failed to provide any evidence of significant peroxisome proliferation in either New (e.g. marmoset) or Old (e.g. rhesus) World monkeys (Table 3 and Rodricks & Turnbull, 1987; Lock *et al.*, 1989; Bentley *et al.*, 1993); however, albeit at high doses, two compounds, ciprofibrate (Reddy *et al.*, 1984) and DL-040 (Lalwani *et al.*,1985), have been reported to produce hepatic peroxisome proliferation in cynomolgus and rhesus monkeys. Studies have also been conducted in human patients treated with several hypolipidaemic agents (all of which are rodent peroxisome proliferators), including ciprofibrate, clofibrate, fenofibrate and gemfibrozil (Bentley *et al.*, 1993; Ashby *et al.*, 1994). While most studies failed to detect any significant changes, clofibrate was reported to produce a small increase in the number of peroxisomes (Hanefeld *et al.*, 1983) and ciprofibrate to produce a small increase in the proportion of the hepatocyte cytoplasm occupied by peroxisomes (cited by Bentley *et al.*, 1993). Owing to the large inter-individual variation in peroxisome morphometrics in these studies, however, together with cell to cell variations and lobular variations, it is difficult to attach any clear biological significance to these findings (Bentley *et al.*, 1993; Ashby *et al.*, 1994).

Generally, peroxisome proliferators have not been reported to produce any significant effects on marker enzyme activities and/or peroxisomes in cultured primate or human hepatocytes (Lock *et al.*, 1989; Bentley *et al.*, 1993; Bieri, 1993; Ashby *et al.*, 1994; Foxworthy & Eacho, 1994). Some examples from the literature are shown in Table 4. All of the compounds listed in Table 4 produced significant concentration-related effects on enzyme activities and/or peroxisomes in rat hepatocyte cultures. When these effects were examined, they were not produced in either guinea pig, primate (marmoset, cynomolgus monkey, rhesus monkey) or human hepatocyte cultures. In many of these studies, the functional viability of the hepatocyte preparation was assessed in parallel experiments in which effects on other end-points were determined. For example, bezafibrate, ciprofibrate and LY 171883 all produced significant, concentration-dependent increases in palmitoyl-CoA oxidation in rat hepatocytes but not in rhesus monkey hepatocytes (Foxworthy *et al.*, 1990). The addition of dexamethasone as a measure of functional viability resulted, however, in significant increases in tyrosine aminotransferase activity in both rat and rhesus monkey hepatocyte cultures.

Although several studies have demonstrated species differences in hepatic peroxisome proliferation, on the basis of measurement of marker enzyme activities and ultrastructural examination, comparatively few investigations have evaluated species differences in cell replication. While both nafenopin and Wy-14,643 are potent mitogens in rat liver, they do not appear to produce any significant stimulation of replicative DNA synthesis in Syrian hamster hepatocytes after either acute or chronic administration (Price *et al.*, 1992; Lake *et al.*, 1993b). In keeping with their known properties *in vivo*, certain peroxisome proliferators have been shown to stimulate DNA synthesis in rat hepatocyte cultures (Elcombe & Styles, 1989; Bieri *et al.*, 1990; Bieri, 1993; Marsman *et al.*, 1993); however, in other studies methylclofenapate did not increase DNA synthesis in guinea pig, marmoset or human hepatocytes (Elcombe & Styles, 1989), and nafenopin was also ineffective in human hepatocytes (Parzefall *et al.*, 1991). The functional viability of the human hepatocytes used in these studies was confirmed by the observation that the addition of epidermal growth factor did result in significant increases in replicative DNA synthesis (Elcombe & Styles, 1989; Parzefall *et al.*, 1991). In one study, nafenopin, which induces palmitoyl-CoA oxidation and replicative DNA synthesis in rat hepatocytes (Bieri *et al.*, 1990), was reported to increase replicative DNA synthesis in marmoset hepatocytes when cultured in the absence, but not in the presence, of serum (Bieri *et al.*, 1988); however, the significance of this result is questionable (Bieri, 1993) in view of the lack of effect of nafenopin in human hepatocytes and of methylclofenapate in marmoset and human hepatocytes. Moreover, nafenopin did not induce palmitoyl-CoA oxidation activity in marmoset hepa-

tocytes cultured in either the presence or absence of serum (Bieri *et al.*, 1988), a finding that is in agreement with findings *in vivo* with this compound (Lake *et al.*, 1989) and with finding both *in vivo* and *in vitro* in this species for several other peroxisome proliferators (Tables 3 and 4).

**Table 4. Some examples of species differences in the effects of rodent peroxisome proliferators in primary hepatocyte cultures**

| Compound[a] | Species[b] | | | | | | References |
|---|---|---|---|---|---|---|---|
| | Rat | Guinea pig | Marmoset | Cynomolgus monkey | Rhesus monkey | Human | |
| Beclobric acid | + | ND | ND | – | ND | – | Blaauboer *et al.* (1990) |
| Benzbromarone | + | ND | ND | ND | ND | – | Bichet *et al.* (1990) |
| Bezafibrate | + | ND | ND | ND | – | ND | Foxworthy *et al.* (1990) |
| Ciprofibrate | + | ND | ND | – | – | – | Allen *et al.* (1987), Foxworthy *et al.* (1990) |
| Clofibric acid | + | – | ND | – | ND | – | Bichet *et al.* (1990), Blaauboer *et al.* (1990), Lake *et al.* (1986) |
| Fomesafen | + | – | – | ND | ND | – | Smith and Elcombe (1989) |
| LY 171883 | + | ND | ND | ND | – | ND | Foxworthy *et al.* (1990) |
| Methylclofenapate | + | – | – | ND | ND | – | Elcombe & Styles (1989) |
| Mono(2-ethylhexyl) phthalate and derivatives | + | – | – | ND | ND | – | Bichet *et al.* (1990), Elcombe & Mitchell (1986), Lake *et al.* (1986) |
| Mono(2-ethylhexyl)-adipate and 2-ethyl-hexanoic acid | + | – | – | ND | ND | ND | Cornu *et al.* (1992) |
| Monoisodecyl and mono isononyl phthalates | + | ND | – | ND | ND | ND | Benford *et al.* (1986) |
| Nafenopin | + | ND | – | ND | ND | ND | Bieri *et al.* (1988, 1990) |
| Trichloracetic acid | + | ND | ND | ND | ND | – | Elcombe (1985) |

[a] Effect of test compound assessed by changes in enzyme activity (e.g. palmitoyl-CoA oxidation) and/or organelle proliferation

[b] Species response defined as; +, significant concentration -related response; –, no or little effect with poor concentration dependency; ND, not determined

While many studies have demonstrated that on prolonged administration peroxisome proliferators may produce liver tumours in rats and mice (Cohen & Grasso, 1981; Reddy & Lalwani, 1983; Bentley *et al.*, 1993), few studies have examined the chronic effects of such compounds in

other species. Table 5 lists available data for Syrian hamsters and primates (marmosets, cynomolgus monkeys and rhesus monkeys). Clobuzarit, which is known to produce peroxisome proliferation in rat and mouse liver (Orton *et al.*, 1984), did not produce tumours in Syrian hamsters in a two-year study (Tucker & Orton, 1995). DEHP administered by either inhalation or intraperitoneal injection was also reported not to produce tumours in this species (Schmezer *et al.*, 1988), but it should be noted that the dose levels and treatment regimes may not necessarily have been appropriate for an assessment of hepatocarcinogenicity. Both nafenopin and Wy-14,643 have been shown to produce liver enlargement and sustained peroxisome proliferation in Syrian hamsters (Lake *et al.*, 1993b); however, while nafenopin (at a fifth of the dietary level given to hamsters) and Wy-14,643 produced liver nodules and hepatocellular carcinoma in rats after 60 and 40 weeks' feeding, respectively, no such liver lesions were observed after 80 weeks in the Syrian hamster (Lake *et al.*, 1993b).

**Table 5. Effects of chronic administration of peroxisome proliferators in Syrian hamsters and primates**

| Species | Compound | Comments | Reference |
|---|---|---|---|
| Syrian hamster | Clobuzarit | No increase in liver tumours after 2 years' treatment at doses of 5–25 mg/kg bw per day | Tucker & Orton (1995) |
| | Di(2-ethyl-hexyl)phthalate | No increase in liver tumours after either chronic 23-month inhalation (total exposure, 7–10 mg/kg bw) or after intraperitoneal dosing (total dose, 24–54 g/kg bw) for 12–23 months | Schmezer *et al.* (1988) |
| | Nafenopin and Wy-14,643 | Increase in liver weight but no increase in liver tumours after 80 weeks' feeding of either 0.25% nafenopin or 0.025% Wy-14,643 in the diet | Lake *et al.* (1993b) |
| Marmoset | Ciprofibrate | No increase in liver weight after 3 years' treatment at doses of 2–20 mg/kg bw per day | Graham *et al.* (1994) |
| | Clobuzarit | No increase in liver weight after 6 months' treatment at doses of 50–100 mg/kg bw per day | Tucker & Orton (1993) |
| | Clofibrate | No increase in liver weight or liver tumour incidence after 6.5 years' treatment at doses of 94–263 mg/kg bw per day | Tucker & Orton (1993) |
| Cynomolgus monkey | Clobuzarit | No increase in liver weight after 6 months' treatment at 10 mg/kg bw per day, but increase at 25 mg/kg bw per day | Tucker & Orton (1993) |
| Rhesus monkey | Clofibrate | Clofibrate administered at 2% in diet for 6 months or at 0.5–2% in diet (co-administered with androsterone) for 2 years. Increase in relative liver weight only in females given 2% clofibrate diet | Tucker & Orton (1993) |

For further details, see individual references

Some chronic studies with ciprofibrate, clobuzarit and clofibrate have been performed in primates (Table 5). Although none of these studies was of lifetime duration, the 6.5-year study with clofibrate in the marmoset covered about half of the expected lifespan of this species (Tucker & Orton, 1993) and the study with ciprofibrate in marmosets is ongoing (Graham *et al.*, 1994). While some increases in liver weight have been reported at some dose levels in some studies (Table 5), no evidence for significant peroxisome proliferation or peroxisome proliferator-induced liver lesions has been reported (Tucker & Orton, 1993; Graham *et al.*, 1994). In summary, the available long-

term studies conducted in Syrian hamsters and primates suggest that peroxisome proliferator-induced liver lesions, typical of those observed in the rat and mouse, may not be produced in these species. While primates appear to be generally refractory to the effects of rodent peroxisome proliferators (Tables 3 and 4), the Syrian hamster is partially responsive in that peroxisome proliferation, but not increased cell replication, is observed (Price *et al.*, 1992; Lake *et al.*, 1993b).

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# B. POSTULATED MECHANISMS OF CARCINOGENICITY MEDIATED BY PEROXISOME PROLIFERATORS

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## Introduction

The conversion of a normal cell into a malignant phenotype is a complex, multistage process induced by a variety of chemical, viral, physical and hormonal agents. It is now widely accepted that most cancers are the result of one or more heritable alterations at the level of the individual tumour cell (Furth & Sobel, 1947). The concepts of multistage carcinogenesis have emanated from experimental studies of chemical carcinogenesis; in particular, the operationally divisible principles of initiation, promotion and progression have been clearly established from investigations with chemical carcinogens that are genotoxic or mutagenic. The majority of chemical carcinogens characterized thus far are clearly genotoxic, in that they exert genetic damage that can be detected by routinely used tests such as the Salmonella assay and clastogenic assays. There is substantial evidence that genotoxic chemical carcinogens induce gene mutations and that some such mutations are 'carcinogen-specific', correlating with the known metabolism and DNA-binding properties of the chemical and the geographic distribution of specific types of tumours after exposure (Balmain & Brown, 1988; Hsu *et al.*, 1991). This straightforward approach—assuming that carcinogens are mutagens and that carcinogens have to be 'initiators' in the typical initiation–promotion experimental protocols—has proven to be too simplistic for dealing with classes of complete carcinogens, such as the peroxisome proliferators, that are non-genotoxic or non-mutagenic, in that they do not interact with or damage DNA directly or after metabolic activation (Reddy & Rao, 1992).

During the past two decades, there has been a steady rise in the number, types and use of such non-genotoxic chemical carcinogens, mostly owing to concerted efforts to avoid, eliminate or minimize exposure to genotoxic chemicals in response to increased awareness of the comparatively greater carcinogenic hazard for humans. It is therefore to be anticipated that pharmaceutical, agricultural, industrial and other uses of non-genotoxic xenobiotics, including agents with properties similar to those of peroxisome proliferators, such as diethylstilboestrol, *d*-limonene, 2,3,7,8-tetrachlorodibenzo-*para*-dioxin (TCDD) and others, will dominate in the future. Concerted efforts must thus be made to elucidate the mechanism(s) by which non-genotoxic agents cause cancer. An understanding of such mechanisms is of paramount importance because it will allow the development of short-term bioassays and delineation of biomarkers that can serve to distinguish a non-genotoxic carcinogen from a (non-genotoxic) noncarcinogen. In this review, we will briefly discuss the possible mechanisms of hepatocarcinogenesis induced by peroxisome proliferators, a novel class of non-genotoxic hepatocarcinogens.

## Peroxisome proliferators

Peroxisome proliferators are a broad group of compounds including synthetic and naturally occurring compounds, such as certain hypolipidaemic drugs, phthalate ester plasticizers, industrial solvents, herbicides, food flavours, leukotriene D4 antagonists and hormones (Hess *et al.*, 1965; Reddy & Krishnakantha, 1975; Reddy & Lalwani, 1983; Vainio *et al.*, 1983; Elcombe *et al.*, 1985; Ikeda *et al.*, 1985; Lake *et al.*, 1987; Roffey *et al.*, 1990; Rao *et al.*, 1992a). When these agents, with ostensibly dissimilar structures and pharmacokinetic properties, are administered to rodents and non-rodents, including primates, they cause profound proliferation of peroxisomes in hepatic parenchymal cells and marked increases in the activities of the enzymes required for peroxisomal β-oxidation of fatty acids, namely fatty acyl-coenzyme A (CoA) oxidase, enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase bifunctional enzymes, and 3-ketoacyl-CoA thiolase (Lazarow & de Duve, 1976; Hashimoto, 1982; Reddy & Lalwani, 1983; Reddy *et al.*, 1986a). Despite their structural diversity and differences in their potencies, these agents induce qualitatively predictable pleiotropic responses characterized by hepatomegaly, an increase in the number of peroxisomes in liver parenchymal cells and rapid induction of the mRNA levels of all three enzymes of the peroxisomal β-oxidation system due to a coordinate increase in the rates of transcriptional activation of the genes that encode those enzymes (Reddy *et al.*, 1986b). The common singular effect of these structurally different compounds is the induction of peroxisome proliferation in hepatocytes; hence the designation peroxisome proliferators (Reddy *et al.*, 1975).

