From:<laura.n.winks@exxonmobil.com>
To: <coshita@oehha.ca.gov>
Date: 5/5/2009 2:52 PM
Subject: ExxonMobil Chemical submission to OEHHA: Prioritization Comments Part III
Attachments: KlaunigEtAl2003.pdf

Dear Ms. Oshita:

This is the third of three emails containing comments from ExxonMobil Chemical regarding the prioritization of chemicals to be considered at the May 29 meeting of the Carcinogen Identification Committee. These comments pertain specifically to diisononyl phthalate (DINP).

The content of the three emails is as follows:

1st email: The first email has attached Part I of ExxonMobil's comments, which is a summary of the pertinent data.

2nd email: The second email has attached Part II of our comments and its attachments, which provide greater detail on the scientific data.

3rd email: This third email has attached a copy of Klaunig et al. (2003), which is a review of the peroxisome proliferation mechanism, based on data for DINP and other chemicals. Other expert body reviews of the DINP data (CPSC CHAP, EU Risk Assessment, NTP CERHR) have been provided to the CIC by OEHHA and/or are readily available on the Internet. Because Klaunig et al. (2003) is not readily available on the Internet and is not cited by OEHHA, we are providing a copy. It is 6 MB -- if not received please let us know and we will break it down.

ExxonMobil would be pleased upon request to provide a copy of any other study cited in our comments.

If the CIC or OEHHA have any questions or need additional information, please contact me or any of the following scientists:

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Thank you.
(See attached file: KlaunigEtAl2003.pdf)

Regards,
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PPARα Agonist-Induced Rodent Tumors: Modes of Action and Human Relevance‡

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ABSTRACT: Widely varied chemicals—including certain herbicides, plasticizers, drugs, and natural products—induce peroxisome proliferation in rodent liver and other tissues. This phenomenon is characterized by increases in the volume density and fatty acid oxidation of these organelles, which contain hydrogen peroxide and fatty acid oxidation systems important in lipid metabolism. Research showing that some peroxisome proliferating chemicals are nongenotoxic animal carcinogens stimulated interest in developing mode of action (MOA) information to understand and explain the human relevance of animal tumors associated with these chemicals. Studies have demonstrated that a nuclear hormone receptor implicated in energy homeostasis, designated peroxisome proliferator-activated receptor alpha (PPARα), is an obligatory factor in peroxisome proliferation in rodent hepatocytes. This report provides an in-depth analysis of the state of the science on several topics critical to evaluating the relationship between the MOA for PPARα agonists and the human relevance of related animal tumors. Topics include a review of existing tumor bioassay data, data from animal and human sources relating to the MOA for PPARα agonists in several different tissues, and case studies on the potential human relevance of the animal MOA data. The summary of existing bioassay data discloses substantial species differences in response to peroxisome proliferators in vivo, with rodents more responsive than primates. Among the rat and mouse strains tested, both males and females develop tumors in response to exposure to a wide range of chemicals including DEHP and other phthalates, chlorinated paraffins, chlorinated solvents such as trichloroethylene and perchloroethylene, and certain pesticides and hypolipidemic pharmaceuticals. MOA data from three different rodent tissues—rat and mouse liver, rat pancreas,

‡The views expressed in this report are those of the individual authors and do not necessarily reflect the views of ILSI, U.S. EPA, CPSC, or any other organization. Mention of common or trade names of commercial products does not constitute endorsement or recommendation for use.

and rat testis—lead to several different postulated MOAs, some beginning with PPARα activation as a causal first step. For example, studies in rodent liver identified seven "key events," including three "causal events"—activation of PPARα, perturbation of cell proliferation and apoptosis, and selective clonal expansion—and a series of associative events involving peroxisome proliferation, hepatocyte oxidative stress, and Kupffer-cell-mediated events. Similar in-depth analysis for rat Leydig-cell tumors (LCTs) posits one MOA that begins with PPARα activation in the liver, but two possible pathways, one secondary to liver induction and the other direct inhibition of testicular testosterone biosynthesis. For this tumor, both proposed pathways involve changes in the metabolism and quantity of related hormones and hormone precursors. Key events in the postulated MOA for the third tumor type, pancreatic acinar-cell tumors (PACTs) in rats, also begin with PPARα activation in the liver, followed by changes in bile synthesis and composition. Using the new human relevance framework (HRF) (see companion article), case studies involving PPARα-related tumors in each of these three tissues produced a range of outcomes, depending partly on the quality and quantity of MOA data available from laboratory animals and related information from human data sources.

KEYWORDS: carcinogenic mode of action, peroxisome proliferator, PPARα agonist, risk assessment, human relevance of animal tumors, liver tumor, Leydig-cell tumor, pancreatic acinar-cell tumor, DEHP, clofibrate, oxadiazon, perfluorooctanoic acid (PFOA).

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I. INTRODUCTION

A. Scope and Purpose of Document

Data from long-term exposure animal bioassays often are used for identifying potential human carcinogens, since appropriate and adequate human data for most chemicals are lacking. The purpose of this report is twofold: (1) to describe the current understanding of the mode(s) of carcinogenic action of peroxisome proliferator-activated receptor-α (PPARα) agonist-induced tumors and (2) to determine if PPARα agonist-induced rodent tumors should [continue to] be considered relevant and applicable in human cancer hazard/risk assessments of substances belonging to this group of chemicals.

A workshop entitled *Do Peroxisome Proliferating Compounds Pose a Hepatocarcinogenic Hazard to Humans?* was held on December 13–14, 1995, in Washington, DC. The purpose of the workshop, which was held under the auspices of the International Life Sciences Institute (ILSI) Health and Environmental Sciences Institute, was to provide a review of the state of the science at that time on the relationship between peroxisome proliferation and hepatocarcinogenesis and to determine where consensus might be reached regarding the interpretation of these data relative to the assessment of potential

human hazard and risk. Conclusions reached at the workshop and reported in the peer-reviewed scientific literature included information identified as being necessary for characterizing a chemical as a peroxisome proliferator whose carcinogenic mode of action was dependent in some way on this phenomenon and data needed to support a margin of exposure (i.e., nonlinear) risk assessment approach (Cattley et al., 1998). Minimum data identified to characterize a chemical as a nongenotoxic hepatocarcinogen that operates via a PPARα-activation mode of action included measurements of increases in liver weight, size and number of hepatocyte peroxisomes, and replicative DNA synthesis.

Important new information on the mechanism(s) by which peroxisome proliferating chemicals produce certain of the carcinogenic responses in rats and mice, including advances in the understanding of the underlying genetic factors that mediate the biochemical and cellular responses to such chemicals, has emerged since the 1995 workshop. This report presents the deliberations of a workgroup formed in 2001 by the International Life Sciences Institute Risk Science Institute (ILSI RSI) and charged with the task of updating the 1998 report of the 1995 workshop, based on this new information. While there are a number of subclasses of peroxisome proliferators, this document is focused *solely* upon PPARα (*alpha*) agonists. This paper addresses several areas:

- It presents a reassessment, in light of the new data, of the minimum battery of supportive data elements needed to show that the mode(s) of action for hepatocarcinogenicity is/are PPARα driven.
- It presents an analysis of the data available to describe the modes of action by which Leydig-cell and pancreatic acinar-cell tumors are produced in rats by PPARα agonists that also produce liver tumors (i.e., an exploration of the "Tumor Triad").
- It presents principles by which the human relevance of PPARα-driven hepatocarcinogenicity and other tumor responses can be evaluated. This component of the exercise is being conducted using the human relevance framework (HRF) developed by the Framework Subgroup of the ILSI RSI Human Relevance of Animal Tumors Workgroup. The report is structured to address, for each tumor type, the three key questions articulated in the framework (see companion document): (1) Is the weight of evidence sufficient to establish the mode(s) of action in animals? (2) Are the key events in the animal MOA plausible in humans? (3) Taking into account kinetic and dynamic factors, is the animal MOA plausible in humans?
- It includes four chemical-specific case studies as examples of the application of the new knowledge and the new human relevance framework. Three of the case studies focus solely on liver tumors produced by the agent and vary by degree and nature of the data available on that agent to support the argument that the liver tumors occur via the PPARα activation, along with the relevance of this information to human hazard assessment. The fourth case study presents an example of an agent that produces the Tumor Triad in male rats, that is, liver, Leydig-cell and pancreatic acinar-cell tumors. A visual summary of the analysis of these four cases using the human relevance framework can be seen in Figure 1.

B. Overview of the Peroxisome Proliferation/Peroxisome Proliferator-Activated Receptor (PPAR)

Peroxisomes are subcellular organelles found in the cytoplasm of mammalian cells and also in fungi, protozoa and in a modified form in plant cells. They have important metabolic functions that have been studied broadly for several decades (de Duve, 1996; Hashimoto, 1996; Mannaerts and van Veldhoven, 1996). Peroxisomes are known to proliferate under a variety of altered physiological and metabolic states, most notably with the availability of high concentrations of unsaturated and polyunsaturated fatty acids. While these physiologic adaptive responses have been known for some time, greater interest in peroxisome proliferation was generated when extensive peroxisome induction was noted in rodent hepatocytes in response to the administration of certain xenobiotics (e.g., Hess et al., 1965; Reddy et al., 1976; Reddy and Chu, 1996). Based on the observation of peroxisome proliferation, the wide array of chemical and pharmaceutical agents that induce this response have been collectively referred to as "peroxisome proliferators."

Peroxisome proliferation has been identified and characterized traditionally either by morphologic or biochemical techniques (reviewed in Reddy and Chu, 1996). As the term "peroxisome proliferator" implies, there is an increase in the number and volume fraction of peroxisomes in the cytoplasm of cells. This increase is appreciated best by

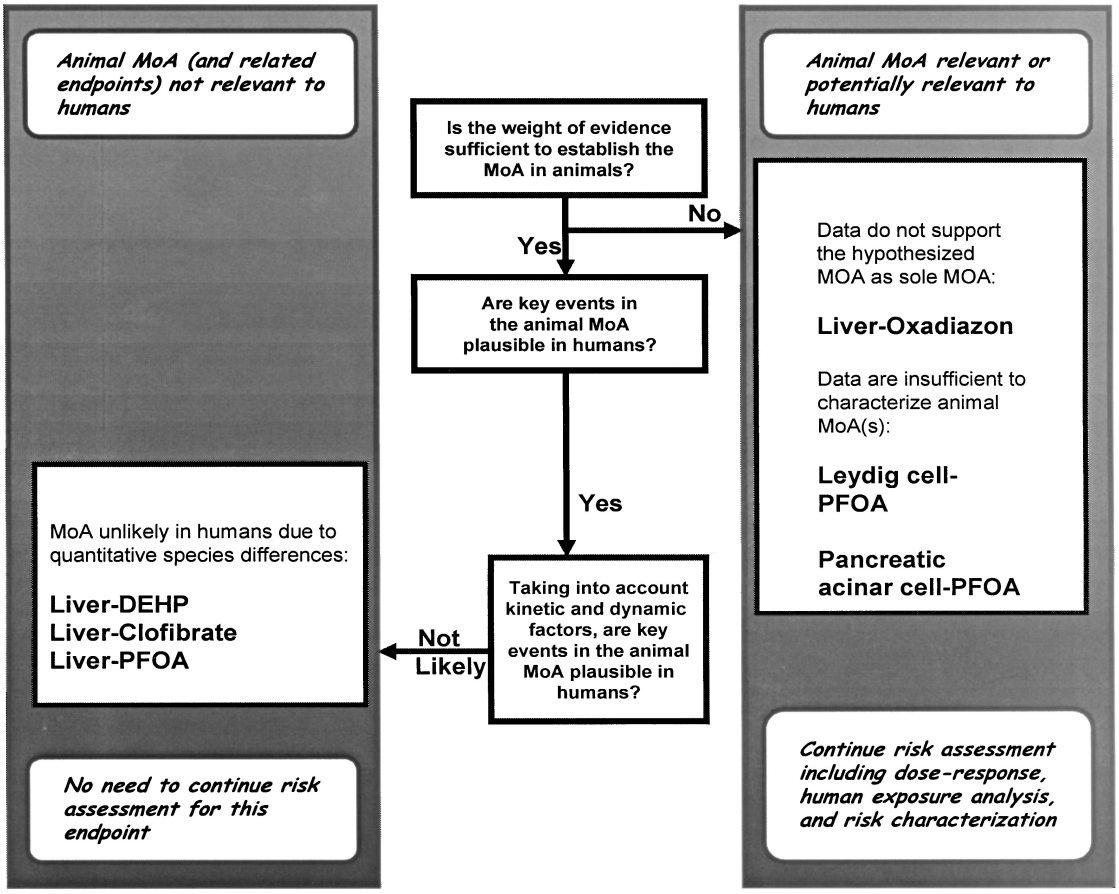

FIGURE 1. Summary of outcomes for all MOAs for four PPARα agonist case studies, analyzed in line with the four-part human relevance framework. The left side depicts data-based findings that certain PPARα agonist-induced liver tumors are irrelevant for human risk assessment because their animal MOA is not likely to have a human counterpart. The right side portrays two outcomes leading to complete risk assessments. One is the product of data-based findings showing oxadiazon appears not to induce liver tumors solely by the MOA hypothesized for PPARα agonists (oxadiazon). The other is the default: When data are insufficient to confidently characterize an MOA for test animals, the animal tumor data are presumed to be relevant to humans and a complete risk assessment is necessary (Leydig-cell and pancreatic acinar-cell, PFOA).

electron-microscopic and morphometric evaluation of peroxisomes. Due to the time and resource requirements to perform electron microscopy, the conclusion that an agent is a peroxisome proliferator is most frequently based on the biochemical evaluation of selected peroxisome enzymes, as measured by the increased metabolism of long-chain fatty acids. The use of biochemical evaluation of a peroxisome-specific enzyme has been validated as a marker of peroxisome induction. It is interesting to note that peroxisome proliferating agents do not uniformly induce all peroxisomal enzymes but selectively induce those peroxisomal enzymes that are associated with the metabolism of fatty acids. While the term "peroxisome proliferator" has been very useful in providing a shorthand term for a group of chemically diverse agents that induce a common pleiotropic response, the term is somewhat misleading since it acknowledges only a very limited component of the responses caused by this group of agents.

Based on the extreme variability in chemical structure of peroxisome proliferating agents, the mechanism of peroxisome induction was clouded for many years. The seminal discovery of the peroxisome proliferator-activated receptor (PPAR) opened important avenues of investigation that subsequently have provided important information on the mechanism of the cellular reponses to peroxisome proliferators (Issemann and Green, 1990). Indeed, the identification of PPAR and the results of the research spawned by this discovery now provide a better understanding of the relationship among the numerous biochemical, physiological, and molecular effects related to exposure to peroxisome

proliferators. The term "peroxisome proliferator" remains in broad use primarily for historical reasons, even though it does not adequately characterize the breadth of biological responses related to the compounds that are included in this classical descriptive term.

Peroxisome proliferation was noted first in rodents (Hess et al., 1965; Reddy et al., 1976). The response is noted frequently in both rats and mice although there are several examples of peroxisome induction noted in one of these species but not the other. There are also examples where the peroxisome proliferative response is greater in one sex compared to the other sex. When species or gender differences are noted in rodents, the basis for the difference is frequently, but not always, the result of variable formation of a peroxisome proliferating metabolite. More importantly, extensive interspecies variability in peroxisome proliferation has been noted when these two rodent species are compared to other species. It is important to note that rats and mice are always the most sensitive species to this response, while guinea pigs, nonhuman primates, and humans are less responsive. Hamsters tend to respond with peroxisome proliferation although the response is less robust than the response in rats or mice. Extensive research has evaluated the basis for this difference and concluded that several possibilities exist. The level of PPARα expression in humans is approximately one-tenth of the level noted in rodents. However, it is not clear if this observation contributes significantly to the explanation for the rodent/human differences in response, since other genes that are known to be regulated by PPARα show similar degrees of regulation in both species. Alternatively, cross-species differences in the capability of a peroxisome proliferator response element (PPRE) to respond to the activation of a PPAR may contribute to the interspecies differences in the peroxisome response.

Peroxisome proliferation is highly tissue specific in addition to being highly species specific (reviewed in Stott, 1988). Peroxisomes first were noted in kidney tubular epithelial cells of rodents; they frequently proliferate in these cells in response to peroxisome proliferating agents. Due to the equal, if not greater, response in the liver, in addition to the somewhat greater homogeneity of cell types in the liver, much of the early investigation of peroxisome induction has focused on the rodent liver. Lesser responses in several other tissues have been well established. The tissue response difference is apparently based on the variability of the levels of PPAR in various tissues, with the peroxisome response being closely related to the level of PPAR in the tissue.

Species Differences in the Response to Peroxisome Proliferators

The hepatic changes associated with exposure to peroxisome proliferators include hepatomegaly, increases in the number and size of peroxisomes and enhanced activity of peroxisomal marker enzymes such as catalase, cytochrome P-450s (e.g., CYP4A1 and 4A3), and acyl coenzyme A (CoA) oxidase. Table 1 shows species differences in peroxisome proliferation in vivo measured by palmitoyl CoA oxidizing systems for some representative substances. As noted earlier, rat and mouse are most sensitive, with hamster showing an intermediate response and guinea pig, monkey, and human appearing to be relatively insensitive or nonresponsive at dose levels that produce a marked response in rats and mice (e.g., Ashby et al., 1994; Bentley et al., 1993; Cattley et al., 1998; IARC, 1995b; Lake et al., 1999).

Short-term in vivo studies evaluating the mechanisms of PPARα agonist-induced alterations in liver also have revealed marked species differences. Replicative DNA synthesis is increased significantly in the livers of rats or mice treated with PPARα agonists but the increase in cell replication is not observed in similarly-treated Syrian hamsters (Lake et al., 1993). In vitro analysis using cells from different species support many of the in vivo observations. Cultured rodent hepatocytes respond to PPARα agonists with increased replicative DNA synthesis, suppression of apoptosis, increased expression of marker mRNAs and proteins, including peroxisomal acyl CoA oxidase and peroxisome proliferation (Bichet et al., 1990; Cornu-Chagnon et al., 1995; Duclos et al., 1997; Elcombe 1985; Elcombe et al., 1996; Goll et al., 1999; Hasmall et al., 1999, 2000a, 2000b; Perrone et al., 1998). It is important to point out that while peroxisome proliferation is reported to occur in cultured rodent hepatocytes exposed to these chemicals, a direct comparison of this effect in vitro to that found in vivo has not been reported. In contrast to observations made in cultured rodent hepatocytes, human and nonhuman primate hepatocytes cultured in the presence of PPARα agonists do not exhibit increased replicative DNA synthesis, suppression of apoptosis, increased expression of marker mRNAs and proteins including peroxisomal acyl CoA oxidase, or peroxisome proliferation (Bichet et al., 1990; Cornu-Chagnon et al., 1995; Duclos et al., 1997; Elcombe, 1985; Elcombe et al., 1996; Goll et al., 1999; Hasmall et al., 1999, 2000b; Perrone et al., 1998). Thus, in vivo and in vitro data strongly support the idea that humans are refractory

TABLE 1
Species Differences in Response to Representative Peroxisome Proliferators in vivo[a]

Chemical	Mouse	Rat	Hamster	Guinea pig	New World monkey	Old World monkey	Human
Bezafibrate	++	++	±	±		−	
Ciprofibrate	++					+	−[b]
Clobuzarit	+	+	−		−		
Clofibrate	++	++	±		−	−	±
Di-(2-ethyl-hexyl) phthalate (DEHP)	+	++	+(+) Chinese ± Syrian	−	−		
DL-040						+	
Fenofibrate		+	±	−		− ±[c]	−
Gemfibrozil		+(+)					−
Methylclofenopate		+	+	−			
Nafenopin		+	+	−	±		
LY171883	++	++	±	−		−	
Tiadenol		++		±			
Trichloroacetic acid (TCA)	+	+		−			

[a]Summarized from Bentley et al. (1993) and Moody et al. (1991); increased activity of palmitoyl CoA oxidase associated with peroxisome proliferation in liver: ++ = strongly positive, >10-fold; + = positive response, >3-fold; ± = weakly positive response, 1.5- to 3-fold; − = negative response, <1.5-fold.
[b]30% Increase in volume density of hepatic peroxisomes.
[c]Qualls et al. (2003).

to many of the effects induced by PPARα agonists in rodents. However, a reduction in serum lipids, which is the primary therapeutic value of these chemicals in humans, occurs in all species tested to date. Since this effect clearly is mediated by the PPARα in mice (Peters et al., 1997b), this suggests that humans have a functional PPARα capable of modulating gene expression in a manner similar to that in rodents.

A recent study evaluated the effects of two PPARα agonists in cynomolgus monkeys at clinically relevant doses (Qualls et al., 2003). Four males per group were treated either with fenofibrate (0, 250, 1250, or 2500 mg/kg/day) or ciprofibrate (0, 3, 30, 150, or 300 mg/kg day) for 15 days. Their findings included: significant hepatic (up to 2-fold) weight increase and hepatocyte hypertrophy (qualitative), increased peroxisome numbers (up to 2.7-fold) and mitochondria numbers (up to 2.5-fold) at exposures roughly 4 times the human clinical dose (i.e., 2500 mg/kg/day for fenofibrate and 150 mg/kg/day for ciprofibrate). At the same time, there were only slight or no increases in key peroxisomal enzyme activities (i.e., palmitoyl CoA oxidase, catalase, carnitine acetyltransferase, and enoyl CoA hydratase).

Two studies in humans showed a marginal increase in peroxisomes in the liver. A morphological analysis of liver biopsy tissue obtained from hyperlipidemic patients receiving a therapeutic dose level of clofibrate (approximately 20–40 mg/kg/day) revealed a 1.5-fold increase in the number and a 23% increase in the volume density of hepatic peroxisomes (Hanefield et al., 1983). Similarly, studies were conducted in seven patients who were biopsied before and after 6 to 24 months of treatment with 2 mg ciprofibrate/kg body weight/day. A morphometric analysis revealed a slight (30%) increase in the volume density of hepatic peroxisomes (Bentley et al., 1993; Hinton et al., 1986). However, no increase in the number of peroxisomes in the livers of hyperlipidemic patients receiving therapeutic doses of fenofibrate or gemfibrozil was observed (Bentley et al., 1993).

C. Summary of Existing Carcinogenicity Bioassay Data

PPARα agonists form a unique class of chemical carcinogens. They represent a large number of structurally and chemically diverse compounds. Out of almost 100 PPARα agonists identified to date, about 30 have been evaluated in long-term studies and shown to induce peroxisome proliferation, the induction of peroxisomal enzymes and hepatocellular carcinoma in rats and/or mice (Ashby et al., 1994; Bentley et al., 1993; Rao and Reddy, 1996). Some substances that increase peroxisome volume density

or peroxisomal enzyme activity in these two species have not demonstrated carcinogenicity in both of them. For some substances that induce peroxisome proliferation, long-term exposure has resulted in liver tumors in mice but not rats. In addition, there are cases in which tumors were detected in only one strain of rat. Understanding whether or not this lack of tumorigenesis is a result of differences in mechanism, potency, or kinetics may improve our ability to evaluate if humans are at risk, since mice and rats should not differ in their sensitivity to these agents as the PPARα is identical in both species.

A number of the 30 PPARα agonists tested in long-term studies also induce non-hepatic tumors. Some of these exhibited the "Tumor Triad," that is, induction of liver, Leydig-cell, and pancreatic acinar-cell tumors in male (non-F344) rats. In a few cases, either the Leydig cell or the pancreatic acinar cell tumor was observed, but not both, along with the liver tumor. It should be noted that, with one exception (cinnamyl anthranilate), neither the Leydig-cell nor pancreatic acinar-cell tumor was observed to occur in the absence of concomitant induction of liver tumors. These chemical substances do not induce peroxisome proliferation in Leydig cells (LCs) or in pancreatic acinar cells (PACs) and hence may induce these extrahepatic tumors via different mechanisms than in the liver where peroxisome proliferation also is seen (Biegel et al., 1992, 2001; Obourn et al., 1997a, 1997b, 1997c). Limited studies of PPARα agonists in other animal species such as the hamster and nonhuman primates did not show any carcinogenic responses. Human clinical and epidemiology data collected following PPARα agonist exposure are scant.

1. Rat and Mouse Data

a. Hepatic Tumors

1) Summary of bioassay results

The PPARα agonists became a novel class of carcinogens when Reddy and coworkers (reviewed in Reddy and Lalwani, 1983) conducted a series of studies in rats and mice in the mid-1970s to investigate the long-term effects of several drugs used for the control of hyperlipidemia in humans. Almost 100% of animals developed hepatocellular carcinomas when groups of 14–35 male F344 rats and/or 9–12 male and female acatalasemic mice (CS^b) were fed 0.1–0.5% nafenopin, methylclofenapate, WY 14,643, tibric acid, BR-841, or clofibrate in the diet for 16–20 months. Similar findings were observed when the authors tested several other peroxisome-proliferating hypolipidemic drugs (e.g., gemfibrozil, fenofibrate, bezafibrate, and ciprofibrate) in rats and/or mice.

The hepatic effects of phthalate ester plasticizers and related compounds on peroxisome proliferation in rodents were found to be similar to those resulting from treatment with clofibrate and other hypolipidemic drugs (Moody and Reddy, 1978). Chronic administration of many of these compounds such as di-(2-ethylhexyl) phthalate (DEHP), di-(2-ethylhexyl) adipate (DEHA), diisononyl phthalate (DINP), or 2-ethylhexanol (2-EH) in rats and/or mice also resulted in increased incidences of hepatocellular carcinomas/adenomas in treated animals (Astill et al., 1996; Butala et al., 1997; Kluwe et al., 1985).

In addition to phthalate ester plasticizers and hypolipidemic drugs of the fibrate and nonfibrate groups, a growing number of structurally dissimilar compounds used as pesticides (e.g., 2,4-D, diclofop-methyl, haloxyfop, lactofen, oxidiazon), solvents (e.g., perchloroethylene, trichloroethylene), and other industrial chemicals (e.g., HCFC-123, perfluorooctanoic acid [PFOA]) have been identified as peroxisome proliferators and shown to be hepatocarcinogenic in rodents (Gold and Zeiger, 1997; Hollander and Wiegand, 1978; Malley et al., 1995; Shirasu 1987a, 1987b; U.S. EPA, 2000a, 2000b; also, unpublished data summarized in the Oxadiazon Case Study).

A summary of the positive and negative responses observed for the liver following long-term exposure of rats and mice to some of the PPARα agonists is shown in Table 2.

2) Possible explanations for different responses in rats and mice

As already noted, some substances that increase peroxisome volume density or peroxisomal enzyme activity in these two species have not demonstrated carcinogenicity in both of them. Several reviews have evaluated the relationship between tumors and peroxisome proliferation comparing the increase in peroxisome proliferation with the tumor incidence (Ashby et al., 1994; Lake, 1995a; Stott, 1988). These comparisons included a wide array of substances, some of which are metabolized differently in rats and mice, leading to species-dependent formation of proximate PPARα agonists. Such differences in metabolism confound the evaluation. Phthalate esters and di(2-ethylhexyl) adipate (DEHA) are hydrolyzed by both rats and mice; thus, these

TABLE 2
Summary of Hepatocarcinogenic Potential of Some PPARα Agonists in Rats and Mice[a]

	Rat			Mouse		
Chemicals	Strain	M	F	Strain	M	F
Aciflurofen	F344	−	−	CD-1	+	+
				B6C3F$_1$	+	+
Bezafibrate	SD-CHW	+				
	???	−	−			
BR 931	F344	+		CS		+
Chlorinated paraffins (C$_{12}$)	F344	+	+	B6C3F$_1$	+	+
Cinnamyl anthranilate	F344	−	−	B6C3F$_1$	+	+
Ciprofibrate	F344	+		C57B1	+	
Clobuzaril (ICI-55897)	Wistar	−		C57B1	+	
Clofibrate	CD; F344	+	+	Swiss	−	
				Alderley-Park	−	−
				C57B1/10J	−	−
Dichloroacetic acid (DCA)				B6C3F$_1$	+	
2,4-Dichlorophenoxyacetic acid (2,4-D)				B6C3F$_1$	+	+
Diclofop-methyl	Wistar	+	+	HOENMRK *f*	+	+
Di(2-ethylhexyl) adipate (DEHA)	F344	−	−	B6C3F$_1$	+	+
Di(2-ethylhexyl) phthalate (DEHP)	F344	+	+	B6C3F$_1$	+	+
Diisononyl phthalate (DINP)	F344	+	+	B6C3F$_1$	+	+
	CD	+	+			
2-Ethylhexanol	F344	−	−	B6C3F$_1$	−	+
Gemfibrozil	CD	+	+	CD-1	−	−
Haloxyfop	F344	−	−	B6C3F$_1$	−	+
HCFC-123	CD	+	+			
Lactofen	CD	−	−	CD-1	+	+
LY 171883				B6C3F$_1$		+
Methylclofenapate	Wistar	+	+			
Nafenopin	F344	+		CS	+	+
Oxadiazon	F344	+	−	CD-1	+	+
	Wistar	+	−	ICR-JCL	+	+
Perchloroethylene (PCE)	F344	−	−	B6C3F$_1$	+	+
Perfluorooctanoic acid (PFOA)	CD	+				
Tibric acid	F344	+				
Trichloroacetic acid (TCA)				B6C3F$_1$	+	
Trichloroethylene (TCE)	F344	−	−	B6C3F$_1$	+	+
2,4,5,-Trichlorophenoxyacetic acid (2,4,5-T)	Wistar; CD	+	+	B6C3F$_1$	+	+
WY 14,643	CD; F344	+		CS	+	

[a]See text for references; M = male, F = female; + = positive in adequate carcinogenicity studies; − = negative in adequate carcinogenicity studies; blank = not tested.

substances allow for evaluation of potency differences among species.

Metabolic differences between species

For PPARα agonists such as trichloroethylene, tetrachloroethylene, and cinnamyl anthranilate, differences in metabolism between rats and mice play a significant role in determining tumorigenesis (Lake, 1995b). These substances do not cause tumors in rats apparently because metabolism to the ultimate peroxisome proliferator is slower in rats than in mice (Ashby et al., 1994; IARC, 1995a, 2000) or hydrolysis to a nonactive form is more efficient in rats than mice.

Trichloroethylene (TCE)

Trichloroethylene (TCE) has been tested for carcinogenic potential in several studies following oral or inhalation exposure using different strains of rats and mice. The results of these studies and the species-specific metabolism of TCE were reviewed by IARC (1995a). NTP conducted an oral chronic study for 104 weeks using F344 rats and $B6C3F_1$ mice (NTP, 1990). While the results for the F344 male rats were considered by NTP to be inadequate because of low survival, there was no indication of liver tumors or preneoplastic foci in either sex of rats, whereas $B6C3F_1$ mice had increased incidences of hepatocellular carcinomas/adenomas. A similar lack of hepatocellular neoplasia was reported for ACI, August, Marshall, and Osborne-Mendel strains of rat treated by oral gavage for 2 years (NTP, 1988) and for Sprague-Dawley rats exposed via inhalation for 104 weeks (Maltoni et al., 1988).

The lack of liver tumors in rats compared with their induction in mice has been evaluated relative to the potency of TCE as a peroxisome proliferator and relative to species-specific metabolism. Palmitoyl CoA (PalCoA) oxidation activity was studied in F344 rats following treatment with TCE, perchloroethylene (PCE), and trichloroacetic acid (TCA) (Goldsworthy and Popp, 1987), and in Osborne-Mendel and Wistar (AP) rats and $B6C3F_1$ and Alderley Park mice treated with TCE (Elcombe et al., 1985). The data demonstrate that rats treated with TCE (or PCE) do not show increases in peroxisomal enzyme activities, while rats treated with TCA have significant increases in peroxisomal enzyme activity. On the other hand, mice were responsive to treatment with TCE, exhibiting increases in peroxisomal volume density and induction of peroxisomal enzyme activities, catalase and palmitoyl CoA oxidation. The accepted explanation for this species difference in sensitivity is a difference in metabolism. TCE is metabolized by cytochrome P-450s to tri- and dichloroacetic acids. Goldsworthy and Popp (1987) clearly demonstrated that TCA was a peroxisome proliferator in rats. Yet rats metabolize TCE more slowly than mice and metabolism appears to be saturable (Green and Prout, 1985; Green et al., 1997; Prout et al., 1985). Thus, the lack of tumorigenesis in rats compared with mice may be explained by a difference in metabolism to the active metabolite.

Cinnamyl anthranilate

Long-term studies of cinnamyl anthranilate (CA) were conducted using F344 rats and $B6C3F_1$ mice. At the termination of 103 weeks of administration in the diet, liver tumors were observed in mice but not rats (IARC, 2000). This species difference in tumors is reflected in the degree of peroxisome proliferation in the two species and in the metabolism of CA by the two species. Lake et al. (1997a) measured PalCoA activity in F344 rats and $B6C3F_1$ mice treated with varying doses of CA. Mice responded with significant increases in PalCoA activity, but rats were unresponsive. Again, metabolism plays a key role in this species difference. Hydrolysis of CA leads to inactivation of the peroxisome proliferator. In mice, hydrolysis is a saturable process, whereas in rats, hydrolysis is not saturable (IARC, 2000). Thus, the lack of tumors in rats again appears to be the result of species metabolic differences.

Dose-response relationships between peroxisomal enzyme activity and tumor induction

Butylbenzyl phthalate (BBP) and DEHA are substances for which the appearance of tumors or peroxisome proliferation in rats or mice is not influenced by metabolism. DEHA, like other phthalate ester plasticizers, is hydrolyzed by pancreatic lipases (Albro and Thomas, 1973; Lake et al., 1977; White et al., 1980, 1983), and there is no apparent quantitative species difference between rats and mice in hydrolytic activity (Lake et al., 1984a). Liver tumors were observed in $B6C3F_1$ mice, but not in F344 rats at equivalent dietary levels, or, in the case of BBP, not in either species. Instead, the difference in, or lack of, tumorigenesis appears to reflect the degree of peroxisome proliferation and shows that tumorigenesis generally is not observed at doses where peroxisome proliferation is not induced or induced only at low levels. Furthermore, there are examples in which a low, but statistically significant, level of peroxisome proliferation is not followed by increases in tumor incidence. This would suggest that a minimum amount of peroxisome proliferation is necessary before a tumorigenic response can be elicited. A nonlinear dose-response relationship for this class of substances has been hypothesized, although the existence of a true threshold has not been proven empirically.

TABLE 3
PalCoA Activity Following DEHA Exposure

Rats			Mice		
Dose level (ppm)	mg/kg/day	PalCoA[a]	Dose level (ppm)	mg/kg/day	PalCoA[a]
0	0	1.7	0	0	3.30
1500	144	2.06	1500	343	4.99*
3000	282	1.96	3000	808	4.36*
6000	577	3.37*	6000	1495	8.10*
12,000	1135	6.16*	**12,000**[b]	3075	29.07*
25,000	2095	23.25*	**25,000**[b]	5330	46.51*
40,000	3140	67.82*			

Note. Boldface dose levels used in the chronic study. Asterisk indicates significantly different from control, $p \leq .05$.
[a]Activity expressed as nmol NADH formed/mg protein/min.
[b]Significant increase in female mouse liver tumors at this dose level.

Di(2-ethylhexyl) adipate (DEHA)

NTP conducted a bioassay in F344 rats and B6C3F$_1$ mice at dietary concentrations of 12,000 (2659 and 3222 mg/kg/day for male and female mice, respectively; 697 and 860 mg/kg/day for male and female rats, respectively) and 25,000 ppm (6447 and 8623 mg/kg/day for male and female mice, respectively; 1509 and 1674 mg/kg/day for male and female rats, respectively) (NTP, 1982a). A statistically significant increased incidence of liver tumors was observed in female mice at both dose levels, but not in male mice[1] or either sex of rat at either dose. Lake et al. (1997b) investigated peroxisomal enzyme changes in *female* rats and mice exposed to DEHA in the diet for up to 13 weeks. The PalCoA activity at various dose levels is presented in Table 3.

These data suggest that minor increases in peroxisome proliferation, such as those seen in the F344 rat, are insufficient to trigger an observable tumorigenic effect. This observation is supported further by data from some phthalate esters.

Butylbenzyl phthalate (BBP)

IARC has reviewed the carcinogenicity of BBP (IARC, 1999). NTP conducted three studies with BBP using F344 rats and B6C3F$_1$ mice. The first study exposed animals to dietary concentrations of 6000 or 12,000 ppm (NTP, 1982b). There were no increases in liver tumors observed in mice or rats, although survival of treated male rats rendered that portion of the study inadequate. A second feeding study exposed male rats to 3000, 6000, or 12,000 ppm, while female rats were exposed to 6000, 12,000 or 24,000 ppm (NTP, 1997a). Again, no increases in liver tumors were noted. An additional study of feed-restricted rats at 12,000 ppm (males) or 24,000 ppm (females) also showed no evidence of an increased incidence of liver tumors (NTP, 1997b).

Although BBP is a weak peroxisome proliferator, the dose-response data for PalCoA in F344 rats indicate significant increases in activity (Barber et al., 1987; BIBRA, 1985). In the BIBRA 21-day study employing doses that included those used in the cancer studies, five males and five females were used per dose group. The results are shown in Table 4. The data for the males are on

TABLE 4
PalCoA Activity Following BBP Exposure

F344 Rats		
Dose level (ppm)	mg/kg/day[a]	PalCoA[a,b]
0	0	4.08–5.33
6000	639–679	7.07*–5.52
12,000	1277–1346	10.2*–6.43
25,000	2450–2628	18.0*–15.2*

Note. Asterisk, significantly different from control, $p \leq .05$.
[a]Data for males on left; data for females on right.
[b]Activity expressed as nmol NADH formed/mg protein/min.

[1]There was a significant trend for male mice, but the incidence was not greater than historical controls for the laboratory.

the left, and for the females, on the right, within each cell of the table.

After 21 days of treatment, PalCoA activity was increased at doses that were without evidence of liver tumors in the long-term studies. This observation begs the question as to what level of increased PalCoA activity is the threshold for tumorigenesis. Dose-response data for DEHP and DINP provide some indication of the minimum level of PalCoA activity required for an observable tumorigenic effect.

DEHP, DINP, and tumorigenic dose responses

Ashby et al. (1994) compared the relative induction of peroxisomal activity with tumorigenesis. They concluded that, across the class of PPARα agonists, there was a correlation of 70–80% for increased PalCoA activity and tumor formation. It appears that a minimal level of peroxisomal enzyme induction is required to elicit an increase in tumor incidence, at least in rats, and several investigators have proposed that there is an apparent threshold for tumor induction by these agents (Ashby et al., 1994; Cattley et al., 1998; Huber et al., 1996). Additional insight can be gained by comparing the dose-response enzyme data for DEHP from David et al., (1999) and DINP to increases in tumor incidence. Table 5 compares the enzyme data with tumor incidence for rats and mice following DEHP exposure. The data for the males are on the left, and for the female, on the right, within each cell of the table.

Based on these dose-response data for DEHP, significant increases in liver tumors are observed at enzyme activities of about 25 nmol product formed/mg protein/min for rats and 15 nmol product formed/mg protein/min for mice. The data for DINP also support these values. Using data for F344 rats and B6C3F$_1$ mice, a comparison of tumorigenic dose levels with peroxisome proliferation (Barber et al., 1987; Kaufman et al., 2002) suggests roughly the same threshold for rats (CPSC, 2001) (see Table 6). For mice, it may be somewhat lower (about 9 nmol/mg protein/min), as a statistically significant increase in liver tumors was observed in females at the 1500 ppm dose level. The data for the males are on the left, and for the females on the right, within each cell of the table.

These data for DEHP and DINP indicate that peroxisomal activity is proportional to the exposure level. Increases in tumor incidence also may be proportional to exposure level (at least in mice), but the increases generally are not above background or control levels at low exposures.

Figure 2 compares the incidence of hepatocellular tumors with the dose of DINP received in the two-year study. Figure 3 compares PalCoA enzyme activity with tumor incidence in the DINP two-year study. Both figures illustrate the variability (scatter) in the tumor incidence. They also illustrate the difficulty in determining empirically whether there is a true threshold for tumor induction, at least in mice. The lack of a statistically significant tumor response at the low dose(s) is not sufficient to establish the existence of a threshold for tumorigenesis. The observed response is consistent with either a threshold or nonthreshold dose-response model.

The value of using DEHP and DINP data to compare tumor responses and palmitoyl CoA activity is that both substances have robust dose-response data for both tumors and palmitoyl CoA and the method used to determine palmitoyl CoA activity was the same for both substances. It is difficult to

TABLE 5
PalCoA Activity Following DEHP Exposure

F344 Rats			B6C3F$_1$ Mice		
Dose Level (ppm)	mg/kg/day	PalCoA[a]	Dose Level (ppm)	mg/kg/day	PalCoA[a]
0	0	16.4–12.1	0	0	7.1–3.75
500	28.9–36.1	11.7–10.5	**100**	19.2–23.8	7.1–4.9
2500[b]	146.6–181.7	28*–23.3*	**500**[c]	98.5–116.8	15.2*–15.3*
12,500[b]	789.0–938.5	58.6*–61.7*	**1500**[b]	292.2–354.2	19.1*–25.3*
			6000[b]	1266.1–1458.2	53.9*–56.3*

Note. Asterisk, significantly different from control, $p \leq .05$.
[a] Activity expressed as nmol NADH formed/mg protein/min.
[b] Significant increase in liver tumors at this dose level.
[c] Significant increase in liver tumors only for one sex.

TABLE 6
PalCoA Activity Following DINP Exposure

F344 Rats			B6C3F$_1$ Mice		
Dose level (ppm)	mg/kg/day	PalCoA[a]	Dose level (ppm)	mg/kg/day	PalCoA[a]
0	0	6.0–7.0	0	0	4.0–5.3
6000	639–607	10–11.3	**500**	115–142	6.1*–5.9
12,000[b]	1192–1193	27.1*–26.3*	**1500**[c]	352–441	9.3*–8.9*
25,000	2195–2289	62.1*–77.3*	**4000**[b]	951–1192	23.1*–22.1*
			8000[b]	2022–2509	43.4*–43.9*

Note. Asterisk, significantly different from control, $p \leq .05$.
[a]Activity expressed as nmol NADH formed/mg protein/min.
[b]Significant increase in liver tumors at this dose level.
[c]Significant increase in liver tumors in females.

apply these transition points to other PPARα agonists because sufficient dose-response data often are not available for those other chemicals, but a few examples exist. Bendele et al. (1990) observed tumors in B6C3F$_1$ mice exposed to the experimental drug, LY171883. Females exposed to 0.0225 ppm in the feed demonstrated an increased incidence of hepatocellular tumors and both sexes had increased incidences of tumors at 0.075 ppm. The transition point for palmitoyl CoA activity and tumors observed for DEHP and DINP is not apparent for LY171883 for unknown reasons, although there may be substantial differences in the method of palmitoyl CoA measurement for LY71883 compared with that for the two phthalates (see Table 7).

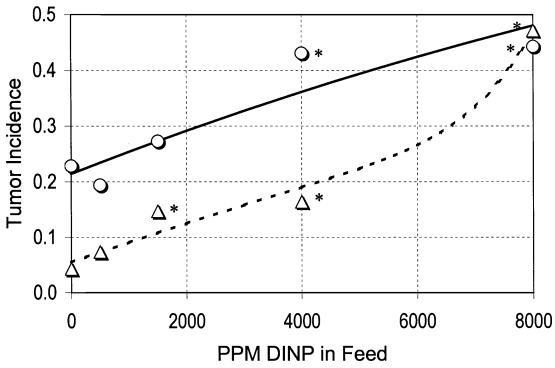

FIGURE 2. Incidence of hepatocellular tumor in B6C3F$_1$ mice exposed to DINP for 2 years: circles, males, observed; solid line, males, maximum likelihood estimate (MLE) of risk, multistage model; triangles, females, observed; broken line, females, MLE risk, multistage model. Asterisk, significantly different from control, $p < .05$.

The data for the males are on the left, and for the females on the right, within each cell of the table.

Hartig et al. (1982) reported the tumor incidence for clofibrate in Sprague-Dawley-CHW rats fed 0, 1500, 4500, or 9000 ppm in the diet for two years. The data for the males are on the left, and for the females, on the right, within the columns of Table 8 showing dose (mg/kg/day) and tumor incidence. Makowska et al. (1990) measured palmitoyl CoA in male F344 rats treated for 26 weeks at some of the same dose levels. In a later study, enzyme activity was measured in male F344 rats treated with 2 or 20 mg/kg/day after 14 days of exposure (Makowska et al., 1992).

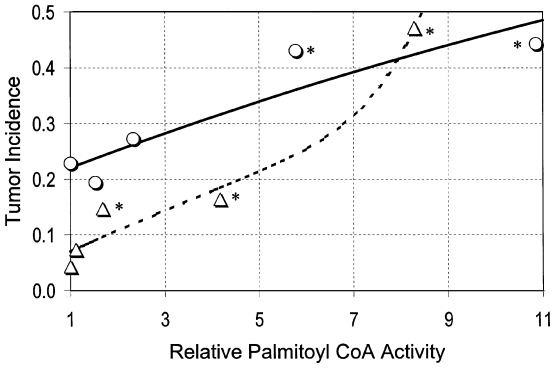

FIGURE 3. Incidence of hepatocellular tumor plotted against palmitoyl CoA oxidase activity (nmol NADH formed/mg protein/min) in B6C3F$_1$ mice exposed for 2 years to DINP in feed: circles, males, observed; solid line, males, MLE risk, multistage model; triangles, females, observed; broken line, females, MLE risk, multistage model. Asterisk, significantly different from control, $p < .05$.

TABLE 7
LY171883 in B6C3F$_1$ Mice

Dose level (ppm)	Dose (mg/kg/d)	PalCoA[a]
0	0	4.1–6.4
0.0075	7.3–9.2	5.0–5.9
0.0225[c]	22.5–27.5	4.2–5.9*
0.075[b]	80.5–95.9	7.9*–14.0*

Note. Asterisk, significantly different from control, $p \leq .05$.
[a]Activity expressed as nmol NADH formed/mg protein/min.
[b]Significant increase in mouse liver tumors at this dose level.
[c]Significant increase in mouse liver tumors, females only.

Reddy et al. (1986a, 1986b) measured PalCoA activity and tumor incidence in separate experiments. The tumor incidence for the lowest dietary concentration (10 ppm) was not reported. Table 9 summarizes the data.