Although all peroxisome proliferators induce qualitatively similar hepatic pleiotropic effects, significant quantitative differences are seen according to the type of chemical and the species studied (Reddy *et al.*, 1984; Grey & de la Iglesia, 1984; Lake *et al.*, 1984; Reddy *et al.*, 1986a). Peroxisome proliferators exert a minimal or no effect in other tissues (Nemali *et al.*, 1988). Extensive studies have been carried out on peroxisome proliferator-induced hepatic changes in rats and mice, the most responsive species, using hypolipidaemic drugs and phthalate ester plasticizers (Reddy & Lalwani, 1983). In these two species, the peroxisome proliferator-induced pleiotropic responses, although dependent upon the dose and duration of exposure, are clearly divided into two phases: short-term treatment results in adaptive changes characterized by hepatomegaly and hepatic peroxisome proliferation with attendant increases in selected enzyme activities, and long-term treatment results in the development of liver tumours (Reddy *et al.*, 1982; Reddy & Lalwani, 1983). Short-term administration of peroxisome proliferators also results in the induction of microsomal cytochromes P450IVAV6 (fatty acid ω-hydroxylase) and cytosolic epoxide hydrolase (Moody & Reddy, 1976; Cohen & Grasso, 1981; Ciriolo *et al.*, 1982; Gibson *et al.*, 1982; Reddy & Lalwani, 1983; Moody *et al.*, 1985; Lock *et al.*, 1989). Alterations in the levels of different peroxisomal enzymes are highly variable: catalase and urate oxidase activities are increased by two- to threefold, whereas the hydrogen peroxide generating peroxisomal fatty acid β-oxidation enzyme activities increase by 20–40-fold (Nemali *et al.*, 1988). It is important to note that the changes in peroxisome volume density and enzyme activities persist at the steady-state level as long as the peroxisome proliferators are administered; reversal of these alterations is dependent upon the half-lives of the induced enzymes and of the peroxisome proliferator in the host.

The cell-specific effects of peroxisome proliferators have been documented clearly in studies on induction *in vitro* in primary liver-cell cultures, in hepatocytes transplanted into subcutaneous fat or into the anterior chamber of the eye of rats, and in rats with transdifferentiated pancreatic hepatocytes that emerge after a regimen of copper depletion and repletion (Reddy & Rao, 1987; Usuda *et al.*, 1988; Nemali *et al.*, 1989). The results of these studies show that hepatocytes are highly responsive to the inductive effects of peroxisome proliferators. On the basis of the tissue-specific induction of peroxisome proliferation, the rapidity of response, the demonstration of a reversible peroxisome proliferator-binding moiety in liver cytosol, and the transcriptional activation of genes of the β-oxidation enzyme system and cytochrome P450IVA4, among others (Gibson *et al.*,

1982; Reddy *et al.*, 1986b; Hardwick *et al.*, 1987; Reddy & Rao, 1987), it has been proposed that peroxisome proliferators exert their pleiotropic responses *via* a receptor-mediated signal transduction mechanism (Reddy & Lalwani, 1983; Reddy & Rao, 1986; Lalwani *et al.*, 1987).

Support for this concept was provided by the identification of peroxisome proliferator-binding protein(s) and characterization of a peroxisome proliferator- activated receptor (PPAR) in mouse liver, which is a member of the nuclear hormone receptor superfamily (Lalwani *et al.*, 1987; Issemann & Green, 1990). The PPAR was subsequently shown to be activated by medium- and long-chain fatty acids and to regulate the expression of genes for the peroxisomal β-oxidation system, mitochondrial medium-chain acyl-CoA dehydrogenase, and cytochrome P450IVA6 (Gottlicher *et al.*, 1992; Kaikaus *et al.*, 1993). Subsequently, frog, human and rat PPAR counterparts were characterized (Dreyer *et al.*, 1992; Gottlicher *et al.*, 1992; Sher *et al.*, 1993; Zhu *et al.*, 1993). A good correlation has been observed between ability to activate the PPAR and induction of peroxisome proliferation by peroxisome proliferators (Green, 1992). The PPAR appears to be associated with a 72-kDa heat-shock protein in the cytoplasm, and ligand-mediated translocation of PPAR to the nucleus results in heterodimerization with the retinoic X receptor (RXR). This complex binds to peroxisome proliferator responsive elements and initiates transcriptional activation of specific genes (Huang *et al.*, 1994). To date, three isoforms of PPAR have been described in frogs (xPPARα, xPPARβ and xPPARγ) and three in mice (mPPARα, mPPARγ and mPPARδ). After isolation of mPPARγ cDNA from mouse liver (Zhu *et al.*, 1993), an isoform, designated mPPARγ2, was described which exhibits adipocyte specificity in mice (Tontonoz *et al.*, 1994). mPPARγ2 cDNA encodes an additional 30 amino acids, N-terminal to the first ATG codon of mPPARγ1 (formerly mPPARγ); it also differs in the 5′-untranslated sequence.

The restricted tissue distribution of xPPARγ, mPPARγ1 and mPPARγ2 and the identification of other PPAR isoforms raise important questions about the physiological role of this PPAR subfamily and the structural basis of its diversity (Kliewer *et al.*, 1994). The tissue-specific effects and species variation of peroxisome proliferators may be dependent on the presence of a specific PPAR isoform(s), heterodimerization partner(s), the potency and pharmacokinetics of a given proliferator and differences in the peroxisome proliferator responsive elements of the genes in different species. It is of interest to note that the mPPARγ gene spans more than 105 kilobases and gives rise to two mRNA species, mPPARγ1 and mPPARγ2, with distinct 5′-exons that are spliced onto common downstream sequences (Zhu *et al.*, 1995). These two mRNAs arise as a result of alternative promoter use, the mPPARγ1 promoter being active primarily in the liver and the mPPARγ2 promoter primarily in adipose tissue (Zhu *et al.*, 1995). Thus, the existence of multiple PPAR isoforms transcribed from different promoters could increase the diversity of ligand- and tissue-specific transcriptional responses and could account for species differences in the magnitude of response to peroxisome proliferators.

## Peroxisome proliferators and hepatocarcinogenesis

The hepatocarcinogenic effects of different types of peroxisome proliferators have been abundantly demonstrated in rats and mice (Reddy *et al.*, 1980; Kluwe *et al.*, 1982; Reddy & Lalwani, 1983; National Toxicology Program, 1990; Rao & Reddy, 1991; Rao *et al.*, 1992b). These compounds are unequivocally established as complete carcinogens (Rao *et al.*, 1984a; Rao & Reddy, 1987; Cattley & Popp, 1989; Kraupp-Grasl *et al.*, 1990). The carcinogenic potency of peroxisome proliferators is closely related to their ability to induce peroxisome proliferation: liver tumours develop in a high percentage of animals treated with potent peroxisome proliferators, generally within 12 months of treatment with comparatively low dose levels, whereas after treatment with weak peroxisome proliferators, tumours develop at a lower incidence after a long latency (Reddy *et al.*, 1979; Rao *et al.*, 1984a; Kluwe *et al.*, 1982; Rao *et al.*, 1987).

Despite some claims of a lack of correlation between the level of peroxisome proliferation and hepatocarcinogenicity (Marsman *et al.*, 1992), it has been shown that any peroxisome proliferator administered at a dose that can induce a 15–25% increase in peroxisome volume density in hepatocyte cytoplasm can induce a nearly 100% incidence of liver tumours in rats and mice (Reddy, 1990). Under physiological conditions, peroxisomes occupy 1.5–2.0% of the cytoplasmic volume in hepatocytes (Moody & Reddy, 1976; Grey & de la Iglesia, 1984), whereas administration of peroxisome proliferators such as ciprofibrate, Wy-14,643, nafenopin and methyl clofenapate can increase the peroxisome volume density to about 18–25% (Reddy & Krishnakantha, 1975; Moody & Reddy, 1976).

The morphological features of liver tumours induced by peroxisome proliferators in rats and mice are those of hepatocellular carcinomas, and the process of carcinogenesis induced by these non-genotoxic carcinogens in liver is similar to that resulting from exposure to genotoxic carcinogens (Rao *et al.*, 1984a). Nevertheless, tumours induced by peroxisome proliferators have some distinct phenotypic properties that contrast strongly with those of tumours induced by genotoxic carcinogens (Rao *et al.*, 1988a; Rao & Reddy, 1994). Peroxisome proliferator-induced tumours lack the γ-glutamyltranspeptidase and glutathione-*S*-transferase P enzymes, which are characteristically expressed in genotoxic carcinogen-induced liver tumours (Hanigan & Pitot, 1985; Sato, 1988). The absence of these two proteins is attributed to failure of derepression of the genes that encode them (Rao *et al.*, 1988a).

A variety of short-term assays, such as Ames' mutagenicity test with various strains of *Salmonella typhimurium*, observation of $^{3}$H-thymidine incorporation into replicating DNA of proliferating lymphocytes, DNA strand breaks and unscheduled DNA synthesis in hepatocytes and covalent binding to macromolecules in hepatocytes, and demonstration of DNA adduct formation in hepatocytes by $^{32}$P-postlabelling analysis have clearly shown the non-genotoxic and non-mutagenic nature of several peroxisome proliferators (Warren *et al.*, 1980; Reddy *et al.*, 1983; Goel *et al.*, 1985; Gupta *et al.*, 1985; Nilsson *et al.*, 1991). Peroxisome proliferators also give negative results in a recently developed, sensitive test for intrachromosomal deletion and recombination in yeast (Schiestl & Reddy, 1990).

## Possible mechanisms of tumour induction by peroxisome proliferators

As pointed out above, the general lack of demonstrable mutagenicity or genotoxicity of peroxisome proliferators led to a re-evaluation of the paradigm that all chemical carcinogens must be mutagens and should be able to serve as 'initiators' in a typical 'initiation–promotion' experiment. Thus, a single dose of a carcinogenic chemical should be able to initiate carcinogenesis, so that cells can be promoted with a known promoter. By this criterion, many, if not all, genotoxic chemical carcinogens are initiators, but non-genotoxic carcinogens, such as peroxisome proliferators, cannot induce detectable initiation after a single dose (Cattley *et al.*, 1988) or after short-term administration (Glauert & Clark, 1989). This is neither surprising nor unexpected, because non-genotoxic carcinogens do not interact with or damage DNA, either directly or after metabolic activation (Gupta *et al.*, 1985; Nilsson *et al.*, 1991).

In order to appreciate the mechanism(s) by which peroxisome proliferators and other non-genotoxic chemical carcinogens cause cancer, a paradigm shift should be considered: that DNA damage and mutations can be induced by chemicals, such as peroxisome proliferators, not directly but indirectly, as a result of biological alterations to intermediary metabolism (e.g. free-radical generation), and that such non-genotoxic carcinogen-mediated 'initiation' may be an insidious, slow process or quantitatively subtle, like 'death by a thousand paper cuts'. Thus, any genetic alteration that eventually occurs during chronic exposure to peroxisome proliferators should be attributable to pleiotropic responses and the consequences of the interaction of the peroxisome proliferator with the receptor—so-called receptor-mediated carcinogenesis.

Three mechanisms have been proposed for the hepatocarcinogenesis induced by peroxisome proliferators: (1) oxidative stress, resulting from disproportionate increases in the levels of hydrogen peroxide, which generates and degrades peroxisomal and cytosolic enzymes; (2) increased cell proliferation; and (3) promotion of spontaneously initiated lesions. The first two mechanisms are not mutually exclusive, and some confluence could very well occur if the massive increases in hydrogen peroxide generating peroxisomal fatty acyl-CoA oxidase results in sustained production of excessive DNA-damaging reactive oxygen intermediates in liver parenchymal cells, to cause continued initiation that can be augmented and enhanced by cell proliferation (Reddy & Rao, 1986).

## Oxidative DNA damage and carcinogenesis

Active oxygen species, such as the superoxide radical anion ($O_2^{\cdot}$), hydrogen peroxide and hydroxyl radical ($OH^{\cdot}$), are continuously generated in different cytoplasmic organelles and are highly reactive with proteins, lipids and DNA. All mammalian cells are equipped with protective antioxidant defences that prevent cellular injury; however, when the defence mechanisms are overwhelmed, either by excessive generation of active oxygen species or decreased levels of antioxidant enzymes or a combination of the two, several pathological conditions, including cancer, can result (Trush & Kensler, 1991; Janssen *et al.*, 1993). Active oxygen species have been shown to induce a variety of DNA lesions, such as strand breaks, cross-linking of DNA and base damage (Meneghini, 1988; Poot, 1991). On the basis of these genotoxic effects, it is generally believed that oxidative stress plays an important role in the induction of spontaneous and carcinogen-induced tumours (Janssen *et al.*, 1993).

Peroxisomes contain a variety of enzymes, including the marker enzyme catalase and at least one hydrogen peroxide-generating flavin oxidase (de Duve & Baudhuin, 1966; de Duve, 1973; Tolbert, 1981). Peroxisomes appear to act in mammalian cells in several ways, including ß-oxidation of fatty acids (del Rio *et al.*, 1990; Tolbert, 1981). Under normal physiological conditions, peroxisomes generate hydrogen peroxide, most of which is degraded effectively by the catalase present in this organelle (de Duve, 1973). In liver in which peroxisomes have been induced by peroxisome proliferators, there are disproportionate increases in the levels of catalase and of hydrogen peroxide-producing peroxisomal fatty acyl-CoA oxidase: catalase levels are increased by 1.5–2-fold and those of fatty acyl-CoA by over 20–40-fold (Reddy *et al.*, 1986b; Nemali *et al.*, 1989). Hydrogen peroxide can react with $O_2^{\cdot}$ radicals through the metal-catalysed Haber–Weiss reaction (Halliwell & Gutteridge, 1990). Increased levels of hydrogen peroxide and $OH^{\cdot}$ radicals were clearly demonstrated in hepatocytes after treatment with peroxisome proliferators (Elliott *et al.*, 1986; Tomaszewski *et al.*, 1986), and greater amounts of lipofuscin and high levels of conjugated dienes in the livers of rats treated chronically with peroxisome proliferators are further evidence for lipid peroxidation caused by oxygen free radicals (Reddy *et al.*, 1982; Goel *et al.*, 1986; Lake *et al.*, 1987; Conway *et al.*, 1989; Marsman *et al.*, 1992). In addition, treatment with peroxisome proliferators results in a considerable decrease in the levels of antioxidant enzymes, further increasing the susceptibility of liver to peroxidative risk (Ciriolo *et al.*, 1982; Awasthi *et al.*, 1984; Lake *et al.*, 1987). DNA damage has been induced *in vitro* in SV40 DNA by peroxisomes isolated from the livers of rats exposed to a peroxisome proliferator (Fahl *et al.*, 1984).

Several new, sensitive techniques are available for evaluating oxidative lesions in DNA. It has been shown with high-performance liquid chromatography and electrochemical detection that 8-hydroxydeoxyguanosine (8-OHdG) adducts are a frequent and consistent DNA lesion caused by $OH^{\cdot}$ (Kasai *et al.*, 1987; Shigenaga & Ames, 1991). 8-OHdG is considered to be an important mutagenic lesion (Janssen *et al.*, 1993). Originally in collaboration with Dr Kasai and Dr Nishimura, we showed that 8-OHdG is formed in the livers of rats treated with ciprofibrate, a potent peroxisome proliferator (Kasai *et al.*, 1989). Takagi *et al.* (1990a,b, 1991) corroborated these findings with different peroxisome proliferators after short- and long-term administration. An additional DNA

alteration caused by peroxisome proliferators is a reduction in the level of I-compounds, which may play a significant role during the initiation phase of hepatocarcinogenesis (Randerath *et al.*, 1991).