In spite of their inadequacies, the data for ciprofibrate and clofibrate appear to be consistent with the observation that a minimal level of peroxisomal enzyme induction is required to elicit an increase in tumor incidence.

Potency

Although the existence of a threshold in the tumor dose response may continue to be debated, there are clear differences in the potency of PPARα agonists. Such differences reflect the doses required to produce either increases in peroxisomal enzyme activity and/or liver tumors. Potencies have been derived experimentally and reported by Ashby et al. (1994), Barber et al. (1987), Doull et al. (1999), Lake (1995b), Reddy et al. (1986a, 1986b), and Stott and Hawkins (1993). Opinions differ somewhat regarding the potency of some strong agonists such as WY 14,643 and ciprofibrate, but there is general agreement on the ranking of weak or very weak agonists such as acetylsalicyclic acid (ASA) and DEHP (Doull et al., 1999; Stott and Hawkins, 1993). Perhaps the most complete ranking for enzyme induction was provided by Barber et al. (1987), using F344 rats. Using their data, Table 10 was constructed which groups agonists into several broad categories.

Thus, clear differences in the potency of PPARα agonists have been noted with agreement on the low potency of many nontherapeutic substances other than ASA. The question remains: How does potency for enzyme induction translate to tumorigenesis? Ashby et al. (1994), Lake (1995b), Reddy et al. (1986a, 1986b), and Stott and Hawkins (1993) all demonstrated that high levels of peroxisomal enzyme activity (greater than a sevenfold increase)

TABLE 8
Clofibrate in Rats

Dose level (ppm)	Dose (mg/kg/d)	Tumor incidence (%)	PalCoA
0	0	0–0.1	2.4[a]
	2	na	~30
	20	na	~42
1500	58–71	0.01–0	12.6
4500[b]	169–218	10–10	nd
9000[b]	364–491	11–26	33.2

Note. na, Not applicable; nd, not determined.
[a]Activity expressed as nmol NADH formed/mg protein/min in F344 rats.
[b]Significant increase in liver tumors in SD rats at this dose level.

TABLE 9
Ciprofibrate in Rats

Dose level (ppm)	Dose (mg/kg/d)	PalCoA
0	0	7.9[a]
10[b]	0.5	41.4
100[c]	5	92.1
200[c]	10	106.4

[a]Activity expressed as nmol NADH formed/mg protein/min.
[b]Tumor incidence at this dose not reported.
[c]Significant increase in liver tumors at this dose level.

TABLE 10
Potency of PPARα Agonists to Produce Peroxisomal Enzyme Induction in F344 Rats

Compound	Rank	Activity[a]
Fenofibrate, clobuzarit	Very strong	>300
Ciprofibrate, CPIB, tibric acid, WY 14,643	Strong	<100, >40
Clofibrate, methyl clofenopate	Moderate	<40, >20
DEHP, DINP, DIDP	Weak	<20, >10
DEHA, ASA, BBP	Very weak	<10

Note. Adapted from Barber et al. (1987).
[a]Palmitoyl CoA oxidase activity (nmol/min/mg)/ (μmol/kg/d) $\times 10^{-3}$.

resulted in liver tumors. At what point in the dose response tumors occur has been determined only for a few weak agonists.

Conclusions

Several PPARα agonists have demonstrated carcinogenicity in mice but not rats. The reverse is true for clofibrate but otherwise has seldom been observed; admittedly, not all of the PPARα agonists have been tested fully in both species. In some cases, the metabolism of the substance in rats and mice is different, leading to inactive or lower levels of active metabolites in rats compared with mice. In addition, it appears that a minimum level of increased peroxisomal enzyme activity is necessary to elicit a statistically significant increase in tumor incidence. Pal-CoA data for various plasticizers are consistent with an apparent threshold for tumor induction. However, due to limitations in the sensitivity of carcinogenicity bioassays in general, the existence of a threshold is difficult to demonstrate empirically.

b. Other types of tumors

As noted earlier, some PPARα agonists induce tumors in rats and/or mice at sites other than the liver. Some examples are listed in Table 11. For instance, statistically significant increased incidences of mononuclear cell leukemias were induced in F344 rats by butylbenzyl phthalate (BBP), diisononyl phthalate (DINP), and perchloroethylene. Chlorinated paraffin (C12), cinnamyl anthranilate, perchloroethylene, and trichloroethylene also caused kidney tumors in the rat. Thyroid tumors were induced by chlorinated paraffin (C12) and

TABLE 11
Summary Data on Nonhepatic Tumor Induction by Representative PPARα Agonists in Rats and Mice[a]

	Rat		Mouse	
Chemicals	Strain	Target organ	Strain	Target organ
Butylbenzyl phthalate	F344	Mononuclear cell Leukemia (F) Pancreas (M, F)		
Chlorinated paraffins (C12)	F344	Kidney (M), thyroid (F)	B6C3F$_1$	Thyroid (F)
Cinnamyl anthranilate	F344	Kidney (M), pancreas (M)	A/He	Liver (M, F)
Clofibrate	F344	Pancreas (M)		
	CD	Testes, pancreas (M)		
2,4-Dichlorophenoxyacetic acid (2,4-D)			B6C3F$_1$	Lung (M, F)
Di (2-ethylhexyl) phthalate (DEHP)	CD	Testes		
	F344	Pancreas (M)		
Diisononyl phthalate (DINP)	F344	Mononuclear cell Leukemia (M, F) Testes?		
Gemfibrozil	CD	Testes, pancreas (M)		
HCFC-123	CD	Testes, pancreas (M)		
Methylclofenapate	Wistar	Testes, pancreas (M)		
Nafenopin	F344	Pancreas (M)		
Perchloroethylene	F344; CD	Testes, kidney (M), Mononuclear cell Leukemia (M)		
Perfluorooctanoic acid (PFOA)	CD	Testes, pancreas (M)		
Trichloroethylene	F344 CD	Testes, kidney (M)	ICR/Swiss	Lung (M)
2,4,5-Trichlorophenoxyacetic acid (2,4,5-T)	CD	Thyroid (F)	B6C3F$_1$	Lung (M, F)
WY 14,643	CD	Testes, pancreas (M)		

[a]See text for references; M = male, F = female.

2,4,5-T in rats and/or mice. The lung also appeared to be a target organ of cinnamyl anthranilate, 2,4-D, 2,4,5-T, and trichloroethylene (NTP, 1993).

A number of PPARα agonists have been reported to induce testicular (Leydig cell) tumors in rats. These include clofibrate, DEHP, DINP, gemfibrozil, HCFC-123, methylclofenapate, perchloroethylene, perfluorooctanoic acid (PFOA), trichloroethylene, and WY 14,643 (Biegel et al., 2001). Interestingly, Leydig-cell tumors were observed only when these compounds were tested in non-F344 male rats. It is known that by 2 years of age, the F344 rat has virtually a 100% incidence of spontaneously occurring Leydig-cell tumors, which would make it difficult, if not impossible, to detect a xenobiotic-induced testicular tumor in this strain. The finding that a relationship appears to exist between PPARα agonists and Leydig-cell tumor formation has led to the speculation that many, if not all, peroxisome proliferators could produce this tumor if tested adequately in a rat strain other than F344.

Clofibrate, HCFC-123, PFOA, and WY 14,643 induced pancreatic acinar cell tumors in addition to liver and Leydig cell tumors (Biegel et al., 2001; Svoboda and Azarnoff, 1979). The data led Biegel and coauthors to hypothesize further that a tumor triad (i.e., liver, Leydig-cell, and pancreatic tumors) may be induced by peroxisome proliferators. In addition, methylclofenapate was found to cause all three tumors in the Wistar rat (Tucker and Orton, 1995). Nafenopin induced only the liver and pancreatic tumors in the F344 rat (Reddy and Rao, 1997a, 1997b). As hypothesized earlier, the lack of the observation of an increase in Leydig cell tumors with nafenopin is undoubtedly due to the fact that the spontaneous incidence in the aging male F344 rat is virtually 100%.

The reader is referred to section III for a more detailed summarization and analysis of the data on chemicals inducing Leydig-cell and/or pancreatic acinar-cell tumors in addition to liver tumors, including a dose-response comparison for each tumor type.

2. Data in other animals

In spite of their carcinogenic activity in rats and mice, peroxisome proliferators have not been shown to be carcinogenic in other animal species, including other rodents. Hepatic peroxisome proliferation was induced by nafenopin or WY 14,643 in the hamster; however, no liver tumors were observed in groups of 8 male Syrian hamsters after 60 weeks of treatment with either compound (Lake et al., 1993). Limited carcinogenicity studies with DEHP in the Syrian hamster also showed no carcinogenic effects (Schmezer et al., 1988).

Tucker and Orton (1995) reported on a series of studies conducted with clofibrate and clobuzarit in nonhuman primates and hamsters. Administration of clofibrate to 11–16 male and female marmosets per group at daily doses of 0, 94, 157, 218, or 263 mg/kg body weight for 6.5 years did not cause increased incidences of liver or other tumors. No neoplasms were observed in groups of 4 male and 4 female marmosets dosed with clobuzarit by gavage at 0, 10, 20, or 40 mg/kg daily for 3 months, or in groups of 5 cynomolgus monkeys given clobuzarit at dose up to 80 mg/kg in a 6-month study. Because of the small number of animals and short periods of exposure, the results of these studies are inadequate to assess fully the carcinogenic potential of the chemical in these species. No increases in tumor incidence were found in a study in which groups of 52 male and 52 female Syrian hamsters were fed a diet containing 0, 5, 12.5, or 25 mg/kg/day clobuzarit for 2 years.

II. HEPATIC TUMORS: IN-DEPTH ANALYSIS

A. Introduction

This section provides an in-depth analysis of (1) the proposed mode(s) of action (MOAs) for rat and mouse liver tumors induced by PPARα-ligands, (2) the plausibility of the MOAs occurring in humans, and (3) the relevance/applicability of this hepatocarcinogenic response in the rodent for human health risk assessment. The section is structured to address the three key questions articulated in the ILSI RSI human relevance framework (see companion document): (1) Is the weight of evidence sufficient to establish the mode(s) of action (MOA) in animals? (2) Are key events in the animal MOA plausible in humans? (3) Taking into account kinetic and dynamic factors, is the animal MOA plausible in humans?

Each of the MOAs is presented in Figure 4 with an accompanying table (Table 12) that lists the key steps in the MOA(s) together with a consideration of degree of association, strength of the evidence and specificity of the key event to a PPARα agonist-induced response. A detailed analysis of the supportive evidence for the postulated MOA(s) follows the table. This analysis is not intended to reflect an exhaustive review of the literature but rather a summation of key evidence in support or otherwise of the postulated MOAs and their key events.

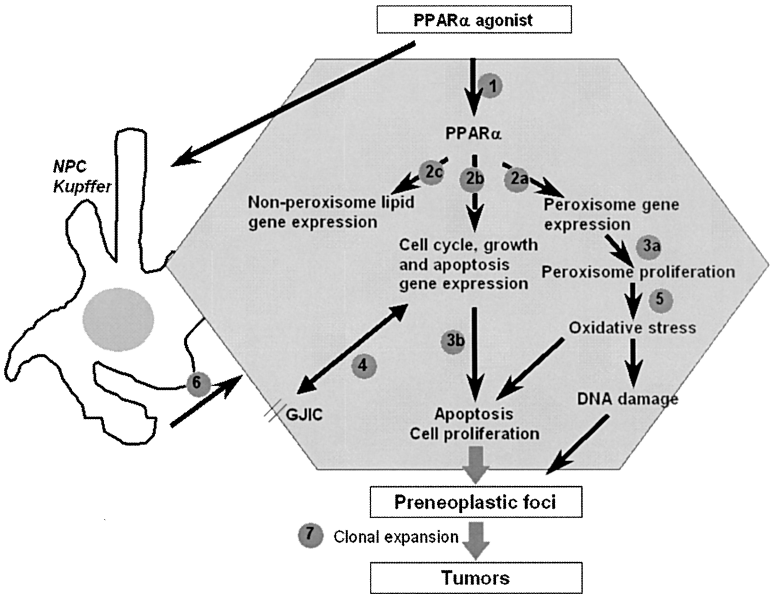

FIGURE 4. Schematic depicting a proposed mode of action (MOA) with key events of rodent liver tumors induced by PPARα ligands. PPARα ligands activate PPARα (key event 1), which then regulates the transcription of genes involved in peroxisome proliferation, cell cycle/apoptosis and lipid metabolism (key events 2a, 2b, and 2c), leading to perturbations in cell proliferation and apoptosis (key event 3b) and peroxisome proliferation (key event 3a). Suppression of apoptosis coupled with a stimulation of cell proliferation allows DNA-damaged cells to persist and proliferate, giving rise to preneoplastic foci and ultimately to tumors via further clonal expansion (key event 7). Peroxisome proliferation may lead to oxidative stress (key event 5), which may contribute to the MOA by causing indirect DNA damage and/or by contributing to the stimulation of cell proliferation. PPARα ligands also inhibit gap junction intercellular communication (GJIC) (key event 4) and stimulate nonparenchymal hepatic Kupffer cells (key event 6); both of these effects also may contribute to the induction of cell proliferation. Numbered key events are listed in the accompanying Table 12 together with an assessment of whether or not the key event is causal in the tumor's etiology or simply associated with it, the strength of the evidence for making that judgment, and whether or not the key event is specific to PPARα agonist MOA or more generally attributed to other carcinogens acting by other MOAs.

B. Proposed Mode of Action: Is the Weight of Evidence Sufficient to Establish the Mode(s) of Action in Rats and Mice?

1. Evidence in Animals/Key Events

a. Peroxisome Proliferator-Activated Receptor-α (PPARα), Target Genes and Receptor Function

In 1990, a receptor termed the "peroxisome proliferator-activated receptor" (PPAR) was cloned from mouse liver using the estrogen receptor DNA binding domain as an anchor for the ligand binding domains of orphan nuclear receptors (Issemann and Green, 1990). The name for this receptor was based on the fact that the receptor is activated by a diverse class of chemicals collectively referred to as PPARα agonists. Since the cloning of that PPAR (now referred to as PPARα), two other PPAR subtypes have been cloned (PPARβ/δ and PPARγ). Further work with PPARα has increased significantly our understanding of how PPARα agonists regulate gene expression and ultimately result in toxicity and cancer. Since the initial discovery in mice, PPARα has been cloned for several species, including other rodents, humans, amphibians, teleosts and cyclostoma (Escher and Wahli, 2000). PPARα is highly expressed in cells that have active fatty acid oxidation capacity, including hepatocytes, cardiomyocytes, enterocytes, and the proximal tubule cells of the kidney. It is well accepted now that PPARα plays a central role in lipid metabolism. PPARα acts as a transcription factor to modulate changes in target

TABLE 12
Key Events for the Mode(s) of Action (MOA) of Rodent Liver Carcinogenesis

Event	Relationship[a]	Weight of evidence	Specificity	Comments
1. Activation of PPARα	Causal	Strong	High	Extensive evidence for PPARα activation Extensive evidence that PPARα null mice are resistant to key events Limited evidence that null mice are resistant to tumors
2a. Expression of peroxisomal genes	Associative	Strong	High	A good biomarker for PP exposure Causal link to cell proliferation and tumors uncertain
2b. PPARα-mediated expression of cell cycle, growth and apoptosis	Associative	Weak	Low	Putative target gene identities are unknown
2c. Nonperoxisome lipid gene expression	Associative	Strong	Low	Mediates hypolipidemia
3a. Peroxisome proliferation	Associative	Strong	High	Causal link to cell proliferation and tumors uncertain Excellent biomarker
3b. Perturbation of cell proliferation (i) and apoptosis (ii)	Causal	Strong	Low	Provides an MOA for carcinogenesis Common to other nongenotoxic carcinogens
4. Inhibition of GJIC	Associative	Strong	Low	Not clear if hepatocyte proliferation regulates GJIC or vice versa GJIC inhibition seen with other nongenotoxic liver carcinogens
5. Hepatocyte oxidative stress	Associative	Weak	Low	Occurs under many circumstances May regulate hepatocyte growth or cause low levels of DNA damage
6. Kupffer cell-mediated events	Associative	Strong	Low	Occurs under many circumstances Independent of PPARα
7. Selective clonal expansion	Causal	Strong	Low	Promotion of spontaneous DNA damage is key event in MOA Seen with all carcinogens

[a]Relationship to hepatic tumors or key events leading to tumors. *Causal*: Required step for PPARα MOA, based on empirical evidence. *Associative*: Events that are occurring but may or may not be causally linked to the MOA. *Weight of evidence (strong, weak): Strong* is normally defined by having several studies which support that MOA, preferably with multiple PPARα agonists from multiple laboratories and with limited evidence of contradiction. *Weak* is normally defined by having a single study with a single PPARα agonist from a single laboratory or a significant amount of contradiction in the literature. *Specificity to PPARα-induced rodent hepatic tumors (high, low): High* is defined as unique to this PPARα MOA. *Low* is defined as not unique to PPARα MOA.

gene expression in response to ligand activation, facilitating increased mobilization, transport, and oxidation of fatty acids, which serve as energy substrates during periods of starvation and/or by pharmacological activation.

Regulation of target gene expression by PPARα

PPARα is a ligand-activated transcription factor that is activated by PPARα agonists as well as by endogenous fatty acids and their metabolites (Dreyer et al., 1992; Gottlicher et al., 1992; Issemann and Green, 1990; Sher et al., 1993). The term "activation" denotes an alteration of the three-dimensional structure of the receptor complex resulting in altered regulation of gene expression. The physical alterations initiated by ligand binding may include events such as loss of heat-shock and chaperone proteins, nuclear translocation, and protein turnover. In various reporter gene expression systems, the formation of a transcriptionally active complex with the addition of ligand is evident by the observation of conformational changes of PPARα and γ using limited proteolysis (Berger et al., 1996; Dowell et al., 1997b). Most receptor activators have been demonstrated to bind to PPAR subtypes with reasonable affinity and several naturally occurring ligands of these receptors have been identified (reviewed in Willson et al., 2000). Many mono- and polyunsaturated fatty acids bind directly to PPARα at physiological concentrations and cause transcriptional activation. Long-chain unsaturated fatty acids such as linoleic acid, polyunsaturated fatty acids (PUFAs) including arachidonic, eicosapentaenoic, and linolenic acids, and the branched-chain fatty acid phytanic acid bind to PPARα with reasonable affinity (in the μM range) (Willson et al., 2000). The eicosanoids 8(*S*)-hydroxyeicosatetraenoic acid (8(*S*)-HETE) and leukotriene B4 (LTB4) are reported to be PPARα ligands. Despite the higher affinity of these compounds in in vitro systems, it is unclear whether the endogenous concentration of 8*S*-HETE or LTB4 is sufficient to cause activation of PPARα in vivo. This has led some investigators to speculate that PPARα has evolved to respond to the cumulative amount of intracellular fatty acids (Willson et al., 2000). There are considerable differences in the ability of various PPARα agonists to bind to PPARα as measured by appropriate ligand binding assays (see Table 13). For example, clofibrate has a relative affinity to bind 50% of PPARα in the range of 100–1000 μM while the more potent PPARα agonist WY 14,643 has a relative affinity ranging from 0.6 to 30 μM. Additionally, the very high affinity ligand for PPARα (GW2331) has a relative binding affinity of 30 nM. Further, relative binding affinity correlates well with the ability to activate PPARα target gene expression (Krey et al., 1997) as measured by transient transfection assays (see Table 14).

Heterodimerization of PPAR and RXRα leads to binding to PPREs and subsequent modulation of target gene transcription. However, if PPAR

TABLE 13
Differences in Ligand Binding Affinity for Representative PPARα Agonists

PPARα agonist	$EC_{50}/IC_{50}/LIC_{50}$[a]	PPARα-species[b]	Assay[c]	Reference
MEHP	50 μM	Mouse	CAT	Issemann and Green (1990)
Clofibrate	100–1000 μM	*Xenopus*	CARLA	Krey et al. (1997)
WY 14,643	30 μM	*Xenopus*	CARLA	Krey et al. (1997)
WY 14,643	0.6 μM	Mouse	LIC	Forman et al. (1997)
WY 14,643	<1 μM	Mouse	LIC	Kliewer et al. (1997)
GW2331	0.15 μM	Human	SPA	Mukherjee et al. (2002)
	0.14–0.39 μM, depending upon ligand	Human	FRET	Mukherjee et al. (2002)
	30 nM	Human	SPA	Moya-Camerana et al. (1999)

[a] EC_{50}: Effective concentration to induce 50% recruitment of co-activator to PPARα (CARLA). IC_{50}: Concentration required to cause 50% inhibition of radiolabeled ligand binding by unlabeled ligand (SPA). $ILIC_{50}$: Concentration required to cause 50% induction of a ligand–receptor complex (LIC).
[b] The species of PPARα origin used for this assay.
[c] The assay used to determine EC_{50}, IC_{50}, or LIC_{50}. CAT: Chloramphenicol acetyl transferase activity assay. CARLA: Co-activator-dependent receptor ligand assay. LIC assay: ligand-induced complex formation. SPA: scintillation proximity assay. FRET: fluorescence resonance energy transfer assay.

TABLE 14
Differences in Transactivation for Representative PPARa Agonists

PPARα agonist	EC_{50}	PPARα species[a]	RE-species[b]	Conc. range[c]	Assay	Reference
MEHP	>500 μM	Mouse	ACO-rat	0–750 μM	Transfection	Hasmall et al. (2000b)
MEHP	>400 μM	Human	ACO-rat	0–750 μM	Transfection	Hasmall et al. (2000b)
MEHP	0.5–1.0 μM	Mouse	BIEN-rat	0–20 μM	Transfection	Maloney and Waxman (1999)
MEHP	~5μM	Human	BIEN-rat	0–20 μM	Transfection	Maloney and Waxman (1999)
Clofibrate	~100 μM	Human	Cyp4a6-rabbit	0–1000 μM	Transfection	Sher et al. (1993)
Clofibrate	10–100 μM	Human	ACO-rat	0–1000 μM	Transfection	Sher et al. (1993)
Clofibrate	55 μM	Human	Gal4-yeast	NP	Transfection–chimeric receptor	Shearer and Hoekstra (2003)
Clofibrate	200 μM	Mouse	ACO-rat	NP	Transfection	Krey et al. (1997)
Clofibrate	50 μM	Mouse	Gal4-yeast	NP	Transfection–chimeric receptor	Shearer and Hoekstra (2003)
WY 14,643	1–10 μM	Human	Cyp4a6-rabbit	0–100 μM	Transfection	Sher et al. (1993)
WY 14,643	1–10 mM	Human	ACO-rat	0–100 μM	Transfection	Sher et al. (1993)
WY 14,643	5 μM	Human	Gal4-yeast	NP	Transfection–chimeric receptor	Shearer and Hoekstra (2003)
WY 14,643	0.63 μM	Mouse	Gal4-yeast	NP	Transfection–chimeric receptor	Shearer and Hoekstra (2003)
Fenofibrate	30 μM	Human	Gal4-yeast	NP	Transfection–chimeric receptor	Shearer and Hoekstra (2003)
Fenofibrate	18 μM	Mouse	Gal4-yeast	NP	Transfection–chimeric receptor	Shearer and Hoekstra (2003)
GW2331	10 nM	Mouse	Gal4-yeast	NP	Transfection–chimeric receptor	Kliewer et al. (1997)
GW2331	50 nM	Human	Gal4-yeast	NP	Transfection–chimeric receptor	Kliewer et al. (1997)
GW409544	2.3 nM	Human	Gal4-yeast	NP	Transfection–chimeric receptor	Xu et al. (2001)
Phenylacetic acid PPARα agonist	16 nM	Human	Gal4-yeast	NP	Transfection–chimeric receptor	Lawrence et al. (2001a)
Phenylacetic acid PPARα agonist	10 μM	Mouse	Gal4-yeast	NP	Transfection–chimeric receptor	Lawrence et al. (2001a)

Note. NP, data not provided.
[a]The species of PPARα origin used for this assay.
[b]The target gene responsive element and species of origin.
[c]The concentration range used for the assay.

interacts with another transcription factor, this complex may no longer associate with DNA and the interacting protein may be removed from its normal site of action. For example, the cross-talk between PPAR and thyroid hormone receptor is due, at least in part, to direct association of the two proteins. PPARα agonists may inhibit thyroid hormone's ability to regulate gene expression by increasing the formation of an inactive PPARα/TR complex (Hunter et al., 1996; Miyamoto et al., 1997). Similarly, PPARα inhibits the effects of C/EBPα (Hollenberg et al., 1997), LXRα (Miyata et al., 1996) and GHF-1 (Tolon et al., 1998) through a non-DNA binding mechanism. PPAR exerts some of its anti-oxidant effects by blocking the association of NF-kappaB, AP1, STAT1α and Ets to their cognate DNA elements (Ricote et al., 1998). A classic example of how PPARα agonists may regulate gene expression via this route was given by Sakai et al. (1995), with reference to glutathione *S*-transferase π (GST-P) expression. PPARα agonists regulate GST-P expression by decreasing activity at the AP-1 site of this gene, presumably the result of a PPARα/jun dimerization (Sakai et al., 1995). The interactions of steroid hormone receptors with the AP-1 complex are well recognized (Mohamood et al., 1997; Zhang et al., 1991) and may have important consequences in the regulation of growth regulatory gene expression.

Steroid hormone receptor co-activators and co-repressors are an important class of proteins that can interact with PPARα. Cofactors directly interact with nuclear receptors and repress (co-repressors) or enhance (co-activators) their transcriptional activities in a receptor-, tissue-, and gene-specific manner (Horwitz et al., 1996). In the absence of ligand, PPARα may bind to co-repressors, leading to decreases in receptor activity. Ligand binding induces a conformational change in PPARα that might favor binding to co-activators. The receptor/coactivator complex then can activate gene transcription by means of recruiting chromatin-modifying enzymes (i.e., histone acetyl transferases) (Horwitz et al., 1996). DNA-bound receptors appear to function as large multiprotein complexes, the constituents and function of which are dependent on the cell and promoter context. A partial list of cofactors that might interact with PPARα include PBP (Zhu et al., 1997), SRC-1 (Zhu et al., 1996), CBP/P300 (Dowell et al., 1997a), RIP140 (Treuter et al., 1998), PGC-1 (Puigserver et al., 1998), TRAP220 (Yuan et al. 1998), PRIP (Zhu et al., 2000), RAP250 (Caira et al., 2000) (co-activators) and NcoR (Dowell et al., 1999) and SMRT (Yan et al., 1998) (co-repressors).

Administration of PPARα agonists causes changes in numerous mRNAs and many of these genes are central to lipid metabolism. For example, mRNAs encoding peroxisomal, mitochondrial and microsomal fatty acid metabolizing enzymes all are increased by PPARα agonists (Bocher et al., 2002; Hihi et al., 2002; Shearer et al., 2003). Further, liver mRNAs encoding proteins involved in lipid transport including fatty acid binding protein, fatty acid transporters, lipoprotein lipase, and apolipoproteins also are regulated by these chemicals (Bocher et al., 2002; Hihi et al., 2002; Shearer et al., 2003). In addition to modifying gene expression essential to lipid metabolism, PPARα agonists also can influence mRNAs encoding proteins that regulate cell proliferation or the acute phase response. Lastly, PPARα can mediate alterations in mRNAs associated with steroid metabolism (17β-HSD), prostaglandin synthesis (COX2), iron metabolism (transferrin), nitrogen metabolism (GOT, GPT), and hepatitis B viral replication (Peters et al., 2002). While it is clear that PPARα is critical in the regulation of lipid metabolism and that this receptor likely regulates the cell proliferation/apoptosis underlying PPARα agonist-induced hepatocarcinogenesis, the precise role for some of these alterations in gene expression is not understood. For many of the target genes that regulate lipid metabolism, functional PPREs have been identified in their respective promoter region, including peroxisomal fatty acid metabolizing enzymes, fatty acid transporter, apolipoproteins, and lipoprotein lipase (Desvergne et al., 1999). Identification of PPREs in target genes is an ongoing research area that will likely confirm the presence of functional responsive elements for many target genes that have not yet been established. However, it is also possible that alterations in mRNA expression are an indirect effect induced by a PPARα-specific target gene that, in turn, regulates expression of a subsequent mRNA. For example, while it is clear that CDK and cyclin expression are increased by PPARα agonists (Peters et al., 1998), data demonstrating the presence of a functional PPRE is lacking. Additionally, there is indirect evidence suggesting alterations in other proteins following PPARα agonist administration, yet confirmation of corresponding increased mRNA is lacking. For example, in vitro data suggest that Cu/Zn superoxide dismutase (SOD) is modulated by PPARα (Yoo et al., 1999). Further, analysis of enzyme activities suggest that acyl CoA carboxylase (Maragoudakis and Hankin, 1971) and enzymes that regulate phospholipid levels (Mizuguchi et al., 1999) also are modulated by PPARα agonists.

Physiological role for PPARα activation

PPARα regulates lipid homeostasis through the modulation of expression of genes involved in fatty acid uptake, activation and oxidation (Desvergne et al., 1998; Desvergne and Wahli, 1999; Schoonjans et al., 1996a, 1996b; Wahli et al., 1995). Activation of PPARα increases expression of lipoprotein lipase, fatty acid binding protein and fatty acid translocase, all of which may contribute to increased intracellular uptake of fatty acids (Besnard et al., 1993; Kaikaus et al., 1993; Lee et al., 1995; Martin et al., 1997; Motojima et al., 1998; Schoonjans et al., 1995; Staels and Auwerx, 1992; Vanden Heuvel et al., 1993). Expression of hepatic apolipoproteins that influence lipoprotein uptake also can be altered by PPARα activation, such as apolipoproteins CII, CIII, AI, AII, and AIV (Andersson et al., 1999; Berthou et al., 1995; Staels et al., 1992, 1995). Long-chain fatty acids typically are activated to CoA thioesters by acyl CoA synthase, another gene product that is regulated by PPARα (Martin et al., 1997; Schoonjans et al., 1995). Interestingly, thioesterases that catalyze the hydrolysis of acyl CoAs to the corresponding free fatty acid and CoA also are responsive to PPARα activation (Hunt et al., 2000). Transport of fatty acids across the mitochondrial membrane by carnitine palmitoyl transferases (CPTs) also is under control of PPARα (Aoyama et al., 1998; Brandt et al., 1998; Mascaro et al., 1998). Oxidation of fatty acids is influenced significantly by PPARα as peroxisomal, microsomal, and mitochondrial fatty acid oxidizing enzymes all are induced by treatment with PPARα agonists (Aoyama et al., 1998; Lee et al., 1995). It is clear that PPARα has a central role in regulating lipid homeostasis by modulating gene expression in response to both exogenous and endogenous activation. For example, many of the changes in target gene expression that modulate fatty acid uptake, activation, and oxidation are facilitated by PPARα in response to treatment with PPARα agonists, dietary fatty acids and fasting. However, alterations in mRNAs encoding lipid-related enzymes such as uncoupling proteins (UCPs), CPTs, and lipogenic proteins observed with dietary fatty acids and fasting are independent of PPARα since these changes are found in both wild-type and PPARα-null mice (Kersten et al. 1999; Leone et al., 1999; Ren et al., 1997).

In addition to being a key regulator of fatty acid metabolism in response to exogenous and endogenous activation, PPARα also has been shown to modulate other physiological functions. It is thought that this receptor regulates the inflammatory response induced by leukotriene B4 by altering catabolism of this endogenous ligand (Devchand et al., 1996). PPARα also inhibits interleukin-1 (IL-1) signaling leading to cyclooxygenase-2 induction by interacting with, and negatively regulating, NF-kappaB (Staels et al., 1998). Given the influence of PPARα in modulating inflammation and fatty acid catabolism, it is not surprising that there is evidence demonstrating a direct role for this PPAR subtype in the molecular mechanisms suppressing atherosclerosis (Neve et al., 2000). Given the central role of this receptor in lipid-related pathways, it also is likely that other lipid dependent functions will be modified as a result of PPARα activation.

Peroxisome proliferation in the absence of PPARα

Subsequent to the demonstration that PPARα-null mice were refractory to peroxisome proliferation induced by the potent agonist WY 14,643 (Lee et al., 1995), it became widely assumed that peroxisome proliferation was mediated exclusively through that receptor. Thus, in the course of developing PPARγ agonists for therapeutic intervention in non-insulin-dependent diabetes mellitus (NIDDM), one of the criteria for identification of leads used by some pharmaceutical companies was a high level of specificity for the γ subtype in order to avoid the liability of rodent liver tumors. However, in the course of routine screening for peroxisome proliferation potential in mice, it was noted that even compounds with a very high degree of specificity for PPARγ over PPARα displayed a significant degree of peroxisome proliferation potential at supra-pharmacological doses (DeLuca et al., 2000). In following up on this observation, these authors demonstrated that agents with γ- or γ/δ-specificity could induce some of the pleiotropic effects of peroxisome proliferation in mice lacking a functional PPARα. Since some of the effects were attenuated in the null mice relative to the wild-type mice, particularly the induction of acyl CoA oxidase (ACO), they concluded that the effects seen in wild-type mice resulted from both "cross-reactivity" of the compounds tested (i.e., weak PPARα agonism) and, more interestingly, functional overlap ("cross-talk") among the PPAR family members.

A retrospective search of the literature reveals that there were some earlier indications of non-PPARα-related peroxisome proliferation. Edvardsson et al. (1999) observed indications of

peroxisome proliferation in the livers of ob/ob mice in response to treatment with rosiglitazone, a PPARγ-selective thiazolidinedione drug. Interestingly, they did not observe this effect in lean mice (the doses used were much lower than in the DeLuca et al., study) and hypothesized that the increased levels of PPARγ2 in the livers of ob/ob mice were responsible for this apparent cross-talk. It is interesting to note that Costet et al. (1998) have observed increased PPARγ expression in the fat-filled hepatocytes that accumulate in the livers of aged PPARα-null mice; however, DeLuca et al. (2000) did not observe the same phenomenon in the young mice they used in their studies.

Indications of cross-talk between the α and γ isoforms also have been observed in the context of adipogenesis (Brun et al., 1996). These authors found that PPARα was capable of weakly stimulating adipogenesis when exposed to strong agonists (but, interestingly, not PPARδ). More recently, other investigators also have begun to observe indications of peroxisome proliferation in PPARα-null mice, generally consisting of modest increases in liver weight and/or increases in expression of acyl-CoA mRNA levels (J. Peters and C. Corton, personal communications). Thus, it would appear that the selectivity of the PPAR family members for their respective DREs can depend on the potency of the agonist and, presumably, the cellular context.

Conclusions

Activation of PPARα is related causally to induction of rodent liver tumors. The weight of evidence is strong and the specificity is high because PPARα-null mice are refractory to tumor formation in response to a prototypical PPARα agonist (WY 14,643). This conclusion would be strengthened by the conduct of additional bioassays with other PPARα agonists. Regulation of peroxisomal acyl CoA oxidase in response to activation by PPARα agonists is associative with rodent tumor formation. The weight of evidence is strong because virtually all PPARα agonists that cause high-level induction of acyl CoA also cause rodent liver tumors. The specificity is high because the induction of acyl CoA requires PPARα. Selective activation of acyl CoA-induced oxidative stress in the absence of increased hyperplastic signaling has not been performed. Regulation of genes that mediate cell cycle progression, growth, and apoptosis in response to activation by PPARα agonists is deemed to be an associative event in the induction of rodent liver tumors. The weight of evidence is weak, and while it is known that changes in genes that regulate the cell cycle in response to PPARα agonists require the PPARα, the putative target gene identities are unknown. Whether or not this effect is due to a direct interaction between PPARα and a functional PPRE in these genes is uncertain. Regulation of lipid metabolizing enzymes in response to activation by PPARα agonists is associative with rodent tumor formation. The weight of evidence is strong because virtually all PPARα agonists that cause induction of genes encoding lipid-metabolizing enzymes also cause rodent liver tumors and these changes in gene expression require the PPARα. The specificity of alterations in gene encoding lipid-metabolizing enzymes is unknown because their role (if any) in modulating changes that lead to liver tumor formation is uncertain, as they have not been examined in an appropriate model system.

b. Cell Proliferation and Apoptosis

Many nongenotoxic carcinogens, including the PPARα agonists, appear to elicit their carcinogenic effects through the induction of cell proliferation. Increased cell replication produced by PPARα agonists may increase the frequency of spontaneous mutations by increasing the frequency of errors in DNA repair or replication, or may alter methylation of the genome leading to a change in gene expression (Cattley et al., 1991, 1998; Huber et al., 1991). Most peroxisome proliferators produce a strong, albeit transient, increase in replicative DNA synthesis during the first few weeks of exposure. This increase in replicative DNA synthesis is restored to baseline levels within 2 to 4 weeks of treatment for all PPARα agonists except WY 14,643, where S-phase persists above control for the duration of treatment. Several studies have demonstrated that peroxisome proliferators promote the growth of chemically and spontaneously induced lesions through enhanced cell replication (Cattley and Popp, 1989; Cattley et al., 1991; Isenberg et al., 1997; Marsman et al., 1988).

The induction of cell proliferation in liver by PPARα agonists is believed to enhance the rate of fixation of DNA damage in the genome, leading to changes in gene expression such as the silencing of tumor suppressor genes or increased expression of oncogenes. These changes facilitate clonal expansion of initiated cells, leading to the formation of hepatic focal lesions (Cattley et al., 1991, 1998; Huber et al., 1991). Once early lesions are formed, continued exposure to PPARα agonists causes a

selective increase in DNA replication of up to approximately 40% in these liver foci, while replication of hepatocytes in the normal surrounding liver is increased only slightly. Furthermore, preneoplastic foci respond to the cell replicative rather than the peroxisomal effects of PPARα agonists, suggesting that the growth stimulus is of particular significance for the carcinogenic action of this class of compounds (Grasl-Kraupp et al., 1993).

In addition to the ability of PPARα agonists to induce rodent liver cell proliferation, there is some evidence that nongenotoxic carcinogens, in general, can suppress the hepatocyte apoptosis that may normally remove DNA-damaged, potentially tumorigenic, cells from the liver (Bayly et al., 1994; Cunningham et al., 1995; James and Roberts, 1996; Oberhammer and Qin, 1995; Roberts et al., 1995; Schulte-Hermann et al., 1995). Apoptosis is a conserved process with several functions in eukaryotic organisms, including maintenance of correct cell number and removal of damaged cells. Cells undergoing apoptosis exhibit a characteristic series of changes, including DNA cleavage, nuclear breakdown, dramatic changes in cell membrane structure, and fragmentation of cytoplasmic organelles (Alison and Sarraf, 1995; reviewed in Wyllie, 1993). Studies conducted in vitro show that nafenopin, methylclofenapate, and WY 14,643 all suppress both spontaneous hepatocyte apoptosis and that induced by the physiological negative regulator of liver growth, transforming growth factor β1 (TGFβ1) (Bayly et al., 1994; Oberhammer and Qin, 1995). In addition, PPARα agonists can suppress apoptosis induced by diverse stimuli such as DNA damage or ligation of Fas, a receptor related to the tumor necrosis factor alpha (TNA alpha) family of cell surface receptors (Gill et al., 1998). These in vitro data are supported by in vivo studies that show suppression of apoptosis after 2 days of dosing with nafenopin or DEHP and also by studies where nafenopin, cyproterone acetate (CPA), and phenobarbital can suppress the apoptosis caused by CPA withdrawal (Bursch et al., 1984). Thus, the cells that would normally be removed by apoptosis may persist for further mitogenic stimulation by nongenotoxic carcinogens, giving rise to tumors.

These data that illustrate the suppression of apoptosis by PPARα agonists refer to the acute phase of PPARα agonist treatment (days 1–20) when the liver is increasing in size. However, once a steady state of liver enlargement is reached, levels of apoptosis are likely to return to background. One report suggests that continued exposure to WY 14,643 results in an increase in apoptosis with time (Burkhardt et al., 2001). However, WY 14,643 is unusual in that the induction of S-phase persists, which is not the case for other PPARα agonists. Thus, it seems reasonable that suppression of apoptosis would persist to compensate in the case of WY 14, 643. Interestingly, both cell proliferation and apoptosis increase in PPARα agonist-induced tumors in the rat compared with normal surrounding tissue, suggesting that cell turnover is increased in tumorigenic lesions (Grasl-Kraupp et al., 1997).

PPARα agonists have been shown to produce multiple tumor precursor effects including liver hyperplasia, sustained elevation of cell turnover, and altered growth in preneoplastic foci (Butterworth, 1990; Marsman et al., 1988). The exact mechanisms by which PPARα agonists induce hepatic cancer in rodents are not fully understood but studies have demonstrated that the response of the liver to peroxisome proliferators, including the suppression of apoptosis and induction of cell proliferation, requires PPARα. Evidence for the involvement of PPARα in replicative DNA synthesis and hepatocarcinogenesis comes largely from work with PPARα-null mice following dietary exposure to WY 14,643. In these studies, it was shown that wild-type mice showed increased hepatic labeling by bromodeoxyuridine (BrDU) compared to untreated controls while no increase in hepatic labeling index was observed in the null (−/−) mice. Also, chronic treatment with WY 14,643 resulted in a 100% incidence of hepatocellular neoplasia in wild type mice while the null mice were unaffected (Peters et al., 1997a, 1997b). Additionally, the ability of PPARα agonists to suppress apoptosis was lost in hepatocytes isolated from PPARα-null mouse livers. Also, changes in cell cycle genes such as CDK-1, CDK-2, CDK-4, PCNA, and c-myc proteins and CDK-1, CDK-4, cyclin D1, and c-myc mRNA seen in wild-type mice fed WY 14,643 were not observed in PPARα-null mice (Peters et al., 1998).

The development of hepatocellular neoplasms following treatment with PPARα agonists is species-specific and appears to correlate with the induction of DNA synthesis (Huber et al., 1996; James and Roberts, 1996). While peroxisome proliferators produce a wide spectrum of effects in rat and mouse liver, other species such as the Syrian hamster and guinea pig are less responsive and appear refractory to the induction of DNA synthesis. A transient increase in hepatic replicative DNA synthesis following administration of tumorigenic doses of DEHP was seen in rats and mice, but not in hamsters (Isenberg et al., 2000). Additionally, several peroxisome proliferators (DEHP, DINP, and clofibrate)

had no effect on DNA synthesis in cynomolgus monkeys (Pugh et al., 2000). Humans also appear to be nonresponsive to the adverse liver effects of PPARα agonists. Several groups have shown that, following treatment with several of these agents, no increase in DNA synthesis was seen in primary human hepatocytes. Collectively, these data provide evidence for species differences in the hepatic response to them and it further has been concluded, based on a lack of induction of DNA synthesis in human hepatocytes, that, unlike mice and rats, human liver cells appear to be refractory to hepatocarcinogenesis induced by PPARα agonists (Hasmall et al., 2000b; Perrone et al., 1998).

c. Role of Hepatic Nonparenchymal Cells in the Rodent Response to Peroxisome Proliferators

Hepatic nonparenchymal cells (NPCs), particularly the dedicated macrophages known as Kupffer cells, have been implicated in the hepatic proliferative response to PPARα agonists. Evidence for this was derived initially from in vivo studies from one laboratory that showed that use of Kupffer cell inhibitors such as methyl palmitate or glycine could prevent the induction of hepatic DNA synthesis in response to WY 14,643 (Rose et al., 1997a, 1997b). Since then, in vitro studies have shown that the removal of hepatic NPCs prevents the proliferative response to the peroxisome proliferators WY 14,643 and nafenopin (Hasmall et al., 2000a; Parzefall et al., 2001). In light of these in vitro results, one should be mindful of the potential meaning of results with respect to responsiveness (or lack thereof) in human hepatocyte assay systems that would have had the Kupffer cells removed during preparation.

Peroxisome proliferators appear to activate Kupffer cells directly through mechanisms involving oxygen radicals, protein kinase C, and the transcription factor nuclear factor-kappa B (NF-kappaB) (Rose et al., 1999). NF-kappaB activation and binding were seen shortly after WY 14,643 administration, but pretreatment with allopurinol, a xanthine oxidase inhibitor and free radical scavenger, suppressed NF-kappaB activation by WY 14,643. It was concluded that NF-kappaB is activated by reactive oxygen species and plays a central role in the mechanism of action of PPARα agonists (Rusyn et al., 1998). WY 14,643 also was shown to produce a rapid oxidant-dependent activation of NF-kappaB in Kupffer cells in vivo and activated superoxide production by isolated Kupffer cells (Rusyn et al., 2000).

Addition of nonparenchymal cells, or conditioned medium from nonparenchymal cell cultures, increased DNA synthesis. DNA synthesis induced by nafenopin and TNFα was prevented by the removal of nonparenchymal cells from normal hepatocyte cultures and was restored by returning nonparenchymal cells to the purified hepatocytes (Hasmall et al., 2000a; Parzefall et al., 2001). This finding provides additional evidence that nonparenchymal cells are permissive for the growth response of hepatocytes to PPARα agonists in vitro, and this may be mediated, at least in part, by TNFα. In addition, it is likely that oxidants play a role in signaling hepatocellular proliferation following exposure to PPARα agonists via activation of NF-kappaB and an increase in mitogenic cytokines such as TNFα. In addition, the activation of NF-kappaB by peroxisome proliferators is species specific. This class of compounds activates NF-kappaB in rat and mouse liver, while they do not induce any changes in hamsters. This may help to explain the differences seen between species in relationship to cell proliferation (Tharappel et al., 2001).

Although it is clear that hepatic NPCs are required for the proliferative response to PPARα agonists, the mechanisms of this dependency are unclear. Activated NPCs, particularly Kupffer cells, are implicated in producing oxygen radicals and cytokines such as tumor necrosis factor α (TNFα) and interleukin-1 (IL1). In support of this, TNFα is able to increase hepatocyte proliferation and suppress apoptosis in cultured rodent hepatocytes (Holden et al., 2000; Rolfe et al., 1997). Furthermore, the hepatocyte growth response to PPARα agonists can be prevented by antibodies to either TNFα (Bojes et al., 1997; Rolfe et al., 1997) or TNFα receptor 1 (TNFR1) (West et al., 1999). One group reported that treatment with PPARα agonists increases TNFα mRNA more than two-fold (Bojes et al., 1997; Rolfe et al., 1997) but this was not seen by others (Anderson et al., 2001; Holden et al., 2000). Thus, perhaps treatment with PPARα agonists may not mediate *de novo* TNFα gene expression, suggesting that the response to this class of compounds may be mediated by bioactivation or release of preexisting TNFα protein from Kupffer cells (Holden et al., 2000). Kupffer cells are thought to produce free radicals via NADPH oxidase, leading to activation of NF-kappaB and production of TNFα leading to the induction of hepatocyte S-phase.

In contrast to these data arguing for a role for TNFα, the proliferative response of hepatocytes

to PPARα agonists is intact both in TNFα- and in TNFα receptor-null mice (Givler et al., 2000; Lawrence et al., 2001b), refuting any specific role for this cytokine in the response to PPARα agonists. Current data suggest that nonparenchymal Kupffer cells are required but not sufficient for the response to PPARα agonists.

As noted previously, the response to PPARα agonists is mediated by PPARα since PPARα-null mice are refractory to PPARα agonist-induced growth perturbation and peroxisome proliferation (Christensen et al., 1998; Hasmall et al., 2000b; Lee et al., 1995; Peters et al., 1997a, 1997b). However, it has been shown that Kupffer cells do not express PPARα (Peters et al., 2000), suggesting that their role in the response to PPARα agonists is independent of PPARα. This was confirmed recently by the demonstration that NPCs isolated from a PPARα-null mouse can restore the response of wild-type isolated hepatocytes to PPARα agonists (Hasmall et al., 2000c). These data suggest that NPCs are required for the response to PPARα agonists. However, NPCs also are implicated in the response to many other liver xenobiotics (Roberts and Kimber, 1999), questioning their specificity to the MOA of PPARα agonists.

While it is generally accepted that stimulation of hepatocellular proliferation by PPARα agonists is a central event in the mechanism of carcinogenesis, little is known about the precise signaling that is involved. In addition to a PPARα-related pathways in hepatocytes, a role for mitogenic cytokines produced by Kupffer cells in PPARα-independent manner has been suggested (Rusyn et al., 2000b). It appears that reactive oxygen species play a role in signaling proliferatory events in both cell types by acting as activators of a transcription factor, nuclear factor (NF-kappaB). In whole liver of both rats and mice, activity of NF-kappaB is increased by a number of peroxisome proliferators as early as 2 hours after the first treatment (Li et al., 1996; Rusyn et al., 1998). Interestingly, the timing of NF-kappaB activation is different in Kupffer and parenchymal cells. It was shown that after a single in vivo dose of WY 14,643, NF-kappaB activity is increased first in Kupffer cells (at 2 hours), and only about 6 hours later, is it increased in parenchymal cells (Rusyn et al., 1998)

The molecular events that occur in Kupffer cells have been studied extensively. It has been shown that oxidants play a role in signaling increases in cell proliferation via a mechanism involving activation of NADPH oxidase, production of superoxide, activation of NF-kappaB, and release of mitogenic cytokines (Rose et al., 2000). Since Kupffer cells are known to be activated by PPARα agonist treatment in vivo (Bojes and Thompson, 1996), it was hypothesized that such activation would result in increased production of oxidants. To address this hypothesis, Kupffer cells were isolated from untreated rats, cultured in the presence of peroxisome proliferators, and superoxide production was assessed. Indeed, WY 14,643 and monoethylhexyl phthalate (MEHP), a key lipophilic metabolite of DEHP, increased superoxide production in isolated Kupffer cells in a dose-dependent manner, indicating that they can affect Kupffer cells directly (Rose et al., 1999). Since NADPH oxidase is a major superoxide-producing enzyme in macrophages, it was hypothesized that it is involved in oxidant-dependent activation of NF-kappaB by the peroxisome proliferator WY 14,643 in Kupffer cells (Rusyn et al., 2000a, 2000b). Indeed, both activation of NF-kappaB and increases in cell proliferation following a single dose of WY 14,643 were prevented completely when rats were pretreated with diphenyleneiodonium, an inhibitor of NADPH oxidase. $p47^{phox}$ is a critical subunit of NADPH oxidase; therefore, $p47^{phox}$ knockout mice were used specifically to address the hypothesis of involvement of NADPH oxidase in the mechanism of PPARα agonist-induced hepatocellular proliferation. In livers of wild-type mice, WY 14,643 activated NF-kappaB, which then was followed by an increase in mRNA for TNFα. Importantly, these changes did not occur in $p47^{phox}$ knockouts. Moreover, when Kupffer cells were treated with WY 14,643 in vitro, superoxide production was induced in cells from wild-type but not $p47^{phox}$-null mice. Finally, when mice were fed a WY 14,643-containing diet for 7 days, the resulting increase in liver weight and cell proliferation in wild-type mice did not occur in $p47^{phox}$-null mice. Combined, these studies shed light on oxidant-mediated signaling events in Kupffer cells that contribute to increased cell proliferation due to peroxisome proliferators.

The role of reactive oxygen species in proliferative signaling in parenchymal cells is less well understood. As was noted above, activation of NF-kappaB was observed in parenchymal cells after treatment with PPARα agonists (Rusyn et al., 1998). One possible explanation for this increase is that mitogenic cytokines produced by Kupffer cells would activate NF-kappaB in the course of their normal signaling through extracellular receptors. Alternatively, it was shown that NF-kappaB can be activated directly by a peroxisome proliferator in a responsive H4IIEC3 rat hepatoma cell line (Li et al., 2000a). Thus, it was suggested that increased

NF-kappaB activity is secondary to the action of H_2O_2-generating enzymes, such as fatty acyl CoA oxidase, since overexpression of this enzyme in COS-1 cells, in the presence of an H_2O_2-generating substrate, can activate an NF-kappaB-regulated reporter gene (Li et al., 2000b).

d. Oxidative Stress

Experimental evidence suggests an intimate association of reactive oxygen species with the mechanism of tumorigenesis by PPARα agonists. It has been hypothesized that such overproduction of oxidants might cause DNA damage and lead to mutations and cancer (Reddy and Rao, 1989; Yeldani et al., 2000). In addition, reactive oxygen species play a signaling role in a rapid increase of parenchymal cell proliferation caused by peroxisome proliferators (Rusyn et al., 2000a, 2000b). Thus, it appears that oxidant-related molecular events are intertwined with other pathways activated by PPARα agonists in vivo in rodent liver, and therefore are important to the understanding of the mechanisms of their action.

Evidence for increased production of reactive oxygen species

In rodent liver, PPARα agonists induce increases in the activity of enzymes that generate and degrade hydrogen peroxide and oxidize DNA, lipids, and other molecules. Numerous studies suggest that possible sources of oxidants in the liver of rats and mice treated with these chemicals are the peroxisome, mitochondria, microsomal oxidases, or NADPH oxidase in Kupffer cells. Reddy and coworkers originally proposed that fatty acyl CoA oxidase in the peroxisome is the enzyme responsible for oxidative stress by PPARα agonists (Nemali et al., 1988); however, their group found that mice lacking this protein, instead of being protected from chemically induced liver cancer, developed liver tumors spontaneously, possibly as a result of a hyperactivation of PPARα by unmetabolized lipids (Fan et al., 1998). Despite the evidence that oxidative stress plays a role in the mechanism of action of these compounds, precise molecular sources of reactive oxygen species that are activated by peroxisome proliferators are still a subject of debate.

A summary of various indirect confirmations for PPARα agonist-initiated increases in oxidants is presented in Table 15. It should be noted, however, that causative relevance of some of this evidence to the carcinogenic effect of PPARα agonists has been questioned; contrasting views are noted in the footnotes.

In addition, in vivo evidence for PPARα agonist-induced production of oxidants was obtained by using the spin-trapping technique and electron spin resonance (ESR) spectroscopy (Rusyn et al., 2001) when rats were given di(2-ethylhexyl) phthalate (DEHP); an ESR-detectable radical adduct signal was detected in bile fractions collected during the first 2 hours of treatment. No later time points were analyzed in this study. The radical species detected in this experiment were shown to be derived from the hydroxyl radical. The formation of ESR-detectable radical adducts was measured prior to detectable increases in proliferation of peroxisomes in parenchymal cells, and it was shown that NADPH oxidase in Kupffer cells was a molecular source of the hydrogen peroxide-derived radical species.

Oxidants and DNA damage in the carcinogenesis of PPARα agonists

Oxidative stress to DNA has been hypothesized to be a common pathway for many non-genotoxic chemical carcinogens (Klaunig et al., 1998). An overwhelming number of studies draw a connection between chemical exposure, DNA damage, and cancer based on measurement of 8-hydroxy-2′-deoxyguanosine (8-OH-dG), a highly mutagenic lesion, in DNA isolated from organs of treated animals in vivo (reviewed in Kasai, 1997). The development of sensitive high-performance liquid chromatography/electrochemical detection (HPLC/ECD) technique for measurements of this adduct (Floyd et al., 1984) resulted in a number of studies showing increases in 8-OH-dG in a variety of disease states and after treatment with a number of diverse toxic compounds. Kasai et al., (1989) showed that treatment with the PPARα agonist ciprofibrate leads to a twofold increase in the number of 8-OH-dG adducts in rat liver genomic DNA. Similar observations were made by other groups for a variety of compounds, doses, and timings of treatment (Tagaki et al., 1990b; Qu et al., 2001). In addition to 8-OH-dG, increases in other oxidative DNA modifications have been reported (Randerath et al., 1991).

With the reported finding that there were increased levels of oxidative stress-induced DNA adducts in livers of rats and mice treated with PPARα agonists, concerns were raised as to whether the increase in oxidative DNA damage was due to

TABLE 15
Evidence for Increased Formation of Oxidants Following Treatment with PPARα Agonists

In Vivo
Increases in expression of hydrogen peroxide-generating fatty acyl CoA oxidase as compared to hydrogen peroxide-degrading catalase[a] (Nemali et al., 1988; Reddy et al., 1986a)
Accumulation of lipofuscin (Reddy et al., 1982; Marsman et al., 1992)
High levels of conjugated dienes[b] (Lake et al., 1987; Goel et al., 1986)
Accumulation of 8-hydroxydeoxyguanosine[c] (Kasai et al., 1989; Takagi et al., 1990b)
Antioxidant treatment reduces tumor incidence[d] (Rao et al., 1984)
Peroxisome proliferator-induced hepatocellular proliferation is prevented in catalase-overexpressing transgenic mouse model (Nilakantan et al., 1998)
Peroxisome proliferator-induced hepatocellular proliferation is abolished in NADPH oxidase-deficient mice (Rusyn et al., 2000b)
Decrease in levels of antioxidant enzymes (Cirilio et al., 1982; Awasthi et al., 1984)
In Vitro
Increased production of oxidants by peroxisomes from livers of animals treated with peroxisome proliferators[e] (Elliott et al., 1986; Tomaszewski et al., 1986)
Induction of expression of hydrogen peroxide-generating fatty acyl CoA oxidase in cultured hepatocytes (Ozasa et al. 1985)
Induction of DNA damage in vitro by peroxisomes from livers of animals treated with peroxisome proliferators[e] (Fahl et al., 1984)
Transformation of mammalian cells by peroxisomal fatty acyl CoA oxidase (Chu et al., 1995)

[a]Given the extremely high rate at which peroxisomal catalase converts H_2O_2 into H_2O and O_2, it should not escape from peroxisomes (Nichols and Schonbaum, 1963).
[b]Lack of increase in hepatic levels of F2-isoprostanes in livers of mice treated with WY 14,643 for 7 days has been reported (Soliman et al., 1997).
[c]It was reported that chronic treatment with peroxisome proliferators does not result in increased adduct levels. (Cattley and Glover, 1993; Hegi et al., 1990) Alternatively, it was suggested that such increases may be due to oxidation of mitochondrial, rather than nuclear, DNA (Sausen et al., 1995).
[d]Modulation of levels of vitamin E had no effect on hepatic tumors induced by peroxisome proliferators (Glauert et al., 1990).
[e]It was shown that treatment with peroxisome proliferators increased H_2O_2 in vitro, but not in the perfused liver because fatty acid supply is rate-limiting for H_2O_2 production in intact cells (Handler and Thurman, 1988; Handler et al., 1992).

preparative artifacts and/or contamination with mitochondrial DNA (Cattley and Glover, 1993; Sausen et al., 1995). Subsequent studies indicate that earlier estimates of ubiquitous persistence of 8-OH-dG in genomic DNA were exaggerated, most likely due to significant artifactual oxidation of guanine bases during isolation of DNA (Collins, 1999). He reported that levels of 8-OH-dG measured in control mammalian cells by either HPLC/ECD or "comet assay" are about $1–2 \times 10^{-7}$ dG when DNA extraction solutions are supplemented with antioxidants. Other data measuring 8-OH-dG and exocyclic DNA adducts in rats and mice treated with WY 14,643 for up to 3 months show no increase over controls with absolute levels nearly identical to the lowest values reported to date (Swenberg et al., unpublished observations). These recent developments raise questions as to whether previous studies of oxidative adducts, especially of 8-OH-dG, in the peroxisome proliferator field should be reconsidered. While oxidative stress to DNA is most likely involved in the mechanism of carcinogenesis by PPARα agonists, the role of oxidative DNA adducts may require further examination.

One possible explanation for the apparent lack of convincing increases in oxidant-modified DNA bases, despite the previously discussed conclusive evidence of increased oxidant production after treatment with PPARα agonists (Rusyn et al., 2001), is that oxidative stress to DNA in a living animal is a chronic effect. Moreover, chronic, relatively low-level insults to DNA caused by PPARα agonists will elicit adaptive responses such as increased DNA repair that will be able to remove the bulk of introduced lesions. A few may escape repair and be potentially converted to mutations.

Changes in expression of DNA repair genes as an in vivo marker of oxidative stress to DNA

DNA repair pathways, notably a base excision repair mechanism, efficiently remove oxidative lesions from DNA via a series of steps. An oxidized base is converted to an apurinic/apyrimidinic site, then to a single strand break, and finally to a sealed reconstructed double helix in a complex process that involves multiple proteins (Wood et al., 2001). It is known that DNA repair enzymes may be induced both in vivo and in vitro following increased production of reactive oxygen species (Tsurudome et al., 1999) or exposure to genotoxic agents (Holt et al., 2000). In view of possible induction of DNA damage by PPARα agonists, DNA repair mechanisms may be an important factor to consider in the mechanism of action of these compounds for a variety of reasons. First, increased repair may mask actual levels of oxidative DNA damage by removing the bulk of the lesions that are introduced by a chemical treatment. Second, in the process of DNA repair by a base-excision mechanism, additional mutagenic lesions, such as abasic sites and single strand breaks, are introduced and if the induction of enzymes that participate at different steps of the repair is uneven, there is potential for accumulation of mutagenic intermediates (Posnick and Samson, 1999). Third, if DNA polymerase β, a repair-specific enzyme that has low fidelity for DNA replication, is present in significant amounts in the nucleus of a proliferating cell, mutations can be introduced by erroneous base pairing by this error prone protein (Canitrot et al., 1998, 1999). An increase in gene expression for oxidative stress-related DNA repair proteins could be used as a sensitive marker for oxidative stress to DNA by nongenotoxic carcinogens that induce secondary production of reactive oxygen species.

The latter is important in view of numerous contradictory reports on the validity of measurements of DNA adducts. The ability of PPARα agonists to induce expression of base excision repair enzymes in liver of rats and mice was examined. WY 14,643 increased expression of an entire long-patch base excision DNA repair pathway in a time-dependent manner (Rusyn et al., 2000a). Importantly, expression of enzymes that do not repair oxidative DNA damage was not changed and the degree of induction of DNA repair gene expression correlated with dose and carcinogenic potency of the compounds from this group. Collectively, these data suggest that DNA base excision repair may be an important factor in PPARα agonist-induced carcinogenesis and that analysis of expression of long-patch base excision repair genes may be a sensitive approach for studying the mechanisms of action of non-genotoxic carcinogens that might provide further evidence supporting a role of oxidative DNA damage by PPARα agonists.

e. Inhibition of Gap Junctions

Gap junctional intercellular communication (GJIC), a process by which cells exchange small molecules, appears to be necessary for normal growth, development, and differentiation of tissues, as well as maintenance of homeostasis in multicellular organisms. The observation that many tumor promoters inhibit GJIC led to the hypothesis that GJIC may play a role in carcinogenesis (Trosko et al., 1990). However, the relationship between inhibition of GJIC and a carcinogenic response remains circumstantial and the mechanism is unclear (Budonova and Slaga, 1994; Yamasaki, 1990). Klaunig et al. (1988) reported that branched phthalate esters could inhibit GJIC in cultured mouse hepatocytes and suggested that this could be relevant to the development of hepatocellular carcinoma in rodents after long-term treatment. This and other observations indicate that PPARα agonists also are inhibitors of GJIC (e.g., Dybing et al., 1995). It also has been hypothesized that reactive oxygen species generated during the peroxisome proliferation process could alter membrane fluidity, affecting GJIC (e.g., Jansen and Jongen, 1996), and, indeed, H_2O_2 inhibits GJIC (Upham et al., 1997). However, others wonder whether other factors may be involved (Cruciani et al., 1997; Upham et al., 1998).

GJIC inhibition is highly correlated with tumor induction by PPARα agonists. One series of studies compared the dose and time dependency as well as specificity of GJIC inhibition and liver tumor induction using DEHP as a model compound (Isenberg et al., 2000). DEHP inhibits GJIC either in rodent liver under in vivo conditions or in rodent hepatocytes under in vitro conditions. The authors reported inhibition of GJIC in both rat and mouse liver at carcinogenic doses but no effects at non-carcinogenic levels. Kamendulis et al. (2002) reported that DEHP inhibited GJIC in rat and mouse hepatocytes.

Inhibition of GJIC by PPARα agonists exhibits species specificity, correlating for the most part with the species specificity seen with the induction of peroxisome proliferation and carcinogenicity. For

example, while GJIC was inhibited in rat and mouse liver following treatment with DEHP, it was not inhibited in hamsters (Isenberg et al., 2000), a species in which there also is no evidence for peroxisome proliferation or liver tumor induction following phthalate treatment (Schmezer et al., 1988). Pugh et al. (2000) reported that DEHP did not induce peroxisome proliferation or inhibit GJIC in cynomolgus monkey liver under in vivo conditions. MEHP, the monoester corresponding to DEHP and the form in which it is absorbed, inhibited GJIC and elevated peroxisomal enzymes in hepatocytes derived from rats and mice but had no effects on liver cells from hamsters, monkeys or humans (Baker et al., 1996; Kamendulis et al., 2002).

The inhibition of GJIC by PPARα agonists also exhibits dose and duration relationships similar to those shown by markers of peroxisomal proliferation, including liver enlargement and induction of β-oxidation enzymes. Isenberg et al. (2001) reported that, following exposure of F344 rats to DEHP, the dose-response relationships for inhibition of GJIC and induction of peroxisome proliferation were similar to that for the tumor induction in the liver; that is, doses that produced tumors also induced peroxisome proliferation and inhibited GJIC, whereas doses that did not increase tumor frequency had no effect on the other parameters. Additionally, it was noted that GJIC remained inhibited as long as treatment continued; liver weights and peroxisomal enzyme levels also remained elevated over the same period. The magnitude of inhibition of GJIC and the indicators of peroxisome proliferation declined within 2 weeks of cessation of exposure.

There are a number of other examples, although few have been studied as comprehensively as DEHP. Ashby et al. (1994) identified 18 substances, including DEHP, for which there were adequate data on peroxisome proliferation and hepatocellular tumor induction to permit meaningful comparisons. There also are GJIC data for eight of these covering a range of chemical classes (see Table 16). In all instances, the GJIC data paralleled the peroxisome proliferation and tumor response data. Among these, DINP induced peroxisome proliferation and liver tumors in rats and mice (Butala et al., 1996, 1997) and inhibited GJIC in the livers of those species (Smith et al., 2000). It also inhibited GJIC in rat and mouse hepatocytes in culture (Kamendulis et al., 2002). Conversely, it did not induce peroxisome proliferation or inhibit GJIC in primate liver (Pugh et al., 2000) or in human or monkey hepatocytes in culture (Kamendulis et al., 2002). WY 14,643 inhibited GJIC in rat and mouse hepatocytes but had no

TABLE 16
Comparison Data on Peroxisome Proliferation, Hepatocellular Tumors, and GJIC Effects

Substance	Peroxisome proliferation	Liver tumors	GJIC inhibition in vitro	GJIC inhibition in vivo	Species specificity
DEHP	Rat + Mouse +	Rat + Mouse +	Rat + Mouse +	Rat + Mouse +	No effects in primate liver or human hepatocytes
DINP	Rat + Mouse +	Rat + Mouse +	Rat + Mouse +	Rat + Mouse +	No effects in primate liver or human hepatocytes
WY 14,643	Rat +	Rat +	Rat + Mouse +	Rat + Mouse +	No effects in primate liver or human hepatocytes
Clofibrate	Rat + Mouse +	Rat + Mouse +	No data	Rat +	No effects in primate liver
Methylclofenopate	Rat +	Rat +	SHE cells + V79 cells + Mouse fibroblasts +	No data	No data
Nafenopin	Rat +	Rat +	Rat +	No data	No effects in guinea pig hepatocytes
Trichloroethylene	Rat − Mouse +	Rat − Mouse +	Rat − Mouse +	No data	No data
Trichloroacetic acid	Mouse +	Mouse +	Rat − Mouse +	No data	No data

Note. + indicates a positive response; − indicates a negative response.

effects in monkey or human hepatocytes tested under in vitro conditions (Kamendulis et al., 2002). Nafenopin inhibited GJIC in rat but not guinea pig hepatocytes (Elcock et al., 1998; Leibold et al., 1994). Trichloroethylene and trichloroacetic acid inhibited GJIC in mouse but not rat hepatocytes, paralleling the in vivo liver tumor response data (Klaunig et al., 1989; Lington et al., 1996). Clofibrate inhibited GJIC in F-344 rat liver (Krutovskikh et al., 1995) but not cynomolgus monkey liver (Pugh et al., 2000) when tested under in vivo conditions. Methylclofenopate inhibited GJIC in Syrian hamster embryo cells (Cruciani et al., 1997) as well as in mouse fibroblasts (Jansen and Jongen, 1996).

2. Conclusions—Overall Evaluation of Mode(s) of Action

Activation of PPARα is causally related to the induction of rodent liver tumors. The weight of evidence is strong because PPARα has been shown to bind to, and be activated directly by, chemicals in this class. Also, PPARα-null mice are refractory to all agonist-induced responses linked to tumor formation as well as tumor formation itself in response to a prototypical PPARα agonist (i.e., WY 14,643). The specificity is high because there is no evidence that other structurally dissimilar nongenotoxic carcinogens require PPARα for liver tumor induction. Additional bioassays with other PPARα agonists in PPARα-null mice will strengthen the link between chronic PPARα activation and the hepatocarcinogenic effects of this class of chemicals.

Increased expression and activity of peroxisomal enzymes including acyl CoA oxidase in response to activation by PPARα agonists is associated with rodent liver tumor formation. The weight of evidence is strong because (1) all PPARα agonists that cause high-level sustained induction of acyl CoA oxidase also cause increases in mouse and rat liver tumors, and (2) acyl CoA oxidase acts like a tumor suppressor in that functional inactivation of the acyl CoA oxidase gene leads to spontaneous liver tumor formation. The specificity is high because the induction of acyl CoA oxidase requires PPARα and generally does not occur with other nongenotoxic carcinogens. Models in which selective activation of acyl CoA oxidase-induced oxidative stress occurs in the absence of increased hyperplastic signaling have not been identified but are crucial for determining any role for acyl CoA oxidase-associated oxidative stress in liver tumor formation.

Alteration in the expression of genes that mediate cell cycle progression in response to activation by PPARα agonists is associated with induction of rodent liver tumors, as alterations in the balance between hepatocyte proliferation and apoptosis depend on changes in the expression of genes involved in conveying mitogenic signals to the nucleus through the cell cycle apparatus. The weight of evidence is weak because, while a number of cell cycle genes expected to be altered by many different mitogenic chemicals have been shown to be altered by PPARα agonists in a PPARα-dependent manner, the precise pathways that mediate the mitogenic signal have not been identified. Specificity is low because these events occur with other nongenotoxic hepatocarcinogens.

Alteration in the expression and activities of nonperoxisomal lipid-metabolizing enzymes in response to activation by PPARα agonists is associated with rodent tumor formation. The weight of evidence is strong because virtually all PPARα agonists that cause induction of genes encoding lipid-metabolizing enzymes also cause rodent liver tumors, and there is no evidence that these changes are mechanistically linked to liver tumor formation. The specificity of these effects is low because while PPARα is required for alteration by PPARα agonists, these genes can be altered by other nongenotoxic carcinogens and physiological states. Determination of the role these changes play in liver tumor formation requires construction of appropriate animal models in which these changes can be studied in isolation from other PPARα-mediated events.

Increased peroxisome proliferation in response to activation by PPARα agonists is associated with rodent liver tumor formation. The weight of evidence is strong because (1) all PPARα agonists that cause high-level sustained peroxisome proliferation also cause increases in mouse and rat liver tumors, but (2) there is no evidence that peroxisome proliferation per se is mechanistically linked to liver tumor formation. The specificity is high because the induction of peroxisome proliferation requires PPARα and generally does not occur with other nongenotoxic carcinogens. Models in which selective induction of peroxisome proliferation in the absence of increased hyperplastic signaling have not been identified but are crucial for determining the contribution of peroxisome proliferation to liver tumor formation.

The alteration in the balance between cell proliferation and apoptosis is causally related to the induction of liver tumors. The weight of evidence is strong because PPARα agonist-induced liver tumors

do not occur in the absence of increases in either short-term or chronic hepatocyte proliferation. Specificity is low because these events occur with other nongenotoxic hepatocarcinogens.

Inhibition of GJIC is associated with increases in PPARα agonist-induced liver tumors. The weight of evidence is strong because all PPARα agonists evaluated inhibited GJIC and there is no evidence that PPARα agonist inhibition of GJIC by itself leads to tumor formation. The specificity is low because GJIC inhibition is likely a common process for other nongenotoxic carcinogens and is not specific for PPARα agonists.

Increases in oxidative stress-induced macromolecular damage are associated with increases in PPARα agonist-induced liver tumors. The weight of evidence is weak because virtually all PPARα agonists that cause induction of markers of oxidative stress other than DNA adducts also cause rodent liver tumors. There is presently no clear relationship between increases in oxidative stress and increases in the levels of DNA adducts after PPARα agonist exposure. The specificity is low because any chemical that induces oxidative stress could increase DNA damage and increase the likelihood of hepatocyte initiation.

Activation of Kupffer cells is associated with PPARα agonist-induced liver tumors. The weight of evidence is strong as PPARα agonist-induced cell proliferation is decreased or abolished by prior inactivation of Kupffer cell activities including NADPH oxidase. However, appropriate animal studies in which Kupffer cell activation is abolished during a PPARα-agonist bioassay have not been carried out. The specificity is low because activation of Kupffer cells occurs under many circumstances and is not specific to PPARα agonist-induced hepatocarcinogenesis.

Selective clonal expansion of altered hepatocytes is related causally to the induction of liver tumors. The weight of evidence is strong because PPARα agonist-induced clonal expansion occurs with PPARα agonists that also induce liver tumors and liver tumors do not occur in the absence of increases in proliferation of hepatocytes within the preneoplastic foci. Specificity is low because these events occur with other nongenotoxic hepatocarcinogens.

3. Data Gaps

While a substantial body of knowledge is available to support the characterization of the mode of action by which liver tumors arise on rats and mice and the relevance of this MOA to the assessment of human cancer hazard, there remain some unanswered questions that would benefit from further investigation. For example, the regulation by PPARα of the critical genes in the rodent regulating cell growth—both cell proliferation and suppression of apoptosis—is not understood fully. As this information becomes available, one then could conduct cross-species comparison studies in nonhuman primates to determine similarities and/or differences. In addition, no data exist on the nonhuman primate homology to the human PPARα.

Also, it would be important to test a potent agonist in the non-human primate, using multiple treatment groups and measuring receptor binding, β-oxidation and cell proliferation rates, and liver weight and histopathology. Other organ sites known to express PPARα, such as the kidney, as well as the organs which form the remainder of the so-called Tumor Triad, (pancreas and testes) should be examined for evidence of peroxisome proliferation and/or other pre-neoplastic and neoplastic changes. It also would be ideal to conduct a parallel study in a responsive species (i.e., rat or mouse). In addition, kinetic data should be collected to determine its role in describing interspecies differences. In connection with these studies, one should assess the comparative potency of the potent PPARα agonist on the nonhuman primate and human receptor using an appropriate transactivation assay.

These data collectively would serve to improve substantially our understanding of whether or not the responses observed in rats and mice could occur in the human.

4. Supportive Data Elements for Evaluation of Proposed Mode(s) of Action for Hepatic Tumors

In order to demonstrate convincingly that a previously uncharacterized compound is acting through the PPARα agonist-induced MOA, it is necessary to generate data addressing a subset of the key events described above. This section presents the key events related to the liver tumor, the most common methodologies used to address these key events, and a brief discussion of the relevance of the data generated to provide a definitive demonstration of the PPARα agonist-induced MOA. Obviously, the analysis given below would apply *only* to compounds that had been previously characterized as not genotoxic by virtue of evaluation for direct DNA

reactivity or interference with mammalian topoisomerases and other measures of mutagenicity/clastogenicity.

PPARα Activation

The most common method used to demonstrate that a compound is a PPARα agonist is through the use of in vitro reporter assays. In these assays, two plasmids, one coding for the receptor and the other an appropriate reporter construct (e.g., a rodent PPRE/luciferase construct), are transfected into a suitable cell line, which is treated subsequently with a compound and assayed for increased reporter expression. Due to patent constraints and other considerations, the receptor construct is frequently a chimera, composed of, for example, a GAL4 DNA binding domain linked to a PPARα ligand binding domain (with the appropriate DRE replacing the PPRE in the reporter construct). These data clearly are highly specific to a PPARα MOA (as evidenced by data derived from PPARα-null mice) and should be considered essential data; however, it cannot be considered definitive in the absence of in vivo data. Finally, it should be noted that peroxisome proliferation-like events have been noted in PPARα-null mice in response to treatment with extremely high doses of ligands specific for other PPAR family members. Thus, it may be possible for compounds with little or no PPARα activity to produce other sequelae of PPARα agonism, such as liver tumors. It seems likely that data requirements for linking such compounds to a PPARα agonist-induced MOA would be more rigorous, but such speculation is beyond the scope of this document.

Gene Expression Changes (Peroxisomal, Growth Regulation, Fatty Acid Metabolism)

A widely used marker for peroxisome proliferation is the measurement of increases in the rate of peroxisomal β-oxidation. One method involves spectrophotometric measurement of the reduction of NAD^+ to NADH in the third step of the four-step β-oxidation cycle. This is complicated by the fact that mitochondria also perform β-oxidation utilizing this reaction, so the mitochondrial reaction must be poisoned with cyanide. An alternative method is to measure the generation of H_2O_2 by acyl CoA oxidase, the rate-limiting enzyme in the cascade; this avoids the above complication since, in mitochondria, the electrons generated in this step feed into the redox cycle instead of reducing O_2. Alternatively, induction can be measured at the RNA level using various molecular techniques such as Northern blot or real-time polymerase chain reaction (PCR) (e.g., TaqMan). This latter technique provides the flexibility to examine expression levels of other peroxisomal genes as well, such as CYP4A, but acyl CoA oxidase remains one of the most widely used and reliable markers for peroxisome proliferation and, therefore, should be considered essential data.

The expression levels of many other genes are known to be influenced by PPARα agonists, and a subset of these, primarily those involved in fatty acid metabolism, have demonstrable PPRE elements. Demonstration of this, usually via determination of transcription levels, also can provide support for a PPARα agonist MOA, but since the functions of the majority of these are unrelated to the phenomenon of peroxisome proliferation, such data should only be considered as supportive.

A number of laboratories have shown that PPARα agonists can influence the expression levels of various growth regulatory genes but, to date, no PPREs have been identified in the regulatory regions of any of them, so such changes could be indirect. Thus, even though it seems likely that such changes participate in the process of PPARα agonist-induced tumorigenesis, they are commonly seen in the course of the tumorigenic process regardless of the MOA involved and therefore are irrelevant to the determination of a PPARα agonist-related MOA.

Peroxisome proliferation

This term refers to the increase in volume percent and density of peroxisomes that is seen subsequent to treatment with PPARα agonists. The most reliable methodology for discerning this involves catalase staining and electron microscopy; however, this is capital-, skill-, and labor-intensive and beyond the capabilities of many laboratories. An alternative approach is to perform light microscopy on liver sections stained immunohistochemically for peroxisomal membrane protein. Since this is the very definition of peroxisome proliferation, it can, in combination with PPARα agonism, be considered definitive proof of a PPARα agonist-related MOA.

Perturbation of cell proliferation and apoptosis

Increased rates of cell proliferation and decreased rates of apoptosis are hallmarks of the liver

growth seen in the early stages of treatment with PPARα agonists. These changes are believed, on fairly solid theoretical grounds, to be integral to the carcinogenic process. Many methodologies for observing these phenomena exist. For increased cell proliferation, they include visual observation of mitotic figures, ^{3H}Tdr (tritiated thymidine) or BrdU (5′-bromo-2′-deoxyuridine) labeling (followed by immunohistochemical staining, IHCS), and IHCS for PCNA or Ki67, though these latter techniques may be somewhat less reliable than labeling with DNA precursors. For apoptosis, they include visual observation of apoptotic foci, TUNEL assay, and IHCS for caspases. As stated earlier, observation of these phenomena constitute very strong evidence of an ongoing carcinogenic process, but since they are general to most, if not all, carcinogenic processes, irrespective of the causative agent, they cannot be considered proof of a PPARα agonist-related MOA. However, in conjunction with one or more of the PPARα agonist specific phenomena already discussed, they would constitute very strong corroborative evidence.

Inhibition of gap-junctional intercellular communication (GJIC)

Disruption of GJIC is a phenomenon that is observed subsequent to treatment with a wide range of "nongenotoxic carcinogens," including PPARα agonists. The most widely used technique for its detection involves the monitoring of Lucifer yellow dye transfer in cultured, anchorage-dependent cells. However, since this is such a widespread phenomenon, it cannot be considered as indicative specifically of a PPARα agonist-related MOA.

Hepatocyte oxidative stress

The disproportionate increase in the level of oxidative versus antioxidative enzyme activities (e.g., acyl CoA oxidase vs. catalase) provides a strong theoretical basis for the hypothesis that peroxisome proliferation results in oxidative stress, as does the long-term accumulation of lipid peroxidation products such as lipofuscin. Nevertheless, it has proven difficult to unambiguously demonstrate increases in oxidative damage to DNA. In addition, induction of oxidative stress is by no means an exclusive or PPARα agonist-specific phenomenon, but rather a general response to a variety of metabolic stressors. For these reasons, this does not represent a good piece of evidence for a PPARα agonist MOA.

Kupffer cell-mediated events

While evidence from a number of laboratories indicates Kupffer-cell involvement in the PPARα agonist MOA process, the evidence for and precise nature of their role remain uncertain, as well as nonspecific, to provide convincing evidence for a role in the PPARα agonist-related MOA.

Selective clonal expansion

This process is believed to lie at the heart of the early neoplastic process and has been demonstrated convincingly to be a chronic response to PPARα agonist treatment. However, its demonstration can be a problematic and labor-intensive endeavor. In addition, it is completely nonspecific, occurring irrespective of the carcinogenic modality and is therefore not particularly useful as evidence for demonstration of a PPARα agonist-related MOA.

Conclusions

Based on the preceding discussion, a minimal set of data elements to support a convincing demonstration that rodent liver tumors have arisen as a result of a PPARα MOA would consist of PPARα agonism combined with light- or electron-microscopic evidence for peroxisome proliferation. Alternatively, evidence for PPARα agonism (i.e., in a receptor assay) combined with increases in liver weight and one (induction of acyl CoA oxidase) or more (e.g., induction of CYP4A) of the specific in vivo markers of peroxisome proliferation would suffice. Additionally, demonstration that liver growth was accompanied by at least transiently increased rates of replicative DNA synthesis and/or decreased apoptosis would significantly strengthen the case. In an earlier examination of this issue (Cattley et al., 1998), the minimum data set was considered to consist of evidence of hepatomegaly, increased peroxisomal volume by morphometry, and enhanced DNA synthesis rates. These cellular changes should take place in a dose responsive manner and correlate with the dose dependency of the tumor formation. It was felt by the current working group that, due to the definitive nature of the PPARα-null mouse data, the demonstration of PPARα agonism was sufficient to abrogate the necessity for some of the more rigorous (and technically demanding) requirements determined by the previous working group. In the presence of overt

necrosis or genotoxicity, alternative modes of action should be considered as well.

Finally, it should be noted that the degree of evidence for the convincing identification of a PPARα agonist in a prospective fashion (e.g., for screening purposes) could probably be less rigorous than that required for retrospective determination in the face of a positive bioassay.

C. Applicability of Animal MOA(s) to Human Health Risk Assessment

1. Overview/Summary of Existing Available Human Literature (In Vitro, Epidemiological, and Clinical)

There is considerable debate concerning the mechanisms by which PPARα agonists cause liver tumors in rodent models and whether or not these chemicals represent a human cancer risk. It is well established that long-term administration (40–50 weeks) of PPARα agonists results in hepatocarcinogenesis in rats and mice (Ashby et al., 1994; Bentley et al., 1993; Lake, 1995a; Reddy and Lalwani, 1983). In contrast, liver tumors are not found in Syrian hamsters after long-term administration (40–60 weeks) of the PPARα agonists WY 14,643 and nafenopin (Lake et al., 1993). Similar investigations in nonhuman primates indicate that these species also are refractory to induction of markers of cancer hazard, including peroxisome proliferation and hepatocyte proliferation. Long-term exposure studies in these species have provided no evidence of PPARα agonist-induced liver cancer (Cattley et al., 1998), although these experiments were not carried out over the full life span of the animals. Consistent with this idea, hepatic peroxisome proliferation also has not been found in nonhuman primates treated with these chemicals (Ashby et al., 1994; Lake, 1995a, 1995b). Liver biopsies from humans treated with gemfibrozil, clofibrate, or fenofibrate for periods ranging from 2 to 156 months (9–28 samples examined per study) did not reveal convincing evidence of peroxisome proliferation (the observed changes were only up to less than 1.5-fold), supporting the hypothesis that humans are not susceptible to liver cancer mediated by PPARα agonists (Blumcke et al., 1983; de la Iglesia et al., 1982; Gariot et al., 1987).

Nonetheless, one study examining the effects of clofibrate showed a 50% increase in peroxisome number and a 23% increase in peroxisome density, responses that were not statistically significant (Hanefeld et al., 1983). Two clinical trials also have examined relative mortality and cancer rates in human males treated with fibrates. In the Helsinki Heart Study, a total of 4081 men aged 40–55 with elevated serum cholesterol were treated with either gemfibrozil or placebo for a 5-year period (Frick et al., 1987; Huttunen et al., 1994). Despite significant lowering of serum lipids, which prevented coronary heart disease in the gemfibrozil-treated group, no differences in total death rate or liver cancer incidence were observed between the groups. It should be pointed out that liver cancer incidence was not reported as a separate endpoint; the incidence was reported either as total deaths from cancer or deaths from liver, gallbladder, and intestinal cancers combined. Except for a borderline statistically significant difference ($p = .062$) in the incidence of basal-cell carcinomas of the skin between 2051 patients treated with gemfibrozil for 5 years and 2,030 subjects receiving a placebo, no differences were found for other cancers. The incidence of cancer mortality in this study, for placebo and fibrate-treated patients, was less than 2% for each group, compared to virtually 100% in PPARα agonist-treated rodents (Ashby et al., 1994; Bentley et al., 1993; Lake, 1995a; Reddy and Lalwani, 1983).

The other randomized clinical trial was conducted by the World Health Organization (WHO) to determine whether clofibrate would lower the incidence of ischemic heart disease (IHD) in men and was carried out in 15,745 men with a treatment group and two control groups (one high and one low cholesterol level) of about 5000 men each, for an average of 5.3 years (CPI, 1978). The treatment period was for 5.3 years; follow-up reports were provided 4.3 and 7.9 years after this period. Clofibrate was reported to cause a statistically significantly higher age-adjusted total mortality as compared with the high cholesterol placebo-treated control groups in this study. The excess mortality was due to a 25% increase in noncardiovascular causes, that is, diseases of the liver, gallbladder, pancreas, and intestines, including malignant neoplasms of these sites (CPI, 1980). However, in the final follow-up study (5.3 years in the treatment phase with 7.9 years follow-up for a total of 13.2 years), neither the difference in the number nor rate of cancer deaths between the clofibrate-treated group and the control groups was statistically significant (CPI, 1984). The reason for the difference in mortality at the earlier timepoint is uncertain. Similar to the Helsinki Heart Study, no data on the incidence of liver cancer alone was provided. It should be noted that, in this final follow-up study, there was a 12% excess of deaths from all

causes other than IHD compared with 25% in the previous studies. Furthermore, the proportional difference between the treated group and the control groups in the final follow-up study was diminished for malignant disease but increased for nonmalignant diseases. The results indicate that the excess in deaths from diseases other than IHD was largely confined to the clofibrate-treatment period (average 5.3 years). However, 7.9 years posttreatment, there were 27 deaths associated with liver, gallbladder, and intestinal cancers in the clofibrate-treated group, compared to 18 and 11 deaths associated with the same endpoints in the high cholesterol and low cholesterol control groups, respectively. Similar to the Helsinki Heart Study, this incidence is less than 1% per group. Lastly, a limited epidemiological study showed no evidence of increased cancer risk as a result of fibrate therapy (Law et al., 1994). However, as with the clinical trials, cancer incidence was not reported specifically for liver cancer. Data concerning the human susceptibility to liver cancer from peroxisome proliferators come primarily from species comparisons of short-term responses, such as proliferation of peroxisomes in liver parenchymal cells, hepatomegaly and the induction of various hepatic enzymes, and of PPARα expression.

Given the importance of PPARα in mediating the short- and long-term effects of PPARα agonist exposure in mice and rats including liver cancer, cancer risk in humans has been gauged in part by comparing the properties of PPARα between susceptible rodent species and humans. Transient transfection studies using a human PPARα cDNA show that this receptor can transactivate reporter constructs, providing indirect evidence that the human PPARα is functional (Sher et al., 1993). Thus, it is not surprising that PPREs have been described in human genes that are transcriptionally regulated by the PPARα, including human apo C-III (Hertz et al., 1995), lipoprotein lipase (Schoonjans et al., 1996a), apo A-I (Vu-Dac et al., 1994), apo A-II (Vu-Dac et al., 1995), carnitine palmitoyl transferase-I (Mascaro et al., 1998), and acyl CoA oxidase (Varanasi et al., 1996). Interestingly, large increases in the expression of marker mRNAs and proteins including peroxisomal acyl CoA oxidase are not found in human and nonhuman primate hepatocytes treated with these chemicals in vitro (Bichet et al., 1990; Cornu-Chagnon et al., 1995; Duclos et al., 1997; Elcombe 1985; Elcombe et al., 1996; Goll et al., 1999; Hasmall et al., 2000b). These observations are consistent with a recent study demonstrating the lack of an increase in acyl CoA oxidase mRNA in human liver samples from 48 patients treated with one of several fibrates (bezafibrate, fenofibrate, or gemfibrozil), despite signficant induction of hepatic apolipoprotein A-I mRNA and lowering of serum lipids following treatment (Roglans et al., 2002). However, dose-dependent induction (<3-fold) of acyl CoA oxidase activity has been observed in human hepatocytes treated with clofibrate and ciprofibrate (Cimini et al., 2000; Perrone et al., 1998) and treatment with perfluorodecanoic acid resulted in significant induction of peroxisomal density and increased acyl CoA oxidase activity in human cells derived from glioblastoma (Cimini et al., 2000). Human PPARα activation is reported to result in increased apolipoprotein A-II and lipoprotein lipase transcription and reduced apolipoprotein C-III, which is key to lowering serum triglycerides (Auwerx et al., 1996; Staels et al., 1997; Vu-Dac et al., 1995).

Because the hypolipidemic effects of PPARα agonists are mediated by the PPARα in rodents and possibly in humans, and the human genome encodes a PPARα gene as well as a number of genes with apparent PPREs, it is of great interest to determine why the target cells for cancer effects in rodents appear to be refractory to these chemicals in humans. Interestingly, it was shown recently that human apolipoprotein C-III can be downregulated by fibrates in cultured human hepatocytes, while no changes in target genes encoding peroxisomal enzymes including acyl CoA oxidase, BIEN and THIOL were detected (Lawrence et al., 2001a). Further, stably transfected HepG2 cells expressing levels of either human or murine PPARα at levels typically reported in rodent liver do not respond to fibrates by increasing mRNAs levels of acyl CoA oxidase, BIEN, or THIOL (Hsu et al., 2001; Lawrence et al., 2001a). Fibrate treatment does cause increased expression of HMG CoA synthase and carnitine palmitoyl transferase-I (CPT-I) in HepG2 cells overexpressing PPARα, all three of which are known target genes in rodents (Hsu et al., 2001; Lawrence et al., 2001a). In contrast to these observations made in cultured cells, evidence from an in vivo model suggests that there may be considerably more similarities in PPARα target genes between humans and rodents. Using a rescue approach of adenoviral infection of PPARα-null rodent livers, it was demonstrated that the human PPARα is indeed capable of activating many of the known target genes regulated by the murine PPARα including ACO, BIEN, THIOL, VLCAD, and MCAD (Yu et al., 2001). Additional support for this was provided by the fact that hPPARα-adenoviral-infected

PPARα-null mice also exhibited peroxisome proliferation (Yu et al., 2001). While there are data available indicating that cultured human hepatocytes do not exhibit increased markers of cell proliferation in response to exposure to PPARα agonists while cultured rodent hepatocytes do, this model system may be inappropriate for evaluating this effect. PPARα agonists do not cause increases in DNA synthesis in purified rat hepatocytes cultured in vitro (Parzefall et al., 2001). However, when purified rat hepatocytres are cocultured in the presence of the Kupffer-cell-derived cytokine TNFα, a significant increase in replicative DNA synthesis was found, suggesting that hepatocytes cultured in vitro may require the presence of Kupffer-cells in order to exhibit similar changes in molecular and biochemical changes induced by PPARα agonists (Parzefall et al., 2001). Combined, the preceding observations demonstrate that there is some overlap in target gene activation between humans and rodents, and that further characterization is required to determine the reasons for the differences. Several possible explanations have been postulated to account for this disparity.