Free radical-induced DNA damage is not an uncommon event in mammalian cells, but under physiological conditions oxidative DNA damage is rapidly repaired (Loeb, 1989). Under conditions of persistent oxidative stress, as are commonly observed with continuous administration of peroxisome proliferators with sustained overproduction of hydrogen peroxide-generating peroxisomal fatty acyl-CoA oxidase, the normal repair mechanisms might be insufficient and overwhelmed. There is increasing experimental evidence that peroxisome proliferators cannot cause DNA damage directly, but the receptor-mediated pleiotropic effects can lead indirectly to DNA damage and other genetic changes essential for hepatocarcinogenesis. It is interesting that simultaneous treatment of rats with peroxisome proliferators and antioxidants results in a marked reduction in tumour incidence, further attesting to the role of oxidative stress in hepatocarcinogenesis (Rao *et al.*, 1984b).

Recently, we examined the role of sustained elevation of hydrogen peroxide-generating peroxisomal fatty acyl-CoA oxidase in the transformation of mammalian cells (Chu *et al.*, 1995). We generated CV-1 cells that stably express rat peroxisomal fatty acyl-CoA oxidase. When these stable transfectants were exposed to a fatty acid for 2–6 weeks, they acquired growth advantage and transformed phenotype. The growth of these cells in soft agar and the formation of tumours when the transformed cells were transplanted into nude mice provide additional evidence for a role in the hepatocarcinogenesis induced by peroxisome proliferators of overexpressed peroxisomal fatty acyl-CoA oxidase in liver.

## Increased cell proliferation and peroxisome proliferator-induced carcinogenesis

The role of cell proliferation in genotoxic carcinogen-induced tumorigenesis is well understood when carcinogen-induced DNA damage is fixed and converted to heritable genetic alterations (Grisham *et al.*, 1983); however, the role of cell proliferation in non-genotoxic-induced carcinogenesis is not clear. It has been hypothesized that chronic mitogenesis is indirectly carcinogenic simply by increasing the probability of that endogenous DNA damage will be converted into mutations (Ames & Gold, 1990; Cohen & Ellwein, 1991). Some investigators have suggested that peroxisome proliferators also induce liver tumours through increased cell proliferation (Butterworth *et al.*, 1987). This hypothesis is based on the finding that all peroxisome proliferators markedly increase cell proliferation during the first two weeks of their administration (Smith-Oliver & Butterworth, 1987; Marsman *et al.*, 1988; Styles *et al.*, 1988; Rao & Reddy, 1989; Yeldandi *et al.*, 1989; Barrass *et al.*, 1993). Nevertheless, evaluation of the cell proliferative effects of many peroxisome proliferators clearly indicates that the cell proliferation is short-lived; it is maximal during the first two weeks and gradually decreases to control levels (Yeldandi *et al.*, 1989; Eacho *et al.*, 1991; Marsman *et al.*, 1992; Barrass *et al.*, 1993). This transient effect clearly shows that it makes no significant contribution to the carcinogenicity of peroxisome proliferators. The decrease in cell proliferation after the initial burst may be due to increased levels of TGF-β, a growth factor that has been shown to inhibit hepatocyte growth (Thoresen *et al.*, 1992; Rumsby *et al.*, 1994).

Other experimental findings also indicate a limited role for cell proliferation in peroxisome proliferator-induced carcinogenesis. These include: a decrease in the number of $2 \times 2$ N hepatocytes (proliferative fraction) and an increase in 4 N (non-proliferative) cells; and induction of a hyperplastic type of cell proliferation (Columbano *et al.*, 1987; Styles *et al.*, 1991). It has been shown that the mitogen-induced hyperplastic type of cell proliferation is less likely to lead to cancer than that resulting from compensatory hyperplasia (Columbano *et al.*, 1987). Unlike compensatory hyperplasia and cell proliferation caused by some mitogens, the liver-cell hyperplasia caused by nafenopin, a peroxisome proliferator, is not associated with activation of c-*myc*, c-*jun* or c-*fos* proto-oncogenes (Cari *et al.*, 1993). The significance of these differences in proto-oncogene activation to hepatocarcinogenesis, if any, is not clear. It is also pertinent that peroxisome

proliferators do not induce tumours in the kidney, an organ in which increased cell proliferation and minimal peroxisome proliferation occur (Ward *et al.*, 1988). Furthermore, liver tumour induction by peroxisome proliferators can be inhibited by antioxidants without a decrease in cell proliferation (M.S. Rao, unpublished data). On the basis of these findings, it is reasonable to assume that cell proliferation caused by peroxisome proliferators is inconsequential for initiation but could play a role in the promotion of lesions initiated by oxidative DNA damage.

## Promotion of spontaneously developed lesions

Another mechanism that has been proposed (Cattley *et al.*, 1991; Kraupp-Grasl *et al.*, 1991) is promotion by peroxisome proliferators of spontaneously occurring hepatic lesions. Those investigators showed a higher incidence of hepatic tumours in aged than in young rats after treatment with a peroxisome proliferator. In the study of Kraupp-Grasl *et al.* (1991), in which rats were treated with nafenopin for 13 months, tumours developed in both young and old rats, although the incidence was higher in the latter. Similarly, in the study of Cattley *et al.* (1991), in which Wy-14,643 was given for 22 weeks, tumours developed in both young and old rats. These investigators ignore two facts, however: (1) all initiators are also promoters, so any spontaneous lesions in the older rats would have been promoted; and (2) the fact that even the younger rats developed tumours after administration of a weak peroxisome proliferator for 13 months or a potent peroxisome proliferator for 22 weeks clearly proves a carcinogenic effect. In our laboratory, studies with ciprofibrate, a potent peroxisome proliferator, yielded similar tumour incidences in six-month- and one-year-old rats (Rao *et al.* 1990).

The development of hepatocellular carcinomas in C57Bl/6 mice, a strain resistant to hepatocarcinogenesis which rarely develop spontaneous lesions, after ciprofibrate administration also clearly indicates that peroxisome proliferators are not simply promotors but complete carcinogens (Rao *et al.*, 1988b). Another strong indication that peroxisome proliferators are complete carcinogens is the finding of a significant difference in the prevalence of H-*ras* mutation in spontaneously developed and ciprofibrate-induced lesions (Fox *et al.*, 1990; Hegi *et al.*, 1993): H-*ras* gene activation was observed in 71% of spontaneously developed lesions and in only 22% of ciprofibrate-induced lesions.

## Conclusions

Peroxisome proliferators are a diverse group of chemicals of therapeutic, industrial and agricultural value. These compounds, although not mutagenic, are hepatocarcinogenic in rats and mice. Short-term assays that have mutagenicity or genotoxicity as the end-point are not particularly useful for identifying certain classes of carcinogens, such as peroxisome proliferators. We postulated that a link exists between peroxisome proliferation and the development of liver tumours (Reddy *et al.*, 1980) and suggested that the morphological phenomenon of peroxisome proliferation in liver cells could be used as a simple, sensitive and valuable short-term biological indicator of this specific class of non-genotoxic chemical, which may be carcinogenic. This indicator could also be used to predict the target organ, i.e. the liver. Short-term biological effects *in vivo* can be evaluated easily by using a variety of simple tests. The available experimental evidence suggests that the ability of these agents to induce carcinogenesis depends on the degree of induction of peroxisome proliferation. There is also increasing support for a role of hydrogen peroxide-generating peroxisomal fatty acyl-CoA oxidase in liver carcinogenesis induced by these agents. Further studies are essential for ascertaining the role of downstream events associated with the enormous increase in the activity of this peroxisomal oxidase, related to overproduction of hydrogen peroxide and other reactive oxygen intermediates in the liver, with significant, sustained increases in peroxisome volume density.

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# C. PEROXISOME PROLIFERATION, GENOTOXICITY AND CARCINOGENICITY

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## 1. Introduction

More than 70 chemicals have been reported in the open literature to have peroxisome proliferator activity (European Chemical Industry Ecology and Toxicology Centre, 1992; Bentley *et al.*, 1993; Ashby *et al.*, 1994). The relationship between peroxisome proliferation and hepatocarcinogenesis was recently reviewed comprehensively (Ashby *et al.*, 1994). Data on long-term carcinogenicity were available for 28 of these chemicals, but for only 18 were both satisfactory long-term carcinogenicity studies and quantitative data on peroxisome proliferation available. The correlation between hepatic peroxisome proliferation and hepatocarcinogenesis was examined, and the genotoxicity of the chemicals was described in detail; the possibility of an indirect genotoxic mechanism via generation of reactive oxygen species was discussed. The present report is a brief description of the findings on hepatocarcinogenicity and genotoxicity for the 18 peroxisome proliferators reported by Ashby *et al.* (1994). In addition, we briefly review the database on the 18 chemicals with respect to carcinogenicity at sites other than the liver.

Many peroxisome proliferators, such as the lipid-lowering fibrates, are derivatives of phenoxyacetic acids, several of which are used as herbicides. Several of them (2,4-D, 2,4,5-T and MCPA) have been reported to have peroxisome proliferation activity (Kawashima *et al.*, 1984; Hietanen *et al.*, 1985), but there are no reports to indicate that these compounds have hepatocarcinogenic potential (Ashby *et al.*, 1994). Phenoxypropionic acids are also produced as herbicides; however, data related to peroxisome proliferation and hepatocarcinogenesis are not available in the open literature. Four phenoxypropionic acid herbicides (fluazifop-butyl, haloxyfop, propaquizafop and quizalafop-ethyl) have been submitted for registration to the pesticide authorities in the Nordic countries. Data on peroxisome proliferation, genotoxicity and hepatocarcinogenicity extracted from the registration documentation for these four herbicides are presented below.

## 2. Data in the open literature on hepatocarcinogenic peroxisome proliferators

### 2.1 Carcinogenicity

Ashby *et al.* (1994) identified 18 chemicals for which there are both adequate carcinogenicity data and relevant quantitative data on peroxisome proliferation in rats and/or mice (Table 1). In addition to the studies referred to in Table 1, adequate data were also available from studies of

clobuzarit and di(2-ethylhexyl)phthalate in hamsters and clofibrate in marmosets; however, neither induction of hepatocellular tumours nor peroxisome proliferation was observed in these studies.

**Table 1. Database for examining the concordance between hepatocellular peroxisome proliferation (PP) and hepatocarcinogenicity (HC) in rats and mice (M, male; F, female)**

| Compound | CAS No. | Rats | | | | | Mice | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Strain | PP | | HC | | Strain | PP | | HC | |
| | | | M | F | M | F | | M | F | M | F |
| Benzylbutyl phthalate | 85-68-7 | F344 | | – | | – | | | | | |
| Cinnamyl anthranilate | 87-29-6 | F344 | – | + | – | – | B6C3F1 | + | + | + | + |
| Ciprofibrate | 52214-84-3 | F344 | + | | + | | C57Bl | + | | + | |
| Clobuzarit | 22494-47-9 | Wistar | + | | – | | C57Bl | + | | + | |
| Clofibrate | 637-07-0 | SD | + | + | + | + | C57Bl | + | | – | |
| | | F344 | + | | + | | Swiss | + | | – | |
| | | Wistar | + | | – | | | | | | |
| Di(2-ethylhexyl)adipate | 103-23-1 | F344 | + | + | – | – | B6C3F1 | + | + | – | + |
| Di(2-ethylhexyl)phthalate | 117-81-7 | F344 | + | + | + | + | | | | | |
| Di-isononyl phthalate | 28553-12-0 | F344 | – | – | – | – | | | | | |
| Gemfibrozil | 25812-30-0 | SD | + | | + | | | | | | |
| Lactofen | 3513-60-4 | | | | | | CD-1 | + | + | + | + |
| LY 171883 | 88107-10-2 | | | | | | B6C3F1 | | + | | + |
| Methylclofenapate | 21340-68-1 | F344 | + | | + | | | | | | |
| Nafenopin | 3771-19-5 | F344 | + | | + | | | | | | |
| Tetrachloroethylene | 127-18-4 | F344 | – | – | – | – | B6C3F1 | + | + | + | + |
| Tibric acid | 37087-94-8 | F344 | + | | + | | | | | | |
| Trichloroacetic acid | 76-03-9 | | | | | | B6C3F1 | + | | + | |
| Trichloroethylene | 79-01-6 | F344 | – | | – | | B6C3F1 | + | | + | |
| | | Osborne-Mendel | – | | – | | | | | | |
| Wy-14,643 | 50892-23-4 | F344 | + | | + | | | | | | |

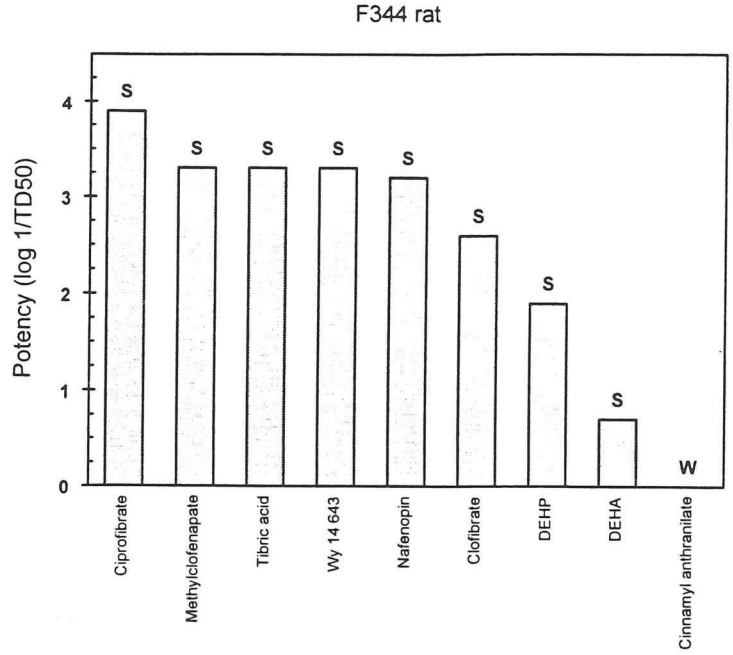
Adapted from Ashby *et al.* (1994)

The numerical correlation between chemicals that induce peroxisome proliferation in the livers of laboratory animal species in short-term studies and hepatocellular tumours in those animals in long-term studies was determined by Ashby *et al.* (1994). When all the species were considered as a group, the correlation between hepatic peroxisome proliferation and hepatocarcinogenesis was 81%. When rats and mice were considered separately, the correlation was 80% in each species.