There are dramatic differences in the expression levels or function of the expressed protein. For example, PPARα mRNA levels in human liver RNA samples are at least 10-fold less than those observed in a limited set of mouse RNA controls (Palmer et al., 1998; Tugwood et al., 1996). In contrast, while the level of human PPARα protein measured in five out of six human liver samples was considerably lower than the receptor in mouse livers, there was one human liver sample that appeared to express PPARα protein at comparable levels to mouse (Walgren et al., 2000). Additionally, guinea pig liver also was reported to contain significantly less PPARα compared to mouse liver (Bell et al., 1998). Combined, these observations suggest that the level of PPARα in the human liver may not be sufficient to activate the plethora of target genes that are altered by PPARα agonists in rodent models, yet may be capable of modulating lipid homeostasis through alteration of expression of genes in the liver or, more likely, other target organs that express higher levels of PPARα. However, given the relatively small number of frozen human liver samples examined, further quantification of PPARα mRNA and protein in addition to other transcription factors to serve as good positive controls would be well received.

While it appears that lower levels of PPARα expression may, in part, contribute to the species differences between rodents and humans, expression of truncated or mutant PPARα, some through expression of alternatively spliced products also have been described. No mutations or polymorphisms have been described to date in rodent species. A dominant negative form of human PPARα has been described and the presence of this protein could signficantly inhibit PPARα activation and subsequent target gene modulation (Gervois et al., 1999). Additionally, two different mutations (L162V and V227A) in the human PPARα have been described by separate laboratories (Flavell et al., 2000; Sapone et al., 2000; Vohl et al., 2000; Yamakawa-Kobayashi et al., 2002). The biological significance of these mutant human isoforms of PPARα is unclear but has been linked to significant differences in serum apolipoproteins (Flavell et al., 2000; Vohl et al., 2000), serum cholesterol (Flavell et al., 2000), and fibrate-induced changes in serum HDL cholesterol (Bosse et al., 2002). Some of the mutant PPARα proteins were shown to act as dominant negative proteins in that they could prevent the normal PPARα from interacting with RXR, binding the PPRE, and activating gene expression. These findings suggest that in addition to reported lower expression levels of human PPARα, mutant variants, in particular a dominant negative isoform, also may contribute to the apparent insensitivity in humans to PPARα agonists. However, since some humans are responsive to fibrate therapy, the hypothesis that altered PPARα protein accounts for the species differences is likely not true for all cell types in humans that express PPARα.

Species differences in sensitivity to PPARα agonists may be explained partly by differences in the structure of the PPREs that regulate the expression of target genes including mutations or polymorphisms. The best example of this is the significant differences between the human and rat apolipoprotein A-I promoter. Fibrate administration to rats causes down regulation of hepatic apolipoprotein A-I mRNA and protein (Staels et al., 1992). In contrast, transcription of the human apolipoprotein A-I gene is induced by fibrates in human liver based on in vivo and in vitro data (Hahn and Goldberg, 1992; Jin et al., 1996; Saku et al., 1985; Tam, 1991; Vu-Dac et al., 1994). The mechanism underlying this species difference was discovered when investigators found that the functional PPRE required to induce human apolipoprotein A-I expression is mutant in the rat promoter, and that the regulatory region responsible for downregulation in rodent apolipoprotein A-I is not functional in humans (Vu-Dac et al., 1998). Another example of possible variation in a PPARα target gene promoter region is reported for acyl CoA oxidase. The PPRE upstream of the human acyl CoA oxidase gene when cloned

in front of a reporter gene is inactive in cells transiently transfected with a PPARα expression vector (Woodyatt et al., 1999). However, another group had previously reported that this PPRE is capable of being transactivated in reporter assays using relatively high concentrations (mM) of PPARα agonists (Varanasi et al., 1996) after clarifying the original reported PPRE sequence (Varanasi et al., 1998). Subsequently, using site-directed mutagenesis of the rat acyl CoA oxidase PPRE, it was reported that the human PPRE sequence is not transactivated in reporter assays (Lambe et al., 1999). Due to this controversy, further analysis of PPREs of other PPARα target genes in humans should be conducted. Lastly, differences in the ability of PPARα-RXRα heterodimers to bind to the acyl CoA oxidase PPRE in rat and human cell extracts also have been suggested to explain the species differences and deserves further investigation (Rodriguez et al., 2000).

It is clear from the published studies that humans possess a functional PPARα, and that the human receptor is activated by peroxisome proliferators. It is clear that some of the genes modulated by these chemicals in humans differ from those regulated by rodent PPARα. The available epidemiological and clinical studies are inconclusive, but, nonetheless, do not provide evidence that peroxisome proliferators cause liver cancer in humans. Further studies are needed to determine the biological significance of inter-individual variability in PPARα sequences, to determine the variation of PPARα mRNA and protein expression in human liver (in particular for young children and older adults, between males and females, and possible racial differences), and to clearly elucidate the mechanisms underlying the apparent species differences in target gene modulation via activation of PPARα between rodents and humans.

2. Conclusions

a. Are Key Events in the Animal MOA Plausible in Humans?

The MOA for PPARα ligand-induced rat/mouse liver tumors is well defined. *The weight of evidence suggests that this MOA would be plausible in humans* since humans possess PPARα at sufficient levels to mediate the human hypolipidaemic response to therapeutic fibrate drugs. (Table 17) This hPPARα is comparable to the rat or mouse PPARα in its affinity for PPARα ligands, and there is nothing to suggest a reduced activity compared with the rat or mouse receptor. Thus, a point in the rat/mouse key events cascade where the pathway is biologically precluded in humans cannot be identified, in principle.

b. Taking into Account Kinetic and Dynamic Factors, is the Animal MOA Plausible in Humans?

Nonetheless, the weight of evidence suggests that this MOA is not likely to occur in humans based on differences in several key steps, when taking into consideration kinetic and dynamic factors (Table 17).

Two papers have described 10-fold less mRNA for PPARα in human liver compared with the rat or mouse (Palmer et al., 1998; Tugwood et al., 1998), and one paper describe the level of human PPARα protein as variable but generally lower than the mouse (Walgren et al., 2000).

The PPARα-mediated peroxisome proliferation response (and associated regulation of peroxisomal genes/proteins and increased peroxisomal β-oxidation) is either absent or much reduced in human liver. Evidence for this comes from cultures of human hepatocytes where there is little (only up to twofold) or no induction of key markers such as acyl CoA oxidase mRNA, acyl CoA oxidase protein, and/or acyl CoA oxidase activity as measured by palmitoyl CoA oxidation and from studies conducted with patients treated with several different hypolipidemic peroxisome proliferators that have shown little or no hepatic peroxisome proliferation. Where seen, the response is inconsistent and very weak compared with the response seen in rat and mouse (reviewed in Doull et al., 1999).

These human data are supported by data from non-human primates where the majority of studies suggest that peroxisome proliferators have little or no effect in both old world and new world monkeys (reviewed in Doull et al., 1999). The maximal response reported was less than two-fold at the maximum tolerated dose (MTD); this can be contrasted with the 20- to 50-fold induction seen in the rat. However, some response (up to 5-fold) has been reported at high doses for clofibrate and DL-040 (an experimental hypolipidemic agent) by one laboratory (reviewed in Doull et al., 1999).

Since the specific PPARα-regulated genes that play a role in the regulation of proliferation and apoptosis in the rat/mouse liver are not yet known, any qualitative difference in the relevant genes between mice/rats and humans cannot be evaluated.

TABLE 17
Comparative Analysis of Rodent and Human Data—Liver Tumors

Rat/mouse MOA key events for liver tumors	Is this key event in the animal MOA plausible in humans?	Taking into account kinetic and dynamic factors, is this key event in the animal MOA plausible in humans?
1. Activation of PPARα	Yes	Yes
2a. Expression of peroxisomal genes	Not likely	Not likely
2b. PPARα-mediated expression of cell cycle, growth, and apoptosis	Unknown	Unknown
2c. Nonperoxisome lipid gene expression	Yes—this is the molecular basis of human therapeutic response to hypolipidemic drugs	Yes
3a. Peroxisome proliferation	Not likely	Not likely—no or weak response in human biopsy material and in non-human primates
3bi. Perturbation of cell proliferation	Not likely—not seen in many independent studies of human hepatocytes in vitro; not measured in humans in vivo; not seen in non-human primates in vivo or in vitro; not seen in hamsters	Not likely
3bii. Perturbation of apoptosis	Not likely—not seen in limited studies of human hepatocytes in vitro; not measured in humans in vivo	Not likely
4. Inhibition of GJIC	Not likely—no inhibition in primates in vitro or in vivo or in human hepatoctyes in vitro	Not likely
5. Hepatocyte oxidative stress	Unknown	Unknown
6. Kupffer cell-mediated events	Unknown	Unknown
7. Selective clonal expansion	Unknown—no response seen in non-human primates	Unknown
8. Liver tumors	Not likely	Not likely

However, immediate early genes such as *myc*, *fos*, and *jun* and cell-cycle genes such as the cyclin-dependent kinases (CDKs) and their inhibitors (CDIs) are regulated in rat and mouse liver by PPARα agonists. This upregulation is common to all cycling cells and thus is an expected but nonspecific effect of peroxisome proliferators in the rodent liver. These immediate early and cell-cycle genes have not been measured in humans.

The regulation of nonperoxisomal genes involved in fatty acid metabolism and transport occurs in human liver and other tissues and is the molecular basis of the therapeutic response to PPARα ligands. Since this occurs in humans in the absence of peroxisome proliferation, this suggests that the therapeutic response is independent of, and unrelated to, peroxisome proliferation. Although the regulation of genes involved in fatty acid metabolism occurs in humans, this is qualitatively different from the rat and mouse in several aspects. For example, some of the apoplipoprotein genes go up in the human but down in the rodent and the ApoA1 gene promoter is PPARα responsive in humans but not rats (reviewed in Roberts and Moffat, 2001).

It is clear that the response of human liver to PPARα ligands is qualitatively and quantitatively different from the response seen in rat and mouse liver. Evidence suggests that this is due to marked interspecies differences in PPARα-mediated gene regulation (different gene promoters, different receptor activities, and different receptor levels). Although the lower levels of PPARα in human liver may contribute to this difference, they do not provide a full explanation due to the complex nature of gene regulation via nuclear hormone receptors. Spare receptors may enable a maximal response without full receptor occupation; conversely, it is possible for a full response to be seen at reduced receptor levels. Thus, even if some human individuals possess high levels of PPARα (comparable to the rodent), it is highly unlikely that they would respond like rodents. Evidence for this comes from studies where genes that are PPARα-responsive in the rat and mouse liver remained nonresponsive in human liver cell lines even after overexpression of hPPARα (Hsu et al., 2001; Lawrence et al., 2001a).

PPARα ligands do not induce cell proliferation or suppress apoptosis in human hepatocytes in vitro (Perrone et al., 1998; Goll et al., 1999; Hasmall et al., 2000b; Williams and Perrone, 1995). Many of these studies include a positive control to ensure that human hepatocytes are of sufficient quality to mount a positive growth response. There are no data on human hepatocyte proliferation in vivo (nor are such data likely to become available). Nonetheless, confidence in a quantitative difference is provided by data from nonhuman primates where no regulation of cell proliferation is seen in vivo and in vitro (reviewed in Doull et al., 1999). Thus, it is not likely that PPARα ligands will regulate apoptosis and proliferation in human hepatocytes.

GJIC is regulated by PPARα ligands in rat and mouse liver. There are several studies to show that GJIC is not regulated by PPARα ligands in humans and nonhuman primates (Kamendulis et al., 2002). Although most of the studies on GJIC originate from the same laboratory, the weight of evidence suggests that regulation of GJIC by PPARα ligands would not occur in humans.

The end point of hepatocyte oxidative stress has not been evaluated in humans or nonhuman primates.

Kupffer-cell-mediated events have not been evaluated in humans or nonhuman primates.

Selective clonal expansion has not been evaluated in humans but does not occur in nonhuman primates.

There is no convincing evidence for human tumorigenicity resulting from exposure to the PPARα agonists DEHP and various hypolipidemic fibrates. The DEHP studies are not considered adequate to demonstrate lack of tumor hazard but do constitute some evidence of absence. In contrast, there are extensive data for some of the hypolipidaemic drugs, particularly clofibrate. IARC concluded that "the mechanism of liver carcinogenesis in clofibrate-treated rats would not be operative in humans" based on the results of extensive epidemiological studies (IARC, 1996), particularly the WHO trial on clofibrate comprising 208,000 man-years of observation (CPI, 1980, 1984). Further, a meta-analysis of the results from six clinical trials on clofibrate also found no excess cancer mortality (Law et al., 1994).

These human data are supported by evidence from nonhuman primate studies that show no evidence of tumors or focal lesions over 6 to 7 years of exposure. However, it is important to point out that many of the molecular and biochemical changes just described have not been evaluated in nonhuman primates using recently developed, high-affinity PPARα agonists. Most of the work to date has been performed and published using PPARα agonists with relatively low (DEHP, DINP) or moderate affinity (fibrate class of hypolipidemic drugs) for activation of PPARα.

In summary, the weight of evidence overall currently suggests that the rodent MOA for liver tumors is not likely to occur in humans, taking kinetic and dynamic factors into account. This conclusion is based upon evaluation of the existing body of evidence and would apply to the consequences of exposure to *known* examples of PPARα agonists. This judgment might warrant amendment if and when new, far more potent (therapeutic) agonists reach the market, as has been suggested to be a possibility (DeLuca, personal communication).

D. Case Studies—PPARα Agonist-Induced Hepatic Tumors

Four case studies illustrate the application of the ILSI RSI Human Relevance Framework to the databases for the liver tumors that these specific chemical agents induce. Three case studies addressing only the liver tumors follow. The fourth case study, which assesses Leydig-cell and pancreatic-cell tumors in addition to liver tumors, can be found at the end of section III. The three liver-only case

studies were chosen on the basis of the amount and nature of the data supporting a hypothesis that the liver tumor was induced following PPARα activation. They represent, in turn, 1) an example of a rich animal database but limited chemical-specific human data; (2) an example of a less robust animal database along with several human epidemiology studies that may shed light on this chemical's human carcinogenic potential, and (3) an example of an animal database which does not support PPARα activation as the sole MOA, even though additional relevant data were generated following the observation of liver tumors in the standard long-term chronic/carcinogenicity bioassay.

1. Robust Animal Data Set/Minimal Human Data

a. Introduction

Di-(2-ethylhexyl) phthalate (DEHP) was selected for the case study representing the Robust Animal Data/Minimal Human Data set. DEHP is a 2-ethylhexanol ester of *ortho*-phthalic acid. This substance is a plasticizer for polyvinyl chloride (PVC) and has specific uses in PVC products for medical devices such as intravenous and hemodialysis tubing, blood bags, and enteral feeding tubes. DEHP also may be used in a variety of consumer products formed from PVC including toys, household products, and interior automobile parts.

The National Toxicology Program conducted lifetime studies in which F344 rats and B6C3F$_1$ mice were provided a diet containing DEHP (NTP, 1982c). Both sexes of both species developed hepatocellular adenomas and carcinomas over the 2-year exposure period. Similar findings were reported by David et al. (1999) using the same strains of rodents but over an expanded dose range to better assess dose response. Dose levels at which tumors were observed are listed in Table 18. Adenomas in the pancreas and testes also have been reported for male rats (Berger, 1995; David et al., 2000a; NTP, 1982c). These nonhepatic lesions, which may be related to PPARα activation, are discussed elsewhere.

Peroxisome proliferators, including DEHP, are known to act on the liver via PPARα. Activation of this receptor leads to a cascade of events that results in tumorigenesis in the liver of rodents. For DEHP, the downstream events examined do not occur in isolated human hepatocytes or nonhuman primates. However, some downstream events of PPARα activation such as those related to fatty acid metabolism have been demonstrated in human hepatocytes or nonhuman primates in vivo exposed to peroxisome proliferators other than DEHP. Therefore, it may not be possible to totally separate, qualitatively, the mode of action for tumorigenesis in humans from that in animals although DEHP is considered to be a weak peroxisome proliferator compared with substances that have been shown to produce even a slight response in humans or non-human primates. Differences in kinetics and dynamics between humans and animals exist that also should be considered in characterizing the hazard for cancer in humans following exposure to DEHP.

TABLE 18
Hepatic Tumor Responses to DEHP in Mice and Rats

Mice			Rats		
Dietary level (%)	Dose (mg/kg)	Tumor incidence	Dietary level (%)	Dose (mg/kg)	Tumor incidence
Control		11%, 4%[a]	Control		7%, 0%[a]
0.01	19.2–23.8	23%, 6%			
0.05	98.5–116.8	32%*, 11%	0.05	28.9–36.1	7%, 2%
0.15	292.2–354.2	42%*, 29%*	0.25	146.6–181.7	17%*, 5%
0.3 (NTP)	672–799	52%*, 24%*	0.6 (NTP)	322–394	12%, 12%*
0.6 (NTP)	1325–1821	58%*, 36%*	1.2 (NTP)	674–774	24%*, 26%*
0.6	1266.1–1458.2	53%*, 63%*	1.25	789.0–938.5	43%*, 31%*

Note. (NTP) = NTP study only; asterisk, significantly higher than control.
[a]Male and females respectively.

b. Is the Weight of Evidence Sufficient to Establish the Mode of Action in Animals?

Postulated mode of action

The postulated mode of action for liver tumors resulting from activation of PPARα is described in detail elsewhere. For DEHP, an initial step in the carcinogenic process is the hydrolysis of the diester to its monoester metabolite, MEHP. Hydrolysis of DEHP occurs in the gastrointestinal tract via pancreatic lipases, in the liver by esterases and in blood via nonspecific esterases. MEHP is a stable metabolite that is more biologically active compared with the diester, DEHP. The remaining 2-ethylhexanol can be oxidized to 2-ethylhexanoic acid, both of which are very weak peroxisome proliferators. Therefore, most studies of DEHP using in vitro systems utilize MEHP as the active metabolite. Activation of PPARα is the initial key event in tumorigenesis that occurs with MEHP. Activation of this receptor then triggers DNA transcription for genes regulating peroxisomal enzymes, cell proliferation, and apoptosis in the hepatocyte. Increases in mRNA for peroxisomal enzyme activity have been demonstrated following exposure of rodents to DEHP or isolated cells to MEHP or MEHP metabolites. Bursts of cell proliferation and inhibition of apoptosis have been demonstrated in rodents exposed to DEHP or isolated cells to MEHP. Increased peroxisomal enzyme activity results in oxidative stress in the hepatocytes may trigger release of cytokines from the Kupffer cells. Increases in peroxisomal enzyme activity have been demonstrated in rodents following exposure to DEHP or isolated cells to MEHP, and release of cytokines has been demonstrated in isolated cells exposed to MEHP. Selective clonal expansion of cells in rodents occurs resulting in adenomas and carcinomas. The promoting properties of DEHP have been demonstrated in rodents.

Key events

An initial step in the toxicology of DEHP is the hydrolysis to its metabolically-active monoester, MEHP. In studies of peroxisome proliferation in isolated hepatocytes, the diester demonstrates little if any peroxisome proliferator activity (Gray et al., 1983; Mitchell et al., 1985a). Hydrolysis can occur in the gastrointestinal tract by pancreatic lipases, in the liver by esterases, or in the blood by nonspecific esterases (Huber et al., 1996). MEHP formation may be a prerequisite for absorption from the GI tract (Albro and Thomas, 1973; Carter et al., 1974; White et al., 1980, 1983).

Once hydrolyzed, MEHP can activate PPARα in the hepatocyte (Hasmall et al., 2000b; Issemann and Green, 1990; Issemann et al., 1993; Maloney and Waxman, 1999). DEHP does not activate the receptor (Maloney and Waxman, 1999). In addition, Ward et al. (1998) showed that treatment of PPAR-null mice with DEHP does not result in downstream key events (mRNA for CYP4A1, CYP4A3, acyl CoA oxidase, peroxisomal bifunctional enzyme, peroxisomal 3-ketoacyl CoA thiolase, increased liver weight, or hepatic lesions associated with peroxisome proliferation).

Activation of PPAR then triggers DNA transcription of genes for peroxisomal enzymes and fatty acid metabolizing enzymes (Reddy et al., 1986a; Ward et al., 1998). Levels of mRNA for these genes have been shown to increase in the livers of rodents exposed to DEHP. Peroxisome proliferation occurs as a result of treatment with DEHP and PPAR activation. Moody and Reddy (1978) demonstrated that rats exposed to DEHP developed an increase in the size and number of peroxisomes in the hepatocyte. These increases accompanied an increase in activity for enzymes associated with peroxisomes and the metabolism (oxidation) of lipids (Huber et al., 1996). Increases in the activities of catalase, carnitine acetyltransferase (CAT), lauric acid hydroxylation, and palmitoyl CoA oxidation (PalCoA) have been observed following treatment with DEHP. Increases in several forms of cytochrome P-450 (CYP) also have been reported (Dirven et al., 1990, 1992; Huber et al., 1996; Ward et al., 1998). The increase in enzyme activity is thought to result in oxidative stress in the hepatocyte with the possible formation of hydrogen peroxide leading to interactions with DNA (Reddy and Lalwani, 1983).

Although not demonstrated for DEHP, transcription of genes for cell replication and inhibition of apoptosis must occur because a burst of cell proliferation and an inhibition of apoptosis are observed following exposure to DEHP or MEHP. Bursts in cell replication have been demonstrated in F344 rats and $B6C3F_1$ mice following exposure of up to 13 weeks (Conway et al., 1989; James et al., 1998; Smith-Oliver and Butterworth, 1987; David et al., 1999) and cell replication was demonstrated, although not sustained over the entire time course, in F344 rats exposed to DEHP for 1 year

(Marsman et al., 1988) and in $B6C3F_1$ mice exposed for 40 weeks (Ward et al., 1988). Concomitant with cell proliferation is an inhibition of apoptosis. James et al. (1998) showed that inhibition of apoptosis occurred in rodent hepatocytes in vitro and in F344 rats and $B6C3F_1$ mice in vivo following exposure to DEHP. Both of these processes lead to a proliferation of cells. While concentration-related increases in cell replication and inhibition of apoptosis have been demonstrated in rat hepatocytes incubated with MEHP (Hasmall et al., 2000b), dose-related changes in cell proliferation and/or inhibition of apoptosis have not been demonstrated in vivo. In addition, the role of the Kupffer cell has been demonstrated. Rose et al. (1999) demonstrated that MEHP could activate the Kupffer cell to release cytokines in vitro, but not after in vivo exposure. The role of oncogenes in the carcinogenicity of DEHP is uncertain, but may be related to the proliferative events. DEHP can activate expression of c-*fos*, c-*jun, egr*-1, NUP475, *fos*B, JE, and KC in vitro (Ledwith et al., 1993, 1996), and c-*fos*, c-*jun* and c-*myc* in vivo (Hasmall et al., 1997).

Exposure to DEHP results in the inhibition of GJIC. Isenberg et al. (2000, 2001) demonstrated that F344 rats and $B6C3F_1$ mice treated with DEHP showed evidence of inhibition of GJIC; this effect was species specific, roughly following the sensitivity of species to peroxisome proliferation. No data on the response of GJIC in PPAR-null mice are available.

Exposure to peroxisome proliferators is thought to result in the induction of oxidative stress as described elsewhere. As noted, conflicting results have been reported following in vivo exposure to DEHP. Work by Takagi et al. (1990) and Cattley and Glover (1993) reported conflicting results in animals treated with DEHP; there are no data for dose-related increases in 8-OHdG with DEHP. On the other hand, Tomaszewski et al. (1986) reported dose-related increases in steady-state hydrogen peroxide levels in rat and mouse liver incubated with DEHP. Whether hydrogen peroxide levels can be equated with oxidative stress is uncertain.

DEHP promotes tumor growth following treatment of cells with various mutagens (Osterle and Deml, 1988; Ward et al., 1983, 1986).

The evidence in animals for the key events associated with DEHP-induced liver tumors is summarized in Table 19.

Strength, consistency, specificity of association of tumor response with key events/dose-response relationship/temporal association

Dose-response data for activation of PPARα by MEHP were generated by Issemann and Green (1990) using concentrations of 1–100 μM. Activation was measured by stimulation of CAT activity in COS1 cells. Activation ranged from 0 to 80% with an ED_{50} of 50 μM. Maloney and Waxman (1999) also demonstrated dose-dependent activation of PPARα by MEHP using COS1 cells transfected with a luciferase reporter gene. Significant increases in luciferase activity occurred at 5 μM MEHP with maximal activation at 20 μM MEHP (higher concentrations were not tested or reported). Activation did not occur following exposure of cells to 2 mM DEHP. Dose-related increases in peroxisomal enzyme activity were observed for PalCoA in vitro and in vivo. Gray et al. (1983), Hasmall et al. (1999, 2000b), and Mitchell et al. (1984, 1985a) all incubated rat hepatocytes with concentrations of 50 to 1000 μM MEHP in tissue culture demonstrating increased enzyme activity beginning at 50–100 μM. These results are consistent with those from Issemann and Green for activation of the PPARα receptor by MEHP. Reddy et al. (1986a) demonstrated that mRNA for peroxisomal enzymes was increased in the hepatocytes of rats exposed for 1 day to DEHP. Ward et al. (1998) showed that PPAR-null mice treated with 20,000 ppm DEHP in the diet do not demonstrate increases in cell proliferation, peroxisomal enzyme activity, or increases in CYP4A mRNA.

Increases in enzyme activity and peroxisome proliferation have been observed in a number of strains of rats and mice after in vivo exposure at the same dose levels that also are tumorigenic. Barber et al. (1987), David et al. (1999), Mitchell et al. (1985b), Moody and Reddy (1978), and Reddy et al. (1986b) all demonstrated increases in enzyme activity in F344 rats following exposure to DEHP. There is a good correlation between increased enzyme activity and increased volume density of peroxisomes (Reddy et al., 1986b). Therefore, increased enzyme activity may be used as a biomarker for peroxisome proliferation. Dose- and time-related changes in PalCoA activity in Wistar rats treated with 50, 200, or 1000 mg/kg DEHP were reported by Mitchell et al. (1985b). Animals showed

TABLE 19
Analysis of DEHP-induced Liver Tumors

Event	Evidence in animals	Key references
Hydrolysis to monoester essential for bioactivation[a]	In vitro for downstream events in primary rodent hepatocytes; in vitro for PPARα activation	Gray et al. (1983), Maloney and Waxman (1999), Mitchell et al. (1985a)
Activation of PPARα[a]	Concentration-related activation *in vitro*; no downstream events in PPAR-null mice in vivo	Hasmall et al. (2000b), Issemann and Green (1990), Maloney and Waxman (1999) in vitro. Ward et al. (1998) in vivo.
PPARα-dependent regulation of genes encoding for peroxisomal enzymes	In vivo increases in mRNA for cytochrome P-450 (CYP) in wild-type versus no increase in PPAR-null mice	Reddy et al. (1986a), Ward et al. (1998)
PPARα-dependent expression of cell cycle growth and apoptosis genes	No data	
PPARα-dependent expression of nonperoxisomal fatty acid metabolism genes	Increase in gene expression for fatty acid metabolism enzymes	Reddy et al. (1986a)
Peroxisome proliferation	Dose-related increases in CYP and peroxisomal enzymes	Barber et al. (1987), David et al. (1999), Dirven et al. (1990, 1992), Huber et al. (1996), Moody and Reddy (1978), Mitchell et al. (1985b), Reddy et al. (1986b)
Perturbation of cell proliferation and/or apoptosis[a]	Bursts of cell replication in vivo	Conway et al. (1989), David et al. (1999), James et al. (1998), Smith-Oliver and Butterworth (1987)
	Prolonged cell replication in vivo	Marsman et al. (1988), Mitchell et al. (1985b), Ward et al., (1988)
	Inhibition of apoptosis in vitro	Hasmall et al. (1999, 2000 b), James et al., (1998)
Inhibition of GJIC	GJIC inhibited in vivo	Isenberg et al., (2000, 2001), Kamendulis et al. (2002)
Hepatocyte oxidative stress	Conflicting data in vivo; increased H_2O_2 levels in vitro	Cattley and Glover (1993), Takagi et al., (1990b), Tomaszewski et al. (1986)
Kupffer-cell-mediated events	Kupffer cell-mediated cell proliferation altered in vitro	Rose et al. (1999)
Selective clonal expansion[a]	DEHP promotes initiated cells in vivo	Osterle and Deml (1988), Ward et al. (1983, 1986)

[a]Causal key event; others are associative key events.

an increase in activity over a 9-month exposure period. Reddy et al. exposed male F344 rats to varying levels in the diet (2500–20,000 ppm) of DEHP for 30 days. Barber et al. exposed male and female F344 rats to varying levels in the diet (100–25,000 ppm) DEHP for 21 days. David et al. (1999, 2000a) exposed male and female F344 rats and B6C3F$_1$ mice to varying levels of DEHP for up to 104 weeks.

Increases in the activities of PalCoA, other peroxisomal enzymes, and proliferation of peroxisomes also have been observed in other strains of rats with similar responses in Sprague-Dawley rats

(Barber et al., 1987; Ganning et al., 1991; Lake et al., 1984b) and Wistar rats (Dirven et al., 1990, 1992).

Several investigators have demonstrated that cytochrome P-450 activity is increased in response to treatment with DEHP. CYP4A is induced in male and female rat liver (Huber et al., 1996). Induction of CYP4A is dependent on PPAR (Ward et al., 1998). Dirven et al. (1990, 1992) reported dose-related increases in cytochrome P-450 4A1 (CYP4A1) following exposure to DEHP.

Bursts in cell replication have been demonstrated in F344 rats and $B6C3F_1$ mice following exposure of up to 13 weeks (Conway et al., 1989; David et al., 1999; James et al., 1998; Smith-Oliver and Butterworth, 1987). Dose-related increases in cell proliferation (based on BrdU labeling) were not seen, however. Increases in cell labeling were observed only at dietary levels of 12,500 ppm in rats and 10,000 ppm in mice. Lower dose levels that were carcinogenic did not demonstrate an increase in labeling index. It is possible, given the weak promotion ability, that BrdU labeling is an insensitive method for detecting low levels of cell proliferation. Dose- and time-related changes in DNA synthesis in Wistar rats treated with 50, 200, or 1000 mg/kg DEHP were reported by Mitchell et al. (1985b). Prolonged cell replication, albeit at a low level, was demonstrated in F344 rats exposed to DEHP for 1 year, although when observed at time points between 1 and 52 weeks, showed no sustained response (Marsman et al., 1988) and in $B6C3F_1$ mice exposed for up to 40 weeks (Ward et al., 1988). Studies conducted in vitro have shown some dose-related increases in DNA synthesis. Hasmall et al. (1999, 2000b) showed concentration-related increases in DNA synthesis in rat hepatocytes incubated with 250–750 μM MEHP, with significant increases at 500 and 750 μM.

Concomitant with cell proliferation is an inhibition of apoptosis. James et al. (1998) demonstrated that inhibition of apoptosis occurred in rodent hepatocytes incubated with 750 μM MEHP in vitro. Concentration-related inhibition of apoptosis was shown in rat hepatocytes incubated with 250–750 μM MEHP (Hasmall et al., 1999, 2000b).

Exposure to DEHP can promote clonal expansion of cells, although DEHP is a weak tumor promoter compared to other substances. Ward et al. (1983, 1986) in mice and Oesterle and Deml (1988) in rats demonstrated that DEHP could promote cells initiated with diethylnitrosamine.

The data for the dose response of key events are consistent with the incidence of tumors. The studies conducted by the National Toxicology Program (NTP, 1982c; Kluwe, 1982) and David et al. (1999) are the most robust in terms of numbers of animals. Both series of studies exposed F-344 rats and $B6C3F_1$ mice to dietary levels of DEHP for 104 weeks. David and coworkers included an interim necropsy after 78 weeks of treatment in which 10 animals per sex were evaluated from the control and highest dose levels and a recovery group that received control diet for the final 26 weeks of the study. The results indicate that dose levels of $\geq$147 mg/kg/day are carcinogenic in F344 rats; dose levels of $\geq$98 mg/kg/day are carcinogenic in $B6C3F_1$ mice. Only hepatocellular adenomas and carcinomas were seen in both species. Increased PalCoA activity measured after 1, 13, 78, and 104 weeks of exposure was observed at these same dose levels (David et al., 1999).

Temporal association and reversibility

The temporal association between the key events and tumors has been explored to a limited degree. Increases in peroxisomal enzyme activity can be detected within days of initiation of treatment (Barber et al., 1987; David et al., 1999; Isenberg et al., 2000; Mitchell et al., 1985b). Levels rapidly reach a maximum that is sustained for as long as treatment is continued (David et al., 1999; Ganning et al., 1991; Isenberg et al., 2001; Mitchell et al., 1985b). Temporal association of cell proliferation/inhibition of apoptosis and tumors is not well established. Bursts in cell replication in rats and mice have been demonstrated within the first 13 weeks of exposure (Conway et al., 1989; David et al., 1999; Isenberg et al., 2001; James et al., 1998; Mitchell et al., 1985b; Smith-Oliver and Butterworth, 1987). Prolonged cell replication, albeit at a low level, was demonstrated in F344 rats exposed to DEHP for 1 year (although time points between 1 week and 52 weeks showed no sustained response) (Marsman et al., 1988) and in $B6C3F_1$ mice exposed for up to 40 weeks (Ward et al., 1988). Cell replication has been demonstrated in animals receiving high dose levels of DEHP. In addition, in vivo data on tumor incidence, liver weight, and peroxisome proliferation indicate that cessation of treatment may arrest or alter the carcinogenic process; the tumor incidences were lower in animals treated for 78 weeks and then placed on the control diet for another 26 weeks than in those animals treated for the full 104 weeks (David et al., 1999).

Thus, the temporal relationship between exposure and tumorigenesis is demonstrable.

Considering the temporal relationships for tumor development following DEHP exposure: (a) The key events cited (PPAR activation, peroxisome proliferation, oxidative stress, cell replication, inhibition of apoptosis) are processes that show initial bursts of activity following the initiation of exposure; (b) there is evidence for sustained activity; and (c) cessation of exposure may have some affect on proliferation of tumors (David et al., 1999). Thus, the mechanism can adequately explain the temporal relationship between the key events and the development of tumors.

Biological plausibility and coherence of the database

DEHP, like most peroxisome proliferators, lacks the genotoxicity associated with other carcinogens. In order to understand the effect on DNA, the genetic toxicity of DEHP has been studied in many test systems under varying conditions. The results from both in vitro and in vivo studies have been negative.

The linkage of DEHP tumorigenesis to PPARα is indirect. Ward et al. (1998) demonstrated that PPARα-null mice treated with DEHP failed to demonstrate signs of peroxisome proliferation. These animals were treated for only 24 weeks. This is the only study of any duration with PPAR-knockout mice treated with DEHP. By association, the results of Peters et al. (1997) with WY 14,643 provides indirect evidence that DEHP tumorigenesis is related to PPARα. In that study, PPAR-knockout mice and wild-type mice were treated with WY 14,643, considered a more potent carcinogen/peroxisome proliferator than DEHP, for 1 year; the wild-type mice developed liver tumors, but the PPAR-knockout mice did not. It is assumed that a long-term study of DEHP using PPAR-knockout mice would provide the same results because WY 14,643 is a more potent peroxisome proliferator that causes the same tumors in the same tissues of rodents through the same key events.

Alternate modes of action

No other modes of action have been proposed for DEHP carcinogenesis. Genotoxic mechanisms can be excluded as a mode of action.

Conclusions—assessment of postulated MOA in animals and statement of confidence

The data supporting the proposed mode of action for DEHP and its reliance on PPARα in the liver are robust. Key elements in the mode of action for PPARα-induced tumorigenesis in rodents have been demonstrated for DEHP over time and dose. There are multiple data sets from different laboratories demonstrating the key events in response to DEHP/MEHP exposure. The dose response for many events is consistent with the observation of tumors in rodent studies. The temporal relationship between the observance of tumors in rodents and the potency of DEHP to promote clonal expansion of initiated cells is consistent. The lack of tumors in nonhepatic tissues, except those that may be related to PPARα activation as discussed elsewhere, and the lack of a number of key downstream events associated with PPARα activation in PPAR-null mice exposed to DEHP support the conclusion that all the effects observed are due only to the activation of this receptor and the downstream events resulting from this activation and that no other modes of action are operant.

c. Are the Key Events in the Animal MOA Plausible in Humans?

Concordance analysis of key events

Concordance of key events for the proposed liver tumor MOA of peroxisome proliferators in humans has been described elsewhere. In general, the receptor is present in human tissue, albeit at what appears to be reduced density compared with rats or mice. Activation of the receptor has been demonstrated for a number of substances. Gene expression and a minimal level of peroxisomal enzyme activity have been demonstrated in human or nonhuman primates with some peroxisome proliferators. The following focuses on the association of key events with exposure to DEHP (or its metabolites).

The initial key events for DEHP-associated tumorigenesis have been demonstrated using human hepatocytes or studies with nonhuman primates. Hydrolysis of the diester to the monoester occurs via lipases and esterases from human tissue (Huber et al., 1996) and data from in vivo studies showed that humans metabolize DEHP to MEHP (Albro

et al., 1982; Anderson et al., 2001; Schmid and Schlatter, 1985). In addition, there are data to indicate that DEHP/MEHP can activate the human PPARα (hPPAR). Maloney and Waxman (1999) demonstrated dose-dependent activation of PPARα by MEHP using COS1 cells transfected with a luciferase reporter gene. Significant increases in luciferase activity occurred at 5 μM MEHP with maximal activation at 20 μM MEHP (higher concentrations were not tested or reported). Similar results of activation of hPPARα in NIH 3T3 cells by MEHP were reported by Hasmall et al. (2000b).

However, studies that evaluated the downstream events of PPARα activation have generally shown no response in isolated human hepatocytes in vitro or nonhuman primate liver in vitro or in vivo. Studies comparing the hepatic effects of DEHP in rats and mice to other species have been conducted since the late 1970s. In general, rats and mice are sensitive, and hamsters are less sensitive, guinea pigs and primates are nonresponders showing no signs of increased peroxisomal enzyme activity, cell proliferation, inhibition of apoptosis, or inhibition of GJIC. For example, Dirven et al., (1993) observed no increase in RNA for CYP4A1 or acyl CoA oxidase in isolated nonhuman primate cells exposed to 100–600 μM MEHP. Hasmall et al. (1999, 2000b) found no increase in DNA replication or inhibition of apoptosis in isolated human hepatocytes incubated with 0.25-2 mM MEHP. Rhodes et al. (1986) treated marmosets with 2000 mg/kg DEHP by oral gavage for 14 days and did not observe any response in the liver. No increases in PalCoA oxidation or 12-lauric acid hydroxylase activities were seen. Kurata et al. (1998) treated male and female marmoset monkeys for 13 weeks with up to 2500 mg/kg DEHP by oral gavage. There was no change in liver weight, PalCoA, or CAT activities and no increase in volume-density of peroxisomes. Similar results have been observed in Old World monkeys. Short et al. (1987) observed no increase in liver weight, PalCoA, CAT, or 12-lauric acid hydroxylase activities in cynomolgus monkeys treated for 25 days with 500 mg/kg by gavage; Pugh et al. (2000) showed no increase in PalCoA oxidation, DNA replication, or change in GJIC in cynomolgus monkeys treated with 500 mg/kg for 14 days. These findings were confirmed by Kamendulis et al. (2002) using rat, nonhuman primate, and human hepatocytes exposed to up to 500 μM MEHP.

Isolated hepatocytes from nonhuman primates (Dirven et al., 1993; Kamendulis et al., 2002) or humans also are not responsive to MEHP compared with rat hepatocytes (Bichet et al., 1990; Elcombe and Mitchell, 1986; Hasmall et al., 1999, 2000b; Kamendulis et al., 2002). These data support the conclusion that primates are nonresponsive to the downstream events of DEHP. Hildebrand et al. (1999) and Goll et al. (1999) reported that rat hepatocytes incubated with DEHP showed evidence of peroxisome proliferation, whereas human hepatocytes did not respond. These studies suggest that DEHP can elicit a response in rat hepatocytes unlike other studies showing that only MEHP elicits a response in vitro. Given the magnitude of the responses observed by Hildebrand et al. and Goll et al. in rat hepatocytes, their data on nonresponsiveness of the human hepatocytes may be inconclusive.

The supporting evidence for concordance is summarized in Table 20.

Conclusion—statement of confidence

The early key events (hydrolysis and receptor activation) for the DEHP tumorigenic MOA have been demonstrated using human tissue. However, there is a lack of concordance for all the downstream events examined. This suggests that toxicodynamic differences exist that make human/primate liver cells insensitive to DEHP since the general processes (regulation of lipid metabolism, cell proliferation, oxidative stress, clonal expansion) are possible in human tissue.

d. Taking into Account Kinetic and Dynamic Factors, is the Animal MOA Plausible in Humans?

As discussed elsewhere, there are differences in the receptor density in human liver compared with rodent liver. Palmer et al. (1998), Tugwood et al. (1996), and Walgren et al. (2000) quantified the receptor population in human liver and demonstrated that humans contained roughly 10 times fewer receptors than did rodents (with the exception of one individual out of a total of 26 who had levels comparable to the mouse). These apparent differences in receptor density may play a role in the toxicodynamic differences that lead to a lack of downstream events in human cells/nonhuman primates following exposure to DEHP/MEHP. On the other hand, receptor population density alone may not explain species differences since HepG2 cells overexpressing hPPARα failed to respond to WY 14,643 (Lawrence et al., 2001a).

TABLE 20
Comparative Analysis of Rodent and Primate Data for DEHP

Event	Evidence in rats and mice	Evidence in primates
Hydrolysis to monoester essential for bioactivation	In vitro for downstream events in primary rodent hepatocytes; in vitro for PPARα activation	Hydrolysis and absorption occur in humans and primates, but the rate of hydrolysis is lower and absorption is lower and saturated at ~200 mg/kg
Activation of PPARα	Concentration-related activation in vitro; no downstream events in PPAR-null mice in vivo	hPPARα can be activated by MEHP, but receptor population is ~10 times lower in human liver than rodent liver
PPARα-dependent regulation of genes encoding for peroxisomal enzymes	In vivo increases in mRNA for cytochrome P-450 (CYP) in wild-type versus no increase in PPAR-null mice	No evidence of increased transcription
PPARα-dependent expression of cell cycle growth and apoptosis genes	No data	No data
PPARα-dependent expression of nonperoxisomal fatty acid metabolism genes	Increase in gene expression for fatty acid metabolism enzymes	No evidence of increased transcription
Peroxisome proliferation	Dose-related increases in CYP and peroxisomal enzymes	No peroxisomal enzyme activity in human cells
Perturbation of cell proliferation and/or apoptosis	Bursts of cell replication in vivo Prolonged cell replication in vivo Inhibition of apoptosis in vitro	No evidence of cell replication or inhibition of apoptosis
Inhibition of GJIC	GJIC inhibited in vivo	No evidence of inhibition of GJIC in primates in vivo, or human or primate hepatocytes in vitro
Hepatocyte oxidative stress	Conflicting data	No data
Kupffer-cell-mediated events	Kupffer-cell-mediated cell proliferation altered in vitro	No data
Selective clonal expansion	DEHP promotes initiated cells in vivo	No data

Kinetic factors

While some of the initial key events have been demonstrated for humans, there are differences in bioavailability which affect the assessment of DEHP. For example, across species there are differences in the rate of formation and absorption of MEHP following ingestion of DEHP. Data for species differences in hydrolysis of DEHP are minimal. However, Nachlas and Seligman (1949) showed that pancreatic esterase and lipase from humans has slightly lower hydrolytic activity than enzyme from rat pancreas depending on the substrate (see Table 21).

TABLE 21
Pancreatic Enzyme Activity in Rat and Human

Substrate	Rat pancreatic enzyme	Human pancreatic enzyme
β-Naphthyl acetate	5.71 ± 2.42	3.17 ± 1.24
β-Naphthyl laurate	2.02 ± 0.85	0.65 ± 0.34
β-Naphthyl stearate-palmitate	0.302 ± 0.065	0.044 ± 0.025

From Nachlas and Seligman (1949). Pancreatic extract incubated with various substrates and the activity measures. Activity expressed as μmol/hr/mg tissue.

In addition, Lake et al. (1977) showed that hydrolysis of DEHP was roughly 10 times slower for human intestinal mucosa than for mucosa from rats (0.0128 nmol/mg protein/h for humans vs. 0.112 nmol/mg protein/h for rats). Data from Astill (1989) support the conclusion that differences in esterase activity exist between rodents and primates. Astill treated cynomolgus monkeys with DEHP and compared the excretion with that of rodents. The data suggest that hydrolysis of DEHP was lower for the primate than for the rat. Thus, differences in the ability to hydrolyze the diester to the monoester suggest that differences in absorption (vide infra) could occur between humans and rodents.

Pharmacokinetic studies in primates and humans have demonstrated that DEHP is absorbed from the GI tract, but Rhodes et al. (1986) showed that the bioavailability of DEHP in marmoset monkeys is less than in rats. Across a wide dose range, rats absorb at least 50% of orally administered DEHP as monoester and excrete it in the urine (Rhodes et al., 1986). In contrast, marmosets absorb a much smaller fraction of high doses of orally administered DEHP. Rhodes and coworkers (1986) estimated that the maximum DEHP dose that could be absorbed by a marmoset was similar to that absorbed by a rat given 150 to 200 mg/kg. Schmid and Schlatter (1985) reported that human volunteers excreted only 11–15% of an orally administered dose of DEHP in the urine, and further studies indicated that, even at very low levels of DEHP administration, humans absorb only 12–14% of orally administered doses (Anderson et al., 2001). A PBPK model for the absorption and metabolism of DEHP has been developed for rats (Keys et al., 1999). This model has not yet been applied to humans.

Dynamic factors

As stated earlier, the ability of MEHP to activate the PPARα receptor has been demonstrated (Hasmall et al., 2000b; Maloney and Waxman, 1999). However, there may be differences in the efficacy for hPPAR relative to mouse PPAR (mPPAR). Maloney and Waxman (1999) showed that MEHP may be a less potent hPPAR agonist compared with mPPAR in COS1 cells transfected with a luciferase reporter gene, although the authors cautioned that direct comparisons may not be valid because of potential differences in plasmid levels.

In contrast to the data of Maloney and Waxman, Hasmall et al. (2000b) found no differences in the activation of hPPAR and mPPAR by MEHP. Both receptors were activated to roughly equivalent levels (in some cases, a greater level for hPPAR) using the ACO reporter gene in NIH 3T3 cells in the presence of a rat promoter (interestingly, neither hPPAR nor mPPAR was activated by MEHP in the presence of human promoter).

It is difficult to reconcile the differences between these two reports on activation. Assuming the data of Maloney and Waxman accurately reflect activation of the human receptor, one possibility is the source of cell lines. COS-1 cells are derived from the African green monkey (nonresponder), while NIH 3T3 cells are derived from the mouse (responder). Another difference between these two studies is in the quantitation of activation. Maloney and Waxman used a luciferin/luciferase reaction to measure activity, while Hasmall and coworkers used acetyl CoA oxidase activity. Which method of quantitation more accurately reflects efficacy is not known, but differential activation of hPPAR and rPPAR by the same peroxisome proliferator has been described, and the cell line used can have an impact on activation (Mukherjee et al., 1994). In addition, a recent study suggests that luciferase gene expression is a good tool to evaluate efficacy of receptor activation (Wurch et al., 2002). Another possibility is that plasmid transfection by Maloney and Waxman did not result in equivalent levels of receptor and their data do not accurately reflect efficacy of hPPAR activation by MEHP. On the other hand, the data of Yu et al. (2001) also showed differences in hPPAR and mPPAR activation by MEHP.

If differences in efficacy of receptor activation by MEHP exist they are, alone, insufficient to explain the lack of biological events in primates and isolated human hepatocytes. Following activation of the receptor in human cells, the resulting gene expression may not take place because of species-specific postactivation deficiencies. Such a hypothesis is supported by data from Gervois et al. (1999), Keller et al. (1997), Lambe et al. (1999), and Woodyatt et al. (1999) suggesting that the receptor itself or the response element may be altered in humans relative to rodents. The data by Hasmall et al. (2000b) in which mPPAR and hPPAR were both inactive when human promoter was transfected into the NIH 3T3 cells suggest that postactivational differences lead to the lack of the response.

Thus, while hPPAR can be activated by DEHP/MEHP, the downstream events may not occur because of dynamic differences in the processes leading to gene expression. Potential kinetic and dynamic differences are summarized in Table 22.

TABLE 22
Potential Kinetic and Dynamic Differences Between Rodents and Humans

Factor	Difference between human (or non-human primate) and rodent	Reference
Hydrolysis by lipase	1.8–8.75 times less in human	Lake et al. (1977), Nachlas and Seligman (1949)
Absorption	25 times less in marmoset	Rhodes et al., (1986)
Activation of PPARα	Potentially 1.5 times lower in human	Maloney and Waxman (1999), Yu et al. (2001)

Further support for the lack of hazard potential may come from the limited epidemiology data available. There are several studies that have examined the incidence of cancer or cancer-related mortality in workers exposed to DEHP. Because DEHP has been manufactured for more than 50 years, workers represent a population exposed for sufficient length of time to evaluate potential risk. Thiess et al. (1978a) evaluated mortality among 221 workers exposed to DEHP during manufacture at the BASF facility in Ludwigshafen, Germany. Measured airborne concentrations of DEHP were 0.003–0.004 ppm in work areas. The subjects, 101 employees, ranged in age from 22 to 60 years of age and had been exposed for 4 months to 35 years (average of 12 years). There were 8 deaths among this group, compared with 15.9 expected deaths in the geographic area and 17.0 expected from the national rate. There was one death from pancreatic cancer (with 0.13 expected) and one bladder papilloma (0.01 expected) in workers with the longest employment. Other investigators have focused on the polyvinyl chloride (PVC) industry and cancer-related deaths (Hagmar et al., 1990; Hardell et al., 1997; Ohlson and Hardell, 2000). Unfortunately, it is not possible to separate exposure to DEHP from exposure to other substances. Therefore, no conclusions about an association can be made between exposure to DEHP and occurrence of cancer.

Exposure to DEHP is highest among patients receiving hemodialysis or infants receiving extracorporeal membrane oxygenation (ECMO) (Huber et al., 1996). The cancer-related mortality among dialysis patients has been investigated. Kantor et al. (1987) studied a cohort of 28,049 patients with chronic renal failure who received dialysis for at least 6 months. The relative risk of cancer was less than 1 (0.9 relative risk) compared with the national incidence. Leukemia, non-Hodgkin's lymphoma, Hodgkin's disease, thyroid cancer, and biliary-tract cancer were moderately increased (relative risk of 1.4–2.2). The relative risk for liver cancer was less than 1 for males (0.4) and higher than 2 for females (2.4) with an overall risk of 0.9. Mailloux et al. (1991) evaluated the cause of death for 532 dialysis patients who were monitored over a 16-year period. Increased cancer-related mortality was not associated with dialysis. On the other hand, Iseki et al. (1993) and Inamoto et al. (1991) found cancer-related mortality higher in 1982 patients and 23,209 patients, respectively. Cancers of the colon, uterus, and breast were higher among dialysis patients in Okinawa (Iseki et al., 1993). Similar results were observed by Inamoto et al. (1991) with the addition that liver cancer was higher than expected in dialysis patients. For several reasons, this finding does not provide sufficient evidence of DEHP-related cancer in humans. First, the organ sites of cancer observed by Inamoto et al. reflected sites in the general population. Tumors in the uterus, colon, and breast would not be expected based on animal studies. Second, there is no concordance between the findings from the Japanese studies and the earlier study by Kantor. Given that the population sizes were comparable, one would expect the sites and incidences of cancer to be comparable unless there were other confounding factors. And third, patients who receive hypolipidemic drugs are not known to have a higher-than expected incidence of liver cancer, suggesting that more potent peroxisome proliferators have failed to show a similar cancer rate.

Therefore, the limited epidemiology data indicate that there is no evidence of carcinogenic effects of DEHP in humans, which provides support to the conclusion that downstream events are not likely to occur in humans and that there would be no cancer hazard.

e. Statement of Confidence

Nonhuman primate and human cells in culture are refractory to the peroxisome proliferating effects of DEHP. This lack of response has been tested in

several laboratories over several years in old world and new world primates and in isolated human hepatocytes. The confidence is high that primates and humans do not respond to DEHP exposure as do rodents. This may be explained by a combination of lower PPARα receptor population in human tissue, mutant PPARα isoforms, and/or PPREs in target genes, lower efficacy of hPPAR activation by MEHP, the inability to trigger downstream events, and lower bioavailablity of MEHP at the target tissue from lower absorption and/or hydrolysis. Any one factor may not be responsible for the lack of response. Instead, each factor may contribute. In addition, the lack of cell proliferation in primates exposed to rodent tumorigenic dose levels of DEHP indicates a lack of total concordance for this key event for this substance and suggests that humans may be refractory to the carcinogenic effects of all PPARα agonists.

f. Implications

Using the human relevance framework, the data are sufficient to support the conclusion that a mode of action for the DEHP-induced liver tumor has been established in animals. Comparing the key events of this MOA to humans (and nonhuman primates), the key event of perturbation of cell proliferation and/or apoptosis does not occur in nonhuman primates. In addition, some associative events such as increased peroxisomal enzyme activity and inhibition of GJIC do not occur. This leads to a conclusion that key events of the MOA are not plausible in nonhuman primates (and humans). The strength of this conclusion rests on only one study of a causal key event and several studies of associative events. However, dynamic and kinetic factors contribute to this decision. The data lead to a conclusion that a carcinogenic response induced via the MOAs for liver tumorigenesis in the rodent is not likely to occur in humans following exposure to DEHP.

2. Modest Animal Data/Modest Human Data

a. Introduction

Clofibrate was selected to represent the modest animal data/modest human data case study. Clofibrate (ethyl chlorophenoxyisobutyrate) is a colorless to pale yellow liquid. Clofibrate is a therapeutic agent used for the treatment of hyperlipidemias in humans. It is taken orally as capsules at the usual daily dose of 2 g (20–30 mg/kg body weight) and has been used in the prophylaxis of ischemic heart disease.

Clofibrate was tested for carcinogenicity by dietary administration in several experiments in rats and mice, and in one experiment in marmosets by gastric instillation for 6.5 years. It induced liver tumors, Leydig-cell tumors, and pancreatic acinar-cell tumors in rats; this phenomenon is characterized as the "Tumor Triad." No increase in tumor incidence was reported in mice or marmosets. In addition to the animal data, this case study is unique in having some limited human data. This case study on clofibrate explores the mode of action information available on the liver tumor ONLY (not the Leydig cell and pancreatic acinar-cell tumors).

b. Is the Weight of Evidence Sufficient to Establish the Mode of Action in Animals?

Postulated mode of action

The postulated mode of action for liver tumors resulting from activation of PPARα is described in detail elsewhere. PPARα activation is involved in clofibrate-induced liver growth and carcinogenesis in the rat. Not all of the key events observed in rats following clofibrate administration also are seen in humans. Clofibric acid is the active (and primary) metabolite of clofibrate; conversion of the parent to this metabolite precedes receptor activation, since the ethyl ester is rapidly hydrolyzed to the acid (Thorp, 1962). In light of this, the majority of data on mode of action (MOA) have been derived using the metabolite rather than the parent compound.

Evidence in animals/key events

There are data supporting PPARα activation in clofibrate-induced liver growth and carcinogenesis in the rat. Issenman and Green (1990) showed concentration-related activated reporter constructs in vitro, exhibiting less potency than the other fibrates tested (WY 14,643, nefenopin, and methylclofenopate). PPARα-null mice failed to show the increases in liver weight, hepatic peroxisome proliferation, and mRNA levels exhibited by wild-type mice when given clofibrate at 0.5% in the diet for 2 weeks (Lee et al., 1995).

In one study, male and female Sprague-Dawley rats were given clofibrate in the diet (1500–9000 ppm or approximately 90–540 mg/kg body weight) for up to 13 weeks. Increases in peroxisomal fatty acyl CoA oxidase activity were observed after 1 week (at all doses in males and only at the highest dose in females) and after 13 weeks in animals treated with 4500 or 9000 ppm. In male rats that ingested 9000 ppm clofibrate for 13 weeks, both peroxisomal fatty acyl CoA oxidase activities and peroxisomal volume densities were increased approximately sevenfold (IARC, 1996). Incubation of primary cultures of rat hepatocytes with clofibric acid at 50–250 μM for up to 72 h resulted in an up to sixfold increase in the induction of peroxisomal β-oxidation (Foxworthy and Eacho, 1986), which is consistent with in vivo data. Combined, it is clear that clofibrate can cause peroxisome proliferation in rodents and cultured rat primary hepatocytes.

There is evidence that clofibrate can induce increases in hepatic cell proliferation in rodents and cultured primary rat hepatocytes. In rats fed 0.5% clofibric acid for 1 to 26 weeks, BrdU-labeling indices (an indirect measure of cell replication) were approximately 10 times higher in the treated group than in controls in the first week of treatment, but this increase was not found at later time points compared to other more potent PPARα agonists such as methylclofenapate or WY 14,643 (Barrass et al., 1993; Eacho et al., 1991; Marsman et al., 1992). Clofibrate (i.e., 50–250 μM clofibric acid) also has been shown to cause mild increases in BrdU labeling ($\sim$25%) in cultured primary rat hepatocytes after 48 h of culture (Goll et al., 1999). In contrast, others report no difference in ^{3}H-thymidine incorporation into primary rat hepatocytes cultured for 24–48 h in 100–1000 μM clofibric acid (Perrone et al., 1998). This disparity could be due to the different methods used to assess replicative DNA synthesis. In any case, there are both in vivo and in vitro data showing that clofibric acid can cause increased replicative DNA synthesis.

Clofibrate induction of peroxisome proliferation and associated effects are likely mediated by PPARα. PPARα-null mice failed to show the increases in liver weight, hepatic peroxisome proliferation, and mRNA levels for peroxisomal enzymes exhibited by wild-type mice when given clofibrate at 0.5% in the diet for 2 weeks (Lee et al., 1995).

Oxidative stress and liver damage, as evidenced by accumulation of hepatic lipofuscin, have been reported in Fischer 344 rats given 5000 ppm clofibric acid in the diet ($\sim$300 mg/kg body weight) for up to 22 weeks. In male Sprague-Dawley rats treated with 500 mg/kg body weight clofibrate in the diet for 22 days, increases in hepatic lipofuscin (three- to four-fold) were completely prevented when the antioxidant vitamin E was fed simultaneously with clofibrate; however, peroxisomal β-oxidation activity was still increased (Marsman et al., 1992; Stanko et al., 1995). A slight (two- to threefold) increase was observed in levels of 8-hydroxydeoxyguanosine, a marker of oxidative DNA damage, in hepatic DNA of male Fischer 344 rats fed 0.5% clofibric acid for 22 weeks in the diet, and a similar small increase in this adduct was observed in rats treated at this dose with clofibrate for one month, but this may have been due to the method of DNA isolation which likely included mitochondrial DNA in the analysis (Cattley and Glover, 1993). Levels of 8-hydroxydeoxyguanosine in rats fed clofibrate (0.5%) for up to 1 year also were increased after 2 or 12 months of feeding as compared to controls (Takagi et al., 1990a).

In studies of the effects of clofibrate given in combination with known carcinogens, clofibrate enhanced the hepatocarcinogenicity of *N*-nitrosamines in rats and hamsters (Cattley et al., 1994; Hosokawa et al., 1989; Mizumoto et al., 1988; Mochizuki et al., 1983).

The key events associated with clofibrate hepatocarcinogenesis in rats are summarized in Table 23.

Conclusions—assessment of postulated and alternative MOAs in animals and statement of confidence

Studies in PPARα-null mice have demonstrated that the induction of peroxisome proliferation, hepatic cell proliferation and liver tumor formation by WY 14,643 is mediated through PPARα (Lee et al., 1995; Peters et al., 1997a, 1998). There are data showing that the hepatocarcinogenic effect of clofibrate in the rat is associated with peroxisome proliferation and increased cell proliferation (IARC, 1996). Short-term studies using PPARα-null mice have shown that PPARα activation is required for clofibrate-induced liver growth, peroxisome proliferation and target gene activation (Lee et al., 1995). No long-term studies have been conducted in PPARα-null mice, and two studies in mice fed clofibrate ranging from 1000 to 5000 ppm for 18 months provided no evidence of increased liver tumor formation as a result of treatment (reviewed in IARC, 1996). There is only indirect evidence that hepatocarcinogenesis of clofibrate in the rat is

TABLE 23
Key Events Associated with Clofibrate-Induced Hepatocarcinogenesis in Rats

Event	Evidence in animals	Key references
Hydrolysis to clofibric acid[a]	Rapidly hydrolyzed by tissue and serum esterases in rats	Cayen et al. (1977), Thorp (1962)
Activation of PPARα[a]	Concentration-related activation of reporter constructs in vitro; no target gene expression in PPARα-null mice	Issemann and Green (1990), Lee et al. (1995)
PPARα-mediated regulation of genes encoding peroxisomal enzymes	Increased expression of genes encoding peroxisomal enzymes are found in wild-type mice but not in clofibrate-treated PPARα-null mice	Lee et al. (1995)
PPARα-mediated regulation of genes of cell proliferation and apoptosis	No data	
PPARα-mediated regulation of fatty acid metabolism genes	Liver fatty acid binding protein and CYP4A are induced in wild-type mice but not in clofibrate-treated PPARα-null mice	Lee et al. (1995)
Peroxisome proliferation	Peroxisome proliferation found in wild-type mice but not in clofibrate-treated PPARα-null mice	Lee et al. (1995)
Perturbation of cell proliferation and/or apoptosis[a]	Clofibrate causes increased replicative DNA synthesis in vivo and in vitro	Barrass et al. (1993), Eacho et al. (1991), Goll et al. (1999), Marsman et al. (1992)
Inhibition of GJIC	GJIC inhibited in F344 rat liver	Krutovskikh et al. (1995)
Hepatocyte oxidative stress	Increased measures of oxidative damage (lipofuscin accumulation, 8OH-dG) in clofibrate-treated rats or rat hepatocytes	Lake et al. (1987), Marsman et al. (1992), Qu et al. (2001), Stanko et al. (1995), Takagi et al. (1990)
Kupffer cell mediated cell proliferation	No data	
Selective clonal expansion[a]	Enhanced hepatocarcinogenicity of *N*-nitrosamines in rats and hamsters	Cattley et al. (1994), Hosokawa et al. (1989), Mizumoto et al. (1988), Mochizuki et al. (1983)

[a]Causal key event; others are associative key events.

mediated through PPARα, informed by the absence of tumorigenesis in PPARα-null mice treated with agonists that do produce tumors in wild-type mice. The precise mechanism for the induction of cell proliferation in the rat liver is uncertain.

Clofibrate has been tested for genotoxicity in many assay systems, and the results have been uniformly negative (reviewed in IARC, 1996). Induction of liver tumors in rodents by clofibrate does not likely involve a direct genotoxic mechanism. Inhibition of gap-junctional intercellular communication at high concentrations of clofibrate was reported in F344 rat liver (Krutovskikh et al., 1995); however, the significance of this event is uncertain. Activation of PPARα and subsequent downstream events appears to be the only biologically plausible mode of action for liver carcinogenesis of clofibrate in rats.

c. Are the Key Events in the Animal MOA Plausible in Humans?

PPARα is expressed in human liver (Palmer et al., 1998; Tugwood et al., 1996). The human PPARα cDNA has been cloned and appears to be indistinguishable from that of the rodents in overall structure; the DNA binding domains of the human and rodent PPARα are virtually 100% homologous (Mukherjee et al., 1994; Sher et al., 1993). The pharmacological reduction in serum triglycerides and cholesterol in patients treated with clofibrate has been well documented and is consistent with activation of PPARα (IARC, 1996). Indeed, clofibrate or clofibric acid can effectively trans-activate reporter constructs (luciferase or CAT activity) containing mouse PPREs in Hepa cells transiently transfected with human PPARα (Pineau et al., 1996; Sher et al., 1993). Other reports also have demonstrated that human PPARα is either equally or significantly less effective in trans-activating reporter constructs in transfected cells compared to the murine PPARα in response to a variety of other PPARα agonists ranging from weak (DEHP) to more potent (e.g., WY 14,643, nafenopin) PPARα agonists (Hasmall et al., 2000b; Maloney and Waxman, 1999). It is noteworthy that relative to other PPARα agonists such as WY 14,643 or nafenopin, clofibrate/clofibric acid is a relatively weak ligand for PPARα and a relatively weak peroxisome proliferator. Additionally, significant differences in modulation of gene expression are reported to occur between human and rodent cells in response to clofibrate and other PPARα agonists.

Studies examining the extent of peroxisome proliferation by clofibrate in humans have been conducted. Liver biopsies from human patients treated with gemfibrozil, clofibrate or fenofibrate for periods ranging from 2 to 156 months (9–28 samples examined per study) did not reveal convincing evidence of peroxisome proliferation (Blumcke et al., 1983; de la Iglesia et al., 1982; Gariot et al., 1987). One study examining the effects of clofibrate in human liver showed a statistically significant 50% increase in peroxisome number and a statistically nonsignificant 23% increase in peroxisome density (Hanefeld et al., 1983). Since the measurement of volume density rather than the number of peroxisomes is indicative of peroxisome proliferation, these results indicate a negative human response (Cattley et al., 1998).

There are no human data demonstrating alterations in PPARα target gene expression in vivo. However, there are data available from both human primary hepatocytes and human hepatoma cell lines that have examined changes in peroxisomal enzyme activity, fatty acid metabolizing enzymes and replicative DNA synthesis in response to clofibric acid/clofibrate or other PPARα agonists. Most studies using primary human hepatocytes or human hepatoma cell lines have shown no changes or minimal induction in palmitoyl CoA oxidation and/or peroxisome proliferation in response to clofibrate or clofibric acid, while induction of these endpoints is found in clofibrate- or clofibric acid-treated rat primary hepatocytes (reviewed in Doull et al., 1999). In contrast, significant dose-dependent induction (<3-fold) of acyl CoA oxidase activity has been observed in human hepatocytes treated with clofibrate and ciprofibrate (Perrone et al., 1998), and treatment with perfluorodecanoic acid resulted in significant induction of peroxisomal density and increased acyl CoA oxidase activity in human cells derived from glioblastoma (Cimini et al., 2000). Human PPARα activation also is reported to result in increased apolipoprotein A-II and lipoprotein lipase transcription, reduced apolipoprotein C-III, which are likely key to lowering serum triglycerides (Auwerx et al., 1996; Staels et al., 1997; Vu-Dac et al., 1995). Combined, there is evidence that clofibrate can cause modulation of PPARα target genes that regulate lipid metabolism in human liver cells, but there are some notable species differences.

The effect of clofibrate on replicative DNA synthesis in primary human hepatocytes has been examined in at least two studies. In the first, culturing human primary hepatocytes in clofibric acid (250 μM) for 48 h caused no change in BrdU labeling indices as compared to a marginal increase ($\sim$25%) in rat primary hepatocytes cultured in the same concentration of clofibric acid (Goll et al., 1999). In a second independent study, [^{3}H]thymidine incorporation was not affected in human primary hepatocytes cultured for 48 h in the presence of 100–1000 μM clofibric acid, while [^{3}H]thymidine incorporation was not different in similarly treated rat primary hepatocytes (Perrone et al., 1998). However, a number of other PPARα agonists have been shown to induce cell proliferation in rat, but not human, primary hepatocytes (reviewed in Doull et al., 1999). It should be noted that the magnitude of induction of cell proliferation is generally lower in hepatocyte cultures compared to measures obtained in vivo. Inhibition of apoptosis also is reported to occur in response to PPARα agonists and could contribute to hepatomegaly and/or proliferation of mutant liver cells leading to tumor formation. Consistent with this observation, primary rat hepatocytes cultured

in the presence of 50–250 μM clofibric acid exhibit significantly lower levels of apoptotic cells, while primary human hepatocytes cultured under the same conditions exhibit no change in the number of cells undergoing apoptosis (Goll et al., 1999). These observations are consistent with other reports examining the effects of another PPARα agonist, MEHP, on apoptosis in primary rat or human hepatocytes (Hasmall et al., 2000b). However, in a second independent study, the number of apoptotic cells in primary rat hepatocytes cultured in the presence of 100–1000 μM clofibric acid was not different than controls, while measures of apoptosis increased in primary human hepatocytes cultured under the same conditions as compared to controls (Perrone et al., 1998). Nevertheless, these studies provide strong evidence that rat and human primary hepatocytes respond significantly differently to clofibrate with respect to changes in replicative DNA synthesis and apoptosis. Collectively, these studies provide supporting evidence for a lack of sensitivity to clofibrate of human liver cells compared to rats. While there are clear species differences noted in cultured primary hepatocytes, there is no conclusive evidence reported to date identifying the specific target genes that mediate increased hepatic cell proliferation in response to clofibrate or other PPARα agonists.

In summary, PPARα is present in human liver and high sequence homology exists between humans and rats. Clofibric acid is capable of activating human PPARα. While there is no direct evidence of increases in replicative DNA synthesis or inhibition of apoptosis in human liver cells treated with clofibric acid as is seen in rat primary hepatocytes, specific PPARα target genes that cause increases in hepatic cell proliferation have not been identified. Nonetheless, the MOA for clofibrate hepatocarcinogenesis in rodents is not likely to occur in humans, especially when considering other quantitative species differences.

The comparative analysis of rodent and human data is summarized in Table 24.

d. Taking into Account Kinetic and Dynamic Factors, is the Animal MOA Plausible in Humans?

Data on species differences

While the MOA for clofibrate hepatocarcinogenesis in ras is unlikely to occur in humans based upon a qualitative evaluation, one also must consider whether there are quantitative species differences. Data are available that describe quantitative differences between rats, nonhuman primates, and humans in some pharmacokinetics and key events of the MOA.

The absorption, distribution, metabolism, and excretion of clofibrate in rats and in humans have been reviewed (IARC, 1996). The absorption and distribution of orally administered clofibrate in rats appear to be similar to humans. As in the rat, clofibrate also is rapidly hydrolyzed by tissue and serum esterases to clofibric acid in humans (Cayen et al., 1977; Thorp, 1962). In both species, clofibric acid is eliminated in urine as the glucuronide, with small amounts of free clofibric acid (about 15% in rats and 2% in humans) (Emudianughe et al., 1983). Elimination half-lives in rats and humans were estimated to be 4.1 and 15–18 h, respectively (Baldwin et al., 1980; Gugler and Hartlapp, 1978). Therefore, clofibrate is about four times more persistent in human tissue compared to rats. The higher elimination rate in rats is thought to be due to lower binding of clofibric acid to serum protein in rats than in humans.

PPARα is expressed in human liver at levels reported to be approximately 10% compared to expression levels found in the liver of rodents (Palmer et al., 1998; Tugwood et al., 1996). Further, guinea pig liver also is reported to contain significantly less PPARα compared to mouse liver (Bell et al., 1998). In contrast, it is reported that while the level of human PPARα protein was considerably lower in five out of six human liver samples, one human liver sample appeared to express PPARα protein at levels comparable to mouse liver (Walgren et al., 2000). Combined, these observations suggest that the level of PPARα in the human liver may not be sufficient to activate some of the plethora of target genes that are altered by PPARα agonists in rodent models, yet may be capable of modulating lipid homeostasis through alteration of expression of other genes in the liver or, perhaps, more likely other target organs that express higher levels of PPARα. However, the hypothesis that reduced levels of PPARα in human liver accounts for the apparent species difference in response to PPARα agonists has not been tested adequately to date and deserves attention, since a reduced number of nuclear hormone receptors could still be capable of activating critical target genes in response to ligand activation. There are several other possible differences that could contribute to species differences in PPARα activation. The studies describing these differences were described in detail earlier.

Alterations in target gene expression in response to PPARα activation are significantly

TABLE 24
Comparative Analysis of Rodent and Human Data for Clofibrate

Event	Evidence in animals	Evidence in humans
Hydrolysis to clofibric acid[a]	Rapidly hydrolyzed by tissue and serum esterases in rats	Rapidly hydrolyzed by tissue and serum esterases in humans
Activation of PPARα[a]	Concentration-related activation of reporter constructs in vitro; no target gene expression in PPARα-null mice	Activation of reporter constructs by clofibric acid in vitro
PPARα-mediated regulation of peroxisomal genes	Robust increase in expression of genes encoding peroxisomal enzymes are found in rats and in wild-type mice but not in clofibrate-treated PPARα-null mice; relatively large increase in acyl CoA oxidase activity in primary rat hepatocytes cultured in clofibric acid	No induction of acyl CoA oxidase in human liver from patients treated with fibrates; primary human hepatocytes show no or minimal induction of palmitoyl CoA oxidase in response to clofibric acid
PPARα-mediated regulation of genes of proliferation and apoptosis	No data	No data
PPARα-mediated regulation of fatty acid metabolism genes	Liver fatty acid binding protein and CYP4A are induced in rats and in wild-type mice but not in clofibrate-treated PPARα-null mice	Human hepatocytes and cell lines show altered gene expression of Apo CIII, Apo AII and LPL in response to other PPARα agonists; no data available for clofibrate. Reduction in serum triglycerides and cholesterol in patients treated with clofibrate suggests other lipid-related target genes might be regulated similarly
Peroxisome proliferation	Peroxisome proliferation is found in rats and in wild-type mice but not in clofibrate-treated PPARα-null mice; primary rat hepatocytes exhibit peroxisome proliferation in response to clofibrate	Human biopsies from patients treated with clofibrate are essentially negative; primary human hepatocytes exhibit no peroxisome proliferation in response to clofibrate
Perturbation of cell proliferation and/or apoptosis[a]	Clofibrate causes increased replicative DNA synthesis in vivo and in vitro; clofibric acid and other PPARα agonists inhibits apoptosis in primary rat hepatocytes	Primary human hepatocytes treated with clofibrate and other PPARα agonists exhibit no evidence of increased DNA synthesis, and no changes or increases in apoptosis
Inhibition of GJIC	GJIC inhibited in F344 rat liver	Does not inhibit GJIC
Hepatocyte oxidative stress	Increased measures of oxidative damage (lipofuscin accumulation, 8-OH-dG) in clofibrate-treated rats or rat hepatocytes	No data
Kupffer-cell-mediated cell proliferation	No data	No data
Selective clonal expansion[a]	Enhanced the hepatocarcinogenicity of *N*-nitrosamines in rats and hamsters	No data

[a] Causal key event; others are associative key events.

different between rodents, nonhuman primates, and humans based on both in vivo and in vitro studies. The studies describing these differences were described in detail earlier.

There is additional indirect evidence from numerous studies that measured changes in enzyme activity of PPARα-responsive genes showing significant species differences in the response elicited by clofibrate. For example, studies using primary human hepatocytes or human hepatoma cell lines have shown no changes or minimal induction in palmitoyl CoA oxidation and/or peroxisome proliferation in response to clofibrate or clofibric acid, while induction of these endpoints is found in clofibrate-treated rat primary hepatocytes (reviewed in Doull et al., 1999). In contrast, significant dose-dependent induction (<3-fold) of acyl CoA oxidase activity has been observed in human hepatocytes treated with clofibrate and ciprofibrate (Perrone et al., 1998), and treatment with perfluorodecanoic acid resulted in significant induction of peroxisomal density and increased acyl CoA oxidase activity in human cells derived from glioblastoma (Cimini et al., 2000). A comparative study of the effects in the rat hepatoma FaO and the human hepEBNA2 cell lines showed a dose- and time-dependent induction of acyl CoA oxidase activity (a marker enzyme for peroxisome proliferation) as well as the level of acyl CoA oxidase mRNA in the human cell line by clofibrate although a significant species difference was observed since the maximum stimulation of acyl CoA oxidase activity was 2.4- to 3-fold for the human hepEBNA2 cell line compared to 6- to 11-fold for the rat hepatoma FaO cell line (Scotto et al., 1995). Since many of these changes in protein expression have been shown to require a functional PPARα (using a null mouse model), these observations collectively provide additional support for the idea that activation of PPARα results in significantly different modulation of target genes between rodents and humans.

Differences in specific target gene regulation of proteins involved in both peroxisomal fatty oxidation and other lipid-related enzymes may be due to differences in DNA-responsive elements or other receptor–protein interactions. Data supporting these observations were described earlier.

In addition to significant differences in responsive target genes induced via activation of PPARα observed in a variety of species, there also is evidence that the hallmark response of peroxisome proliferation resulting from clofibrate exposure is species-specific. For example, rats and mice are highly responsive to clofibrate-induced peroxisome proliferation and induction of fatty-acid-metabolizing enzymes, while Syrian hamsters exhibit an intermediate response and guinea pigs, marmosets, cynomolgus monkeys, and rhesus monkeys are essentially nonresponsive at high dosage (reviewed in Doull et al., 1999). Additional studies examining similar endpoints using other PPARα agonists including bezafibrate, ciprofibrate and nafenopin have demonstrated similar findings showing that rats and mice respond to these agents by hepatic peroxisome proliferation and induction of fatty acid metabolizing enzymes, while Syrian hamsters and rabbits exhibit an intermediate response, and guinea pigs, marmosets and rhesus monkeys are nonresponsive (Graham et al., 1994; Lake et al., 1989; Makowska et al., 1992; Watanabe et al., 1989). Short-term studies in marmosets and cynomolgus monkeys also have shown no hepatic effects when clofibrate (250 mg/kg/day for 14–90 days) was administered and compared to other peroxisome proliferators (Kurata et al., 1998; Pugh et al., 2000).

Comparative studies of peroxisome proliferation by clofibrate in humans and rats also have been conducted. A number of studies showed that while primary rat hepatocytes exhibit peroxisome proliferation in response to clofibrate or other PPARα agonists, no induction of peroxisome proliferation is found in cultured primary human hepatocytes treated with clofibrate or related PPARα agonists (reviewed in Doull et al., 1999). Data from human liver biopsies of patients treated with clofibrate also indicate that humans do not respond to clofibrate by exhibiting peroxisome proliferation as typically observed in rodents. Liver biopsies of humans treated with gemfibrozil, clofibrate or fenofibrate for periods ranging from 2 to 156 months (9–28 samples examined per study) did not reveal convincing evidence of peroxisome proliferation (Blumcke et al., 1983; de la Iglesia et al., 1982; Gariot et al., 1987). In one study comparing biopsies of 16 patients before and after clofibrate therapy (2 g/day, ~29 mg/kg bw, for 3–94 months), the numerical density of peroxisomes was increased significantly by 50% and the volume density was nonsignificantly increased by 23% (Hanefeld et al., 1983). Since the measurement of volume density rather than the number of peroxisomes is indicative of peroxisome proliferation, these results indicate a negative human response (Cattley et al., 1998). Collectively, these studies demonstrate a significant species difference in response to clofibrate and related PPARα agonists and show that while rodents are highly responsive to peroxisome proliferation, humans appear to be refractory to this effect.

There is also evidence for a species difference in hepatic-cell proliferative events in response to clofibrate and related PPARα agonists. Administration of PPARα agonists to rodents is known to cause an increase in measures of hepatic replicative DNA synthesis (Marsman et al., 1988) that requires a functional PPARα (Peters et al., 1998), although this increase in hepatic replicative DNA synthesis is not found in nonhuman primates treated with PPARα agonists (Pugh et al., 2000). Cultured primary human hepatocytes essentially are refractory to clofibric acid-induced increased replicative DNA synthesis based on two independent reports (Goll et al., 1999; Perrone et al., 1998). Additionally, a number of other PPARα agonists have been shown to induce cell proliferation in rat, but not human primary hepatocytes (reviewed in Doull et al., 1999). It should be noted that the magnitude of induction of cell proliferation is generally lower in hepatocyte cultures compared to measures obtained in vivo.

Inhibition of apoptosis also is reported to occur in response to PPARα agonists and could contribute to hepatomegaly and/or proliferation of mutant liver cells leading to tumor formation. While primary rat hepatocytes cultured in the presence of clofibric acid exhibit significantly lower levels of apoptotic cells, primary human hepatocytes cultured under the same conditions exhibit no change in the number of cells undergoing apoptosis (Goll et al., 1999), which is consistent with other reports examining the effects of another PPARα agonist, MEHP, on apoptosis in primary rat or human hepatocytes (Hasmall et al., 2000b). In other studies, measures of apoptosis actually increased in cultured human primary hepatocytes in contrast to reductions observed in cultured rat primary hepatocytes (Perrone et al., 1998); however, in combination, these studies provide strong evidence that rat and human primary hepatocytes respond differently to clofibrate with respect to changes in replicative DNA synthesis and apoptosis. Collectively, these studies provide supporting evidence for a lack of sensitivity to clofibrate in human liver cells compared to rats. While there are clear species differences noted in cultured primary hepatocytes, there is no conclusive evidence reported to date identifying the specific target genes that mediate increased hepatic cell proliferation in response to clofibrate or other PPARα agonists.

Human clinical/epidemiology data

Two clinical trials have examined relative mortality and cancer rates in male humans treated with fibrates. In the Helsinki Heart Study, a total of 4081 men aged 40–55 with elevated serum cholesterol were treated with either gemfibrozil or placebo for a 5-year period (Frick et al., 1987; Huttunen et al., 1994). Despite significant lowering of serum lipids which prevented coronary heart disease in the gemfibrozil-treated group, no differences in total death rate or liver cancer incidence were observed between treatment groups. However, liver cancer incidence was not reported as a single endpoint; the incidence was either reported as total deaths from cancer, or deaths from liver, gallbladder, and intestinal cancers grouped together. No statistically significant differences were found for any class of cancers examined following this relatively brief exposure and follow-up period (Frick et al., 1987). Importantly, the incidence of cancer mortality in this study, for placebo and fibrate-treated patients, was less than 2% for each group, compared to greater than 50% in PPARα agonist-treated rodents (Ashby et al., 1994; Bentley et al., 1993; Lake, 1995; Reddy and Lalwani, 1983).

The other randomized clinical trial was conducted by the World Health Organization (WHO) to determine whether clofibrate would lower the incidence of ischemic heart disease (IHD) in men and was carried out in 15,745 men with a treatment group and two control groups (one high and one low cholesterol level) of about 5000 men each, for an average of 5.3 years (CPI, 1978). The treatment period was for 5.3 years and follow-up reports were provided 4.3 and 7.9 years after this period. Clofibrate was reported to cause a statistically significantly higher age-adjusted total mortality as compared with the high cholesterol placebo-treated control groups in this study. The excess mortality was due to a 25% increase in noncardiovascular causes from diseases of the liver, gall bladder, pancreas and intestines, including malignant neoplasms of these sites (CPI, 1980). However, in the final follow-up study (5.3 years in the treatment phase with 7.9 years follow-up for a total of 13.2 years), neither the number nor rate of cancer deaths in the clofibrate-treated group was statistically different from the control groups (CPI, 1984). The reason for the difference in mortality at the earlier time point is uncertain. Similar to the Helsinki Heart Study, no specific data on the incidence of liver cancer was provided. It should be noted that in this final follow-up study there was an excess of only 12% deaths from all causes other than IHD, compared with 25% in the previous studies. Furthermore, the proportional differences between the treated group and the control groups in the final follow-up study

was diminished for malignant disease but increased for nonmalignant diseases. The results indicate that the excess in deaths from diseases other than IHD was largely confined to the clofibrate treatment period (average 5.3 years). However, 7.9 years post-treatment, there were 27 deaths associated with liver, gallbladder, or intestinal cancers in the clofibrate-treated group, compared to 18 and 11 deaths associated with the same endpoints in the high cholesterol and low cholesterol control groups, respectively. Similar to the Helsinki Heart Study, this incidence is less than 1% per group.

Lastly, a limited epidemiological study showed no evidence of increased cancer risk as a result of fibrate therapy (Law et al., 1994). However, as with the clinical trials, cancer incidence is not reported specifically for liver cancer. Combined, the clinical trials and epidemiological study do not provide direct evidence that clofibrate or a related PPARα agonist fenofibrate cause hepatocarcinogenesis in humans treated with these therapeutic drugs. One must keep in mind, however, that these studies are limited by relatively brief exposure and follow-up periods.

e. Statement of Confidence

There are significant quantitative differences in the elimination rate and in some of the key events for the MOA of clofibrate hepatocarcinogenesis between rats and humans. Clofibrate is eliminated about four times faster in rats than in humans, meaning that clofibrate is about four times more persistent in human tissues compared to rat. The PPARα receptor is expressed in human liver at relatively lower levels ($\sim$10%) compared to rodents, although the significance of this difference is unclear. The presence of dominant negative isoforms of PPARα in humans may, in part, contribute to the relative refractory response to clofibrate in vivo and in vitro, and the significance of reported mutations also could significantly influence activation of this receptor and subsequent target gene modulation. There is good evidence showing that modulation of target gene expression induced by clofibrate and related PPARα agonists is significantly different between rodents and humans, and some of these differences may be due to mutations or polymorphisms in PPREs of related target genes. There are significant differences noted in replicative DNA synthesis between rodents and nonhuman primates, and the induction of replicative DNA synthesis is mediated by PPARα in mice. Available data demonstrate that rats and mice are highly responsive, Syrian hamsters exhibit an intermediate response, and that guinea pigs, nonhuman primates (both Old World and New World primates), and humans are refractory to peroxisome proliferation in response to clofibrate administration. Evidence from two clinical trials and one epidemiological study provide no indication of increased human liver cancer incidence as a result of exposure to clofibrate or a related PPARα agonist. Combined, these observations demonstrate significant quantitative dynamic differences between humans and rodents in response to clofibrate, and strongly suggest that the MOA for clofibrate hepatocarcinogenesis in rats is not likely to occur in humans exposed to clofibrate, taking into account these kinetic and dynamic factors.

f. Implications

The data lead to the conclusion that a carcinogenic response induced via the MOA for liver cancer in the rodent is unlikely to occur in humans following exposure to clofibrate.

3. Equivocal Evidence

a. Introduction

Oxadiazon was selected to represent the equivocal evidence case study example. Oxadiazon is a hepatocarcinogenic herbicide that has been hypothesized to be a PPARα agonist. Key events supporting the characterization of a nonmutagenic hepatocarcinogen as a PPARα agonist include evidence of hepatomegaly, peroxisome proliferation, cell proliferation as shown by enhanced replicative DNA synthesis, and increased levels of enzymes involved in peroxisomal fatty acid metabolism, especially acyl or palmitoyl CoA oxidase (Cattley et al., 1998). Causal evidence that a chemical is a PPARα agonist includes data showing (a) activation of PPARα (e.g., absence of a response in null mice), (b) perturbation of cell proliferation and apoptosis, and (c) selective clonal expansion (i.e., promotion of spontaneous DNA damage).

No epidemiologic or toxicity data are available on the effects in humans following exposure to oxadiazon. Hepatocellular tumors (adenomas and carcinomas), but no other tumor responses, have been observed following administration of oxadiazon to male Fischer and Wistar rats and to male and female CD-1 and ICR-JCL mice. It has been hypothesized that the neoplastic responses are associated with

the activation of PPARα. Chemical-specific data are available from animal studies for some key events in the hypothesized mode of action, but the overall weight of evidence is equivocal and does not support PPARα activation as being the only initial key event in the mode of action for the neoplastic responses in the liver.

b. Is the Weight of Evidence Sufficient to Establish the MOA in Animals?

Postulated mode of action

There is consistent evidence of the hepatocarcinogenicity of oxadiazon, based on the results of unpublished investigations that have been conducted with Fischer 344 and Wistar rats and CD-1 and ICR-JCL mice. Hepatocellular adenomas and carcinomas (combined) were increased at 50.9 mg/kg/day (male Fischer rats), 3.5 mg/kg/day (male Wistar rats), 10.6 mg/kg/day (male ICR-JCL mice), and 12.0 mg/kg/day (CD-1 male and female mice), and in a second study with CD-1 mice there was an increase in hepatocarcinomas at 153 mg/kg/day. It has been hypothesized that the liver tumor response in rats and mice induced by treatment with oxadiazon, a nonmutagenic carcinogen, is the result of a mode of action involving activation of PPARα, which is associated with peroxisome proliferation, a biomarker for PPARα activation that is not causally linked with hepatocarcinogenicity.

Oxadiazon produces increases in absolute and relative liver weights in both sexes of several strains of rats and mice and in dogs. Electron microscopy of mouse liver sections shows a qualitative and dose-dependent increase in peroxisomes (Reichert et al., 1996). Increased levels of enzymes associated with liver toxicity (ALP, SGOT, SGPT, or LDH) have been reported in subchronic and chronic dietary studies with rats, mice and dogs. Dose-related increases in palmitoyl CoA oxidation (PAO), palmitoyl carnitine transferase, and acetyl carnitine transferase (ACT) were reported in a short-term oral, mechanistic study with mice. However, a dose-related decrease in catalase activity was observed in this study. In vitro studies conducted with oxadiazon showed concentration-dependent increases in both PAO and ACT in primary rat hepatocytes but no effect was observed in cultured human hepatocytes. Oxadiazon is not structurally related to other herbicides that also are peroxisome proliferators (Richert et al., 1996). However, a wide variety of chemical classes has been shown to have the potential to induce peroxisome proliferation.

Data obtained from metabolism studies with rats show that oxadiazon is completely absorbed and extensively metabolized (i.e., 18 metabolites have been identified; Price, 1991). However, the moiety that may be associated with the neoplastic responses in rodents has not been identified.

Oxadiazon is neither mutagenic nor clastogenic but does cause neoplastic cell transformation in vitro. Bacterial assays with 97.49% oxadiazon were negative for gene mutations in *Salmonella typhimurium* and *Escherichia coli* (unpublished data in U.S. EPA MRID Nos. 00069893 and 41871701). Similarly, neither 95.5% oxadiazon nor recrystallized oxadiazon (100%) was mutagenic or clastogenic in cultured mammalian cells and did not cause unscheduled DNA synthesis (UDS) in primary rat hepatocytes. There is, however, evidence that both formulations induced neoplastic transformation in Syrian hamster kidney cells both in the presence and in the absence of S9 activation. The finding of positive cell transformation supports the evidence from mouse bioassays (unpublished data in U.S. EPA MRID Nos. 00444322, 00115733, and 40993301) and the rat long-term studies (unpublished data in U.S. EPA MRID Nos. 00149003/00157780 and 40993401) of liver tumor induction.

Evidence in animals/key events

Oxadiazon produces increased liver weights in rats at $\geq$200 mg/kg/day (14 days), 51 mg/kg/day (6 months), and 6 mg/kg/day (24 months). In mice, increases in liver weights were observed at doses of 113 mg/kg/day (52 weeks) and $\geq$12 mg/kg/day (104 weeks). No increases in liver weights were observed in beagle dogs after 28 days of treatment with oxadiazon.

In a 14-day oral mechanism study with oxadiazon using Sprague-Dawley rats, electron micrographs showed an increase in peroxisomes at 500 mg/kg/day. Livers from lower dose groups were not examined microscopically. However, electron microscopy of CD-1 mouse liver sections in the study of Reichert et al. (1996) showed a qualitative and dose-dependent increase in peroxisomes at 20 mg/kg/day (minimal in 5 of 11 mice), 100 mg/kg/day (moderate in 7 of 7 mice), and 200 mg/kg/day (severe in 9 of 9 mice). Eight of 10 Sprague Dawley rats administered 500 mg/kg/day oxadiazon also showed a "severe" increase in peroxisomes.

As stated earlier, there is ample evidence of increased relative liver weights; however, no study has been submitted on the effects, if any, of oxadiazon on replicative DNA synthesis. It is of note that oxadiazon was tested in two in vitro assays for UDS in primary rat hepatocytes. Both studies were negative for UDS, and cells undergoing DNA replication rather than DNA repair were reported to be evenly distributed among all groups (unpublished data in U.S. EPA MRID Nos. 00115723 and -27).

Increased levels of enzymes associated with liver toxicity (ALP, SGOT, SGPT, and/or LDH) were observed in subchronic dietary studies lasting 90 days in rats as well as chronic studies lasting 1–2 years in rats, mice, or dogs. In a 14-day oral mechanism study (unpublished data in U.S. EPA MRID No. 42310001) with Sprague-Dawley rats, dose-related increases in palmitoyl CoA oxidation (PAO), palmitoyl carnitine transferase, and acetyl carnitine transferase (ACT) were reported at 20, 200, and 500 mg/kg/day; effects at 200 mg/kg/day were statistically significant. Catalase activity, which is generally increased in the presence of peroxisome proliferation, however, was significantly reduced at 200 and 500 mg/kg/day. In agreement with the peroxisomal enzyme activity results from the submitted 14-day study, Reichert et al. (1996) found a significant ($p < .05$) and dose-related increase in rat PAO and ACT at 200 and 500 mg/kg/day. In mice, the same investigators noted that increases in PAO were achieved at 100 (174% of control) and 200 mg/kg ($p < .05$). ACT also was significantly increased at these levels. No biochemical assays in dogs were performed. In vitro studies conducted as part of these investigations showed concentration-dependent increases in both PAO and ACT at 2.5–10×10^{-5} M oxadiazon in primary rat hepatocytes; no effects were seen in cultured human hepatocytes at comparable doses.