The peroxisome proliferating carcinogens included in Table 1 vary both in their tumorigenic potency and in their ability to induce liver tumours in comparison with a given increase in peroxisome proliferation. In male Fischer 344 rats, for example, ciprofibrate, a highly effective hepatocarcinogen, induced tumours in the livers of all the animals at a dietary concentration of 250 mg/kg (ppm), whereas di(2-ethylhexyl)adipate induced tumours in about 5% of animals given feed containing 25 000 mg/kg (ppm) of the substance. Thus, the tumorigenic potency of these two extreme examples, expressed as the logarithm of the inverse of the dose that induces tumours in 50% of animals, differed by a factor of about 1500.

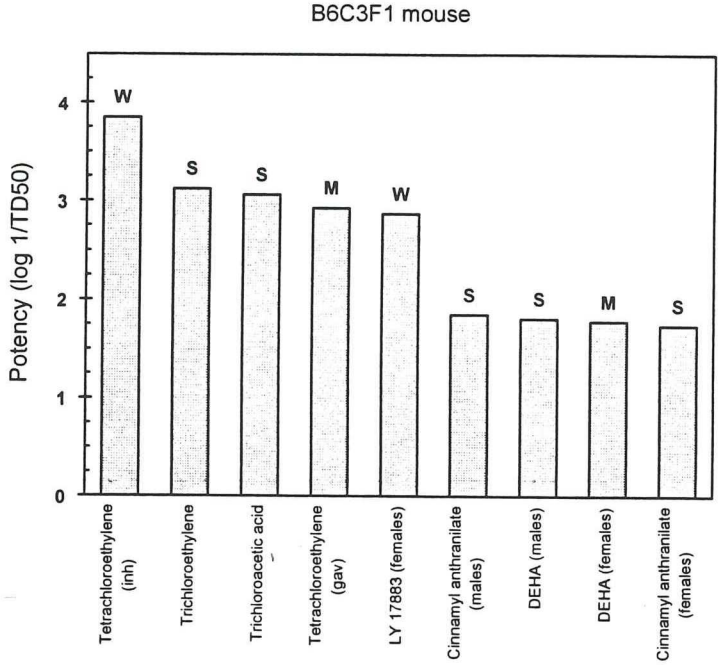
Figures 1 and 2 show the calculated potency values for several peroxisome proliferators in Fischer 344 rats and B6C3F1 mice, the only two animal strains for which there are sufficient data

**Figure 1. Hepatocarcinogenic potency in male Fischer 344 rats**




Adapted from Ashby *et al.* (1994)
The letters above the columns indicate whether the substance is a strong (S) or a weak (W) peroxisome proliferator.

**Figure 2. Hepatocarcinogenic potency in B6C3F1 mice**




Adapted from Ashby *et al.* (1994)
The letters above the columns indicate whether the substance is a strong (S), moderate (M) or a weak (W) peroxisome proliferator.

to permit this type of comparison. Any variation in tumour latency associated with the various peroxisome proliferators was not considered because of insufficient data. In Fischer 344 rats (Figure 1), all chemicals at the highest dose induced a more than sixfold enhancement in the numbers of peroxisomes and caused hepatocellular tumours in more than 75% of the animals. The only exception was cinnamyl anthranilate, which did not induce liver tumours in rats (in contrast to mice) and which enhanced the numbers of peroxisomes in female Fischer 344 rat liver by less than threefold at a dietary concentration of 30 000 ppm. In B6C3F1 mice (Figure 2), inhalation of tetrachloroethylene (perchloroethylene) induced hepatocellular tumours in about 80% of the animals, while the number of peroxisomes was increased by less than threefold. When tetrachloroethylene was given by gavage, however, the number of peroxisomes increased by more than fourfold and the tumour frequency was 50–60%. With LY 17883, the increase in peroxisome proliferation was less than threefold and the tumour incidence was about 30%. Di(2-ethylhexyl)adipate and cinnamyl anthranilate were of similar tumorigenic potency in B6C3F1 mice of each sex, whereas di(2-ethylhexyl)phthalate was less effective in causing peroxisome proliferation in females than in males and less effective than cinnamyl anthranilate in animals of each sex.

Some of the peroxisome proliferators listed in Table 1 did not induce hepatocellular tumours in all long-term studies. It is important to analyse this lack of response in more detail in order to define better the relationship between peroxisome proliferation and hepatocarcinogenesis. The compounds that did not induce hepatocellular tumours in some long-term studies can be divided into two categories:

1. Compounds that do not induce peroxisome proliferation under the conditions in which no carcinogenicity is observed: Two of the chemicals (benzylbutyl phthalate and di-isononyl phthalate) are not peroxisome proliferators at the doses at which the carcinogenicity studies were carried out. Tetrachloroethylene and trichloroethylene, although they can induce peroxisome proliferation, do not do so in rats because that species has a metabolic inability to form sufficient quantities of the active metabolite, trichloroacetic acid. For these two chemicals, the species differences in hepatocarcinogenicity are explained by species differences in peroxisome proliferation. Clofibrate, clobuzarit and di(2-ethylhexyl)phthalate also do not induce peroxisome proliferation in marmosets and hamsters, supporting the correlation between peroxisome proliferation and hepatocarcinogenesis.

2. Compounds that cause peroxisome proliferation but do not induce hepatocellular carcinomas in long-term studies: In all of the examples examined, the proportion of peroxisome proliferators that are not carcinogens was 20%. The absence of carcinogenic activity of the compounds in this category cannot be explained by their ineffectiveness as peroxisome proliferators in short-term studies. The chemicals in question are clobuzarit (male Wistar rat), clofibrate (male C57B1 mouse, male Swiss mouse, male Wistar rat), di(2-ethylhexyl)adipate (male and female Fischer 344 rats, male B6C3F1 mouse) and cinnamyl anthranilate (male Fischer 344 rat). Several explanations are possible: (i) Peroxisome proliferation and hepatocarcinogenesis are not related. (ii) The relationship between peroxisome proliferation and hepatocarcinogenesis is quantitative, and small changes may be insufficient to initiate tumours. (iii) Different species, strains and sexes of rodent may have different sensitivities to tumour induction for similar changes in peroxisomal numbers in the liver. (iv) Factors other than, or in addition to, peroxisome proliferation are also important.

A pertinent question to be addressed is whether the hepatocarcinogenic peroxisome proliferators can induce tumours by other mechanisms and in other organs than the liver. Table 2 lists the non-hepatic tumorigenic responses of the 18 peroxisome proliferators in the database presented in Table 1. Benzylbutyl phthalate and tetrachloroethylene have been found to induce mononuclear-cell leukaemia in Fischer 344 rats. Cinnamyl anthranilate, clofibrate and gemfibrozil induced tumours in

**Table 2. Induction of non-hepatic tumours in rats and mice with peroxisome proliferators included in the database in Table 1**

| Compound and tumour type (reference) | Species | Sex | Dose and route of administration | No. of animals with tumours (%) | | |
|---|---|---|---|---|---|---|
| | | | | Control | Low-dose | High-dose |
| **Benzylbutyl phthalate** | | | | | | |
| Mononuclear cell leukaemia (National Toxicology Program, 1982) | F344 rat | F | 6000, 12 000 ppm in diet | 7/49 (14%) | 7/49 (14%) | 18/50 (36%) |
| **Cinnamyl anthranilate** | | | | | | |
| Acinar-cell adenoma or carcinoma of the pancreas | F344 rat | M | 15 000, 30 000 ppm in diet | 0/42 (0%) | 0/49 (0%) | 3/35 (7%) |
| Adenoma or adenocarcinoma of the renal cortex (National Toxicology Program, 1980) | | | | 0/48 (0%) | 0/50 (0%) | 4/49 (8%) |
| **Clofibrate** | | | | | | |
| Acinar-cell carcinoma of the pancreas (Reddy & Qureshi, 1979) | F344 rat | M | 5000 ppm | 0/14 (0%) | 2/11 (18%) | |
| **Gemfibrozil** | | | | | | |
| Pheochromocytoma of the adrenal glands | CD rat | M | 30, 300 mg/kg bw/day gavage | 3/50 (6%) | 13/50 (26%) | 9/50 (18%) |
| Interstitial-cell tumour of the testes | | | | 1/50 (2%) | 8/50 (16%) | 17/50 (34%) |
| Acinar-cell adenoma or carcinoma of the pancreas (Fitzgerald *et al.*, 1981) | | | | 0/50 (0%) | 6/50 (12%) | 2/50 (4%) |
| **Tetrachloroethylene** | | | | | | |
| Mononuclear-cell leukaemia | F344 rat | M | 200, 400 ppm | 28/50 (56%) | 37/50 (74%) | 37/50 (74%) |
| | | F | inhalation | 18/50 (36%) | 30/50 (60%) | 29/50 (58%) |
| Renal tubular adenoma or adenocarcinoma (National Toxicology Program, 1986) | | M | | 1/49 (2%) | 3/49 (6%) | 4/50 (8%) |
| **Trichloroethylene** | | | | | | |
| Renal tubular adenocarcinoma | F344 rat | M | 500, 1000 mg/kg bw gavage | 0/48 (0%) | 0/49 (0%) | 3/49 (6%) |
| Peritoneal mesothelioma | | | | 1/50 (2%) | 5/50 (10%) | 1/49 (2%) |
| Harderian gland adenoma | B6C3F1 mouse | M | 1000 mg/kg bw | 0/50 (0%) | | 4/50 (8%) |
| (National Toxicology Program, 1990) | | F | gavage | 0/48 (0%) | | 3/49 (6%) |

rat pancreas, and cinnamyl anthranilate, tetrachloroethylene and trichloroethylene induced renal tumours in male rats.

A number of studies have been carried out to investigate possible promotor activity in two-stage carcinogenesis models. Thus, di(2-ethylhexyl)phthalate has been reported to act as a renal tumour promoter (Kurokawa *et al.*, 1988) and as a second-stage promoter in mouse skin carcinogenesis with SENCAR mice (Diwan *et al.*, 1985). Clofibrate was found to promote urinary bladder carcinogenesis in Fischer 344 rats, while no such activity was found with di(2-ethylhexyl)phthalate (Hagiwara *et al.*, 1990).

## 2.2 Clinical trials

The potential carcinogenic risk of hypolipidaemic therapy with fibrates has been evaluated in two randomized clinical trials (WHO, 1986; Huttunen *et al.*, 1994; Law *et al.*, 1994). In the WHO Clofibrate Study (WHO, 1986; Law & Thompson, 1991), 72 deaths from cancer were observed during the five-year trial among subjects treated with clofibrate and 54 among those given a placebo ($p = 0.12$). The difference disappeared during follow-up of the cohort after the intervention period. In contrast, no difference in the incidence of or mortality from cancer was found in the Helsinki Heart Study during five years of treatment with gemfibrozil, but an excess of deaths from cancer (20 vs. 7) was observed among those who originally received this drug during the 3.5-year follow-up period, when about 60% of the participants in both trial groups were taking gemfibrozil (Huttunen *et al.*, 1994). The difference disappeared again when the follow-up was extended to 10 years.

## 2.3 Cell transformation and intercellular communication

Assays for cell transformation *in vitro* are generally regarded as the systems that most closely resemble the early steps of chemical carcinogenesis *in vivo* (Heidelberger *et al.*, 1983). In contrast to most other short-term tests *in vitro*, assays for cell transformation and intercellular communication may also respond to agents that are usually regarded as nongenotoxic carcinogens.

Table 3 is a summary of the activity of hepatocarcinogenic peroxisome proliferators in assays for morphological cell transformation and gap junctional intercellular communication. A more detailed presentation of individual studies is presented in **Annex A**. Di(2-ethylhexyl)phthalate is the peroxisome proliferator that has been studied most often in cell transformation systems. In a collaborative study (Ashby *et al.*, 1985), di(2-ethylhexyl)phthalate was tested in five different laboratories using six different transformation systems. There was good agreement, as positive responses (weak or strong) were reported with five of the six systems (McGregor *et al.*, 1985). Di(2-ethylhexyl)phthalate has consistently been found to induce transformation in the Syrian hamster embryo cell system. It was also found to induce anchorage-independent growth in the mouse epidermal JB6 cell line (Diwan *et al.*, 1985), which has been shown to be malignant. Di(2-ethylhexyl)-phthalate also induced anchorage-independent growth in Fischer rat embryo cells infected with Rauscher leukaemia virus in some experiments (Suk & Humphreys, 1985). Negative results were obtained in three studies: one in C3H10T1/2 cells (Sanchez *et al.*, 1987) and two in BALB/c-3T3 cells (Matthews *et al.*, 1985, 1993). The compound did not act as a complete transforming agent, as an initiator-like agent (promotion by 12-*O*-tetradecanoylphorbol 13-acetate [TPA]) or as a promoter-like agent (initiation with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine) in C3H10T1/2 cells; however, it should be noted that in both systems either short exposures were combined with long expression periods, or di(2-ethylhexyl)phthalate was used at a low concentration when the cells were exposed in the promotion phase. Thus, the negative results may be a consequence of the exposure regimens.

Clofibrate was found to be active in the Syrian hamster embryo cell system by Mikalsen *et al.* (1990a,b) but to be inactive by Tsutsui *et al.* (1993); however, different exposure schemes were used

in the two studies. Clofibrate did not act as a complete transforming agent but gave a promoter-like response after initiation with 3-methylcholanthrene (Lillehaug *et al.*, 1986).

**Table 3. Overall activity of hepatocarcinogenic peroxisome proliferators in assays for morphological cell transformation and gap junctional intercellular communication**

| Compound | Cell transformation | Intercellular communication |
|---|---|---|
| Clofibrate | + | + |
| Di(2-ethylhexyl)adipate | – | |
| Di(2-ethylhexyl)phthalate | + | + |
| Methylclofenapate | | – |
| Nafenopin | | + |
| Trichloroethylene | + | + |
| Trichloroacetic acid | | + |
| Wy-14, 643 | + | + |

\+ Most of the assays performed with the compound gave a positive response (i.e. induced cell transformation or decreased intercellular communication).
– Most of the assays performed with the compound gave a negative response (i.e. did not induce cell transformation or did not decrease intercellular communication).

Trichloroethylene was found to induce transformation and malignancy in FRE cells (Price *et al.*, 1978). Trichloroethylene also induced transformation in BALB/c-3T3 cells (Tu *et al.*, 1985) and a low frequency of transformed colonies in Syrian hamster embryo cells (Amacher & Zelljadt, 1983). The potent peroxisome proliferator, Wy-14,643, induced statistically significant increases in the transformation of Syrian hamster embryo cells only in the presence of a metabolic system (Tsutsui *et al.*, 1993).

The relationship between peroxisome proliferation and cell transformation was investigated by two groups using the Syrian hamster embryo cell system. Tsutsui *et al.* (1993) found no clear relationship between peroxisome proliferation and induction of transformation. Mikalsen *et al.* (1990a,b,c) observed small increases in catalase activity and peroxisomal ß-oxidation, but no increase in the number of peroxisomes after exposure to peroxisome proliferators. Thus, peroxisome proliferation is apparently not involved in the peroxisome proliferator-induced morphological transformation of Syrian hamster embryo cells.