Some of the key events associated with oxadiazon hepatocarcinogenesis in male mice are summarized in Table 25.

Strength, consistency, specificity of association of tumor response with key events/dose-response relationship/temporal association

While increases in the activity of two enzyme markers for PPARα activation followed exposure to oxadiazon, the concordance between the dose response for lipid metabolizing enzymes and liver tumor induction is not strong. A dose of 113 mg/kg/day induced significant ($p < .01$) increases in adenomas (+23%), carcinomas (+41%), and adenomas/carcinomas combined (+63%) in male ICR-JCL mouse livers, while a comparable dose (100 mg/kg/day) caused only a moderate increase in the number of peroxisomes and a less than twofold increase in PAO. At 10.6 mg/kg/day in the ICR-JCL mouse carcinogenicity study, adenomas (+17%), carcinomas (+16%) and adenomas/carcinomas combined (+33%) also were significantly increased. However, only a slight increase in the number of peroxisomes and no increases in PAO were seen.

Similar evidence of a weak response in peroxisomal enzymes was noted in male Sprague-Dawley rats dosed with either 200 or 500 mg/kg/day oxadiazon in the submitted mechanistic study or the published results of Richert et al. (1996). Increases in PAO ranged only from 1.4- to 2.1-fold (200 or 500 mg/kg/day, respectively) over control, while liver tumor induction was achieved at 3.5 mg/kg/day in a carcinogenicity study with Wistar rats. ACT activity, which is distributed not only in the peroxisomes but also in endoplasmic reticulum and mitochondria, only reached a 6-fold increase in male rats at 500 mg/kg/day. Thus, although data on enzyme activities were from short-term (14-day) studies, increased enzyme activities were not observed at doses near the tumor doses. It is unlikely that measurements of enzyme activities following treatment of rats with oxidiazon for a longer duration (i.e., >14 days) would show that changes in enzyme activities occur at lower doses. PPARα agonists typically induce enzyme changes at any dose that results in tumor induction shortly after initiation of treatment (i.e., 14 days or less). It is concluded that the findings with oxadiazon indicate that tumorigenic doses were substantially lower than levels that induced lipid-metabolizing enzymes. Table 26 summarizes the data showing a lack of concordance between precursor events and tumor formation in male ICR-JCL mice. Although oxidiazon induces an increase in peroxisomes and peroxisomal enzyme activities in rats, the evidence is not sufficient to evaluate the role of peroxisome proliferation in tumor induction because (1) increases in peroxisomes were measured only at a single dose (500 mg/kg/day) and (2) enzyme measurements were conducted on higher dose groups than the lowest dose that induced liver tumors in male Wistar rats. Enzyme measurements (palmitoyl CoA oxidase and acetyl carnitine transferase) were conducted on dose groups of 20 mg/kg/day or greater and effects on these enzymes were observed at

TABLE 25
Key Events Associated with the Hepatocarcinogenesis of Oxadiazon in Mice

Key event	Evidence in animals
Activation of PPARα[a]	No data
PPARα-mediated regulation of peroxisomal genes	Dose-related increases in palmitoyl CoA oxidation (Price 1991)
PPARα-mediated regulation of genes of proliferation and apoptosis	No data
PPARα-mediated regulation of fatty acid metabolism genes	No data
Peroxisome proliferation	Dose-dependent increases in peroxisomes Dose-related increases in palmitoyl carnitine transferase and acetyl carnitine transferase (Richert et al., 1996)
Perturbation of cell proliferation and/or apoptosis	No in vivo cell proliferation data; indirect evidence of cell proliferation as shown by increases in absolute and relative liver weights (MRID Nos. 00444322, 00115733, 40993301, 00149003/00157780 and 40993401) Two in vitro studies were negative for UDS and cells undergoing DNA replication rather than DNA repair were evenly distributed among all groups, including control (MRID Nos. 00115723 and -27)
Inhibition of GJIC	No data
Hepatocyte oxidative stress	Decreased catalase activity may lead to increased levels of H_2O_2 (Reichert et al., 1996)
Kupffer cell mediated cell proliferation	No data
Selective clonal expansion	Hepatocellular tumors only evidence

[a]Causal key event; others are associative key events.

200 mg/kg/day and 500 mg/kg/day. Increases in liver tumor incidences were reported for the 3.5- and 39-mg/kg/day dose groups in the Wistar male rat carcinogenicity study. Thus, peroxisome increases and increases in peroxisomal enzyme activities cannot be compared with the lowest doses that led to the induction of liver tumors in male rats, and a PPARα-associated mode of action for liver tumors in male rats cannot be evaluated. It should be noted, however, that increases in palmitoyl CoA oxidase and acetyl carnitine transferase activities in Sprague-Dawley rats occurred at doses well above the doses that induced liver tumors in Wistar rats.

Biological plausibility and coherence of the database

Based on the weight of the evidence, there are sufficient data to classify oxadiazon as a nongenotoxic hepatocarcinogen. The available data also provide suggestive evidence of peroxisome proliferation. There are, however, weaknesses in the database that preclude acceptance of PPARα activation as the sole mode of action for oxadiazon-induced liver tumors; these include:

1. No cell proliferation data were reported for rats or mice; hence, mitogenesis could not be mechanistically linked to activation of PPARα.
2. There was a lack of concordance between the dose response for activation of PPARα, as measured by increases in lipid-metabolizing enzymes, and tumor formation in both rats and mice.
3. Evidence of decreased catalase activity, which generally increases in the presence of a proxisome proliferator, is not consistent with the postulated mode of action.
4. There was a lack of concordance between the dose response for increases in the number of peroxisomes, a biomarker for PPARα activation, and tumor formation
5. There is no direct evidence that oxadiazon activates PPARα.
6. There is evidence that the mode(s) of action for liver tumor induction in rats or mice also may involve cytotoxicity rather than solely

TABLE 26
Summary of Peroxisomal Effects and Liver Tumor Induction in Male Mice Administered Oxadiazon

		Peroxisomal enzyme activities		Neoplasms		
Dose (mg/kg/day)	Number of peroxisomes	Palmitoyl CoA oxidase (% over control)	Acetyl carnitine transferase (% over control)	Adenomas	Carcinomas	Adenomas/ carcinomas combined
0	0	ND	ND	2/69 3%	3/69 4%	5/69 7%
0.3	ND	ND	ND	7/71 10%	1/71 1%	8/71 11%
1.1	ND	ND	ND	2/71 3%	4/71 6%	6/71 8%
10.6	ND	ND	ND	12/69[b] 17%	11/69[a] 16%	23/69[b] 33%
20	Slight (6/11)	106	113	ND	ND	ND
100	Moderate (7/7)	174	389[a]	ND	ND	ND
113	ND	ND	ND	16/71[b] 23%	29/71[b] 41%	45/71[b] 63%
200	Severe (9/9)	259[a]	459.5[a]	ND	ND	ND

Note. ND, no data.
[a]Significantly different from control ($p < .05$).
[b]Significantly different from control ($p < .01$).

the mitogenic response that is characteristic of PPARα agonists.

Alternative modes of action

As stated previously, oxadiazon is not mutagenic, and, with the exception of PPARα activation and peroxisome proliferation, no other mode of action has been hypothesized for oxadiazon. However, a possible alternative mode of action may involve cytotoxicity rather than the mitogenic response that is characteristic of PPARα agonists. Treatment of Fischer 344 rats for 2 years with 51 mg/kg/day of oxidiazon increased activities of LDH, alkaline phosphatase, SGOT, and SGTP. Diffuse hepatocellular necrosis was observed at the lowest dose level that induced liver tumors in Fischer rats, 51 mg/kg/day. In a carcinogenicity study with ICR-JCL mice, SGOT, SGTP, alkaline phosphatase, and blood urea nitrogen (BUN) were increased at a dose level of 113 mg/kg/day. SGTP and liver weights were increased at 11 mg/kg/day. Diffuse hepatocellular necrosis also was observed at a dose level of 11 mg/kg/day, the lowest dose that induced an increase in the incidence of liver tumors in mice. Hence, it appears that there may be at least two possible MOAs occurring. At lower doses, cytotoxicity appears to be responsible for tumorigenesis, while at the higher doses, cytotoxicity and PPARα activation both appear to play a role in the carcinogenic response.

Conclusions—assessment of postulated modes of action and statement of confidence

The available data are insufficient to support the view that oxadiazon-induced liver tumors are the result solely of a PPARα activation mode of action. While there is some indication that activation of PPARα may play a role, existing data are inadequate as a basis for delineation of a plausible sequence of events leading to cancer. In vivo data on potential key events to serve as a basis for consideration of the weight of evidence for the hypothesized mode of action also are limited. For example, in vivo data are lacking that show that enhanced replicative DNA synthesis is a key event which precedes tumor formation. The dose-response results from mechanistic studies also are inconsistent with those of the cancer bioassays, based on the hypothesized mode of action. On the other hand, there are results from carcinogenicity studies with both rats and mice

that indicate that the induction of liver tumors may involve cytotoxicity because of the increase in activities of enzymes that are indicators of hepatotoxicity and the observation of hepatic necrosis at doses that induce liver tumors. It is concluded that the available information is equivocal and does not support PPARα activation as being the sole nongenotoxic mode of action for the hepatocarcinogenic effects of oxadiazon.

Postulated mode of action: human data and analysis

There are no data available on the effects in humans exposed to oxadiazon.

Are the key events in the animal MOA plausible in humans?

The available data on the mode of action for the formation of hepatocellular tumors in rodents administered oxadiazon do not support PPARα activation as being the sole mode of action. There is some evidence for receptor activation, but another, additional, mode of action involving cytotoxicity cannot be excluded. Further investigation is necessary in order to resolve fully these uncertainties regarding the etiology of oxadiazon-induced liver tumors. As a result, a definitive assessment of the human relevance of oxadiazon's mode(s) of action is precluded at this time.

III. LEYDIG-CELL AND PANCREATIC ACINAR-CELL TUMORS: IN-DEPTH ANALYSIS

A. Introduction

This section provides an in-depth analysis of the proposed modes of action (MOAs) for the Leydig-cell and pancreatic acinar-cell tumors often induced in rats by PPARα–ligands, along with liver tumors (the "Tumor Triad"), the plausibility of the proposed MOAs occurring in humans, and the relevance/applicability of these tumor responses in the rat for human health risk assessment. Although the number of PPARα agonists tested in mice is limited when compared with those evaluated in rats, these two extrahepatic tumors seen in rats have not been observed in mice. Each of the two tumor types is addressed separately within this section, the Leydig-cell tumor (LCT) in part B, the pancreatic acinar-cell tumor (PACT) in part C. This section is structured to address, for each tumor type, the three key questions articulated in the human relevance framework: (1) Is the weight of evidence sufficient to establish the mode(s) of action? (2) Are the key events in the animal MOA plausible in humans? (3) Taking into account kinetic and dynamic factors, is the animal MOA plausible in humans?

Each of the proposed MOAs is presented in a figure with an accompanying table that lists the key steps in the MOAs together with a consideration of degree of association, strength of the evidence and specificity of the key event to a PPARα agonist-induced response. A detailed analysis of the supportive evidence for the postulated MOA(s) follows the table. This analysis is not intended to reflect an exhaustive review of the literature but rather a summation of key evidence in support or otherwise of the postulated MOAs and their key events.

As noted earlier, roughly 30 PPARα agonists have been evaluated in long-term studies in rats and/or mice and found to cause liver tumors in one or both species. A number of these exhibited the "Tumor Triad," that is, induction of liver, pancreatic acinar-cell, and Leydig-cell tumors in non-F344 male rats. Others have produced liver tumors and one, but not, both of the other two tumor types. The following PPARα agonists have been shown to induce the tumor triad (9 out of 15 tested to date): clofibrate (Svoboda and Azarnoff, 1979; Tucker and Orton, 1995), DEHP (Berger, 1995; David et al., 1999), fenofibrate (PDR, 2002), gemfibrozil (Fitzgerald et al., 1981), HCFC-123 (Malley et al., 1995), methylclofenapate (Tucker and Orton, 1995), ammonium perfluorooctanoate (PFOA) (Biegel et al., 2001; Sibinski, 1987), tibric acid (Faccini et al., 1979), and Wyeth 14,643 (Biegel et al., 2001). Cinnamyl anthranilate (NTP, 1980) and nafenopin (Reddy and Rao, 1977a, 1977b) produced liver and PAC tumors; these studies were conducted using F344 rats. Butylbenzyl phthalate (BBP), a weak peroxisome proliferator, produced PACTs, but not LCTs or liver tumors in rats or mice (NTP, 1997). DINP (Bio/dynamics, 1986; Butala et al., 1996; Lington et al., 1997), perchloroethylene (Mennear, 1986), and trichloroethylene (Maltoni, 1988; Mennear, 1988) have been shown to induce liver and LCTs in non-Fischer 344 rats. A few points are worth noting from these results. PPARα agonists have clearly been shown to induce LCTs in Wistar and Sprague-Dawley rats, but it is difficult to detect this tumor type in F344 rats due to this strain having a high spontaneous incidence (Cook

et al., 1999). Hence, BBP, cinnamyl anthranilate, and nafenopin might have induced LCTs if they had been evaluated in a non-F344 strain of rat. In contrast, PPARα-induced PACTs have been observed in Wistar, Sprague-Dawley, and F344 rats, since all three strains have a low spontaneous incidence of this tumor type.

Comparison of the dose-response relationships between the tumor types has shown a few trends as well. For instance, the liver and pancreatic acinar-cell (PAC) tumors generally occur at the same dosages except for HCFC-123 and ethylclofenapate. In contrast, there are several instances where the Leydig-cell tumors (LCTs) occurred at lower levels than the liver tumors (i.e., HCFC-123, methyl clofenapate, PFOA, and TCE). DINP and gemfibrozil induced liver and LCTs at the same dosage levels. In addition, the LCT and PAC tumors (PACT) were observed at levels that produced significant liver peroxisomal enzyme induction for the compounds: clofibrate, DEHP, gemfibrozil, HCFC-123, methylclofenapate, PFOA, and WY 14,643. The dose-response information coupled with the data described later suggest that, like the liver, these extrahepatic tumors also may be mediated by the PPARα receptor. Table 35, which provides a detailed display of the data from the long-term studies in rats for the PPARα agonists that produce both extrahepatic and hepatic tumors, can be found at the end of this section following the Tumor Triad case study.

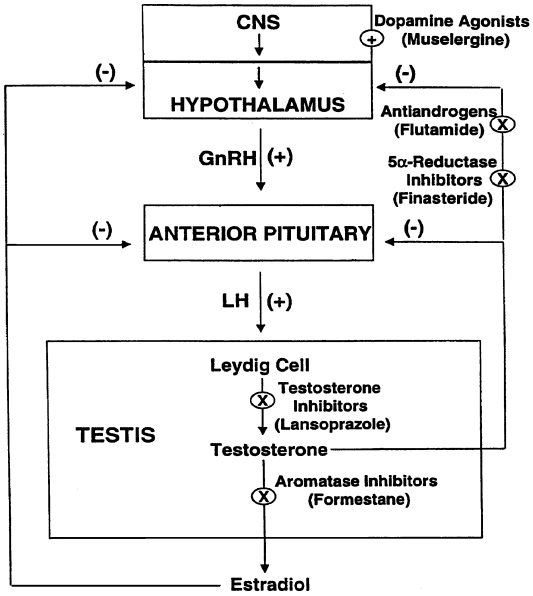

FIGURE 5. Regulation of the hypothalamic–pituitary–testicular (HPT) axis and control points for potential disruption. Symbols: (+) feedback stimulation; (−) feedback inhibition; ⊕ receptor stimulation; ⊗ enzyme or receptor inhibition. Figure from Cook et al. (1999).

B. Leydig-Cell Tumors

1. Proposed Mode of Action: Is the Weight of Evidence Sufficient to Establish the Mode(s) of Action in Rats?

a. Common Mechanisms for Leydig-Cell Tumorigenesis

Figure 5 illustrates the five most common mechanisms by which chemicals can disrupt the hypothalamus–pituitary–thyroid (HPT) axis and produce Leydig-cell tumors (LCTs) in rats (Cook et al., 1999). Each mechanism is described briefly to illustrate concepts; however, additional examples of compounds and detailed descriptions can be found elsewhere (Cook et al., 1999). Dopamine agonists such as muselergine decrease serum prolactin levels, which causes downregulation of luteinizing hormone (LH) receptors on Leydig cells (LCs) (Prentice et al., 1992). This receptor downregulation decreases testosterone production and results in a compensatory increase in LH, which drives the development of LCTs. Androgen receptor antagonists such as flutamide compete with testosterone and dihydrotestosterone (DHT) for binding to the androgen receptor (Simard et al., 1986). This competition reduces the net androgenic signal to the hypothalamus and adenohypophysis resulting in an increase in LH with a concomitant elevation of testosterone (Cook et al., 1993; Viguier-Martinez et al., 1983a, 1983b). Flutamide has been shown to produce LCTs in rats within 1 year of treatment (PDR, 1995a). 5α-Reductase inhibitors such as finasteride block the conversion of testosterone to DHT (PDR, 1995c; Prahalada et al., 1994; Rittmaster et al., 1992). DHT amplifies the androgenic signal through several mechanisms: (1) Unlike testosterone, DHT cannot be aromatized to estrogen and thus its effects are purely androgenic, and (2) DHT binds to the androgen receptor with greater affinity and stability than does testosterone (DeGroot et al., 1995). Hence, 5α-reductase inhibitors decrease DHT levels, which reduces the net androgenic signal received by the hypothalamus and pituitary and thereby causes a compensatory

increase in LH levels. Testosterone biosynthesis inhibitors such as lansoprazole decrease testosterone levels and increase LH levels resulting in the development of LCTs (Fort et al., 1995; Meikle et al., 1994). Aromatase inhibitors such as formestane and letrozole block the conversion of testosterone to estradiol, resulting in a decrease in estradiol and an increase in LH levels (Walker and Nogues, 1994). In summary, the common thread in all of these mechanisms is that a sustained increase in LH levels leads to LC hyperplasia and/or LCTs in rats.

As noted earlier, several PPARα agonists have been shown to induce LCTs in rats. One of the earlier hypotheses for why PPARα agonists induce LCTs was that this class of compounds increased peroxisomes in LCs in a manner similar to the liver (Biegel et al., 1992). This hypothesis was based on the similarity between hepatocytes and Leydig cells; both have abundant smooth endoplasmic reticulum; hepatocytes utilize this organelle for xenobiotic metabolism, while Leydig cells utilize this structure for steroid biosynthesis. In two strains of rat, WY 14,643 did not induce peroxisomes in Leydig cells based upon biochemical and electron microscopy criteria, yet abundant peroxisome induction was present in the liver (Biegel et al., 1992). In addition, PFOA and WY 14,643 did not induce peroxisomes in Leydig cells throughout a 2-year bioassay (Biegel et al., 2001). These data demonstrate that PPARα agonists do not induce peroxisomes in LCs, and hence, may induce LCTs via a different mechanism than that for liver tumors. Consistent with the findings with PFOA and WY 14,643, the PPARα agonist ciprofibrate did not increase peroxisome volume, but did increase mRNA levels of genes encoding peroxisomal β-oxidation enzymes in Leydig cells (Nemali et al., 1988). These data would be consistent with PPARα being expressed within Leydig cells. Collectively, the data demonstrate the Leydig cells do not respond similarly to hepatocytes by producing peroxisome proliferation.

To investigate whether the induction of LCTs by PPARα agonists could be due to an endocrine-related mechanism, PFOA and WY 14,643 were used as model compounds and a series of studies was performed to investigate potential mechanisms. During this time, substantial evidence for the role of PPARα in mediating steroidogenesis has been published that provides a plausible MOA by which PPARα agonists induce LCTs in rats (Figure 6 and Table 27).

b. Evidence in Animals/Key Events

Event 1. PPARα expression has been demonstrated in Leydig cells from rats and humans (Braissant et al., 1996; Gazouli et al., 2002; Kazeto et al., 2001; Schultz et al., 1999). Its expression was lower than that found in liver and could be upregulated following FSH administration, suggesting a role in steroidogenesis (Schultz et al., 1999). More recently, PPARα agonists (bezafibrate, MEHP, and WY 14,643) have been shown to inhibit testosterone steroidogenesis in rats and mice, possibly by reducing levels of the peripheral-type benzodiazepine receptor (PBR) (Gazouli et al., 2002). In addition, PPARα agonists did not inhibit testosterone steroidogenesis in PPARα-null mice, confirming the role of PPARα in inhibiting testosterone steroidogenesis (Gazouli et al., 2002). It is noted that PPARα agonists have not been shown to induce LCTs in mice, which is likely due to differences in the HPT axis (Cook et al., 1999). Nonetheless, the demonstration that PPARα agonists inhibit testosterone biosynthesis in both species provides compelling evidence that the PPARα receptor mediates the inhibition of testosterone biosynthesis. In addition, PPREs also have been identified in aromatase from zebrafish (Kazeto et al., 2001). These data provide evidence that PPARα agonists affect aromatase and the testosterone biosynthetic pathway via PPARα.

LCTs also have been observed at levels that produced significant peroxisomal enzyme induction for the following compounds: clofibrate, DEHP, gemfibrozil, HCFC-123, methylclofenapate, PFOA, and WY 14,643. The best characterized example is PFOA where administration of PFOA to adult male rats by gavage for 14 days was shown to increase serum estradiol levels only at levels that increased β-oxidation (Cook et al., 1992). Liu and coworkers (1996a, 1996b) then examined the PFOA dose-response (treatment doses = 0, 0.2, 2, 20, or 40 mg/kg body weight/day for 14 days) for several key endpoints that were judged to be the initiating events leading to the liver, pancreas and testis tumors. These endpoints included liver weight, hepatic β-oxidation, hepatic aromatase (CYP19A1), and hepatic total cytochrome P-450. The no-observed-effect level (NOEL) for these endpoints was found to be 0.2 mg/kg/day, with significant changes observed at $\geq$2 mg/kg/day for all the endpoints. In the first carcinogenicity study, PFOA was administered at 30 and 300 ppm, which was equivalent to approximately 1.5 and 15 mg/kg body weight/day

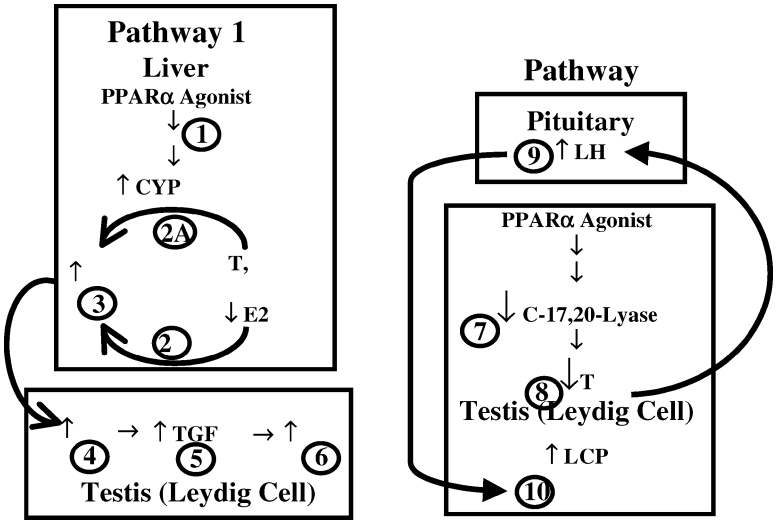

FIGURE 6. Schematic depicting the proposed modes of action with key events for rat Leydig-cell tumors (LCTs) induced by PPARα agonists. A, androstenedione; E2, 17β-estradiol; LCP, Leydig cell proliferation; T, testosterone. Experimental evidence suggests that PPARα agonists may induce LCTs in rats by two potential pathways: enhancement of growth factor expression within the testis (Pathway 1) and/or inhibition of testosterone biosynthesis (Pathway 2) (reviewed in Cook et al., 1999) (Table 27). Both pathways are likely to contribute to the induction of LCTs in rats. In addition, the hormonal fingerprint seen with PPARα agonists (i.e., increased estradiol, decreased testosterone) is distinct from the commonly known mechanisms described in Figure 5. In Pathway 1, PPARα agonists have been shown to increase cytochrome P-450 19A1 (aromatase) activity (Event 1), which enhances the conversion of testosterone (T) or androstenedione (A) to 17β-estradiol (E2) (Event 2a). PPARα agonists also have been shown to decrease E2 metabolism (Event 2b). Hence, Events 2a–b increase serum E2 levels (Event 3) and subsequently lead to increased E2 levels within the interstitial fluid of the testis (Event 4). The increase in interstitial fluid E2 levels has been shown to correlate with an increase in interstitial fluid TGFα levels (Event 5), which would increase Leydig cell proliferation (LCP) (Event 6). In Pathway 2, PPARα agonists decrease peripheral-type benzodiazepine receptor (PBR) levels and/or C-17, 20-lyase activity (Event 7), critical enzymes in the testosterone biosynthetic pathway, which results in a decrease in testosterone levels (Event 8). Decreases in serum testosterone levels increase LH levels (Event 9). LH binding to the LH receptor on Leydig cells increases Leydig cell proliferation (LCP) (Event 10).

(Sibinski, 1987). The Tumor Triad was observed at 300 ppm, but not at 30 ppm (Biegel et al., 2001; Sibinski, 1987). Based on the work by Liu and coworkers (1996a, 1996b), the 300 ppm dose level in the carcinogenicity studies would have increased β-oxidation and serum estradiol levels, while the 30 ppm level would have been around the threshold for these biochemical endpoints. This dose-response information coupled with the data described that follow suggest that these extrahepatic tumors are likely mediated by the PPARα receptor.

Two MOAs are hypothesized by which PPARα agonists induce LCTs. Pathway 1 appears secondary to liver induction where aromatase is induced in the liver which then increases estradiol levels. Pathway 2 describes how PPARα agonists inhibit testosterone biosynthesis. It is possible that both of these mechanisms contribute to the development of LCTs.

Pathway 1—secondary to liver induction (events 2a–6)

Event 2a. The PPARα agonist PFOA has been shown to induce hepatic aromatase activity (Biegel et al., 1995; Liu et al., 1996a, 1996b). Additionally, Leydig cells treated with PFOA in culture have been shown to increase estradiol levels providing indirect evidence that PFOA can induce aromatase in Leydig cells (Liu et al., 1996a, 1996b). The induction of aromatase is consistent with the increase in serum estradiol levels. While a PPRE has not been identified in the 5′ flanking regions of mammalian aromatase,

TABLE 27
Table of Key Events—Leydig-Cell Tumors

Event	Relationship[a]	Weight of evidence	Specificity	Comments
1. Activation of PPARα	Causal—Pathway 1; Associative—Pathway 2—one or both of which may contribute to LCTs	Moderate to weak	Low	PPARα is expressed in liver and LCs of rats and humans; PPREs have been shown to regulate testosterone biosynthesis; PPREs also have been identified in aromatase from zebrafish
		Pathway 1—Secondary to liver induction		
2a. ↑CYP 19A1 (Aromatase)	Associative	Weak	Low	Potential biomarker for peroxisome proliferation [Established with PFOA (in vitro/in vivo)]
And/or				
2b. ↓ Estradiol metabolism	Associative	Weak	Low	Shown with clofibrate, DEHP, gemfibrozil, DBP and WY 14,643
3. ↑ Serum estradiol levels	Associative—a potential biomarker for PP	Weak	Low	Shown in vivo with clofibrate, DEHP, PFOA and WY 14,643; in vitro with 8 out of 11 PPARα agonists
4. ↑ Interstitial fluid estradiol levels	Associative	Weak	Moderate	Shown with PFOA (in vivo)
5. ↑ TGFα levels	Associative	Weak	Moderate	Shown with PFOA (in vivo)
6. ↑ LC proliferation & LCTs	Associative	Weak	Low	TGFα is known to induce LC proliferation in vitro, but has not been shown directly to produce LCTs following in vivo administration

(Continued on next page)

TABLE 27
Table of Key Events—Leydig-Cell Tumors *(Continued)*

Event	Relationship[a]	Weight of evidence	Specificity	Comments
Pathway 2—Direct inhibition of testosterone biosynthesis at level of testis				
7. ↓ Testosterone biosynthesis either by ↓ PBR protein levels and/or $C_{17,20}$-lyase activity	Associative	Moderate	Low	Occurs with other compounds known to directly inhibit testosterone biosynthesis (e.g., ketoconazole)
8. ↓ Testosterone levels	Associative	Moderate	Low	Established with PFOA (in vitro/in vivo) and with 12 other PPARα agonists in vitro (13 out of 13); occurs with other compounds known to directly inhibit testosterone biosynthesis (e.g., ketoconazole)
9. ↑ LH	Associative	Weak	Low	Difficult to establish with PPARα agonists, possibly due to attenuation by the ↑ serum estradiol levels; occurs with majority of compounds known to induce LCTs in rats
10. ↑ LCTs	Associative	Weak	Low	Seen with all compounds that ↑ LH; a well-established mechanism for induction of LCTs

[a]Relationship to Leydig-cell tumors or key events leading to tumors: *Causal:* Required step for PPARα MOA based on data. *Associative*: Events that are occurring but may or may not be causally linked to the MOA. *Weight of evidence (strong, moderate, weak)*: *Strong* is normally defined by having several studies preferably with multiple PPARα agonists from multiple laboratories which support that MOA, with limited evidence of contradiction. *Moderate* spans the data continuum between weak and strong. *Weak* is normally defined by having a single study with a single PPARα agonist from a single laboratory or a significant amount of contradiction in the literature. *specificity (high, moderate, low)*: *High* is defined as unique to this PPARα MOA. *Moderate* spans the continuum between high and low for its association with the PPARα MOA. *Low* is defined as not unique to PPARα MOA.

the aromatase gene from zebrafish (CYP19A2) has been shown to contain a PPARα/retinoid X receptor heterodimer-response element (Kazeto et al., 2001). Interestingly, estrogen has been shown to play a role in the hepatic expression of genes involved in fatty acid β-oxidation and in maintaining lipid homeostasis based on work using an aromatase knockout mouse (Toda et al., 2001). It would have been helpful if the authors had measured serum estradiol levels since there are several aromatase forms which are encoded by distinct genes (Corbin et al., 2001; Graddy et al., 2000); hence, it is possible that bezafibrate may have induced another form of aromatase to contribute to the decrease in hepatic lipids.

Event 2b. Clofibrate, DEHP, gemfibrozil, DBP, and WY 14,643 have been shown to reduce estradiol metabolism, which would increase serum estradiol levels (Corton et al., 1997; Eagon et al., 1994a; Fan et al., 1998; Rao et al., 1994). Increases in estradiol may occur through altered (i.e., decreased) expression of genes that regulate estradiol metabolism (Corton et al., 1997). The expression of the male-specific gene, CYP2C11, that catalyzes the hydroxylations of estradiol at the 2 and 16 alpha positions was either decreased or completely abolished in male rat livers after a 3-week treatment with gemfibrozil or WY 14,643, respectively. The downregulation of CYP2C11 by WY 14,643 could be reproduced in cultured rat hepatocytes, indicating the downregulation does not require other liver cell types (Corton et al., 1997, 1998). Because a CYP2C11 ortholog has not been identified in the mouse, it has not been possible to determine if the decrease in expression is dependent upon PPARα.

Event 3. Administration of PFOA to adult male rats by gavage for 14 days was shown to decrease testosterone levels and increase serum estradiol levels (Cook et al., 1992). The increase in estradiol occurred at the same dose levels as the increase in β-oxidation, suggesting that it may be mediated by PPARα (Cook et al., 1992; Liu et al., 1996b). The decrease in testosterone levels is discussed under Event 8. PFOA and WY 14,643 also have been shown to produce a sustained increase in serum estradiol levels over a 2-year exposure regimen; these increases correlate with their potency to induce LCTs in rats (Biegel et al., 1995, 2001). In addition, DEHP and clofibrate have been shown to increase the serum concentration of estradiol in adult male rats (Eagon et al., 1994a; Rao et al., 1994). Eight out of 11 other PPARα agonists have been shown to increase estradiol production using isolated Leydig cells (Liu et al., 1996a). Collectively, these data suggest that many PPARα agonists can increase estradiol levels by induction of aromatase (Event 2a) and/or by reducing estradiol metabolism (Event 2B) (Corton et al., 1997; Eagon et al., 1994a; Fan et al., 1998; Rao et al., 1994).

Event 4. If an increase in serum estradiol is playing a role in LCT tumorigenesis, then interstitial fluid estradiol levels should be increased. Interstitial fluid bathes the Leydig cells and seminiferous tubules and provides a window to the endocrine milieu of the testis. An increase in interstitial fluid estradiol levels was observed in PFOA-treated rats when compared to control values, which is consistent with estradiol playing a role in LCTs (Biegel et al., 1995).

Event 5. It has been proposed that PPARα agonists may be inducing LCTs via a non-LH type mechanism whereby estradiol modulates growth factor expression within the testis to produce LC hyperplasia and neoplasia (Biegel et al., 1995; Cook et al., 1992). An increase in interstitial fluid TGFα levels was observed in PFOA-treated rats when compared to control values, which is consistent with this hypothesis (Biegel et al., 1995).

Event 6. The increase in TGFα levels within the testis could increase LCTs as TGFα has been shown in vitro to stimulate Leydig-cell proliferation (Cook et al., 1999; Khan et al., 1992). A similar relationship has been proposed for the mammary gland. In the mammary gland, estradiol has been shown to stimulate TGFα secretion, and overexpression of TGFα has been associated with the proliferation of mammary epithelial cells and subsequent neoplasia (Liu et al., 1987). TGFα stimulates thymidine incorporation into LC precursors and appears to be a LC stimulant (Khan et al., 1992; Liu et al., 1996a).

Conflicting evidence exists for the role of estrogens in the development of LCTs in rats. Estrogenic compounds did not induce LCTs in rats when given at doses that produce testicular atrophy, which can confound detection of LC hyperplasia (Gibson et al., 1967; Marselos and Tomatis, 1993; Schardein, 1980; Schardein et al., 1970). These earlier studies also were limited by small sample size and reduced survival. Interestingly, gonadotropin-releasing hormone (GnRH) agonists induce LCTs at low doses, but do not induce LCTs at higher doses where LH levels are suppressed and testicular atrophy occurs (Donabauer et al., 1987; Hunter et al., 1982; PDR, 1995b, 1995d, 1995e). Hence, these negative bioassays with estrogenic compounds may be due to suppression of LH, which, to date, is the

primary demonstrated "driver" of LCTs. Estradiol does appear to play a role in the enhancement of LC tumorigenesis based on data from aging studies. In F344 rats, which have a high spontaneous incidence of LCTs, there is an age-related increase in serum estradiol that correlates with the development of LC hyperplasia and tumor formation (Turek and Desjardins, 1979). However, in the Sprague-Dawley rat, which has a low spontaneous incidence of LCTs, serum estradiol decreases with age (Biegel et al., 2001). In a 2-year rat mechanistic bioassay, PFOA and WY 14,643 produced a sustained increase in serum estradiol levels that correlated with the potency of the two compounds to induce LCTs (Biegel et al., 2001). These studies suggest that estradiol plays a role in enhancement of LCTs in the rat, and that PPARα agonists induce LCTs via a non-LH type mechanism. Whether estradiol plays a role in the induction of LCTs by PPARα agonists can be determined only from an estradiol bioassay conducted at dosing levels that do not induce testicular atrophy or reduce LH levels.

Pathway 2—direct inhibition of testosterone biosynthesis at the level of the testis (events 7–10):

Event 7. Administration of PFOA to adult male rats by gavage for 14 days was shown to decrease testosterone levels (Cook et al., 1992). In vitro and in vivo human chorionic gonadotropin (hCG) challenge studies demonstrated that the PFOA inhibition of the testosterone biosynthetic pathway was occurring at the $C_{17,20}$-lyase step (Cook et al., 1992). More recently, PPARα agonists have been shown to decrease peripheral benzodiazepine receptor (PBR) mRNA (Gazouli et al., 2002). The resulting decrease in PBR protein, which controls the amount of cholesterol transported to the cytochrome P-450 scc (CYP 450scc) (a rate-limiting step), leads to a reduction of the amount of testosterone formed by Leydig cells. These events were observed in both mouse and rat Leydig cells. It then was established that PPARα agonists did not reduce testosterone production or PBR expression within the testis of PPARα-null mice, supporting the hypothesis that these events were mediated by PPARα (Gazouli et al., 2002). Thus, two independent laboratories (Cook et al., 1992; Gazouli et al., 2002) have confirmed that PPARα agonists inhibit testosterone biosynthesis.

Event 8. Several PPARα agonists have been shown to reduce testosterone biosynthesis. For instance, administration of PFOA to adult male rats by gavage for 14 days was shown to decrease testosterone levels (Biegel et al., 1995; Cook et al., 1992). Eleven other PPARα agonists have been shown to inhibit testosterone production using isolated Leydig cells (Liu et al., 1996a). In addition, a second laboratory has confirmed that bezafibrate, MEHP and WY 14,643 inhibit testosterone biosynthesis in rat and mouse Leydig cells (Gazouli et al., 2002). In total, 13 out of 13 PPARα agonists have been shown to inhibit testosterone biosynthesis using cultured Leydig cells.

Event 9. Inhibition of testosterone biosynthesis would disrupt the HPT axis resulting in an increase in LH levels. However, in a mechanistic bioassay, serum testosterone and LH levels were not altered significantly at the levels of PFOA and WY 14,643 tested (Biegel et al., 2001; Cook et al., 1992). The inability to detect an increase in LH may be due to the increased estradiol levels from Pathway 1, which would attenuate the LH increase and reduce the ability to detect such a change. This role of an increase in LH needs to be examined with other PPARα agonists.

Event 10. If LH levels were increased, an increase in LCT would occur. Such a mechanism is well established with other compounds that induce LCTs (Cook et al., 1999).

c. Conclusions—Overall Evaluation of Animal Mode(s) of Action

As described earlier, the mechanistic data from studies using PFOA and WY 14,643 suggest that PPARα agonists induce LCTs by inhibiting testosterone biosynthesis and/or by inducing aromatase, thereby increasing estradiol levels. It is likely that both of these mechanisms contribute to the development of LCTs. If only the latter mechanism were responsible, then the LCTs would be secondary to changes in the liver. However, using isolated Leydig cells, 13 out of 13 PPARα agonists were shown to inhibit testosterone production and 8 out of 11 agonists increased estradiol levels when incubated for longer durations (Gazouli et al., 2002; Liu et al., 1996a). The inhibition of testosterone biosynthesis may contribute to the development of Leydig cell tumorigenesis through disruption of the HPT axis, which would increase LH levels while the increase in estradiol levels may promote LCTs via increasing growth factor expression within the testis (Cook et al., 1999). In a mechanistic bioassay with PFOA and WY 14,643, LH levels were not significantly

increased, yet estradiol levels were increased at several time points (Biegel et al., 2001). The absence of an increase in LH would be consistent with estradiol attenuating the elevation of LH from the inhibition of testosterone biosynthesis.

d. Data Gaps

There are several data gaps that need to be explored. First, the degree to which there may be PPARα control of mammalian aromatase is a critical data gap and the establishment of the existence of PPREs upstream of the aromatase gene would be a valuable first step. Second, while 13 out of 13 PPARα agonists have been shown to inhibit testosterone biosynthesis in vitro (Gazouli et al., 2002; Liu et al., 1996a, 1996b), only two PPARα agonists (i.e., DEHP and PFOA) have been tested and shown to inhibit testosterone levels in vivo (Cook et al., 1992; Eagon et al., 1994a). Examining this relationship for other PPARα agonists in vivo will improve the assessment of the relative contributions of these two pathways for LCT induction. Third, the amount of mechanistic and comparative information for PPARα agonist induction of LCTs is moderate to low when compared to PPARα agonist induction of liver tumors. The development of a knock-out rat (but not an F344) followed by an experiment using WY 14,643 (and, subsequently, other PPARα agonists) is probably the most compelling experiment to test whether the PPARα receptor mediates the induction of LCTs. Short of development of this research tool, additional studies with additional agonists in rat strains other than the F344 would be critical.

e. Supportive Data Elements for Evaluation of Proposed Mode(s) of Action for Leydig Cell Tumors

Section II described the type of data needed to establish that a compound induces hepatic tumors via the PPARα receptor. The following describes data needed to demonstrate that LCTs are due to a compound being a PPARα agonist. As a first step, one must show that the compound does not directly interact with DNA, exhibiting mutagenic or genotoxic characteristics. Secondly, one could demonstrate that a compound is a PPARα agonist through the use of in vitro reporter assays using both liver and LC tissue using strains of rat other than the F344. In addition, the compound should be shown to induce hepatic aromatase and/or inhibit testosterone biosynthesis. Aromatase activity can be measured by an enzymatic assay (e.g., tritiated water release), measurement of protein levels (e.g., Western), or measurement of the message level (e.g., Northern). Using isolated Leydig cells is probably the easiest method to establish that a compound inhibits testosterone biosynthesis. Alternatively, measuring serum estradiol and testosterone levels after at least 2 weeks of treatment is an alternative in vivo method. However, appropriate attention to study design is critical for hormonal measurements (O'Connor et al., 2002).

f. Conclusions

Two modes of action (MOAs) have been proposed to describe the etiology of LCTs in PPARα agonist-treated (non-F344) rats. As noted in Table 27, the weight of evidence available to date to support virtually all of the postulated key events is weak overall, and moderate at best for only two or three of the postulated key events. This judgment on the weight of evidence was based primarily on the fact that the majority of the work has been conducted in two or three laboratories with only a few chemicals, and hence requires confirmation in other laboratories and with a more robust set of chemicals. For instance, the effects on testosterone inhibition and enhanced production of estradiol have been shown in two separate laboratories with up to 13 PPARα agonists in vitro. However, the hormonal changes have been demonstrated with only four agonists in vivo. It is critical that additional in vivo studies be conducted with a much larger set of chemicals to support and confirm this relationship for the class as a whole. *Therefore, one must conclude, at this time, that the answer to the question "Is the weight of evidence sufficient to establish the mode(s) of action for Leydig-cell tumors in rats?" is "No."* That having been said, the postulates are plausible, given the data at hand, and may be proven correct with further confirmatory experimentation in non-F344 rats and/or PPARα knockout rats, as noted earlier. In addition, quite a lot is known about the basic reproductive biology of the rat testis and common mechanisms in the etiology of LCTs, in general. In summary, the characterization of the MOA(s) for Leydig-cell tumorigenesis is less well understood than for the liver. However, data are accumulating that establish biological plausibility. Nonetheless, acceptance of the proposed MOA(s) will require additional research to assess whether PPARα agonists produce these hormonal changes in vivo.

2. Applicability of Animal MOA(s) to Human Health Risk Assessment

a. Overview of existing available human literature (in vitro, epidemiological, and clinical)

There are limited epidemiological data with PPARα agonists. The compounds evaluated include clofibrate, gemfibrozil, and PFOA. Of these compounds, only the PFOA studies in humans assessed potential effects of PFOA on the testis and pancreas. Primate studies conducted with clofibrate (3 months of exposure), DEHP (3 months of exposure), and PFOA (6 months of exposure) examined hepatic and extrahepatic changes (testis, pancreas, testosterone, estradiol, and cholecystokinin [CCK]) and found no effect on these extrahepatic endpoints (Butenhoff et al., 2002; Kurata et al., 1998). In a 2-week primate study conducted with clofibrate, DEHP, and DINP, there were no histopathological findings in the liver, kidney or testes (Pugh et al., 2000).

PFOA epidemiological studies

Occupational monitoring studies have focused on endpoints identified from toxicology studies to assess whether processes similar to those observed in rats may be occurring in humans. For instance, Olsen and coworkers (1998) conducted medical surveillance, measured selected hormone levels, and assessed exposure by measuring serum PFOA levels from workers in a PFOA product plant. PFOA levels from the blood of workers ranged from nondetectable to approximately 100 ppm. The human levels were comparable to those measured in some animal studies, but were several orders of magnitude higher than concentrations typically measured in the blood of nonoccupationally exposed individuals (average around 0.006 ppm) (Hansen et al., 2001).

In 1998, the results from two cross-sectional studies were reported of male production workers employed at a PFOA production plant (Olsen et al., 1998). The first study, conducted in 1993, evaluated 111 workers, while the second study, conducted in 1995, evaluated 80 workers (68 workers participated in both studies). Olsen and coworkers (1998) found no significant associations between plasma PFOA levels and the levels of reproductive hormones in employees from the PFOA production facility. Specifically, there was no significant association between PFOA exposure and estradiol or testosterone (free or bound) levels. They concluded that these "results provide reasonable assurance that, in this production setting, there were no significant hormonal changes associated with PFOA at the serum levels [of PFOA] measured."

Thus, measured levels of PFOA exposure in humans do not appear to be associated with the marked hepatic toxicity or other effects that are seen in mice or rats. It must be noted that the occupational exposure levels were significantly lower than the predicted exposures in the carcinogenicity studies in rats with PFOA.

Epidemiological studies with other PPARα agonists

Human epidemiological studies are available on a limited number of compounds that induce peroxisomes in rodents. These compounds include clofibrate (Oliver et al., 1978) and gemfibrozil (Frick et al., 1987). The epidemiologic studies of clofibrate and gemfibrozil are of limited usefulness for evaluation of carcinogenic potential because of their relatively short exposure duration and/or limited follow-up period; clearly (re)evaluation of individuals who have used either of these two drugs, preferably for longer periods of time, would be valuable.

b. Conclusions

1) Are the key events in the animal MOA(s) plausible in humans?

For the purpose of discussion, and to encourage evolution of the state of the science as expeditously as possible, the analysis will proceed as if the answer to question 1 were a "Yes" (Table 28).

The proposed animal MOAs—induction of aromatase secondary to liver induction (Pathway 1) and the direct inhibition of testosterone biosynthesis (Pathway 2)—are plausible mechanisms and could occur in humans. If PPARα is mediating the induction of aromatase, this mechanism could occur in humans due to the expression of PPARα in human liver. The inhibition of testosterone biosynthesis by PPARα agonists is better established than the induction of aromatase and is also plausible, as PPARα is present in human Leydig cells. The pathways for the regulation of the HPT axis of rats and humans also are similar, in that compounds that decrease testosterone will increase LH levels. Hence, compounds that induce LCTs in rats by disruption of the HPT axis pose a potential risk to human health.