A number of tumour promoters of diverse chemical structures have been shown to inhibit gap junctional intercellular communication in one or several types of cells. Measurement of this commuication has been discussed as a functional assay for tumour promoters (Barrett *et al.*, 1986). As it may be involved in growth control (Loewenstein, 1979), derangement of this process could be of importance in tumour development (Trosko *et al.*, 1990). As for cell transformation, di(2-ethylhexyl)phthalate seems to be the peroxisome proliferator most extensively studied in intercellular communication assays (Table 3). In most studies, the compound was found to inhibit intercellular communication, and the decrease was sustained for at least one week in Syrian hamster embryo cells (Mikalsen & Sanner, 1993). In one study with negative results (Kornbrust *et al.*, 1984), the concentration of di(2-ethylhexyl)phthalate applied was very low.

Trichloroethylene has been studied in both rat and mouse hepatocytes (Klaunig *et al.*, 1989) and found to cause a slight inhibition of intercellular communication in mouse but not rat hepatocytes. Similarly, trichloroacetic acid was found to inhibit gap junctional intercellular commu-

nication in mouse but not in rat hepatocytes. These findings may account partially for the observed species differences in susceptibility to trichloroethylene-induced liver carcinogenesis.

Clofibrate inhibited intercellular communication in Syrian hamster embryo cells, but the inhibition was rapidly reversed, even in the continued presence of the compound (Rivedal, E., Mikalsen, S.-O. & Sanner, T., unpublished data). The potent peroxisome proliferator methylclofenapate did not inhibit metabolic cooperation in V79 cells, while Wy-14,643 and nafenopin did, but no details were presented about these experiments (Trosko *et al.*, 1982). Wy-14,643 and nafenopin have also been reported to inhibit intercellular communication in rat hepatocytes. It is possible that different peroxisome proliferators inhibit intercellular communication by different mechanisms (Leibold *et al.*, 1994).

Altered preneoplastic foci in rat liver generated by Wy-14,643 and ciprofibrate showed decreased connexin 32 expression; however, this is not necessarily related to the faster inhibition of intercellular communication described above, since a decrease in connexin 32 seems to be a general marker in these foci, as it is also generated by other regimens (Neveu *et al.*, 1990).

For several reasons, it is likely that the inhibition of intercellular communication by peroxisome proliferators is independent of peroxisome proliferation: (i) Inhibition of gap junctional intercellular communication by trichloroethylene and trichloroacetic acid in mouse hepatocytes and by clofibrate in Syrian hamster embryo cells was transient; it was maximal before any peroxisome proliferative response had had time to develop. (ii) The $IC_{50}$ values for inhibition of intercellular communication by nafenopin and Wy-14,643 are not in accordance with their potencies as inducers of acyl-coenzyme A oxidase and as hepatocarcinogens. (iii) The potent peroxisome proliferator methylclofenapate did not inhibit metabolic cooperation in V79 cells, while Wy-14,643 and nafenopin did.

## 2.4 Genotoxicity

A total of 142 genotoxicity assays have been performed on 17 of the 18 hepatocarcinogenic peroxisome proliferators reported by Ashby *et al.* (1994). Trichloroethylene was not included in this overall assessment, since the test data on this compound are confounded by a lack of reporting of potentially genotoxic impurities and addition of mutagenic stabilizers. The assays included the *Salmonella* mutagenicity test, unscheduled DNA synthesis *in vitro*, various tests in *Drosophila melanogaster*, sister chromatid exchange *in vitro*, chromosomal aberrations *in vitro*, cell transformation *in vitro*, rodent bone-marrow clastogenicity and rodent liver DNA damage. Of these 142 assays, 108 (76%) gave negative results, 14 (10%) equivocal results and 20 (14%) positive results. This indicates that, as a group, peroxisome proliferators are predominantly non-genotoxic. The database is, however, very uneven, in that genotoxicity data are lacking or exist from only one assay for some chemicals, whereas there is a plethora of data for other chemicals (Table 4). In addition, some of the substances show definite genotoxic activity *in vitro*.

All of the 18 peroxisome proliferators are either organic carboxylic acids or their esters, a pseudo-carboxylic acid (LY 171883) or, as for trichloroethylene and tetrachloroethylene, can be metabolized to an organic carboxylic acid (trichloroacetic acid). As such, these compounds have no structural alerts indicative of electrophilicity and thereby DNA-binding potential; they are mainly nucleophilic. The only exception is trichloroethylene, which during its metabolism to trichloroacetic acid forms a potentially reactive epoxide.

No data on the genotoxicity of clobuzarit and LY 171883 exist in the open literature (Table 4), and single negative results in mutagenicity tests in *Salmonella* have been reported for di-isononyl phthalate, gemfibrozil and tibric acid. Benzylbutyl phthalate and methylclofenapate have been tested extensively and are devoid of any genotoxicity. Lactofen gave negative results in most tests *in vitro* but has not been tested *in vivo*. Di(2-ethylhexyl)adipate may also generally be regarded as nongenotoxic. Clofibrate and cinnamyl anthranilate have been extensively tested, and they show

some evidence of inducing cytogenetic damage and cell transformation *in vitro*; they are not genotoxic *in vivo*.

**Table 4. Genotoxicity of carcinogenic peroxisome proliferators**

| Compound | CAS No. | Overall genotoxic activity |
|---|---|---|
| Benzylbutyl phthalate | 85-68-7 | Negative in a number of tests *in vitro*; negative in mouse dominant lethal test |
| Cinnamyl anthranilate | 87-29-6 | Negative in a number of tests *in vitro*, but both negative and positive in cell transformation tests. Negative in test for mouse bone-marrow micronuclei and rat liver unscheduled DNA synthesis |
| Ciprofibrate | 52214-84-3 | Positive in rat hepatocytes in tests for sister chromatid exchange, chromosomal aberrations and micronuclei *in vitro*; no data *in vivo* |
| Clobuzarit | 22494-47-9 | No data available |
| Clofibrate | 637-07-0 | Negative in a number of tests *in vitro*, positive in one test for chromosomal aberrations and one for sister chromatid exchange *in vitro*, negative in one and positive in two cell transformation tests. Negative *in vivo* for sister chromatid exchange, chromosomal aberrations and single-strand breaks |
| Di(2-ethylhexyl)-adipate | 103-23-1 | Negative in a number of tests *in vitro*; negative in test for mouse bone-marrow micronuclei and weakly positive in mouse dominant lethal test |
| Di(2-ethylhexyl)-phthalate | 117-81-7 | Negative in many tests *in vitro*, positive in several cell transformation tests; negative in tests for mouse blood micronuclei, rat bone-marrow chromosomal aberrations and mouse dominant lethal mutation |
| Di-isononyl phthalate | 28553-12-0 | Negative in one *Salmonella* test, no other data available |
| Gemfibrozil | 25812-30-0 | Negative in one *Salmonella* test, no other data available |
| Lactofen | 3513-60-4 | Negative in several tests *in vitro*; positive in one *Salmonella* test; no in-vivo data available |
| LY 171883 | 88107-10-2 | No data available |
| Methylclofenapate | 21340-68-1 | Negative in several tests *in vitro*; negative in tests for mouse bone-marrow micronuclei, rat liver single-strand breaks, rat liver unscheduled DNA synthesis, MutaMouse *lac*Z and Big Blue *lac*I |
| Nafenopin | 3771-19-5 | Negative in *Salmonella* and rat hepatocyte unscheduled DNA synthesis tests *in vitro*; positive in rat hepatocytes in tests for sister chromatid exchange, chromosomal aberrations and micronuclei *in vitro*; negative in a test for rat liver unscheduled DNA synthesis *in vivo*. |
| Tetrachloroethylene | 127-18-4 | Negative in many tests *in vitro*; negative *in vivo* for rat kidney unscheduled DNA synthesis and rat dominant lethal mutation; negative or equivocal in rat bone-marrow chromosomal aberration tests |
| Tibric acid | 37087-94-8 | Negative in one *Salmonella* test; no other data available |
| Trichloroacetic acid | 76-03-9 | Negative in several tests *in vitro*; negative and positive in mouse bone-marrow micronuclei tests; negative in rat and mouse bone-marrow chromosomal aberration tests |

**Table 4 (contd)**

| Compound | CAS No. | Overall genotoxic activity |
|---|---|---|
| Trichloroethylene[a] | 79-01-6 | Negative in many tests *in vitro*; positive in many tests *in vitro* for point mutation, DNA repair and cell transformation; negative in many tests *in vivo*; negative and positive in mouse bone-marrow micronuclei tests |
| Wy-14,643 | 50892-23-4 | Positive in many chromosomal tests *in vitro*; positive in one cell transformation test; negative in *Salmonella* and rat hepatocyte unscheduled DNA synthesis tests; negative in rat liver unscheduled DNA synthesis test *in vivo* |

Adapted from Ashby *et al.* (1994)
[a] Often lack of information on chemical purity and/or presence of mutagenic stabilizers

A very large database is available on the genotoxicity of di(2-ethylhexyl)phthalate. It has been shown repeatedly to induce cell transformation *in vitro*, but there are no other indications of genotoxic activity *in vitro* or *in vivo*. Wy-14,643 is a clear genotoxicant *in vitro* in that it induces clastogenicity and cell transformation; however, it does not react with DNA. Ashby *et al.* (1994) note that the compound has a potentially genotoxic aromatic substituent, unlike the other 17 chemicals, and they also mention the possible reactivity of the pyrimidine chlorine group in Wy-14,643.

The two peroxisome proliferators nafenopin and ciprofibrate reproducibly induced cytogenicity in rat hepatocytes *in vitro*, whereas nafenopin did not cause DNA damage in the same cells *in vitro* or in the liver *in vivo*. The data on the cytogenicity of trichloroacetic acid *in vivo* are conflicting, in that both positive and negative results have been obtained in tests for bone-marrow micronuclei. There is no indication that it reacts with DNA, and it did not induce chromosomal aberrations in either mouse or rat bone marrow. Tetrachloroethylene has been tested in numerous assays *in vitro* and *in vivo*; the results are essentially negative, but this compound, which is metabolized to trichloroacetic acid, has not been tested in the mouse bone-marrow micronucleus test.

The database on trichloroethylene is both large and conflicting, with positive and negative results found for genotoxicity *in vitro*. Most of the results of tests *in vivo* are negative, but micronuclei were reported in mouse bone marrow. As stated above, an evaluation of the possible genotoxicity of trichloroethylene is hampered by the dearth of information on impurities and stabilizers in the test material.

DNA damage is known to be induced by sufficient concentrations of reactive oxygen species, such as hydrogen peroxide, and after exposure to radiation (Kasai *et al.*, 1986; Imlay & Linn, 1988). Small increases in the frequency of altered bases, such as 8-hydroxydeoxyguanosine, have been reported after repeated administration of several peroxisome proliferators (Table 5); however, these increases have been detected only in unfractionated liver and not in isolated nuclei, indicating that the damage occurs in extranuclear DNA, such as in mitochondria. The observed base alterations do not show any correlation with hepatocarcinogenic potential nor with carcinogenic potency. Further, there is no evidence of oxidation-induced strand breaks after long-term treatment with peroxisome proliferators.

## 2.5 Evaluation of data in the open literature

A detailed analysis of the extensive database on peroxisome proliferation and hepatocellular carcinoma has shown a good, but incomplete, correlation between hepatic peroxisome proliferation in short-term studies and hepatocarcinogenesis in long-term animal studies. Non-hepatic carcino-

**Table 5. Base alterations in livers of rats treated with carcinogenic peroxisome proliferators**

| Compound | Base alteration[a] | Dosage | Observation |
|---|---|---|---|
| Ciprofibrate | 8-OHdG | Single gavage of 250 mg/kg bw | No increase |
| | | 0.025% in diet for up to 40 weeks | Up to 210% increase |
| Clofibrate | 8-OHdG | 0.5% in diet Al salt for 1–12 months | Up to 80% increase |
| Di(2-ethylhexyl)adipate | 8-OHdG | 2.5% in diet for two weeks | Up to 60% increase |
| Di(2-ethylhexyl)phthalate | 8-OHdG | 1.2% in diet for two weeks | Up to 60% increase |
| Nafenopin | 8-OHdG | 1000 ppm in diet for up to seven days | No change |
| Nafenopin | HMdU | 0.01% in diet for up to seven weeks, with partial hepatectomy | Equivocal |
| WY-14643 | 8-OHdG | 0.1% in diet for 22 weeks | Up to 250% increase |

Adapted from Ashby *et al.* (1994)
[a] 8-OHdG, 8-hydroxydeoxyguanosine; HMdU, 5-hydroxymethyl-2'-deoxyuridine

genic effects have been reported for benzylbutyl phthalate, cinnamyl anthranilate, clofibrate, gemfibrozil, tetrachloroethylene and trichloroethylene.

Two epidemiological studies of the use of fibrates showed nonsignificant increases in cancer mortality rates—one during the trial period and the other during the 3.5-year follow-up after the intervention period. Thus, there is no clear epidemiological evidence that peroxisome proliferators are associated with an increased risk of cancer in humans.

Although some hepatocarcinogenic peroxisome proliferators cause morphological cell transformation and inhibit gap junctional intercellular communication, these cellular effects appear to be independent of the process of peroxisome proliferation.

An overall assessment of the genotoxicity of the hepatocarcinogenic peroxisome proliferators indicates that they are predominantly non-genotoxic. The group of 18 chemicals has no common genetic toxicity, however, some of them have genotoxic activity *in vitro*. The clastogenicity and cell transforming activity of Wy-14,643 may be specific to that chemical, although nafenopin and ciprofibrate cause clear, but weak, cytogenic effects in cultured rat hepatocytes. It is less likely that the finding of small increases in the frequency of altered DNA bases after repeated treatment with some peroxisome proliferators is relevant for hepatocarcinogenesis.

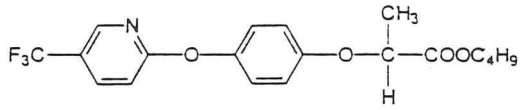
## 3. Review of unpublished data on phenoxypropionic acid herbicides

### 3.1 Background

Fluazifop-butyl, haloxyfop, propaquizafop and quizalafop-ethyl, which are derivatives of phenoxypropionic acids (Figure 3), have been submitted for registration as grass herbicides to the pesticide authorities in the Nordic countries. The present evaluation is based on the available documentation submitted with the application for registration; any other data that the producers may have is not included.

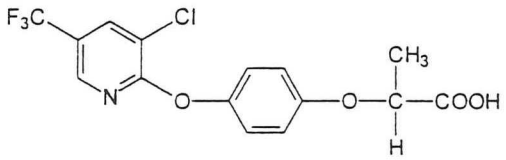
Of the four phenoxypropionic acid herbicides proposed for marketing in the Nordic countries, three exist as racemates; only propaquizafop is the active R-enantiomer (Figure 3). Some of the available data on the genotoxicity of fluazifop-butyl were, however, obtained with the active enantiomer fluazifop-*para*-butyl (CAS No. 79241-46-6).