TABLE 28
Hypothetical Comparative Analysis of Rodent and Human Data for LCTs

Rat MOA key events for Leydig-cell tumor	Is this key event in the MOA plausible in humans?	Taking into account kinetic and dynamic factors, is this key event in the MOA plausible in humans?
1. Activation of PPARα		
In liver?	Yes	Yes
In leydig cell?	Yes	Yes
Pathway 1 and/or		
2a. ↑CYP 19A1 (Aromatase) and/or	Yes	No data
2b. ↓ Estradiol metabolism	Yes	No data
3. ↑ Serum estradiol levels	Yes	Unlikely, based on primate and human data
4. ↑ Interstitial fluid estradiol levels	Yes	No data
5. ↑ TGFα levels	Yes	No data
6. ↑ LC proliferation/LCTs	Yes	Unlikely, based on human disease states
Pathway 2		
7. ↓ Testosterone biosynthesis either by ↓ PBR protein levels and/or $C_{17,20}$-lyase activity	Yes	No data
8. ↓ Testosterone levels	Yes	Unlikely, based on primate and human data
9. ↑ LH	Yes	
10. ↑ LC proliferation/LCTs	Yes	Unlikely, based on human disease states

Therefore, for Pathway 2, the central issue becomes the relative sensitivity between rat and human LCs in their response to increased LH levels.

2) Taking into account kinetic and dynamic factors, is the animal MOA plausible in humans?

In Pathway 1, the critical initiating event is the induction of hepatic aromatase. There is limited evidence that the aromatase induction is mediated by PPARα. If aromatase is regulated by PPARα, there are a number of reasons why humans would be insensitive to this effect: (1) Levels of PPARα mRNA in human liver RNA samples appear to be 10-fold less than mouse RNA controls (Palmer et al., 1998); (2) PPARα protein is lower in humans than mice, although one human sample had levels similar to the mouse (Walgren et al., 2000); (3) truncated or mutant PPARαs appear to act as dominate negative proteins and thereby contribute to the human insensitivity to PPARα agonists (Gervois et al., 1999; Palmer et al., 1998; Sapone et al., 2000; Tugwood et al. 1996; Vohl et al., 2000); and (4) some limited (and conflicting) evidence suggests that differences in PPREs may contribute to differential gene expression across species. Recently, three fibrates were shown to activate PPARα and induce a hypolipidemic effect in humans but not increase the hepatic expression of acyl CoA oxidase (Roglans et al., 2002). These data are consistent with humans being refractory to peroxisome proliferation. Hence, it is unlikely that PPARα agonists would increase aromatase and, thus, estradiol levels in humans. PFOA is the only PPARα agonist that has been evaluated in (occupationally exposed) humans for altering reproductive hormone levels (testosterone, estradiol, folliele-stimulating hormone [FSH], luteinizing hormone [LH], thyroid-stmulating hormone [TSH], prolactin); no effects on reproductive hormone levels were observed (Olsen

et al., 1998). In addition, estradiol levels were not increased in primates administered clofibrate, DEHP, or PFOA (Butenhoff et al., 2002; Kurata et al., 1998). While these data are limited, the primate data are consistent with the expectation that humans would be refractory to aromatase induction by PPARα agonists.

In Pathway 2, PPARα agonists decrease testosterone biosynthesis, which would result in an increase in LH levels. Several lines of evidence exist that suggest that human LCs are quantitatively less sensitive than rats in their proliferative response to LH, and hence, in their sensitivity to chemically induced LCTs (reviewed in Cook et al., 1999). This evidence includes the following:

- The human incidence of LCTs is much lower than the spontaneous incidence in rodents even when corrected for detection bias.
- Several comparative differences exist between rat and human LCs that contribute, at least in part, to the greater susceptibility of the rat to both spontaneous and xenobiotic-induced LCTs. For instance, rat and human LCs have been shown to respond differently to human chorionic gonadotropin (hCG), a hormone equivalent to LH in its action on LCs. Rat LCs respond to hCG stimulation in a proliferative manner, while the human LC response is limited to hypertrophy (Christensen and Peacock, 1980; Simpson et al., 1987). This comparative difference is attributed, in part, to the 13-fold greater number of LH receptors/cell in rats compared to human LCs (Huhtaniemi, 1983). The greater number of LH receptors on rat versus human LCs should, by the spare receptor theory, make the rodent LCs, more sensitive to the same level of circulating LH than human LCs, and this appears to be the case, at least in vitro (Simpson et al., 1987).
- Endocrine disease states in humans (such as androgen insensitivity syndrome and familial male precocious puberty [FMPP]) underscore the marked comparative differences that exist between rats and humans in the responsiveness of their LCs to proliferative stimuli. In FMPP, a mutation results in a state of constant LH receptor activation where the constitutive activation is estimated to be 42% of maximal hCG stimulation (Shenker et al., 1993), thus mimicking the primary mechanisms of LCT formation in rats. If this condition existed in rats, it could reasonably be predicted that they would develop LCTs, yet biopsies from men with FMPP report only LC hyperplasia (Holland, 1991; Rosenthal et al., 1983; Schedewie et al., 1981).
- Several human epidemiology studies are available on a number of compounds that induce LCTs in rats (1,3-butadiene, cadmium, ethanol, lactose, lead, nicotine), which demonstrate no association between human exposure to these compounds and induction of LC hyperplasia or adenomas (summarized in Cook et al., 1999).

Collectively, these data demonstrate that PPARα agonists would be unlikely to induce LCTs in humans via a testosterone biosynthesis mechanism because humans are quantitatively less sensitive than rats to LH stimulation. Such a conclusion regarding the role of LH has been reached by several other investigators (Alison et al., 1994; Bar, 1992; Bosland, 1996; Cook et al., 1999; Prentice and Meikle, 1995). In summary, even if a PPARα agonist produced either testosterone inhibition or estradiol induction secondary to the liver changes, it is unlikely to pose a significant cancer risk to humans because of the comparative differences between rodents and humans discussed above. The most compelling is the human disease state FMPP, where men have a mutated LH receptor that is activated throughout their life and LCTs are not seen.

Overall conclusions

Using the human relevance framework for analysis, MOA(s) have been proposed for LCTs, although the weight of evidence is moderate to low when compared with the robustness of the database available for the liver. *Therefore, it is premature to conclude that the MOA(s) are adequately understood.* That determination notwithstanding, and to encourage the development of confirmatory evidence, we have proceeded beyond Question 1 in the human relevance framework as if we did have sufficient data to support these two MOAs to speculate on possible answers to Questions 2 and 3 (see Table 28). Given what we do know and what we would expect to find from additional experimentation, we would conclude that the MOA(s) could plausibly occur in humans as the same pathways that exist in the rat exist in humans (Question 2: Are the key events in the animal MOA(s) plausible in humans?). However, the key events in the MOA(s) do not appear to be plausible in humans when taking into account kinetic and dynamic factors (Question 3: Taking into account kinetic and dynamic factors, are the MOA(s) plausible in humans?).

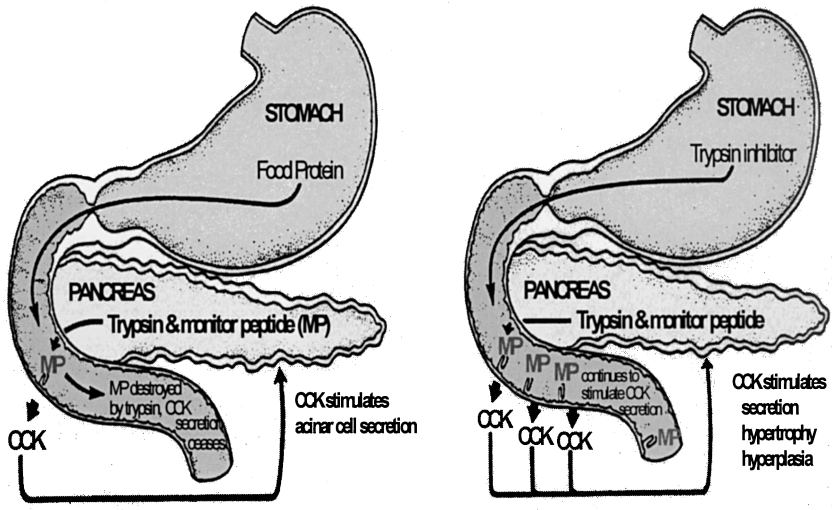

FIGURE 7. Left panel shows the physiological control of CCK (Longnecker, 1987). When a meal is ingested, the physical presence of food within the stomach sets up a neural signal that stimulates the release of CCK from the gut mucosa. CCK is produced in the M(I) cells in the duodenal mucosa and is released into the bloodstream in response to the presence of food in the duodenum (Johnson, 1987). CCK then binds to CCK_A receptors on pancreatic acinar cells and stimulates release of pancreatic enzymes into the gut. The pancreatic juice contains a small protein called the monitor peptide (MP), which binds to receptors in the duodenum that stimulate CCK release into the bloodstream (Iwai et al., 1988). Trypsinogen also is found in pancreatic juice and is cleaved into the proteolytic enzyme trypsin inside the gut. When there is no food remaining in the gut, trypsin degrades the MP ceasing further release of CCK. Right panel illustrates the consequences of consuming a diet that contains a trypsin inhibitor. The presence of a trypsin inhibitor results in an enhanced neutralization of trypsin allowing more molecules of monitor peptide to bind to its receptor. As a result of the increased monitor peptide binding, there is an increase in the amount of CCK released into the bloodstream, which, if sustained, results in acinar-cell hypertrophy, hyperplasia, and adenomas.

C. Pancreatic Acinar-Cell Tumors

1. Proposed Mode of Action: Is the Weight of Evidence Sufficient to Establish the Mode(s) of Action in Rats?

a. Common mechanisms for pancreatic acinar-cell carcinogenesis

The development of pancreatic acinar-cell hypertrophy, hyperplasia, and adenomas in the rat has been shown to be modified by several factors such as steroid hormones (testosterone and estradiol), growth factors such as cholecystokinin (CCK), growth factor receptor overexpression (CCK_A receptor), and diet (fat) (Bell et al., 1992; Longnecker, 1983, 1987; Longnecker and Sumi, 1990; Povoski et al., 1993). For instance, the incidence of spontaneous and induced neoplasms of the exocrine pancreas is higher in male than in female rats. Castration, ovariectomy, and hormone replacement with estradiol and testosterone have been shown to influence the growth of carcinogen-induced preneoplastic foci in the azaserine-rat model of pancreatic carcinogenesis (Longnecker and Sumi, 1990). It appears that testosterone supports the growth of preneoplastic foci and carcinomas, whereas estrogen inhibits such growth.

Growth factors such as CCK have been shown to stimulate normal, adaptive and neoplastic growth of pancreatic acinar cells in rats (Bell et al., 1992; Longnecker, 1987; Povoski et al., 1993). The mechanisms for elevating CCK levels include ingestion of protease inhibitors such as those found in raw soybean flour (Booth et al., 1960; Goke et al., 1988; Johnson, 1981; Oates et al., 1986), impaired bile flow (Gomez et al., 1988; Kurosawa et al., 1989; Ohta et al., 1990; Toriumi et al., 1993) and altered bile acid composition (Gomez et al., 1988; Koop et al., 1989; Nustede et al., 1993). Another potential

mechanism for increasing CCK levels is ingestion of fat. In two-year rat bioassays, corn oil used as a vehicle has been shown to increase pancreatic acinar tumors (Longnecker, 1987), possibly mediated by increased CCK levels. Figure 7 illustrates the normal physiology of CCK regulation (Figure 7A) and the effects of ingestion of a trypsin inhibitor on CCK release (Figure 7B).

Several PPARα agonists have been shown to produce PACTs. A number of potential mechanisms have been investigated in a series of in vitro and in vivo (subacute, subchronic, and carcinogenicity) studies using PFOA and WY 14,643 (Obourn et al., 1997a, 1997b). In the in vitro studies, a CCK receptor competitive binding assay and a trypsin inhibition spectrophotometric assay evaluated the potential for PFOA and WY 14,643 to act as direct CCK agonists or trypsin inhibitors, respectively. In the subchronic study, the potential for WY 14,643 to produce pancreatic effects similar to a known trypsin inhibitor and to modulate gut fat levels was investigated. The carcinogenicity study was designed to assess pancreatic effects produced secondary to liver effects as well as potential CCK_A receptor overexpression. In these in vivo studies, plasma CCK levels were monitored as well as the weights of the pancreas and liver, and pancreatic acinar and hepatic cell proliferation. The carcinogenicity study also investigated clinical pathology markers for cholestasis and hepatic cell injury and changes in bile flow and composition.

PFOA and WY 14,643 were shown not to inhibit trypsin or to bind to the CCK_A receptor in vitro (Obourn et al., 1997a). In the subchronic 56-day feeding study, rats fed 100 ppm WY 14,643 produced no pancreatic weight gain, pancreatic acinar cell proliferation, or increased CCK levels (Obourn et al., 1997a), while rats fed a compound with trypsin inhibitor activity (DPX-A8947, a herbicide) exhibited all three responses (Obourn et al., 1997c). The absence of any early changes in the pancreas in the subchronic study is consistent with the in vitro data, which demonstrated that WY 14,643 is not a CCK_A agonist or a trypsin inhibitor. In contrast to the subchronic effects on the pancreas, effects on the liver were pronounced and immediate. Statistically significant changes in mean relative liver weight, β-oxidation rates of liver microsomal enzymes, and hepatocyte cell proliferation occurred within 7 days, yet, in separate experiments, pancreas β-oxidation rates were not elevated (Obourn et al., 1997a).

In humans, plasma CCK concentrations have been reported to reach higher levels compared to controls after oral administration of fat to patients with subtotal gastrectomy (Goke et al., 1988; Hopman et al., 1984). In 2-year bioassays, rats gavaged with corn oil vehicle exhibit pancreatic acinar-cell tumors, which was proposed to be mediated by increased CCK levels (Longnecker, 1987). However, animals fed a high-fat diet failed to produce an increase in CCK when measured by radioimmunoassay (RIA) (Roebuck et al., 1987). Aliphatic dicarboxylic acids have demonstrated hypolipidemic activity and the ability to increase fecal fat content (Izydore et al., 1991). WY 14,643 was designed originally as a hypolipidemic agent (Santilli et al., 1974). This lipid-lowering effect of WY 14,643 initially was predicted to increase the amount of fat excreted in the feces. However, concentrations of fecal fat from the WY 14,643-treated group showed no increase compared to the control group at the 56-day time point (Obourn et al., 1997a). Therefore, an increase in fecal fat excretion is not predicted and increases in dietary fat would not be responsible for the resultant pancreatic changes observed in the carcinogenicity studies with PPARα agonists.

These series of experiments eliminated all of the commonly known mechanisms by which PPARα agonists could induce PACTs except for alterations in bile acid synthesis and composition. In an 18-month mechanistic study, WY 14,643 decreased bile flow that resulted in cholestasis and a sustained increase in CCK levels (Obourn et al., 1997a, 1997b). During this time, substantial evidence for the role of PPARα in regulating bile acid synthesis has been published that provides a plausible MOA for induction of PACTs by PPARα agonists (Lu et al., 2000; Marrapodi and Chiange, 2000; Post et al., 2001). Hence, PPARα agonists appear to induce PACTs by decreasing bile acid synthesis and/or altering bile acid composition resulting in cholestasis. The decreased bile acid synthesis increases CCK levels, which binds to the CCK_A receptor and stimulates acinar cell proliferation. The evidence for this MOA is summarized in Figure 8 and Table 29, and the evidence supporting each key event is discussed next.

b. Evidence in Animals/Key Events

Event 1. Using polyclonal antibodies to PPARα, β, and γ, PPARs were found to be similarly expressed in the exocrine (i.e., acinar cells) and endocrine (i.e., islet cells, also referred to as β cells) cells of the pancreas (Braissant et al., 1996). PPARβ

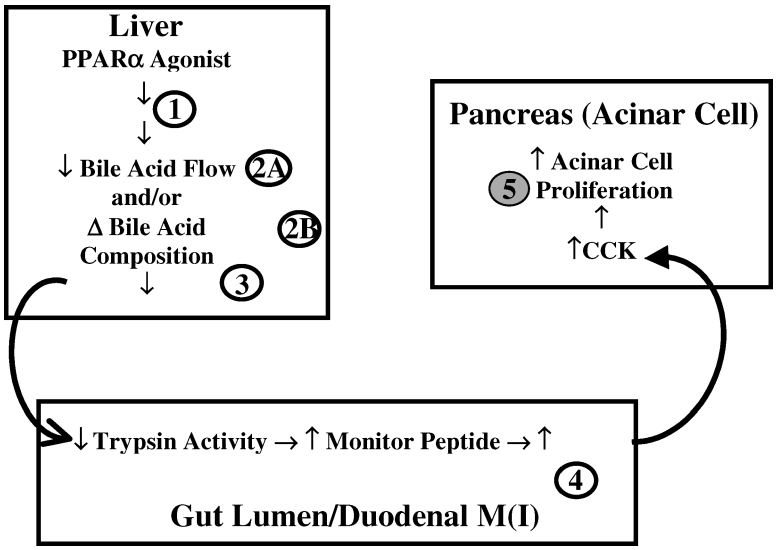

FIGURE 8. Schematic depicting the proposed mode of action (MOA) with key events for pancreatic acinar cell tumors (PACTs) induced by PPARα agonists. Experimental evidence suggests that PPARα agonists induce PACTs in rats by increasing CCK levels secondary to reduced bile acid synthesis and/or alterations in bile acid composition (Obourn et al., 1997a, 1997b). PPARα agonists have been shown to decrease transcription of cholesterol 7α-hydroxylase, the rate-limiting step of bile acid synthesis, via the PPARα/RXRα heterodimer reducing hepatocyte nuclear factor-4 (HNF-4) binding to the DR-1 sequence, which regulates this gene (Marrapodi and Chiange, 2000) (Event 1). Consistent with this inhibition of cholesterol 7α-hydroxylase, PPARα agonists have been shown to reduce bile flow (Event 2a) and alter bile acid composition (Event 2b). Cholestasis is known to occur as a consequence of reduced bile flow and/or composition (Event 3). Bile acids are known to enhance trypsin activity (Longnecker, 1987); hence, reducing bile acid flow or altering bile acid composition reduces trypsin activity and increases CCK release from the M(I) cells in the duodenal mucosa (Event 4). Increasing CCK binding on acinar cell CCK_A receptors increases acinar cell proliferation in rats (Event 5).

was the most prominent form, whereas PPARα and PPARγ had low expression in these two cell types. The role of PPARα and PPARγ in islet cells has been studied (Dubois et al., 2000; Eibl et al., 2001; Roduit et al., 2000; Sugden et al., 2001), but no reports of the role of PPARs in acinar cells have been published to date. While a direct role for PPARα agonists on the acinar cells cannot be excluded, the available data suggest that the PPARα agonists are inducing PACTs secondary to changes in the liver. This conclusion is based in part on using β-oxidation as a marker of acinar cell responsiveness to PPARα agonists and not seeing an increase in this biomarker (Obourn et al., 1997a). In addition, PPARα agonists appear to alter bile acid synthesis and composition. There is growing evidence for the regulation of bile acid synthesis by nuclear receptors including PPARα (Hunt et al., 2000, 2001; Lu et al., 2000). For instance, fibrates have been shown to suppress bile acid synthesis in the liver via PPARα-mediated downregulation of cholesterol 7α-hydroxylase and sterol 27-hydroxylase gene expression (Marrapodi and Chiange 2000; Post et al., 2001).

Event 2a. Fibrates have been shown to suppress bile acid synthesis in the liver via PPARα-mediated down-regulation of cholesterol 7α-hydroxylase and sterol 27-hydroxylase gene expression (Post et al., 2001). In addition, it has been shown that PPARα/RXRα reduces HNF-4 binding to the DR-1 sequence, which decreases the transcription of cholesterol 7α-hydroxylase (CYP7A1), the rate limiting step of bile acid synthesis (Marrapodi and Chiange 2000). In addition, the murine and human cholesterol 7α-hydroxylase gene promoters have been shown to be differentially responsive to PPARα agonists due to the additional PPARα/RXRα binding site in the murine CYP7A1 gene promoter (Cheema and Agellon, 2000). It is plausible that this

TABLE 29
Table of Key Events—Pancreatic Acinar-Cell Tumors

Event	Relationship[a]	Weight of evidence	Specificity	Comments
1. Activation of PPARα in liver	Causal	Moderate	High	PPARα/RXRα reduces HNF-4 binding to the DR-1 sequence, which decreases the transcription of cholesterol 7α-hydroxylase, the rate-limiting step of bile acid synthesis (shown with ciprofibrate and WY 14,643)
2a. ↓ Bile acid flow	Associative	Weak	Low	↓ Bile acid flow has been shown to ↑ CCK; seen with WY 14,643 in 18-month bioassay
And/or				
2b. Altered bile acid composition	Associative	Moderate	High	Altered bile acid composition has been shown to ↑ CCK; altered bile composition has been shown with clofibrate, clofibride, nafenopin and WY 14,643
3. Cholestasis	Associative	Weak	Low	Event occurs as a consequence of Steps 2a,b; ↓ trypsin activity, which ↑ monitors peptide binding → ↑ CCK; WY 14,643 shown to produce event in18-month bioassay; clinical pathology evidence of cholestasis also seen with gemfibrozil, methylclofenopate, and tibric acid
4. ↑ CCK	Causal	Weak	Low	↑ CCK → ↑ acinar cell proliferation; seen with WY 14,643 in 18-month bioassay
5. ↑ Acinar-cell tumors	Associative	Strong	Low	Seen primarily with compounds that act as trypsin inhibitors

Note. Causal: Required step for PPARα MOA based on data *Associative*: Events that are occurring but may or may not be causally linked to the MOA. *Weight of evidence (strong, moderate, weak)*: *Strong* is normally defined by having several studies, preferably with multiple PPARα agonists from multiple laboratories and with limited evidence of contradiction which support that MOA. *Moderate* spans the data continuum between weak and strong. *Weak* is normally defined by having a single study with a single PPARα agonist from a single laboratory or a significant amount of contradiction in the literature. *Specificity (high, low)*: *High* is defined as unique to this PPARα MOA. *Low* is defined as not unique to PPARα MOA.
[a]Relationship to pancreatic acinar cell tumors or key events leading to tumors.

could be true of the rat as well. Hence, it appears the PPARα agonists can regulate bile acid synthesis (Barbier et al., 2002; Hunt et al., 2000, 2001). PPARα also has been shown to regulate the apical sodium-dependent bile acid transporter via binding to a DR-1 motif (Jung et al., 2002).

In the 18-month carcinogenicity study with WY 14,643, bile flow and bile acid concentration were examined (Obourn et al., 1997a, 1997b). Bile flow was expressed relative to liver weight in order to normalize the two- to three-fold increases in liver weight induced. Bile flow was decreased at all time points in the treated rats: 3 months (76% of control), 6 months (71% of control), 8 months (59% of control), 10 months (20% of control), 12 months (30% of control), 15 months (32% of control), and 18 months (49% of control). Using bilary diversion or bile-duct ligation, it has been demonstrated that decreasing bile acid flow will increase CCK levels (Gomez et al., 1988; Kurosawa et al., 1989; Ohta et al., 1990; Toriumi et al., 1993).

Event 2b. In the 18-month carcinogenicity study with WY 14,643, total bile acids were measured to assess effects on bile acid composition and were decreased at 3, 6, 8, and 12 months in the treated rats: 3 months (81% of control), 6 months (74% of control), 8 months (74% of control), 10 months (119% of control), 12 months (86% of control), 15 months (112% of control), and 18 months (111% of control). Other PPARα agonists (i.e., nafenopin, clofibrate, and clofibride) also have been shown to alter bile acid composition, and with the exception of clofibride, all have been reported in the literature to produce PACTs. Nafenopin ablated

chenodeoxycholic and muricholic acids and decreased hydrodeoxycholic acid in rats (Delzenne et al., 1994). Clofibrate and clofibride decreased biliary concentrations of bile salts, phospholipids, and cholesterol (Roze et al., 1977). WY 14,643 has been shown to significantly reduce chenodeoxycholic acid in rat (Neptun et al., 1988). This decrease in chenodeoxycholic acid may be due to increasing sterol 12α-hydroxylase, which would increase formation of cholic acid and decrease formation of chenodeoxycholic acid (Hunt et al., 2000).

The decrease in bile acid output coupled with potential changes in bile acid composition appears to be responsible for the increase in CCK output from the intestine. Altering bile acid composition has been shown to increase CCK levels (Gomez et al., 1988; Koop et al., 1989; Nustede et al., 1993). Clearly, the combination of bile flow measurements, bile acid measurements, and clinical chemistry data demonstrates the presence of cholestasis and suggests a possible role for altered bile flow/composition in the pancreatic lesions induced by WY 14,643 (Obourn et al., 1997a, 1997b).

Event 3. WY 14,643 also increased the levels of serum markers that indicate clinical cholestasis (i.e., bile acids, alkaline phosphatase, and bilirubin). All increases were statistically significant at all sampling time points (3, 6, 8, 10, 12, 15, and 18 months), demonstrating that cholestasis was sustained throughout the 18-month carcinogenicity study (Obourn et al., 1997a, 1997b). These clinical chemistry endpoints strongly suggest a reduced capacity of liver function and cholestasis. Other PPARα agonists have been shown to alter clinical pathology in a manner consistent with the presence of cholestasis (e.g., gemfibrozil-effect on bile acids [NTP, 1996], methylclofenapate—effect on alkaline phophatase [Tucker and Orton, 1995], HCFC-123—effect on alkaline phosphatase [Dr. L. A. Malley, DuPont Haskell Laboratory, personal communication], and tibric acid—effects on alkaline phosphatase and bilirubin [Facinni et al., 1979]).

Decreasing bile acid flow (Event 2a) and/or altering bile acid composition (Event 2b) have been shown to increase CCK levels. This increase in CCK levels occurs as bile acids are thought to enhance the effectiveness of trypsin, and reductions in bile acids would reduce the effectiveness of trypsin. By reducing trypsin activity, an increase in monitor peptide binding to M(I) cells in the duodenal mucosa leads to increased CCK release (Figure 7; reviewed in Longnecker, 1987).

Support for the proposed MOA also comes from experiments using biliary diversion and cholestyramine. Biliary diversion has been shown to increase CCK levels and produce PACTs (Stace et al., 1987). Cholestyramine, which binds bile acids, has been shown to increase CCK and produce PACTs (Brand and Morgan, 1982; McGuinness et al., 1985; Morgan et al., 1990). These data illustrate that if Event 3 occurs with PPARα agonists, then the induction of PACTs by such an MOA could occur.

Event 4. In the WY 14,643 carcinogenicity study, cholestasis correlated with increases in plasma CCK levels (Obourn et al., 1997a, 1997b). Plasma CCK levels statistically increased after 3 months (133% of control), 6 months (144% of control), 8 months (166% of control), 10 months (134% of control), 15 months (228% of control), and 18 months (215% of control) of exposure. Pancreas weight increased modestly above control at most of the time points. Pancreatic acinar cell proliferation mirrored plasma CCK and pancreatic weight gain at 3 months by increasing 1.6-fold. However, at 6 months, pancreatic acinar cell proliferation returned to control levels (107% of control) and remained similar throughout the duration of the study. The assessment of acinar cell proliferation in this study excluded foci of proliferation from the analysis.

Event 5. It is well established that increasing CCK levels will increase acinar cell proliferation (Longnecker, 1987). The most well-studied example of disruption of CCK regulation is from ingestion of trypsin inhibitors from raw soy flour. In rats, long-term feeding of raw soy flour produces PACTs by inhibiting trypsin and increasing CCK levels (McGuinness et al., 1980, 1987). WY 14,643 did not increase acinar cell proliferation when assessed from 1 to 8 weeks of exposure (Oburn et al., 1997a). However, Ohmura and coworkers (1997) demonstrated that a single dose of the PPARα agonist BR931 induced DNA synthesis in pancreatic acinar cells but not ductal or islet cells.

c. Conclusions—Overall Evaluation of Animal Mode(s) of Action

WY 14,643 does not appear to produce pancreatic acinar cell tumors through direct CCK_A receptor agonism, through direct trypsin inhibition, or through increasing dietary fat content. Available data demonstrate that WY 14,643 may be acting via an indirect trypsin-inhibition mechanism whereby reduced bile flow and/or changes in bile

composition are responsible for increased CCK levels. The PPARα agonists nafenopin, clofibrate, clofibride, and WY 14,643 all have been shown to alter bile acid composition, and with the exception of clofibride, all have been reported to produce PACTs (Event 2b).

Hence, WY 14,643 may be acting via an indirect trypsin-inhibition mechanism where reduced bile flow and/or changes in bile composition are responsible for increased CCK levels. The magnitude of the CCK changes by PPARα agonists appears consistent with the described effects on bile flow and composition. For comparison purposes, rats treated for 7 days with the herbicide DPA-A8947 possessing trypsin inhibitor activity had plasma CCK levels and pancreas weights that were increased to 250 and 220% of control, respectively, when compared to the pair-fed control (Obourn et al., 1997c). After 7 days of complete bile flow obstruction, rats had a 290% of control increase in plasma CCK levels but no change in pancreas weight (Kurosawa et al., 1989). Since complete bile flow obstruction represents the most severe form of cholestasis, the modest increases in plasma CCK levels and pancreas weights following WY 14,643 treatment are consistent with a lesser degree of cholestasis and mild changes in bile acid flow seen with WY 14,643. Bile acids enhance the effectiveness of trypsin (Ohta et al., 1990). Recent data have demonstrated that fibrates suppress bile acid synthesis in the liver via PPARα-mediated downregulation of cholesterol 7α-hydroxylase, the rate-limiting step of bile acid synthesis (Marrapodi and Chiange, 2000; Post et al., 2001). If altered bile acid composition and/or altered bile flow are responsible for the increase in CCK, the ability of PPARα agonists to induce rat PACTs would be secondary to changes in the liver that lead to a sustained increase in CCK levels.

d. Data Gaps

There are several data gaps that need to be explored. First, PPARα agonists in addition to WY 14, 643 that induce PACTs need to be evaluated for their ability to induce cholestasis, decrease bile acid flow, and/or alter bile acid composition. These data could be collected in short-term experiments, probably 1 month in duration. Second, these other PPARα agonists that induce PACTs need to be evaluated for their ability to increase CCK levels, which is an essential element for establishing this MOA; however, such experiments would require dosing for at least 3 months, and the absence of a commercially available RIA for CCK makes this a difficult experiment. Third, the amount of mechanistic and comparative information for PPARα agonist induction of PACTs is limited when compared to PPARα agonist induction of liver tumors. The development of a knockout rat followed by an experiment using WY 14,643 (and, then, additional PPARα agonists) would be the most compelling experiment to test whether the PPARα receptor mediates the induction of PACTs in rats.

e. Supportive Data Elements for Evaluation of Proposed Mode(s) of Action for Pancreatic Acinar-Cell Tumors

Section II describes the type of data needed to establish that a compound induces hepatic tumors via PPARα receptor activation. As a first step, one must show that the compound does not directly interact with DNA, exhibiting mutagenic or genotoxic characteristics. Second, one could demonstrate that a compound is a PPARα agonist through the use of in vitro transactivation assays. The following describes data needed to demonstrate that PACTs are due to a compound being a PPARα agonist. In addition to inducing the key events in the liver related to its hepatocarcinogenic potential, the compound should be shown to induce cholestasis (e.g., increase in total serum bile acids, alkaline phosphatase, and bilirubin). These endpoints are relatively simple additions to the standard clinical pathology battery. Alternatively, measurement of bile flow via bile-duct cannulation and measurement of bile acid composition from the collected bile require additional skill not normally available at most industrial laboratories (Obourn et al., 1997a). Measurement of plasma CCK levels would be ideal but is more labor intensive than measurement of bile acid flow. The CCK RIA assay is not commercially available and would require collaboration with a laboratory that routinely measures CCK. Alternatively, the CCK RIA can be developed in-house if one can locate a laboratory willing to provide reagents. Obourn and coworkers (1997c) provide detailed methods for the CCK RIA. In addition, the study duration would have to be at least 3 to 6 months to detect an increase in CCK in rats.

f. Conclusions

A mode of action (MOA) has been proposed to describe the etiology of PACTs in PPARα agonist-treated rats. As noted in Table 29, the weight of evidence based upon evaluation of the available data to support the postulated MOA is weak. This

judgment on the weight of evidence was based primarily on the fact that the majority of the work was conducted by a single laboratory with only two compounds. Hence, confirmation is required by additional laboratories with other compounds. *Therefore, one must conclude, at this time, that the answer to Question 1 in the ILSI RSI human relevance framework ("Is the weight of evidence sufficient to establish the mode of action for pancreatic acinar-cell tumors in animals?") is "No."* As noted earlier, the most expedient way to resolve the remaining questions is the development of a PPARα knockout rat and then testing a potent agonist such as WY 14,643 as well as additional compounds of varying (lesser) potency. In summary, the evidence to support the proposed MOA for the pancreatic acinar tumor is the least well characterized of the three components of the Tumor Triad. Additional studies are needed to assess whether choletasis is the potential initiating event leading to an increase in CCK.

2. Applicability of Animal MOA to Human Health Risk Assessment

a. Overview of Existing Available Human Literature (In Vitro, Epidemiological, and Clinical)

PFOA is the only known PPARα agonist that has been evaluated for pancreatic function in a human epidemiological study. Primate studies conducted with clofibrate (3 months of exposure), DEHP (3 months of exposure), and PFOA (6 months of exposure) examined hepatic and extrahepatic changes (testis, pancreas, testosterone, estradiol, and CCK) and found no effect on these extrahepatic endpoints (Butenhoff et al., 2002; Kurata et al., 1998).

PFOA epidemiological study

In a study of 115 occupationally exposed male PFOA workers, hepatic enzymes were within clinically acceptable ranges and no workers had diagnoses of hepatic disease or signs consistent with hepatic disorders (Gilliland and Mandel, 1996). In a study where male workers employed at a PFOA plant were evaluated in 1993 ($n = 111$), 1995 ($n = 80$), and 1997 ($n = 74$), it was concluded that there was no significant hepatic toxicity detected in the workers exposed to PFOA. In addition to the evaluation of hepatic clinical chemistry parameters, Olsen and coworkers (2000) measured CCK levels in the PFOA workers that were evaluated in 1997, since the PACTs seen in rats have been hypothesized to be due to increased CCK levels (Obourn et al., 1997a). CCK levels were found to be lower in the workers with the highest PFOA exposure. Further, none of the CCK levels were outside the normal limits (Olsen et al., 2000).

b. Conclusions

1) Are the key events in the animal MOA plausible in humans?

As with the LCTs, for the purpose of discussion, and to encourage evolution of the state-of-the-science as expeditously as possible, the analysis will proceed as if the answer to Question 1 were "Yes," given that quite a lot is known about common mechanisms in the etiology of PACTs in general.

The proposed MOA for PPARα agonist-induced PACTs in rats could plausibly occur in humans as similar pathways are present. The critical pathways in this MOA are the PPARα regulation of bile acid synthesis and the CCK regulation of pancreas exocrine secretion. Additionally, human cholecystokinin A and B receptors are differentially expressed in normal pancreas and pancreatic ductal adenocarcinoma, suggesting that these receptors could be upregulated during tumorigenesis, although these findings have been difficult to replicate (Ji et al., 2001; Moonka et al., 1999; Smith et al., 1994; Weinberg et al., 1997). Hence, the key issue becomes the relative sensitivity between rodents and humans regarding these two pathways (Table 30).

2) Taking into account kinetic and dynamic factors, is the animal MOA plausible in humans?

The available data suggest that PPARα agonists induce PACTs by an indirect trypsin-inhibition mechanism where reduced bile flow and/or changes in bile composition produce an increase in CCK levels. A sustained increase in CCK levels appears responsible for the development of PACTs in rats. If this is the case, then the rat PACTs are likely secondary to changes in the liver. Therefore, the critical pathways in this MOA are the PPARα regulation of bile acid synthesis within the liver and the CCK regulation of pancreas exocrine secretion. The key issue becomes the relative sensitivity between rodents and humans in these two pathways. While humans express PPARα and CCK_A receptors, their expression is much lower than in rodents. In

TABLE 30
Hypothetical Comparative Analysis of Rodent and Human Data for Pancreatic Acinar-Cell Tumors

Rat MOA key events for pancreatic acinar cell tumor	Is this key event in the MOA plausible in humans?	Taking into account kinetic and dynamic factors, is this key event in the MOA plausible in humans?
1. Activation of PPARα in liver	Yes	Yes
2a. ↓ Bile acid flow and/or	Yes	Not likely based on absence of liver effects in primates and humans
2b. Altered bile acid composition	Yes	Not likely based on absence of liver effects in primates and humans
3. Cholestasis	Yes	Not likely based on primate data with three PPARα agonists and human data with PFOA
4. ↑ CCK	Yes	Not likely based on primate data with three PPARα agonists and human data with PFOA
5. ↑ Acinar cell tumors	Yes	Not likely because of the physiological differences in the site of CCK action between rodents and primates/humans; consistent with this conclusion, acinar cell tumors are almost exclusively seen in rodents while ductal tumors are seen in humans

addition, humans regulate pancreas exocrine secretion via a neuronal pathway rather than through direct binding of CCK to acinar CCK_A receptors. These points are summarized next and support the conclusion that PPARα agonists are unlikely to induce tumors in humans via the MOA hypothesized for induction of PACTS in rats:

- The regulation of bile acid synthesis by PPARα appears to be similar in rodents and humans and occurs in the livers of both species (Cheema and Agellon, 2000; Post et al., 2001). Humans have a 10-fold lower expression of PPARα in liver than do rodents, which results in an attenuation of the pleiotropic responses induced by PPARα agonists. These data suggest that the ability of PPARα agonists to alter bile acid synthesis in humans would also be attenuated compared to the rodent. Since humans are believed to be refractory to the hepatic effects of PPARα agonists, PPARα agonist-induced pancreatic lesions probably have low relevance to humans. However, even if bile acid synthesis was altered in humans sufficiently to increase CCK levels, the differences in regulation of pancreas exocrine secretion and CCK_A receptor expression suggest that elevated CCK levels would be unlikely to stimulate acinar cell proliferation in humans.
- The location and amount of expression of CCK_A receptors are different across species. Human and monkey CCK_A receptors are expressed prominently in the gallbladder and stomach and have low expression in the brain and pancreas (Holicky et al., 2001). Other studies have confirmed a low expression of CCK_A receptors and a preponderance of expression of CCKB receptors in humans (Gavin et al., 1997; Nishimori et al., 1999). Mice and rats prominently express CCK_A receptors in the pancreas, while CCKB receptors are not expressed (Bourassa et al., 1999; Gavin et al., 1997). Ji and coworkers (2001) demonstrated that human pancreatic acinar cells do not respond to CCK receptor agonists as assessed by the expected functional parameters (i.e., amylase secretion) and this absence of functional response is related to the low CCK_A and CCK_B receptor expression. The lower expression of CCK receptors in the human pancreas would attenuate the trophic response to CCK relative to the rodent.
- Another key difference between the rodent and human is the regulation of exocrine secretion in the pancreas (Ji et al., 2001; Longnecker, 1987). In the mouse and rat, CCK is a potent secretagogue for the exocrine pancreas by

binding to the CCK_A receptors on acinar cells (Bourassa et al., 1999). In rats it appears that two mechanisms are responsible for the CCK-mediated exocrine secretion: (1) an indirect mechanism where CCK binds to CCK_A receptors on afferent neurons on the vagal–vagal loop that increase pancreatic secretion, with the final mediator being acetylcholine acting at m3 muscarinic (acetyl) cholinergic receptors (m3 AChR), which are responsive to carbachol; and (2) a direct mechanism where CCK binds to CCK_A receptors on acinar cells to increase pancreatic secretion (Adler, 1997; Ji et al., 2001; Li et al., 1997; Owyang et al., 1996). In humans, CCK appears to stimulate exocrine pancreatic secretion via a neuronal CCK_A receptor (i.e., indirect mechanism) rather than through binding to an acinar cell receptor (Adler et al., 1995; Go et al. 1993; Ji et al. 2001; Owyang et al., 1996; Soudah et al., 1992). These data illustrate that PPARα agonist-induced increases in CCK levels would be unlikely to produce acinar cell proliferation in humans, as exocrine secretion is regulated only by a neuronal pathway rather than at both the neuronal and acinar cell level as in rodents.

- The cynomolgus monkey CCK_A receptor has a molecular structure (98% homology to the human receptor), hormone binding and signaling, and organ distribution similar to the human receptor; this similarity supports the relevance of the cynomolgus species for preclinical testing of drugs acting on the CCK_A receptor (Holicky et al., 2001). In primate studies, clofibrate (3 months of exposure), DEHP (3 months of exposure), and PFOA (6 months of exposure) did not increase CCK levels or produce histopathological changes in the pancreas (Butenhoff et al., 2002; Kurata et al., 1998). In an 18-month rat carcinogenicity study with WY 14,643, CCK levels were elevated by 3 months of treatment and remained elevated for the remainder of the study (Obourn et al., 1997a, 1997b) demonstrating that the primate studies were of sufficient duration to detect a change in CCK levels. If increased CCK levels mediate the PACTs induced by PPARα agonists in rats, the high homology between the human and primate suggests that humans would be refractory to an increase in CCK levels.
- If PPARα agonists are inducing PACTs via a sustained increase in CCK secondary to liver changes, then epidemiological data from humans consuming trypsin inhibitors would provide a relevant assessment of potential risk to humans since trypsin inhibitors produce greater elevations in CCK than PPARα agonists (5-fold versus <2-fold, respectively). Infants on soymilk formula, vegetarians, and patients using soybean protein diets to reduce cholesterol levels have been cited as populations at greatest risk if the response of the human pancreas is similar to that of the rat (Liener, 1986; McGuinness et al., 1980, 1987). However, human populations with high levels of dietary protease inhibitors have decreased rates of pancreatic cancer rather than increased incidences (Howatson and Carter, 1985; Messina and Messina, 1991; Miller, 1978). It also has been reported that increased levels of CCK have either no effect (Andren-Sandberge et al., 1984; Meijers et al., 1990; Pour et al., 1988) or a protective effect on pancreatic cancer (Johnson et al., 1983; Pour et al., 1988). Therefore, based on the epidemiology data from human populations that consume diets high in protease inhibitors, potential increases in CCK levels would not appear to pose a significant risk to humans for pancreatic acinar-cell tumors.
- Lastly, there is a difference between rodents and humans in the cellular origin of pancreas tumors. Rat pancreatic tumors are predominately acinar cell in origin, while human pancreatic tumors are primarily ductal in origin (Cotran et al., 1989).

Collectively, these data suggest that humans would be refractory to PPARα-induced PACTs. Using the human relevance framework for analysis, an MOA has been postulated for PACTs, although the weight of evidence is weak when compared with the liver. The MOA can plausibly occur in humans as the same pathways exist. However, the key events in the MOA do not appear to be plausible in primates or humans when taking into toxicodynamic responses due to the differences in the feedback pathways for CCK.

Overall conclusions

Using the human relevance framework as the basis for analysis, an MOA has been proposed for PACTs although the weight of evidence is predominantly low or moderate for key events when compared with the robustness of the database available for the liver. At this time, this proposed MOA is the least well characterized of those hypothesized for the three tumor types in the Tumor Triad.

Therefore, it is premature to conclude that the MOA for pancreatic acinar-cell tumors is adequately understood. That determination notwithstanding, and to encourage the development of confirmatory evidence, we have proceeded beyond Question 1 in the human relevance framework as if we did have sufficient data to support this MOA to speculate on possible answers to questions 2 and 3. Given what we do know and what we would expect to find from additional experimentation, we would conclude that the MOA could plausibly occur in humans, as the same pathways that exist in the rat exist in humans (Question 2: Are the key events in the animal MOA(s) plausible in humans?). However, the key events in the MOA would not appear to be plausible in humans when taking into account kinetic and dynamic factors (Question 3: Taking into account kinetic and dynamic factors, are the MOA(s) plausible in human?). Table 30 presents a hypothetical comparative analysis of rodent and human data in an attempt to answer the three framework questions.

D. Case Study—PPARα Agonist-Induced Leydig-Cell, Pancreatic Acinar-Cell, and Hepatic Tumors—The "Tumor Triad"

1. Introduction

In rats, a number of PPARα agonists produce Leydig-cell and/or pancreatic acinar-cell tumors in addition to liver tumors. This phenomenon has been characterized as the "Tumor Triad." PFOA (ammonium perfluorooctanoate) was selected for the case study to illustrate the application of the Human Relevance Framework for an agent producing these three tumors. Additionally, PFOA was selected because it has the most information regarding the potential MOAs for the LCTs and PACTs in rodents and because the human epidemiological and nonhuman primate studies have focused on endpoints that relate to the MOAs proposed for the liver, LCTs and PACTs.

PFOA is a derivative of perfluorocarboxylic acid, which has been used in a wide variety of industrial applications. It is not metabolized and is considered to be non-DNA-reactive. Dietary studies in Sprague-Dawley rats revealed increased incidences of liver, Leydig-cell, and pancreatic acinar-cell tumors in males and a marginal increase in liver and mammary (perhaps not treatment related) tumors in females. The available human occupational monitoring and medical surveillance data do not reveal any adverse impacts upon liver or pancreatic function or reproductive hormone levels.

a. Is the Weight of Evidence Sufficient to Establish the Animal Modes of Action?

Two dietary carcinogenicity studies have been conducted with PFOA in Sprague Dawley rats (Table 31). In the first study, PFOA was administered to male and female rats at 0, 30 (1.5 mg/kg/day) or 300 ppm (15 mg/kg/day). PFOA produced a dose-dependent increase in LCTs and hepatocellular carcinomas in males and hepatocellular carcinomas and mammary-gland fibroadenomas in females, although the latter was interpreted by the investigators as not being treatment related (Cook et al., 1992; Sibinski, 1987). PFOA did not produce PACTs, but did produce a dose-dependent increase in acinar-cell hyperplasia (Dr. S. R. Frame, DuPont Haskell Laboratory, personal communication). In the second carcinogenicity study, PFOA was administered at 0 or 300 ppm (15 mg/kg/day) to male rats only. PFOA again was shown to induce LCTs and hepatocellular adenoma/carcinomas (Biegel et al., 2001). PFOA also increased the incidence of pancreatic acinar-cell adenomas (PACTs). PFOA has been shown to be a PPARα agonist and produces a sustained increase in peroxisome proliferation in the liver (Biegel et al., 2001; Cook et al., 1992; Liu et al., 1996b; Maloney and Waxman, 1999; Pastoor et al., 1987).