**Figure 3. Chemical structures and CAS numbers of the phenoxypropionic herbicides considered**



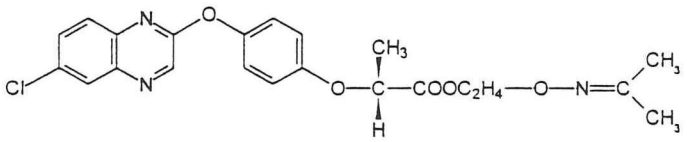
Butyl-(R,S)-2-[4-(5-trifluoromethyl-2-pyridinyloxy)phenoxy]propionate

FLUAZIFOP-BUTYL
CAS No. 69806-50-4



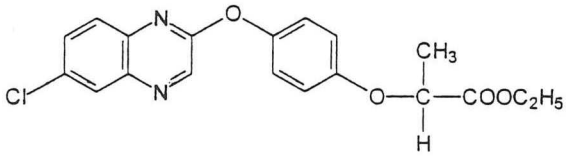
(R,S)-2-[4-((3-chloro-5-(trifluoromethyl)-2-pyridinyl)oxy)phenoxy]propionic acid

HALOXYFOP
CAS No. 69806-34-4



2-[(Isopropylideneamino)oxy]ethyl-(R)-2-[4-((6-chloro-2-quinoxalinyl)oxy)phenoxy]propionate

PROPAQUIZAFOP
CAS No. 111479-05-1



Ethyl-(R,S)-2-[4-((6-chloro-2-quinoxalinyl)oxy)phenoxy]propionate

QUIZALAFOP-ETHYL
CAS No. 76578-14-8

## 3.2 Carcinogenicity

Evidence for induction of hepatic peroxisome proliferation and hepatocarcinogenesis in rats and mice by the phenoxypropionic acid herbicides is summarized in Table 6. Haloxyfop shows clear evidence of hepatocarcinogenicity in mice, whereas propaquizafop is carcinogenic in the livers of both rats and mice. Quizalafop-ethyl induced hepatocellular carcinomas in female rats and in male mice with statistically significant positive trends. The long-term studies with the phenoxypropionic acids were performed with very low doses, especially in the case of fluazifop-butyl and haloxyfop,

and for these compounds, as well as for quizalafop-ethyl, no clear evidence was found that a maximal tolerated dose had been reached. A more detailed description of the long-term studies and a tabular presentation of the data on hepatocarcinogenicity are given in **Annex B**.

**Table 6. Evidence for induction of hepatic peroxisome proliferation and hepatocarcinogenesis in rats and mice by phenoxypropionic acid herbicides**

| Compound | Rats | | | | | Mice | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Strain | Peroxisome proliferation | | Hepatocarcinogenesis | | Strain | Peroxisome proliferation | | Hepatocarcinogenesis | |
| | | M | F | M | F | | M | F | M | F |
| Fluazifop-butyl | Wistar[a] | - | - | - | - | Alderley Park[a] | –[b] | – | –[c] | – |
| Haloxyfop | Fischer 344[a] | + | + | - | - | B6C3F1[a] | + | + | + | + |
| Propaquizafop | Wistar | + | ND | + | + | NMRI | + | ND | + | + |
| Quizalafop-ethyl | CD[a] | - | - | - | (+)[d] | CD-1[a] | –[b] | –[b] | (+)[d] | – |

ND, No data
[a] A clear maximal tolerated dose was not reached.
[b] High incidence of pigment/lipofuscin deposition
[c] Borderline significant positive trend
[d] Significant positive trend

Haloxyfop clearly causes peroxisome proliferation in rats and mice of each sex, as evidenced by increased peroxisome volume density after four weeks of oral treatment with 0.5–10 mg/kg bw per day (Table 7; Dow Chemical, 1985). Increased cyanide-insensitive fatty acyl-coenzyme A ß-oxidation was seen in the livers of male rats given 0.5 mg/kg bw per day or more, and a slight increase in this enzyme activitity was seen in the livers of female rats ingesting 1.0 mg/kg bw per day or more. No such increases were seen in the livers of mice. No ultrastructural evidence of peroxisome proliferation was seen in the livers of male cynomolgus monkeys treated orally with haloxyfop at doses of 5 or 20 mg/kg bw per day for four weeks (Merrell Dow Pharmaceuticals, 1985).

Propaquizafop is a potent peroxisome proliferator in the liver of male rats (Ciba-Geigy, 1991). Electron microscopic examination of liver samples from animals treated orally for two weeks with 200 mg/kg bw per day revealed a striking increase in the number and size of peroxisomes. Cultured primary male rat hepatocytes treated with 100 µmol/L propaquizafop for 48 h showed a marked increase in lauric acid 12-hydroxylation (Table 8; Ciba-Geigy, 1992). In addition, cyanide-insensitive fatty acyl-coenzyme A ß-oxidation was found to be induced to levels comparable to those found after treatment with 100 µmol/L of the potent peroxisome proliferator bezafibrate. Lauric acid hydroxylation was also increased after treatment of cultured male mouse hepatocytes with propaquizafop. In contrast, very little evidence of increased enzyme activity was found after treatment of cultured male guinea-pig or marmoset hepatocytes with propaquizafop or bezafibrate.

No morphological evidence of peroxisome proliferation was reported in the available documentation after subchronic treatment of rats and mice with fluazifop-butyl or quizalafop-ethyl; however, a high incidence of deposition of the pigment lipofuscin was reported in male mice treated with fluazifop-butyl and in male and female mice given quizalafop-ethyl. An established feature of hepatocytes from animals treated with peroxisome proliferators is the deposition of lipofuscin

(Ashby *et al.*, 1994), which is believed to be associated with free radical and lipid peroxidation reactions.

**Table 7. Proliferation of hepatocellular peroxisomes in Fischer 344 rats and B6C3F1 mice treated orally with haloxyfop for four weeks**

| Dose (mg/kg bw per day) | Rats — mean volume, % | | Mice — mean volume, % | |
|---|---|---|---|---|
| | Males | Females | Males | Females |
| 0 | 1.1 (3) | 2.5 (3) | 2.4 (3) | 2.0 (3) |
| 0.1 | 1.3 (3) | 2.6 (2) | – | – |
| 0.5 | 3.9 (2) | 1.2 (2) | 2.6 (3) | 2.0 (1) |
| 1.0 | 6.8 (1) | 4.0 (3) | 5.2 (3) | 2.6 (3) |
| 10.0 | 13.1 (1) | 7.5 (2) | 18.5 (3) | 28.4 (1) |

Mean volume, percentage of cytoplasmic volume occupied by peroxisomes; number of animals in parentheses

### 3.3 Genotoxicity

The genotoxicity profiles of fluazifop-butyl, haloxyfop, propaquizafop and quizalafop-ethyl are shown in Figure 4a-d. None of the pure phenoxypropionic acid herbicides showed any genotoxic activity.

The racemic compound fluazifop-butyl was tested *in vitro* in *S. typhimurium* (ICI, 1980), for gene mutation at the *tk* locus of P388 mouse lymphoma cells (ICI, 1981) and for cell transformation in baby hamster kidney cells (ICI, 1980). The active R-enantiomer fluazifop-*para*-butyl was also tested for mutagenicity in *Salmonella* (ICI, 1983) and in *Schizosaccharomyces pombe* (Istituto di Richerche Biomediche 'Antoine Marxer', 1985a), for chromosomal aberrations in human lymphocytes (Istituto di Richerche Biomediche 'Antoine Marxer', 1986) and for unscheduled DNA synthesis in HeLa cells (Istituto di Richerche Biomediche 'Antoine Marxer', 1985b). Both the racemate and the active enantiomer were evaluated *in vivo* for their ability to induce micronuclei in mouse bone marrow (ICI, 1993), and the racemate was studied for chromosomal aberrations in rat bone marrow (Inveresk Research International, 1980a) and for dominant lethality in mice (Inveresk Research International, 1980b).

Haloxyfop was studied *in vitro* in the *Salmonella* mutagenicity test (Dow Chemical, 1981), for unscheduled DNA synthesis in rat hepatocytes (Dow Chemical, 1980) and for forward mutations at the *hprt* locus in Chinese hamster ovary cells (Litton Bionetics, 1982). The only study performed with haloxyfop *in vivo* was for micronuclei in rat bone marrow (Dow Chemical, 1982).

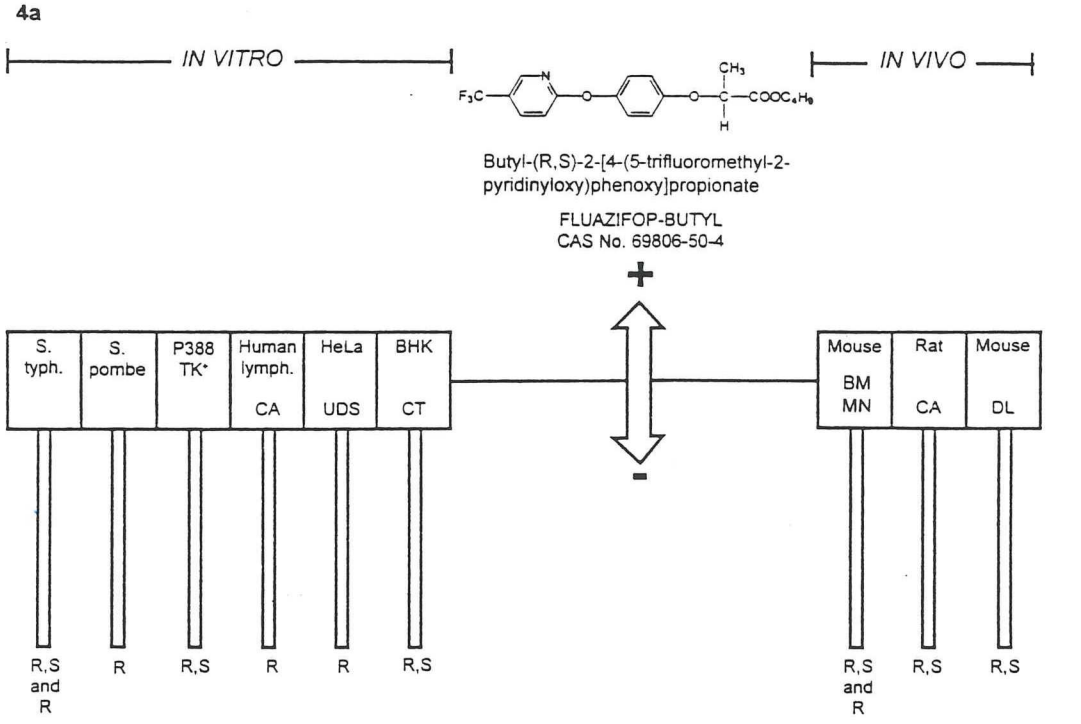
Propaquizafop has been evaluated *in vitro* in the *Salmonella* mutagenicity test (Hoffmann-La Roche & Co., 1989) and in *Saccharomyces cerevisiae* (Hoffmann-La Roche & Co., 1986a), for forward mutations at the *hprt* locus in Chinese hamster V79 cells (Hoffmann-La Roche & Co., 1985a), for chromosomal aberrations in human peripheral blood lymphocytes (Hoffmann-La Roche & Co., 1987a) and for unscheduled DNA synthesis in rat hepatocytes (Hoffmann-La Roche & Co., 1985b). Earlier studies of chromosomal aberrations in hamster bone marrow *in vivo* revealed a clastogenic response (Hoffmann-La Roche & Co., 1987b), but this response was found to be due to an impurity in the tested lots, as purified propaquizafop did not have clastogenic activity. The clastogenic impurity was isolated and characterized as the 7-chloro isomer of propaquizafop (propaquizafop has a chlorine atom at position 6); commercial preparations of propaquizafop do not

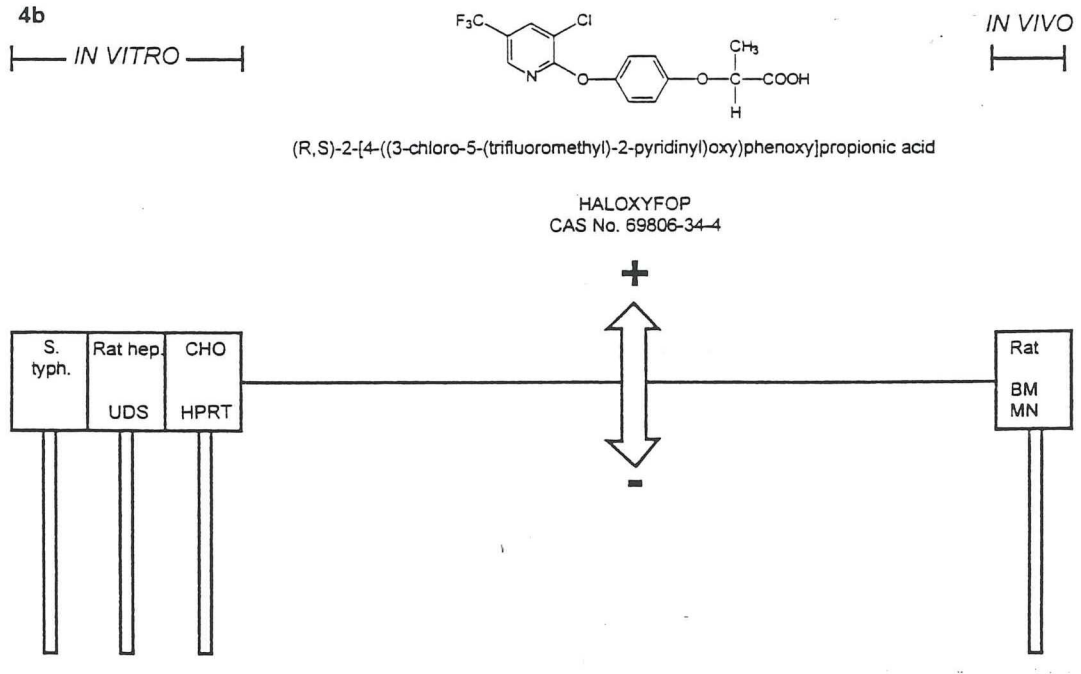
**Table 8. Effect of propaquizafop and bezafibrate on lauric acid 12-hydroxylation and fatty acyl-coenzyme A β-oxidation in primary cultured rat, mouse, guinea-pig and marmoset hepatocytes**

| Compound | Rat | | Mouse | | Guinea-pig | | Marmoset | |
|---|---|---|---|---|---|---|---|---|
| | Lauric acid 12-hydroxylase (pmol/mg protein/h) | Fatty acid coenzyme A β-oxidation (nmol/$10^6$ cells/h) | Lauric acid 12-hydroxylase (pmol/mg protein/h) | Fatty acid coenzyme A β-oxidation (nmol/$10^6$ cells/h) | Lauric acid 12-hydroxylase (pmol/mg protein/h) | Fatty acid coenzyme A β-oxidation (nmol/$10^6$ cells/h) | Lauric acid 12-hydroxylase (pmol/mg protein/h) | Fatty acid coenzyme A β-oxidation (nmol/$10^6$ cells/h) |
| Control | 360 | 65 | 14 | 110 | 33 | 333 | 20 | 47 |
| Propaquizafop (100 µmol/L) | 5010 | 199 | 144 | 107 | 40 | 378 | 21 | 55 |
| Bezafibrate (100 µmol/L) | 9060 | 167[a] | 196 | 122[a] | 71 | 388[a] | 21 | 64[a] |

[a] Normalized against control values in separate experiment with bezafibrate

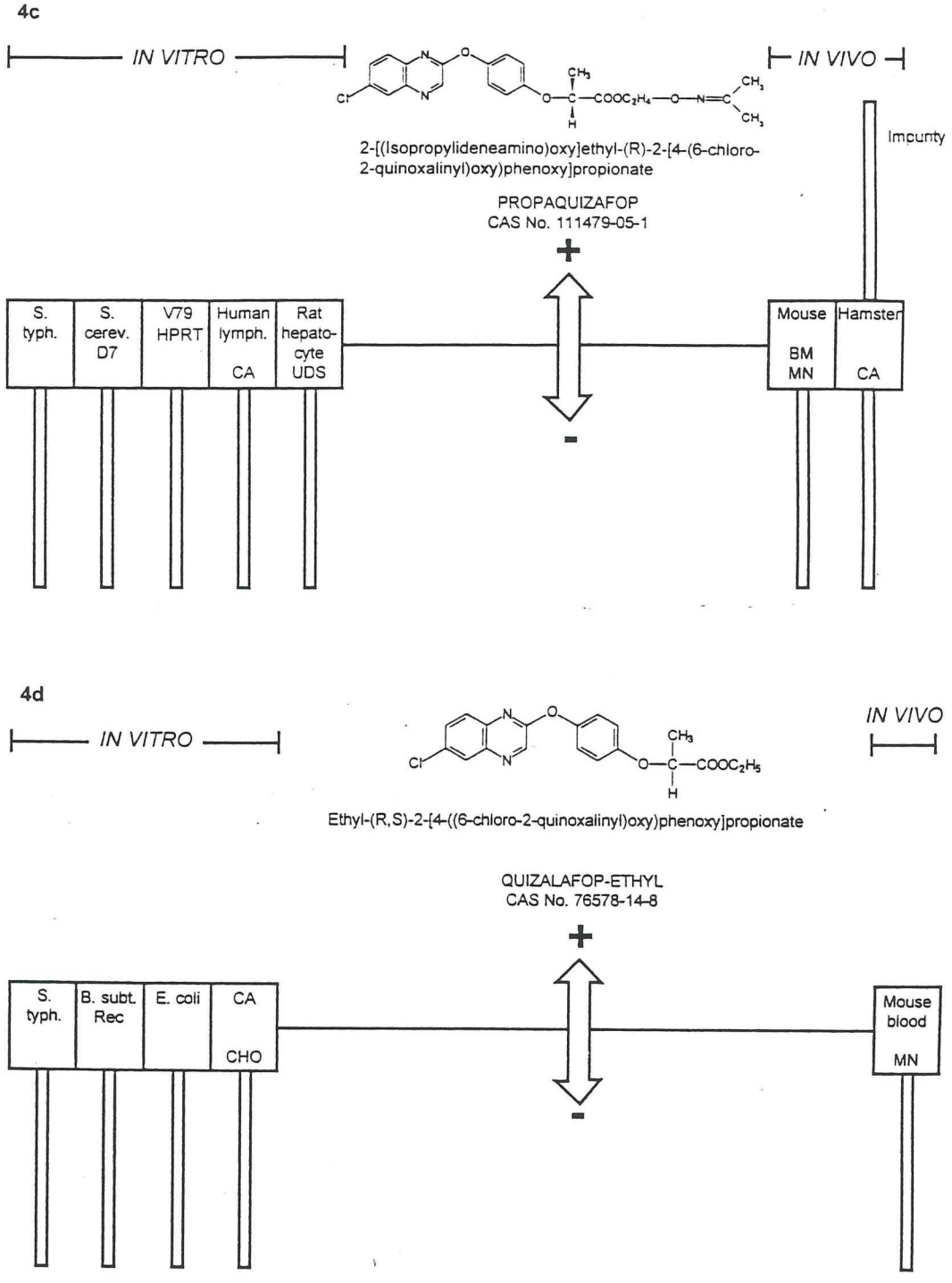
**Figure 4a-d. Genotoxicity profiles for fluazifop-butyl, haloxyfop, propaquizafop and quizalafop-ethyl**




CA, chromosomal aberrations; UDS, unscheduled DNA synthesis; BHK, baby hamster kidney cells; CT, cell transformation; BM, bone marrow; MN, micronuclei; DL, dominant lethal mutation; CHO, Chinese hamster ovary cells

**Figure 4a-d (contd)**




CA, chromosomal aberrations; UDS, unscheduled DNA synthesis; BHK, baby hamster kidney cells; CT, cell transformation; BM, bone marrow; MN, micronuclei; DL, dominant lethal mutation; CHO, Chinese hamster ovary cells

contain this impurity. Propaquizafop did not induce micronuclei in mouse bone marrow (Hoffmann-La Roche & Co., 1986b).

Quizalafop-ethyl has been tested for mutagenicity *in vitro* in the *Bacillus subtilis rec* assay, in *Escherichia coli* and in *Salmonella typhimurium* (Nissan Chemical Industries, undated), and for chromosomal aberrations in Chinese hamster ovary cells (Nissan Chemical Industries, 1983). It has been tested *in vivo* only in the mouse bone-marrow micronucleus test (Nissan Chemical Industries, 1981).

### 3.4 Evaluation of unpublished data on phenoxypropionic acid herbicides

Of the four phenoxypropionic acid herbicides, haloxyfop and propaquizafop are strong peroxisome proliferators; haloxyfop is particularly potent. No clear evidence was found that fluazifop-butyl or quizalafop-ethyl induces peroxisome proliferation; however, an effect is indicated by the finding of lipofuscin deposition in the livers of male mice in long-term experiments. Both haloxyfop and propaquizafop were also hepatocarcinogenic in long-term experiments. Propaquizafop was particularly carcinogenic, but it was tested at much higher doses than haloxyfop. The two other compounds showed slight (quizalafop-ethyl) or very little (fluazifop-butyl) evidence of hepatocarcinogenicity; however, these compounds might have had greater hepatocarcinogenic potential if they had been tested at higher doses, since obvious maximal tolerated doses were not reached in these experiments.

As a group, the phenoxypropionic acid herbicides must be considered to be hepatocarcinogenic peroxisome proliferators, but there are considerable differences in potency between the various members of the group. These herbicides appear to be completely nongenotoxic.

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# ANNEX A

Individual studies of morphological cell transformation and gap junctional intercellular communication with the hepatocarcinogenic peroxisome proliferators are presented in Tables A1 and A2.

**Table A1. Individual studies of the cell transforming activity of hepatocarcinogenic peroxisome proliferators**

| Compound | Concentration[a] | Assay[b] | Response[c] | Reference |
|---|---|---|---|---|
| Clofibrate | 41 μmol/L[d] | SHE[e] | ++ | Mikalsen *et al.* (1990a,b) |
| | 300/300 μmol/L[f] | SHE[g] (±) | –/– | Tsutsui *et al.* (1993) |
| | 5/5 μmol/L[h,i,j] | C3H[k] | –/++ | Lillehaug *et al.* (1986) |
| Di(2-ethylhexyl)-phthalate | 7.7 μmol/L[d] | SHE[e] | ++ | Mikalsen *et al.* (1990a,b) |
| | 77 μmol/L[h] | SHE[e] | ++ | Mikalsen & Sanner (1993) |
| | 10 μmol/L[i] | SHE[e] | ++ | Sanner & Rivedal (1985) |
| | 25.6 μmol/L[i] | SHE[e] | ++ | Barrett & Lamb (1985) |
| | 1300 μmol/L | SHESA7[l] | + | Hatch & Anderson (1985) |
| | +30/–3[d] μmol/L[f] | SHE[g] (±) | ++/+ | Tsutsui *et al.* (1993) |
| | +2.6 mmol/L/–100 μmol/L[f,i] | C3H[m] (±) | +/+ | Lawrence & McGregor (1985) |
| | 100/10 μmol/L[j] | C3H[k] | –/– | Sanchez *et al.* (1987) |
| | +125mmol/L/–157μmol/L[f] | 3T3[n] (±) | –/– | Matthews *et al.* (1985) |
| | 37.7 mmol/L | 3T3[o] | – | Matthews *et al.* (1993) |
| | 2.6 μmol/L[i] | FRERLV[p] | (–)+ | Suk & Humphreys (1985) |
| | 500 ppm (1250 μmol/L)[d,i] | JB6[q] | ++ | Diwan *et al.* (1985) |
| Di(2-ethylhexyl)-adipate | 85.3 mmol/L | 3T3[o] | – | Matthews *et al.* (1993) |
| Trichloroethylene | 1100 μmol/L[d,i] | FRE[r] | ++ | Price *et al.* (1978) |
| | 152 μmol/L[i] | 3T3[n] | + | Tu *et al.* (1985) |
| | 190 μmol/L[i,s] | SHE[e] | + | Amacher & Zelljadt (1983) |
| Wy-14,643 | +3/–100 μmol/L[f] | SHE[g] (±) | +/– | Tsutsui *et al.* (1993) |

[a] For negative results: maximum concentration used; for positive results: minimum concentration that induced a statistically significant response. When the statistics were not reported, the original authors' evaluation of the results was used where possible. The concentrations were sometimes read from graphs and may not be exact.

[b] ±, Experiments done with and without added metabolic system (e.g. hepatocytes, microsomal fractions); SHE, Syrian hamster embryo cell transformation system; C3H, C3H10T1/2 cell transformation system; SHESA7, SHE cells infected with simian agent 7 virus; 3T3, BALB/c-3T3 cell transformation system; FRERLV, Fischer rat embryo cells infected with Rauscher leukaemia virus; JB6, mouse epidermal cell line; FRE, Fischer rat embryo cells; RLE, rat liver epithelial cells; HLEBV, human lymphocytes infected with Epstein-Barr virus.

[c] ++, clear response; +, weak response; +(–), equivocal response; –, negative response. The symbol / is used under two circumstances: (i) when the exposure periods were divided into 'initiation' (left side of /) and 'promotion' (right side of /); and (ii) when the assay was performed with (left) and without (right) an added metabolic system (see assay column).

[d] Lowest concentration studied.

[e] The chemical was added to dishes 24 h after seeding of single cells and left continuously for the next seven days, until the cells were fixed.

[f] First concentration, assay with added metabolic system; second concentration, assay without added metabolic system.
[g] The compound was added to mass cultures of cells for 2 or 3 h in the presence of rat liver microsomal fractions, or for 48 h without. Thereafter, the cells were trypsinized, and a small number of cells were seeded and allowed to grow into colonies during the next seven days.
[h] Only one concentration used, or higher concentrations too cytotoxic.
[i] Significance not given.
[j] First concentration, 'initiation'; second concentration, 'promotion'.
[k] The exposure was divided into an initial 24-h period (initiation) and a six-week promotion (with compound) or expression (without compound) period.
[l] The chemical was added 71–120 h after infection of SHE cells with SA7 virus and was left for 20 h, followed by an expression period of 3-4 weeks.
[m] The chemical was added 24 h after seeding and was left for 24 h, followed by an expression period of six weeks.
[n] The chemical was added 24 h after seeding and was left for 72 h, followed by an expression period of 24 days.
[o] The chemical was added 24 h after seeding and was left for 48 h, followed by an expression period of 3.5 weeks.
[p] The chemical was added 24 h after seeding and left for three days; after three days of recovery, the cells were seeded on soft agar. The number of viable cells was determined four days later.
[q] A cell suspension was seeded in soft agar containing the compound, and the number of anchorage-independent colonies was counted after two weeks.
[r] F1706 cells were treated for 48 h at 50% confluency. The cultures were passaged twice a week, and some of the cells were inspected for foci formation, growth in soft agar and malignancy.
[s] Sum of transformed colonies with three concentrations is given in the reference; this was the highest concentration.

**Table A2. Individual studies of gap junctional intercellular communication with hepatocarcinogenic peroxisome proliferators**

| Compound | Concentration[a] | Assay[b] | Response[c] | Reference |
|---|---|---|---|---|
| Clofibrate | 200 µmol/L | SHE I | + | Rivedal *et al.* (unpublished) |
| Di(2-ethylhexyl)-phthalate | 300 nmol/L | V79 MC | – | Kornbrust *et al.* (1984) |
| | 26 µmol/L[d] | V79 MC | + | Malcolm & Mills (1989) |
| | 512 µmol/L[e] | V79 MC | + | Vang *et al.* (1993) |
| | 77 µmol/L[f] | SHE I | + | Mikalsen & Sanner (1993) |
| | 13 µmol/L[d] | V79 MC | + | Elmore *et al.* (1985) |
| | 500 µmol/L | V79 MC | – | Umeda *et al.* (1985) |
| Methylclofenapate | 1.6 µmol/L[d,g] | V79 MC | – | Trosko *et al.* (1982) |
| Nafenopin | 8 µmol/L[d,g] | V79 MC | + | Trosko *et al.* (1982) |
| | 32 µmol/L[f] | PRH I | + | Leibold *et al.* (1994) |
| Trichloroethylene | 100 µmol/L | PRH I | – | Klaunig *et al.* (1989) |
| | 100 µmol/L | PMH I | + | Klaunig *et al.* (1989) |
| | 1730 µmol/L[h] | PCM I | + | Toraason *et al.* (1992) |
| Trichloroacetic acid | 1000 µmol/L | PRH I | – | Klaunig *et al.* (1989) |
| | 100 µmol/L | PMH I | + | Klaunig *et al.* (1989) |
| Wy-14,643 | 10 µg/ml[d,g] | V79 MC | + | Trosko *et al.* (1982) |
| | 100 µmol/L[f] | PRH I | + | Leibold *et al.* (1994) |

[a] For negative results: maximum concentration used; for positive results: minimum concentration that induced a statistically significant response. When the statistics were not reported, the original authors' evaluation of the results was used where possible. The concentrations were sometimes read from graphs and may not be exact.

[b] The assay system is indicated after the cell type used: I, microinjection of dye; MC, metabolic cooperation; SHE, Syrian hamster embryo cells; V79, Chinese hamster lung fibroblast cell line; PRH, primary rat hepatocytes; PMH, primary mouse hepatocytes; PCM, primary rat cardiac myocytes.

[c] +, The compound inhibited intercellular communication or metabolic cooperation at the indicated concentration at one or more time; –, the chemical did not inhibit intercellular communication or metabolic cooperation.

[d] Statistical significances not given.

[e] Only one concentration used, or higher concentration too cytotoxic.

[f] Lowest concentration used.

[g] Only one, maximal concentration given, with no further details.

[h] Concentration for 50% inhibition of gap junctional intercellular communication.

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# ANNEX B. Carcinogenicity studies with phenoxypropionic acid herbicides

## 1. Fluazifop-butyl

Groups of 60 male and 60 female Wistar rats were given feed containing 0, 2, 10, 80 or 250 ppm fluazifop-butyl (corresponding to doses of 0.08, 0.4, 3.2 or 10.0 mg/kg bw per day for males and 0.1, 0.5, 4.0 and 12.5 mg/kg bw per day for females) for 106 weeks. Ten animals per group were sacrificed at 52 weeks. The rates of mortality during weeks 29–53 of males receiving 80 ppm and rats of either sex receiving 250 ppm suggested an association with treament. The significantly greater number of deaths was probably the result of exacerbation of respiratory disease by the treatment-related geriatric nephropathy. During weeks 14–28, male and female rats receiving fluazifop-butyl at 80 or 250 ppm gained significantly more weight than their respective controls. No pathological changes were seen in the liver and no neoplastic changes occurred that were considered to be associated with treatment with fluazifop-butyl (Table B1; Life Science Research, 1985).

**Table B1. Hepatocellular tumour incidence in rats treated orally with fluazifop-butyl for 106 weeks (mg/kg bw per day)**

| Tumour type | Males | | | | | Females | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Control | 0.08 | 0.4 | 3.2 | 10.0 | Control | 0.1 | 0.5 | 4.0 | 12.5 |
| Hepatocellular adenoma | 0/59 | 0/60 | 0/60 | 0/60 | 0/60 | 1/60 | 1/60 | 0/60 | 0/60 | 0/60 |
| Hepatocellular carcinoma | 0/59 | 0/60 | 0/60 | 0/60 | 0/60 | 0/60 | 0/60 | 0/60 | 0/60 | 0/60 |

Groups of 60 male and 60 female Alderley Park mice were given feed containing 0, 1, 5, 20 or 80 ppm fluazifop-butyl (corresponding to doses of 0.12, 0.6, 2.4 or 9.6 mg/kg bw per day for males and 0.13, 0.65, 2.6 or 10.4 mg/kg bw per day for females) for 97–98 weeks. Twenty animals per group were sacrificed at 52 weeks. There were no deaths that were considered to reflect treatment with the test compound. Body weight gain was unaffected during the first 71 weeks of treatment, but subsequent evaluation was confounded by the effects of mortality and senescence. Treatment-related non-neoplastic changes were seen in the livers of animals of either sex receiving the highest concentration and in male mice receiving 2.4 mg/kg bw. The changes, more marked among male than female mice, were restricted to an apparent increase in the size of the hepatocytes, a slight shift in the deposition of fat and the presence of pigment deposits within hepatocytes. There was a slight increase in the incidence of hepatocellular carcinoma in males at the high dose, which reached marginal statistical significance in the Mantel–Haenszel test for trend (Table B2; Life Science Research, 1983).

Fluazifop butyl is hydrolysed to the acid derivative fluazifop (CAS No. 69335-91-7) in plants and animals. Combined long-term assays for toxicity and carcinogenicity in rats and mice did not provide evidence that fluazifop has carcinogenic potential (Life Science Research, 1981a,b).

**Table B2. Hepatocellular tumour incidence in mice treated orally with fluazifop-butyl for 98 weeks (mg/kg bw per day)**

| Tumour type | Males | | | | | Females | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Control | 0.12 | 0.60 | 2.4 | 9.6 | Control | 0.12 | 0.65 | 2.6 | 10.4 |
| Hepatocellular adenoma[a] | 14/61 | 17/63 | 8/64 | 18/61 | 18/64 | 3/66 | 5/62 | 5/67 | 2/62 | 3/61 |
| Hepatocellular carcinoma[a] | 2/61 | 6/63 | 3/64 | 4/61 | 8/64[b] | 0/66 | 1/62 | 0/67 | 1/62 | 0/61 |

[a] Excludes data from interim sacrifice at 52 weeks of 13–19 animals per group
[b] p = 0.04, Mantel-Haenszel trend test

## 2. Haloxyfop

Groups of 90 male and 90 female rats were given haloxyfop in the diet at concentrations corresponding to doses of 0, 0.1 0.03, 0.065 or 0.10 mg/kg bw per day for males and 0, 0.01, 0.03, 0.065 or 1.0 mg/kg bw per day for females, for 104 weeks. Groups of 20 animals were sacrificed at 6 and 12 months. There was no obvious increase in mortality among animals of either sex and no change in body weight. The incidences of hepatocellular adenomas or carcinomas or of any other tumor type were not statistically significant increased. Treatment-related non-neoplastic effects in the liver (liver enlargement, hepatocellular hypertrophy) were noted at the 6- and 12-month interim sacrifices but not at 24 months (Table B3; Dow Chemical USA, 1984).

**Table B3. Hepatocellular tumour incidence in Fischer 344 rats treated orally with haloxyfop for 104 weeks (mg/kg bw per day)**

| Tumour type | Males | | | | | Females | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Control | 0.01 | 0.03 | 0.065 | 0.1 | Control | 0.01 | 0.03 | 0.065 | 1.0 |
| Hepatocellular adenoma | 4/50 | 2/50 | 0/50 | 5/50 | 1/50 | 2/50 | 1/50 | 1/50 | 0/50 | 0/50 |
| Hepatocellular carcinoma | 0/50 | 1/50 | 0/50 | 0/50 | 0/50 | 0/50 | 0/50 | 0/50 | 0/50 | 0/50 |

Groups of 70 male and 70 female mice were given haloxyfop in the diet at concentrations corresponding to doses of 0, 0.03, 0.065 or 0.6 mg/kg bw per day. Ten animals of each sex at each dose were sacrificed after 6 and 12 months, and the survivors were killed after 24 months. Treatment-related cytoplasmic staining, associated with hepatocellular hypertrophy, in the centrilobular region of the liver and slight increases in liver weight were noted. The body weights of male mice at the high dose were slightly lower than those of their respective controls; this effect was not seen in the females. Mortality rates among male and female mice in the various treatment groups were not statistically different from those in the respective control groups. The indicidence of hepatocellular adenomas was significantly increased in male mice at the high dose, and the incidence of hepatocellular carcinomas was significantly increased in female mice at the high dose (Table B4; Dow Chemical USA, 1985).

**Table B4. Hepatocellular tumour incidence in B6C3F1 mice treated orally with haloxyfop for 104 weeks (mg/kg bw per day)**

| Tumour type | Males | | | | Females | | | |
|---|---|---|---|---|---|---|---|---|
| | Control | 0.03 | 0.065 | 0.6 | Control | 0.03 | 0.065 | 0.6 |
| Hepatocellular adenoma | 8/50 | 10/50 | 15/50 | 19/50[a] | 6/50 | 6/50 | 12/50 | 10/50 |
| Hepatocellular carcinoma | 5/50 | 8/50 | 6/50 | 9/50 | 1/50 | 3/50 | 2/50 | 8/50[b] |

[a] p = 0.01, Fisher's exact test
[b] p = 0.02, Fisher's exact test

## 3. Propaquizafop

Groups of 50 male and 50 female Wistar KFM-Han rats were given propaquizafop in the diet at concentrations corresponding to 0, 5, 25, 50 or 100 mg/kg bw per day for males and 0, 5, 25, 75 or 150 mg/kg bw per day for females, for 104 weeks. Sixty male and 60 female rats served as controls. At the highest dose, overall weight gain was reduced by 62% in females and by 39% in males. A treatment-related increase in absolute and relative (adjusted) liver weight was observed in animals of each sex at doses of 25 mg/kg bw per day and higher. An increased incidence in the liver of basophilic-cell foci was diagnosed in males at the two highest doses and an increased incidence of focal hyperplasia in males at the highest dose, whereas centrilobular hypertrophy was noted in females at 25 mg/kg bw per day and higher. Increased deposition of lipofuscin was observed in animals of each sex at 25 mg/kg bw per day and higher. Significant increases in the incidences of hepatocellular adenomas were seen at the two highest doses in males and at the highest dose in females, and there were significant increases in the incidences of hepatocellular carcinomas at the highest dose in males and females. An increased incidence of Leydig-cell tumours was noted in males at the highest dose (Table B5; Hoffmann-La Roche & Co., 1991a).

**Table B5. Hepatocellular tumour incidence in rats treated orally with propaquizafop for 104 weeks (mg/kg bw per day)**

| Tumour type | Males | | | | | Females | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Control | 5 | 25 | 50 | 100 | Control | 5 | 25 | 75 | 150 |
| Hepatocellular adenoma | 1/60 | 0/50 | 1/48 | 10/48[a] | 13/47[a] | 2/58 | 0/49 | 3/49 | 4/48 | 15/47[a] |
| Hepatocellular carcinoma | 0/60 | 0/50 | 1/48 | 6/47[a] | 12/47[a] | 0/58 | 0/49 | 1/49 | 5/48[b] | 7/47[a] |

[a] p < 0.01, Fisher's exact test
[b] p = 0.02, Fisher's exact test

No indication of carcinogenic potential was found in a 104-week combined chronic toxicity and carcinogenicity study in Wistar rats given propaquizafop in the diet at initial doses of 0, 0.15, 0.5 or 1.5 mg/kg bw per day, or in a group of 30 male and 30 female rats given 5 mg/kg bw per day.

When, after 55 weeks, the doses were increased to 0.45, 1.5, 4.5 and 15 mg/kg bw per day, there was still no effect (Hoffmann-La Roche & Co., 1989).

Groups of 50 male and 50 female NMRI KFRM-Han mice were given diets containing 7.5 (15 from week 15), 30, 100 or 300 mg/kg bw per day of propaquizafop for 80 weeks; 60 male and 60 female mice served as controls. The compound caused dose-related mortality, with 44% of males at the high dose dead by the end of the test. A dose-related increase in liver weight was recorded at the two highest doses. At the time of interim sacrifice, at weeks 24 and 33, marked increases in the incidence and severity of hepatocellular vacuolation, hepatocellular hypertrophy, single-cell necrosis were seen in animals of each sex, with a higher incidence of Kupffer cell and lipoidal granuloma pigment deposits and biliary hyperplasia; a high incidence of severe centrilobular necrosis was seen in males. At terminal necropsy, high incidences were found of severe hepatocellular hypertrophy, hepatocellular necrosis, foci of cellular alteration, lipoid granulomas, haemorrhage/peliosis and cytoplasmic inclusions in animals at the high dose. The incidence of hepatocellular adenomas was significantly increased in male mice at all doses and in females at the high dose. The incidence of hepatocellular carcinomas was significantly increased in male mice at 100 and 300 mg/kg bw per day and in females at the latter dose (Table B6; Hoffmann-La Roche & Co., 1991b).

**Table B6. Hepatocellular tumour incidence in rats treated orally with propaquizafop for 104 weeks (mg/kg bw per day)**

| Tumour type | Males | | | | Females | | | |
|---|---|---|---|---|---|---|---|---|
| | Control | 30 | 100 | 300 | Control | 30 | 100 | 300 |
| Hepatocellular adenoma | 5/50 | 20/49[a] | 16/48[a] | 16/50[a] | 2/50 | 2/48 | 3/49 | 22/50[a] |
| Hepatocellular carcinoma | 0/50 | 4/49 | 7/48[a] | 35/50[a] | 1/50 | 0/48 | 0/49 | 25/50[a] |

[a] $p < 0.01$, Fisher's exact test

## 4. Quizalafop-ethyl

Groups of 50 male and 50 female CD rats were given quizalafop-ethyl in the diet at 0, 25, 100 or 400 ppm (corresponding to doses of 1, 4 or 16 mg/kg bw per day for males and 1.25, 5 or 20 mg/kg bw per day for females) for 104 weeks. Additional groups of 35 animals of each sex were studied for chronic toxicity; 10 of these were killed after 26 and 52 weeks of treatment and the remainder after 78 weeks. The differences in mortality rates between groups were not considered to be related to treatment, and there was no effect on overall rate of growth. An increased incidence of enlarged liver was observed in male rats treated with 16 mg/kg bw per day at the time of the 26-, 52- and 78-week interim kills and in females treated with 20 mg/kg bw per day at the 52-week interim kill. Female rats receiving 20 mg/kg bw per day did not have statistically significant increase in the incidence of malignant liver-cell tumours in comparison with controls, although there was a significant trend with dose (Mantel–Haenszel test). Non-neoplastic changes in the liver consisted of slight generalized hepatocyte enlargement in male rats at the highest dose, slight-to-minimal centrilobular enlargement in rats of each sex receiving the medium and highest doses and cytoplasmic eosinophilia of centrilobular hepatocytes in animals of each sex receiving the highest dose (Table B7; Huntingdon Research Centre, 1985).

Groups of 50 male and 50 female CD-1 mice were given quizalafop-ethyl in the diet for up to 78 weeks at doses of 0, 2, 10, 80 or 320 ppm (corresponding to 0.24, 1.2, 9.6 or 38.4 mg/kg bw per day for males and 0.26, 1.3, 10.4 or 41.6 mg/kg bw per day for females). Additional groups of 10

**Table B7. Hepatocellular tumour incidence in CD rats treated orally with quizalafop-ethyl for 104 weeks (mg/kg bw per day)**

| Tumour type | Males | | | | Females | | | |
|---|---|---|---|---|---|---|---|---|
| | Control | 1.0 | 4.0 | 16 | Control | 1.25 | 5.0 | 20 |
| Hepatocellular adenoma | 0/50 | 1/50 | 1/50 | 2/50 | 3/50 | 1/50 | 1/50 | 1/50 |
| Hepatocellular carcinoma | 1/50 | 1/50 | 3/50 | 1/50 | 0/50 | 0/50 | 0/50 | 4/50[a] |

[a] $p < 0.01$, Mantel-Haenszel trend test

animals were killed after 26 or 52 weeks of treatment. The highest dose approached or exceeded the maximal tolerated dose, in that there was a significant trend of decreased survival in males. Males at the high dose and females at the two highest doses grew more rapidly during the first 52 weeks of the study than their respective controls. The livers of animals at the highest dose were dark and enlarged at 26, 52 and 78 weeks. Increased liver weights were noted at 26 and 52 weeks, particularly in animals at the highest dose and in none at the lower doses. There was a significant trend for an increased incidence of hepatocellular carcinoma in male mice; however, group comparisons revealed no statistically significant difference with respect to hepatocellular adenomas or carcinomas in animals of either sex. Non-neoplastic changes in the liver consisted of diffuse hepatocytic enlargement and hepatocytic pigmentation (brown material in sinusoidal cells, iron-positive at the two highest doses), noted as early as 26 weeks and persisting throughout the study (Table B8; Hazelton Laboratories America, 1985).

**Table B8. Hepatocellular tumour incidence in CD-1 mice treated orally with quizalafop-ethyl for 78 weeks (mg/kg bw per day)**

| Tumour type | Males | | | | | Females | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Control | 0.24 | 1.2 | 9.6 | 38.4 | Control | 0.26 | 1.3 | 10.4 | 41.6 |
| Hepatocellular adenoma | 3/50 | 4/49 | 5/51 | 6/52 | 8/51 | 1/51 | 0/49 | 0/49 | 0/53 | 3/50 |
| Hepatocellular carcinoma | 4/50 | 4/49 | 2/51 | 1/52 | 10/51 | 0/51 | 0/49 | 0/49 | 0/53 | 0/50 |

[a] $p < 0.01$, Mantel-Haenszel test

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