Both the ammonium and sodium salts of PFOA have been tested in a battery of tests for genotoxicity. The weight of evidence from these studies supports the conclusion that PFOA is a nongenotoxic, nonmutagenic compound. Consistent with PFOA being a nongenotoxic hepatocarcinogen, initiation–promotion studies have demonstrated that PFOA is a promoter of liver tumors (Abdellatif et al., 1991; Nilsson et al., 1991).

PFOA appears to induce liver tumors via binding to the PPARα, resulting in peroxisome proliferation and increased liver mitogenesis (Biegel et al., 2001; Maloney and Waxman, 1999; Pastoor et al., 1987). The induction of LCTs by PFOA is postulated to be due to a hormonal mechanism whereby PFOA inhibits testosterone biosynthesis and/or increases serum estradiol levels via induction of hepatic aromatase activity (Biegel et al., 1995, 2001; Cook et al., 1992). Both of these mechanisms appear to be mediated by PPARα. The induction of PACTs is postulated to be secondary to the liver changes, specifically, a sustained increase in plasma cholecystokinin (CCK) secondary to reduced bile

TABLE 31
Summary of Carcinogenicity Studies with PFOA and Magnitude of Peroxisome Proliferation

Tumor incidence and peroxisome proliferation				Reference
Dose level:	0	30	300 ppm	Sibinski et al. (1987)
PP[a]:	—	3×	6×	
Males:				
Liver[a]:	3/50 (6%)	1/50 (2%)	5/50 (10%)	
LCT:	0/50 (0%)	3/50 (6%)	7/50 (14%)	
PACT:		Hyperplasia observed		
Females:				
Liver[a]:	0/50 (0%)	0/50 (0%)	3/50 (6%)	
Mammary:	10/50 (20%)	19/50 (38%)	21/50 (42%)	
Dose level:	0	0 (Pair-fed)	300 ppm	Biegel et al. (2001)
PP:	—	—	6×	
Liver:	2/80 (3%)	3/79 (4%)	10/76 (13%)	
LCT:	0/80 (0%)	2/78 (3%)	8/76 (11%)	
PACT:	0/80 (0%)	1/79 (1%)	8/76 (11%)	

[a]The degree of peroxisome proliferation (PP) was estimated retrospectively for the results in the study by Sibinski and coworkers (1987) using data derived from Liu and coworkers (1996b). The PP was expressed as a mean of the multiple measurements from the study by Biegel and workers (2001).

flow and/or altered bile acid composition resulting in an indirect inhibition of trypsin. The indirect inhibition of trypsin results in an increase in CCK levels (Obourn et al., 1997a, 1997b). There are limited data on the PACT mechanism especially as it relates to PFOA, as much of this mechanism has been elucidated using WY 14,643. PFOA is a surfactant, and this property led to interference in the measurement of CCK; hence, the mechanistic studies were conducted with WY 14,643 since it did not interfere with the CCK radioimmunoassay and was more potent than PFOA in inducing PACTs.

The weight of evidence suggests that these three tumor types are mediated by PFOA binding to the PPARα. The confidence in linking the mechanism to PPARα is high for the liver, moderate for the testis, and weak for the pancreas. This linkage is discussed under the key events for these three tumors.

Liver

The MOA for liver tumors resulting from activation of the PPARα is described in detail in Section II. Regarding PFOA, Maloney and Waxman (1999) have demonstrated that PFOA activates PPARα using COS1 cells transfected with a luciferase reporter gene. Maximal transcriptional activity with PFOA was seen at 10 μM and 20 μM in the mouse and human PPARα, respectively, but PFOA was unable to activate either the mouse or human PPARγ. Both PFOA and WY 14,643 were less potent in activating the human than the mouse PPARα (Maloney and Waxman, 1999). PFOA administered to PPARα knockout mice did not increase β-oxidation unlike that seen in wild-type mice (Yang et al., 2002). In this same study, hepatomegaly unexpectedly was seen in the knockout mice; unfortunately, no histopathological examination was performed and no explanation for this response was offered. PFOA is not metabolized in rats and therefore PFOA metabolites with PPARα activity are unlikely (Kuslikis et al., 1992; Vanden Heuvel et al., 1991). Pastoor and coworkers (1987) were the first to report that PFOA produces peroxisome proliferation. Other investigators have shown that PFOA induction of hepatomegaly, peroxisomal β-oxidation, microsomal 1-acylglycero-phosphocholine acyltransferase, and cytosolic long-chain acyl CoA hydrolase can be blocked in castrated male rats treated with estradiol, demonstrating the sex specific responses to PFOA are related to its rapid elimination in female rats (Kawashima et al., 1989). PFOA also has been shown to decrease levels of HMG-CoA reductase, the rate-limiting step in cholesterol biosynthesis, and acyl CoA cholesterol acyltransferase (ACAT), the enzyme responsible for the esterification of cholesterol (Haughom and Spydevold, 1992).

Liu and coworkers (1996b) characterized the PFOA dose-response (doses of 0, 0.2, 2, 20, or 40 mg/ kg/day for 14 days) for several key endpoints that were judged to be the initiating events leading to the liver, pancreas and testis tumors. These endpoints included liver weight, hepatic β-oxidation,

hepatic aromatase (CYP19A1), and hepatic total cytochrome P450. The NOEL for these endpoints was found to be 0.2 mg/kg/day, with significant changes observed at ≥2 mg/kg/day for all endpoints. In the first carcinogenicity study, PFOA was administered at 30 or 300 ppm, which was equivalent to approximately 1.5 and 15 mg/kg/day (Sibinski, 1987). In this carcinogenicity study, the 30 ppm group was either a NOEL or near a NOEL for tumorigenesis, which is in good agreement with these biochemical endpoints. In the second bioassay with PFOA, the temporal associations of hepatic β-oxidation and cell proliferation were compared to the induction of liver tumors (Biegel et al., 2001). Relative liver weights, hepatic β-oxidation and hepatic cell proliferation were evaluated at 1, 3, 6, 9, 12, 15, 18, 21, and 24 months. Relative liver weights and hepatic β-oxidation were increased at all time points. Hepatic cell proliferation was numerically increased relative to the pair-fed control at 9, 15, 18, and 21 months. It would have been ideal to have characterized the hepatic cell proliferation response at time points less than 1 month since these are generally associated with a maximal response. The liver endpoints (organ weight, β-oxidation and cell proliferation) were all elevated well before the first occurrence of liver tumors, which occurred after 12 months of treatment. WY 14,643 also was evaluated in this study and produced either numerical or statistical increases in hepatic cell proliferation at all time points. Consistent with cell proliferation playing a key role in liver tumorigenesis, WY 14,643 produced a twofold greater incidence in liver tumors than PFOA (Biegel et al., 2001). These data are consistent with the findings of Marsman and coworkers (1988), who demonstrated a similar correlation between cell proliferation and liver tumors for DEHP and WY 14,643.

LCTs

Two endocrine-based MOAs may be responsible for the induction of LCTs by PFOA (Biegel et al., 1995, 2001; Cook et al., 1992). These mechanisms also appear to be applicable for other peroxisome proliferators as well, based on in vitro studies using isolated LCs (Gazouli et al., 2002; Liu et al., 1996a). PFOA (as well as WY 14,643) did not induce peroxisomes in LCs when evaluated throughout a 2-year bioassay (Biegel et al., 2001).

Administration of PFOA to adult male rats by gavage for 14 days was shown to decrease testosterone levels and increase serum estradiol levels (Cook et al., 1992). The increase in estradiol occurred at the same dose levels as the increase in β-oxidation, suggesting that it may be mediated by PPARα (Cook et al., 1992; Liu et al., 1996b). These endocrine changes were hypothesized to play a role in the PFOA induction of LCTs. Subsequently, PFOA has been shown to directly inhibit testosterone production when incubated with isolated LCs, and ex vivo studies demonstrated that this inhibition was reversible (Biegel et al., 1995). Thirteen out of 13 PPARα agonists have been shown to inhibit testosterone production using isolated LCs (Gazouli et al., 2002; Liu et al., 1996a). This inhibition of testosterone biosynthesis appears to be mediated by PPARα (Gazouli et al., 2002) and may contribute to the development of LCT through disruption of the HPT axis. However, in a mechanistic bioassay with PFOA and WY 14,643, serum testosterone and LH levels were not significantly altered at the levels of PFOA and WY 14,643 that were tested (Biegel et al., 2001). This discrepancy may be due to the increase in serum estradiol levels attenuating an increase in LH. Alternatively, high dose levels of these compounds may be needed to detect these hormonal changes, as mechanistic studies for other compounds that induce LCTs have generally used higher dose levels than are used in the traditional bioassay (Cook et al., 1999).

PFOA and WY 14,643 also have been shown to produce a sustained increase in serum estradiol levels over 2 years, and these increases correlate with their potency to induce LCTs in rats (Biegel et al., 2001). Other PPARα agonists (e.g., clofibrate, DBP, DEHP, gemfibrozil) have been shown to increase serum estradiol levels in adult male rats (Eagon et al., 1994; Rao et al., 1994). Eight out of 11 PPARα agonists have been shown to increase estradiol production using isolated LCs (Liu et al., 1996a). Collectively, these data suggest that many PPARα agonists can increase estradiol levels possibly via induction of aromatase. PPREs also have been identified in aromatase from zebrafish (Kazeto et al. 2002) suggesting that an increase in aromatase in mammalian species could be mediated directly by PPARα. It has been proposed that a non-LH type mechanism for the induction of LCTs may be responsible where estradiol modulates growth factor expression within the testis to produce LC hyperplasia and neoplasia (Biegel et al., 1995; Cook et al., 1992, 1999). Several components of the estradiol hypothesis have been tested and are summarized as follows: (1) Peroxisome proliferators induce hepatic aromatase activity (Biegel et al., 1995; Liu et al., 1996b); (2) hepatic aromatase induction increases

serum estradiol levels (Biegel et al., 1995, 2001; Cook et al., 1992; Liu et al., 1996b), which increases testis estradiol levels (Biegel et al., 1995); (3) increased interstitial fluid estradiol levels modulate growth factors, specifically TGFα, within the testis (Biegel et al., 1995); and (4) altered growth factors stimulate Leydig-cell proliferation (Cook et al., 1999). TGFα has been shown to stimulate thymidine incorporation into LC precursors and therefore appears to be able to induce LC mitogenesis (Cook et al., 1999; Khan et al., 1992). There is precedent for TGFα playing a role in tumorigenesis. For instance, in the mammary gland, estradiol has been shown to stimulate TGFα secretion and overexpression of TGFα has been associated with the proliferation of mammary epithelial cells and subsequent neoplasia (Liu et al., 1987).

Estrogenic compounds have been shown to induce LCTs in mice but not rats due to the differential responses between mouse and rat LCs (Cook et al., 1999). Estrogenic compounds do not induce LCTs in rats when given at doses which produce testicular atrophy, which can confound detection of LC hyperplasia (Gibson et al., 1967; Marselos and Tomatis, 1993; Schardein, 1980; Schardein et al., 1970). Interestingly, GnRH agonists induce LCTs at low doses, but do not induce LCTs at higher doses where LH levels are suppressed and testicular atrophy occurs (Donaubauer et al., 1987; PDR 1995a, 1995c, 1995e). Hence, these negative bioassays with estrogenic compounds may be due to suppression of LH, which to date is the primary mechanism by which LCT are produced in rats (Cook et al., 1999). Estradiol does appear to play a role in enhancement of LC tumorigenesis based on data from aging studies. In F344 rats, which have a high spontaneous incidence of LCTs, there is an age-related increase in serum estradiol that correlates with the development of LC hyperplasia and tumor formation (Turek and Desjardins, 1979). However, in the CD rat, which has a low spontaneous incidence of LCTs, serum estradiol decreases with age (Biegel et al., 2001). In a 2-year rat (Sprague-Dawley) mechanistic bioassay, PFOA and WY 14,643 produced a sustained increase in serum estradiol levels that correlated with the potency of the two compounds to induce LCTs (Biegel et al., 2001). These studies suggest that estradiol may play a role in enhancement of LCTs in the rat, and that peroxisome proliferators may induce LCTs via a non-LH type mechanism as well as by inhibition of testosterone biosynthesis. Whether estradiol plays a role in the induction of LCTs by PPARα could be determined by conducting an estradiol bioassay at dose levels that do not induce testicular atrophy or reduce LH levels.

In summary, the existing data demonstrate that the induction of LCTs by PFOA may be attributed to a hormonal mechanism whereby PFOA either inhibits testosterone biosynthesis and/or increases serum estradiol levels via induction of hepatic aromatase activity. Both of these mechanisms appear to be mediated by PPARα.

PACTs

PFOA has been shown to induce PACTs (Biegel et al., 2001). Several other peroxisome proliferators also induce PACTs: butylbenzyl phthalate (BBP) (NTP, 1995), cinnamyl anthranilate (NTP, 1980), clofibrate (Svoboda and Azarnoff, 1979), DEHP (David et al., 1999, 2000), gemfibrozil (Fitzgerald et al., 1981); fenofibrate (PDR, 2002), HCFC-123 (Malley et al., 1995), methylclofenapate (Tucker and Orton, 1995), nafenopin (Reddy and Rao, 1977a, 1977b), tibric acid (Faccini et al., 1979), and WY 14,643 (Biegel et al., 2001). Hence, a subset of peroxisome proliferators induces PACTs. Ohmura and coworkers (1997) demonstrated that the PPARα agonist BR931 induced DNA synthesis in pancreatic acinar cells, but not in ductal or islet cells. The development of pancreatic acinar-cell hypertrophy, hyperplasia, and adenomas in the rat has been shown to be modified by several factors such as steroid hormones (testosterone and estradiol), growth factors such as cholecystokinin (CCK), growth factor receptor overexpression (CCK_A receptor), and diet (fat) (Bell et al., 1992; Longnecker, 1983, 1987; Longnecker and Sumi, 1990; Povoski et al., 1993). These potential mechanisms have been investigated in a series of in vitro and in vivo (subacute, subchronic and carcinogenicity) studies using PFOA and WY 14,643 (Obourn et al., 1997a, 1997b). WY 14,643 was used in the in vivo studies to investigate potential mechanisms rather than PFOA as WY 14,643 produced a threefold greater incidence of PACTs than PFOA. In addition, PFOA interfered with the measurement of CCK, presumably due to its surfactant properties (unpublished data).

PFOA and WY 14,643 were shown not to inhibit trypsin or bind to the CCK_A receptor in vitro (Obourn et al., 1997a). In the 18-month carcinogenicity study with WY 14,643, the observed decreased relative bile flow and bile acid concentration may be responsible for the consistent, albeit modest, increases in plasma CCK levels throughout the study. Consistent with this possible mechanism, fibrates have been shown to suppress bile acid synthesis in the liver via PPARα-mediated downregulation of cholesterol 7α-hydroxylase and sterol

27-hydroxylase gene expression (Post et al., 1999). Subsequently, it has been shown that PPARα/RXRα reduces HNF-4 binding to the DR-1 sequence, which decreases the transcription of cholesterol 7α-hydroxylase (CYP7A1), the rate-limiting step of bile acid synthesis (Marrapodi and Chiange, 2000). This increased output of CCK correlates with the development of pancreatic acinar-cell hyperplasia and tumors. Hence, WY 14,643 may be acting via an indirect trypsin-inhibition mechanism where reduced bile flow and/or changes in bile composition are responsible for increased CCK levels. If this is the case, then the rat PACTs appear to be secondary to changes in the liver. In summary, the mechanisms by which PFOA may induce PACTs are based primarily on data with WY 14,643. This generalization appears reasonable as several other peroxisome proliferators also induce PACTs, which suggests a common mechanism across this class of chemicals. It is noted that this mechanism has the least amount of data relative to the data supporting the liver and Leydig-cell MOAs.

2. Statement of Confidence: Supporting and Limiting Factors

The biological plausibility of any postulated mode of action in animals depends in part on additional considerations such as dose-response and temporal relationships, uncertainties, and the likelihood of alternative modes of action. Based on the preceding information, the dose response for the critical initiating events for the induction of the liver, pancreas, and testis was assessed. PFOA is postulated to induce liver tumors via binding to the PPARα receptor (Maloney and Waxman, 1999). Similarly, the induction of PACTs is postulated to be secondary to the liver changes (i.e., reduce bile acid synthesis). Hence, the most sensitive and reliable marker for liver changes would be hepatic β-oxidation. The induction of LCTs by PFOA is postulated to be due to a hormonal mechanism whereby PFOA inhibits testosterone biosynthesis and/or increases serum estradiol levels via induction of hepatic aromatase activity, both of which are likely to be mediated by PPARα. From the mechanistic bioassay (Biegel et al., 2001), the most sensitive endpoint to address the LCTs would be hepatic aromatase. Liu and coworkers (1996a, 1996b) characterized the PFOA dose-response (doses of 0, 0.2, 2, 20, or 40 mg/kg/day for 14 days) for several key endpoints that were judged to be the initiating events leading to the liver, pancreas and testis tumors. These endpoints included liver weight, hepatic β-oxidation, hepatic aromatase (CYP 19A1) and hepatic total cytochrome P-450. Estradiol levels were increased only at levels which increased hepatic β-oxidation, consistent with the hypothesis that the increase in estradiol secondary to aromatase induction is regulated by PPARα. The NOEL for these endpoints was found to be 0.2 mg/kg/day, with significant changes observed at $\geq$2 mg/kg/day for all the endpoints. In the first carcinogenicity study, PFOA was administered at 30 and 300 ppm, which was equivalent to approximately 1.5 and 15 mg/kg/day (Sibinski, 1987). In this carcinogenicity study, the 30-ppm group was either a NOEL or near a NOEL for tumorigenesis, which is in good agreement with these endpoints.

Regarding the temporal associations, the critical endpoints have been evaluated in two mechanistic bioassays (Biegel et al., 2001; Obourn et al., 1997a, 1997b). For the liver tumors, relative liver weights, hepatic β-oxidation and hepatic cell proliferation were evaluated at 1, 3, 6, 9, 12, 15, 18, 21, and 24 months. Relative liver weights and hepatic β-oxidation were increased at all time points. Hepatic cell proliferation was numerically increased relative to the pair-fed control at 9, 15, 18, and 21 months. It would have been ideal if earlier time points had been measured for this endpoint since more dramatic hepatic effects are likely to have occurred then. However, the focus of this study was the characterization of Leydig-cell proliferation. The liver endpoints were all elevated well before the first occurrence of liver tumors. The induction of LCTs by PFOA is postulated to be due to a hormonal mechanism whereby PFOA inhibits testosterone biosynthesis and/or increases serum estradiol levels via induction of hepatic aromatase activity. In the mechanistic bioassay, PFOA produced either a numerical or a statistical increase in serum estradiol levels at all time points. This hormonal change also occurred well before the induction of the LCTs. The induction of the PACTs is postulated to be secondary to the liver changes; specifically, reduced bile flow and/or composition result in an indirect inhibition of trypsin that produces a sustained increase in plasma CCK. These secondary liver changes correlated with liver enzyme induction. In a mechanistic bioassay with WY 14,643, liver and pancreas weights, hepatic β-oxidation, liver and pancreas cell proliferation, cholestasis markers, bile flow, bile acid concentrations and CCK were evaluated at 3, 6, 8, 10, 12, 15, and 18 months (Obourn et al., 1997a, 1997b). Cholestasis markers were elevated at all time points of the carcinogenicity study and in a subchronic study were elevated at 1 and 2 months (Obourn et al.,

1997a). Plasma CCK levels were statistically elevated at 3, 6, 8, 10, 15, and 18 months. Hence, WY 14,643 appears to increase CCK levels well before the occurrence of the first PACTs tumors, consistent with this endpoint being temporally related to the development of these tumors.

In summary, existing data demonstrate the requisite dose response and temporal associations for the induction of these tumors by PFOA. In addition, a body of literature supports the biological plausibility of these tumors by the proposed modes of action. Similarly, the coherence of the database when comparing the original bioassay data with the two mechanistic bioassays is consistent with the proposed mechanisms. The weight of evidence for the three tumor types is judged to be high for the liver, moderate for the testis, and weak for the pancreas. The weight of evidence was judged to be moderate for the testis as the data are not as robust as for the liver when one compares across other PPARα agonists. The weight of evidence was judged to be weak for the pancreas as this mechanism has only been studied in depth with WY 14,643 and the studies have been conducted in only one laboratory. Regarding the PACTs, the uncertainties lie in the use of the WY 14,643 data to provide mechanistic insights for PFOA-induced PACTs. However, PPARα agonists have been shown to alter clinical pathology data in a manner that is consistent with the presence of cholestasis: gemfibrozil (bile acids) (NTP, 1996), HCFC-123 (alkaline phosphatase) (Dr. L. A. Malley, DuPont Haskell Laboratories, personal communication), methylclofenapate (alkaline phosphatase) (Tucker and Orton, 1995), and tibric acid (alkaline phosphatase, bilirubin) (Faccini et al., 1979). Confirmatory evidence in support of the proposed mode of action for the pancreatic tumor would come from the observation that other PPARα agonists that induce PACTs also inhibit bile acid synthesis and induce cholestasis. Until additional supportive data are generated, one cannot exclude a direct PPARα effect on the pancreatic acinar cells. Since the PPARα agonists produce LCTs and PACTs in the rat but not the mouse, the absolute proof that PPARα is responsible for the induction of tumors would require a PPARα knockout rat. It is unfortunate that this technology is not readily available at this time.

c. Are the Key Events in the Animal Modes of Action Plausible in Humans?

As discussed earlier, the ability of PFOA to induce liver tumors could occur due to the presence of PPARα in human liver. However, when taking into account kinetic ansd dynamic factors, the MOA for PPARα agonist-induced (PFOA, included) liver tumors is not likely to occur in humans; therefore, liver tumors induced via this MOA are not likely to occur.

Regarding the testis, the proposed animal MOAs, that is, induction of aromatase secondary to liver induction and the direct inhibition of testosterone biosynthesis, are plausible mechanisms and could occur in humans. If PPARα is mediating the induction of aromatase, this mechanism could occur in humans due to the expression of PPARα in human liver. The inhibition of testosterone biosynthesis by PPARα agonists is better established than the induction of aromatase and also is plausible as PPARα is present in human Leydig cells. The pathways for the regulation of the HPT axis of rats and humans also are similar, where decreases in testosterone levels will increase LH levels (De Groot et al., 1995). Hence, compounds that induce LCTs in rats by disruption of the HPT axis could pose a potential risk to human health.

The proposed mode of action for PPARα agonist-induced PACTs in rats could plausibly occur in humans as similar pathways are present. The critical pathways in this mode of action are the PPARα regulation of bile acid synthesis and the CCK regulation of pancreas exocrine secretion. Additionally, human cholecystokinin A and B receptors are differentially expressed in normal pancreas and in pancreatic ductal adenocarcinoma, suggesting that these receptors could be upregulated during tumorigenesis, although these findings have been difficult to replicate (Ji et al., 2001; Moonka et al., 1999; Smith et al., 1994; Weinberg et al., 1997).

Therefore, the central issue for the ability of PFOA to induce this tumor triad becomes the relative sensitivity between rat and human in their response to PPARα agonists.

d. Taking into Account Kinetic and Dynamic Factors, Is the Animal Mode of Action Plausible in Humans?

There are marked differences in PPARα levels in human liver compared to rodents which may account for the refactoriness of humans to PPARα agonists. However, receptor concentration alone does not appear to be wholly responsible for the species differences, as HepG2 cells, which overexpress hPPARα, failed to respond to WY 14,643 (Lawrence et al., 2001). These data suggest that coactivators/co-repressors may also be responsible for the marked species differences. These differences in

TABLE 32
Comparative Analysis of Rodent and Primate/Human Data on Liver Tumors Induced by PFOA

Key events	Evidence in rodents	Evidence in humans and/or primates
Activation of PPARα	Concentration-related activation in vitro	hPPARα can be activated by PFOA but is less responsive than rodent PPARα; human receptor concentration is ~10 times lower in human liver than rodent liver
PPARα-mediated regulation of peroxisomal genes	No data	No data with PFOA; recent data with three fibrates demonstrates that lipid lowering occurs without activation of peroxisomal genes
PPARα-mediated regulation of genes of proliferation and apoptosis	No data	No data
PPARα-mediated regulation of fatty acid metabolism genes	No direct measurement of genes but triglycerides were decreased by PFOA. Several key enzymes of cholesterol synthesis are inhibited by PFOA	No data
Peroxisome proliferation	Dose-related increases in CYP and peroxisomal enzymes	Minimal increase in peroxisomal enzyme activity in a 6-month primate study in cynomolgus monkeys
Perturbation of cell proliferation and/or apoptosis	Bursts of cell replication in vivo	No evidence of cell replication in a 6-month primate study in cynomolgus monkeys
Inhibition of GJIC	No data	No data
Hepatocyte oxidative stress	No data	No data
Kupffer cell mediated events	No data	No data
Selective clonal expansion	PFOA shown to increase liver tumor incidence in two carcinogenicity studies	Occupational monitoring has not detected an increase in cancer risk; no increase in hepatocyte labeling index in a 6-month study in cynomolgus monkeys

receptor number and/or co-activators/co-repressors may account in part for either the attenuated or absent PFOA signal in primates or humans. The data summarized in Tables 32, 33, and 34 suggest that PFOA would be unlikely to induce the tumor triad in humans based primarily on toxicodynamic considerations. This conclusion is based on the PFOA primate data, PFOA epidemiological data that assessed the critical endpoints for each target organ, and the species differences in the physiological control of these pathways. In addition, Maloney and Waxman (1999) demonstrated that PFOA activated the mouse PPARα to a greater extent than the human PPARα, suggesting the humans would be less responsive to PFOA than rodents.

Toxicokinetics

PFOA is not metabolized in rats (Kuslikis et al., 1992; Vanden Heuvel et al., 1991). Sex-related differences in the pharmacokinetics of PFOA are seen in rats but not in mice, monkeys or dogs (Griffith and Long, 1980; Hanhijarvi et al., 1988). Male rats are more sensitive to the toxic effects of PFOA than female rats, presumably due to their slower excretion of PFOA (Griffith and Long, 1980; Hanhijarvi et al., 1982, 1987). The half-life of PFOA in female rats has been estimated to be 10 h (Ophaug and Singer, 1980; Hanhijarvi et al., 1987), while the half-life in male rats has been estimated to be 5–7 days (Kennedy et al., 1986). Humans also

TABLE 33
Comparative Analysis of Rodent and Primate/Human Data on Leydig-Cell Tumors Induced by PFOA

Key events	Evidence in rodents	Evidence in humans and/or primates
1. Activation of PPARα	Liver: PFOA activates the rodent PPARα more than the human PPARα	Liver: PFOA activates human PPARα less than the rodent PPARα
	Leydig cell: ciprofibrate increased mRNA levels and peroxisomal β-oxidation and catalase genes	Leydig cell: PPARα present but no data on activation
Pathway 1		
2a. ↑CYP 19A1 (Aromatase) and/or	Seen with PFOA in vivo and in vitro	Normal levels of hepatic enzymes, no hepatic disease in occupationally exposed males
2b. ↓ Estradiol metabolism	No PFOA data, but seen with clofibrate, DEHP, DBP, gemfibrozil, WY 14,643	No data
3. ↑ Serum estradiol levels	Shown in vivo with PFOA, clofibrate, DEHP, WY 14,643; in vitro with 8/11 PPARα agonists	No significant association between PFOA serum levels and increased estradiol levels in occupationally exposed males; no changes seen in 6-month study in cynomolgus monkeys
4. ↑ Interstitial fluid estradiol levels	Shown with PFOA (in vivo)	No data
5. ↑ TGFα levels	Shown with PFOA (in vivo)	No data
6. ↑ LC proliferation & LCTs	Increased tumor incidence observed in two long-term studies	Occupational monitoring has not detected an increase in cancer risk; no increase in labeling index for Leydig cells in a 6-month study in cynomolgus monkeys
Pathway 2		
7. ↓ Testosterone (T) biosynthesis either by ↓ PBR protein levels and/or $C_{17,20}$-lysase activity	↓ PBR protein levels has been shown with bezafibrate, MEHP and WY 14,643; ↓ $C_{17,20}$-lysase activity has been stablished with PFOA (in vitro, in vivo)	No data
8. ↓ Testosterone levels	Established with PFOA (in vitro and in vivo); observed with 13 out of 13 PPARα agonists in vitro	No significant association between PFOA serum levels and decreased testoserone levels in occupationally exposed males; no changes seen in 6-month study in cynomolgus monkeys
9. ↑ LH	No sustained increase in LH with PFOA throughout 2-year bioassay	No significant association between PFOA serum levels and increased LH levels in occupationally exposed males
10. ↑ LCTs	Increased tumor incidence observed in two long-term studies	Occupational monitoring has not detected an increase in cancer risk; no increase in labeling index for Leydig cells in a 6-month study in cynomolgus monkeys

TABLE 34
Comparative Analysis of Rodent and Primate/Human Data on Pancreatic Acinar-Cell Tumors Induced by PFOA

Key events	Evidence in rodents	Evidence in humans and/or primates
1. Activation of PPARα in liver	PFOA activates the rodent PPARα more than the human PPARα	PFOA activates human PPARα less than the rodent PPARα; no changes in liver enzymes or increase in liver disorders/disease in occupationally exposed males
2a. ↓ Bile acid flow and/or	No data[a]	No data
2b. Altered bile acid composition	No data[a]	No data
3. Cholestasis	No data[a]	No evidence of cholestasis in occupationally exposed males; no changes seen in 6-month study in cynomolgus monkeys
4. ↑ CCK	No data[a]	Negative association between PFOA exposure and CCK levels in occupationally exposed males; no changes seen in 6-month study in cynomolgus monkeys
5. ↑ Acinar cell tumors	Increased incidence seen in one long-term study	Occupational monitoring has not detected an increase in cancer risk; no increase in labeling index for acinar cells in a 6-month study in cynomolgus monkeys

[a]No data with PFOA; WY 14,643 data provide the basis for the proposed MOA.

excrete PFOA very slowly. In a man exposed to PFOA, the half-life was estimated to be greater than 18 months (Ubel et al., 1980). The rapid excretion of PFOA by female rats appears to be due to active renal tubular secretion (organic acid transport system), which can be inhibited by probenecid (Hanhijarvi et al., 1982). This renal tubular secretion is believed to be hormonally-controlled, since castrated male rats treated with estradiol have excretion rates of PFOA similar to those of female rats (Ylinen et al., 1989). Consistent with previous reports, Kudo and coworkers (2002) have shown that the slower clearance of PFOA in male rats is due to their lower expression of organic anion transporter 2 (OAT2). In similar experiments, other investigators have shown that PFOA induction of hepatomegaly, peroxisomal β-oxidation, microsomal 1-acylglycero-phosphocholine acyltransferase, and cytosolic long-chain acyl coenzyme A (CoA) hydrolase can be blocked in castrated male rats treated with estradiol (Kawashima et al., 1989), presumably due to the enhanced excretion of PFOA (Ylinen et al., 1989). Kawashima and coworkers (1989) have estimated that male rats are 32–64 times more sensitive to PFOA than female rats based on the induction of these four parameters.

Nonhuman primate data with PFOA

A 6-month toxicity study with PFOA was conducted in male cynomolgus monkeys. PFOA was administered by capsule at 0, 3, 10, or 30 mg/kg/day (Butenhoff et al., 2002). In addition, the 10-mg/kg/day PFOA group also had a subset that was used to assess reversal of any changes after 3 months of no treatment. The primary goals of this study were to assess the general toxicity of PFOA in a primate species as well as to assess potential hormonal changes (testosterone, estradiol, and CCK) that have been associated with the development of LCT and PACT in the male rat (Biegel et al., 2001). The 30-mg/kg/day group subsequently was reduced to 20 mg/kg/day due to hepatotoxicity. Hepatotoxicity, as assessed by clinical pathology changes, was observed only at the high dose. Dose-dependent increases in liver weight were seen in all treatment groups, which correlated with hepatocellular

hypertrophy. The hypertrophy was attributed primarily to mitochondrial proliferation, since peroxisome proliferation, which was assessed by β-oxidation, was elevated less than twofold. There were no other macroscopic and microscopic changes observed other than in the liver, nor were there any other changes noted in the clinical chemistry (i.e., no evidence of cholestasis), urinalysis or hematology. There were no apparent compound-related changes in CCK, estradiol, or testosterone levels, which is consistent with the hypothesis that these extrahepatic tumors are mediated by PPARα (Biegel et al., 2001; Obourn et al., 1997a, 1997b). The elimination half-life of PFOA in male and female cynomolgus monkeys was approximately 1 month (Butenhoff et al., in preparation). The PFOA primate results are similar to the findings with three other peroxisome proliferators assessed in the primate (i.e., clofibrate, DEHP and DINP), none of which demonstrated effects on the primate liver, pancreas or testis (Kurata et al., 1998; Pugh et al., 2000).

Human data with PFOA

A series of PFOA epidemiological studies have been conducted. These studies focused on occupational monitoring incorporating endpoints identified from toxicology studies to assess whether processes similar to those observed in rats may be occurring in humans (Biegel et al., 2001). For instance, Olsen and coworkers (1998) conducted medical surveillance, measured selected hormone levels and assessed exposure by measuring serum PFOA levels from workers in a PFOA product plant. PFOA levels from the blood of workers ranged from non-detectable to approximately 100 ppm. The human levels were comparable to those measured in some animal studies, but were several orders of magnitude higher than concentrations typically measured in the blood of nonoccupationally exposed individuals (average around 0.006 ppm; Hansen et al., 2001).

In a study of 115 occupationally exposed male PFOA workers, hepatic enzymes were within clinically acceptable ranges, and no workers had diagnoses of hepatic disease or signs consistent with hepatic disorders (Gilliland and Mandel, 1996). In a study where male workers employed at a PFOA plant were evaluated in 1993 ($n = 111$), 1995 ($n = 80$), and 1997 ($n = 74$), it was concluded that there were no significant hepatic toxicity detected in the workers exposed to PFOA. In addition to the evaluation of hepatic clinical chemistry parameters, Olsen and coworkers (2000) measured CCK levels in the PFOA workers that were evaluated in 1997 since the PACTs seen in rats have been hypothesized to be due to increased CCK levels (Obourn et al., 1997a, 1997b; Biegel et al., 2001). CCK levels were found to be lower in the workers with the highest PFOA exposure. Further, none of the CCK levels were outside the upper limit of normal. In 1998, the results from two cross-sectional studies were reported with male production workers employed at a PFOA production plant (Olsen et al., 1998). The first study, conducted in 1993, evaluated 111 workers, while the second study, conducted in 1995, evaluated 80 workers (68 workers participated in both years). Olsen and coworkers (1998) found no significant associations between plasma PFOA levels and the levels of reproductive hormones in employees from the PFOA production facility. Specifically, there was no significant association between PFOA exposure and estradiol or testosterone (free or bound) levels.

Thus, PFOA exposure in humans does not appear to be associated with the marked changes seen in the liver, testis, and pancreas of rats. Moreover, as blood PFOA levels increased, CCK levels decreased (Olsen et al., 2000). It is noted that the exposure to PFOA in the workplace is lower than the exposure to rats. However, the PFOA primate study was conducted at an MTD and did not alter the key endpoints identified in rat studies for the liver, testis, or pancreas, consistent with the conclusion that these events are unlikely to occur in humans.

Epidemiological studies with other peroxisome proliferators

Human epidemiological studies are available on a limited number of compounds that induce peroxisomes in rodents. These compounds include clofibrate (Oliver et al., 1978) and gemfibrozil (Frick et al., 1987). However, the epidemiological studies on clofibrate and gemfibrozil are of limited usefulness for the evaluation of carcinogenic potential because of their relatively short exposure duration and/or limited follow-up period; clearly reevaluation of individuals who have used these two drugs would be valuable.

Liver

Medical surveillance of workers exposed to PFOA has not demonstrated any evidence of liver toxicity nor has there been reports of an increased

risk of cancer (Gilliland and Mandel, 1996; Olsen et al., 1998, 2000). These findings are consistent with the PFOA primate data where there was no evidence of hepatic toxicity and minimal increases in hepatic β-oxidation (<2-fold) (Butenhoff et al., 2002).

LCTs

As described earlier, PPARα agonists are postulated to induce LCTs either by inhibiting testosterone biosynthesis and/or by inducing aromatase and thereby increasing estradiol levels, both of which appear to be mediated by PPARα. The rat and human hypothalamic–pituitary–testicular axis responds to inhibition of testosterone with a subsequent increase in LH. Hence, if PFOA inhibited testosterone biosynthesis in humans, an increase in LH could occur. However, a 6-month cynomolgus monkey study with PFOA did not demonstrate any compound-related effects on testosterone or estradiol (Butenhoff et al., 2002). These data suggest that PFOA would not induce LCTs in humans because humans are quantitatively less sensitive than rats to LH stimulation (Cook et al., 1999). Such a conclusion regarding the role of LH has been reached by several other investigators as well (Alison, et al., 1994; Bar, 1992; Bosland, 1996; Prentice et al., 1995). In summary, either testosterone inhibition or estradiol induction is unlikely to pose a significant risk to humans. This conclusion is based on the comparative differences between rodents and humans, as discussed earlier. The most compelling is the human disease state FMPP where men have a mutated LH receptor that is activated throughout their life, but LCTs are not seen (Cook et al., 1999).

PACTs

The available data suggest that PPARα agonists induce PACTs by an indirect trypsin-inhibition mechanism where reduced bile flow and/or changes in bile composition produce an increased in CCK levels. A sustained increase in CCK levels appears responsible for the development of PACTs in rats. If this is the case, then the rat PACTs are secondary to changes in the liver. Therefore, the critical pathways in this mode of action are the PPARα regulation of bile acid synthesis within the liver and the CCK regulation of pancreas exocrine secretion. The key issue becomes the relative sensitivity between rodents and humans in these two pathways. While humans express PPARα and CCK_A receptors, their expression is much lower than in rodents. In addition, humans regulate pancreas exocrine secretion via a neuronal pathway rather than direct binding of CCK to acinar CCK_A receptors as in rodents. Consistent with this conclusion, a 6-month cynomolgus monkey study with PFOA did not demonstrate any compound-related effects on CCK levels or clinical pathology evidence of cholestasis nor an increase in labeling index in acinar cells (Butenhoff et al., 2002). Thus, PFOA exposure in humans does not appear to be associated with the marked changes seen in the liver, testis, and pancreas of rats. Moreover, as blood PFOA levels increased, CCK levels decreased (Olsen et al., 2000).

e. Statement of Confidence

Application of the human relevance framework for the analysis of the human relevance of PFOA-induced rat tumors was useful overall. The laboratory data demonstrate that PFOA produced liver, pancreas, and testis tumors in two carcinogenicity studies in Sprague-Dawley rats (Biegel et al., 2001; Sibinski, 1987). PFOA is not metabolized in rats, suggesting that these tumors are not due to a reactive intermediate (Kuslikis et al., 1992; Vanden Heuvel et al., 1991). The weight of evidence from a comprehensive battery of genetic toxicity tests demonstrates that PFOA is not a genotoxic compound. Hence, PFOA appears to be inducing tumors in the liver, pancreas, and testis via nongenotoxic mechanisms that are likely due to the fact that PFOA is a PPARα agonist.

The summarized data suggest that PFOA induces the Tumor Triad because it is a PPARα agonist. This tumor triad includes liver tumors, LCTs (testosterone biosynthesis inhibition and/or increasing serum estradiol levels), and PACTs (inhibition of bile synthesis resulting in increased CCK levels). All of these changes appear to be mediated via PPARα in the liver. There is high confidence in the proposed MOA for PFOA induction of liver tumors in rats and moderate confidence in the proposed MOAs for LCTs because the data set is not as robust as for the liver when one compares across the number of other PPARα agonists. There is low confidence in the proposed MOA for PACTs, as this has been studied only in depth with WY 14,643 and the studies have been conducted in one laboratory. Regarding the PACTs, the uncertainties lie in the use of the WY 14,643 data to provide insights for PFOA-induced PACTs. Confirmatory evidence in support of the proposed MOA for PACTs would come from the observation that other PPARα agonists that induce PACTs also inhibit bile acid

synthesis and induce cholestasis. Since the PPARα agonists have induced LCTs and PACTs only in the rat, the absolute proof that PPARα is responsible for the induction of tumors would require a PPARα knockout rat. It is unfortunate that this technology is not readily available at this time.

Figure 9 summarizes the outcome of the application of the human relevance framework to the information available for PFOA.

f. Implications

PFOA induces liver tumors, LCTs (testosterone biosynthesis inhibition and/or increasing serum estradiol levels), and PACTs (inhibition of bile synthesis resulting in increased CCK levels) in rats. All of these changes appear to be mediated via PPARα. Humans appear to be refractory to PPARα agonists for several reasons including low PPARα receptor

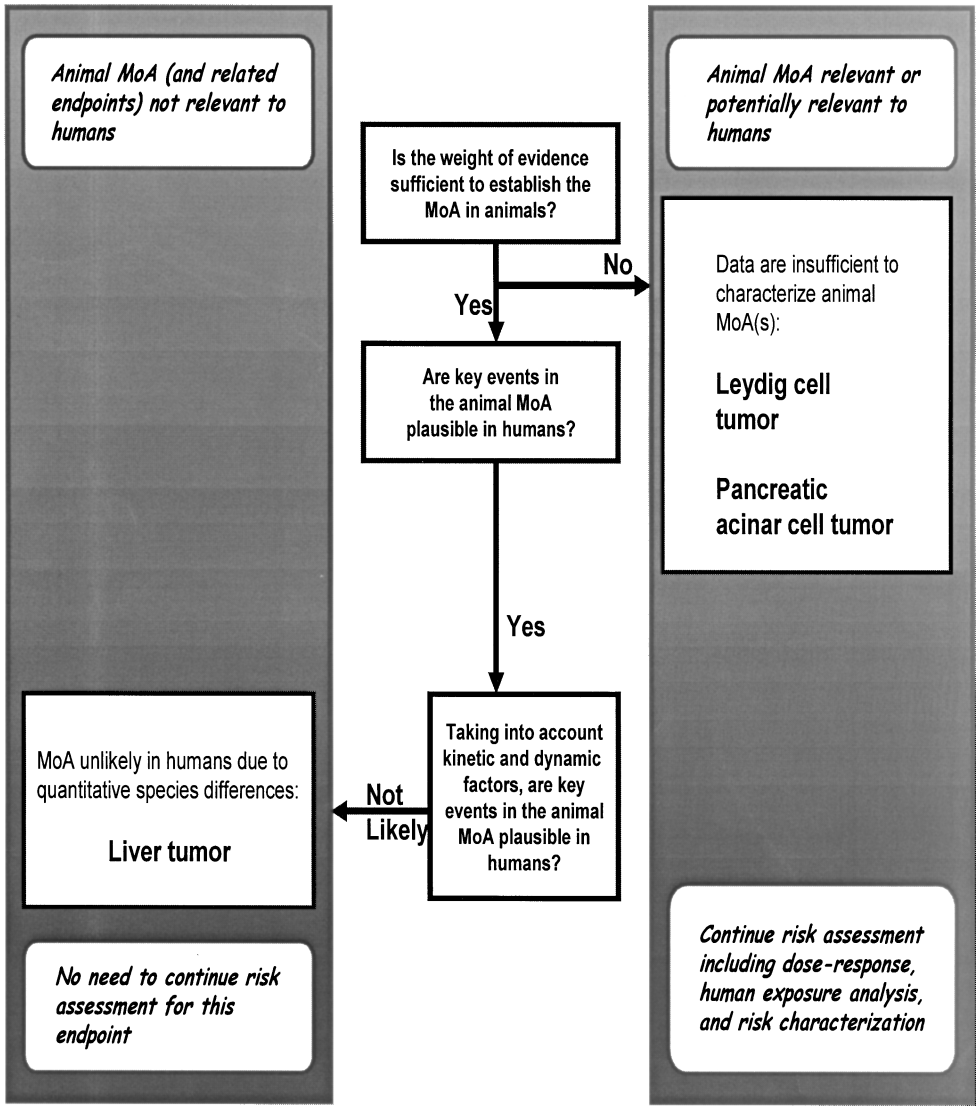

FIGURE 9. Summary of outcomes for all MOAs proposed for the Tumor Triad, analyzed in line with the four-part human relevance framework, using PFOA as the case example. The left side depicts data-based findings that the liver tumors are irrelevant for human risk assessment because their animal MOA is not likely to have a human counterpart. The right side shows that the data for the Leydig-cell and pancreatic-cell tumors are insufficient to confidently characterize the MOA(s) for test animals. Therefore, the animal tumor data are presumed to be relevant to humans and a complete risk assessment is necessary.

TABLE 35
Summary of PPARα Agonists taht Induce Extrahepatic and Hepatic Tumors

Compound–route (strain)	Tumor incidence (tumor percent)						Reference
Cinnamyl anthranilate–diet (F344)	Dose levels:	0	15,000	30,000 ppm			NTP (1980)
	Males:						
	PP:	—	2.5×	2.5×			
	Liver:	1/48 (2%)	4/49 (8%)	4/50 (8%)			
	LCT:	44/47 (94%)	45/50 (90%)	48/50 (96%)			
	PACT:	0/42 (0%)	0/49 (0%)	3/45 (7%)			
	Females:						
	Liver:	1/46 (2%)	2/50 (4%)	0/50 (0%)			
	PACT:	0/50 (0%)	0/50 (0%)	0/50 (0%)			
Clofibrate–NS (Wistar)	Dose levels:	0	200	400 mg/kg			PDR (1995)
	Males:						
	PP:	—	5×	>5×			
	Liver[a]:	NS	+[b]	NS			
	LCT:	NS	NS	+[b]			
Clofibrate–dietary (F344)	Dose levels:	0	0.5%				Svoboda and Azarnoff (1979)
	Males:						
	PP:	—	5×				
	Liver[a]:	0/25 (0%)	4/25 (16%)				
	PACT:	0/25 (0%)	4/25 (16%)				
DEHP–NS (Sprague-Dawley)	Dose levels:	0	30	95	300 mg/kg		
	Males:						
	PP:	—	—	—	5×		
	LCT:	NS	NS	NS	NS		Berger (1995)[c]
DEHP–dietary (F344)	Dose levels:	0	100	500	2500	12,500 ppm	David et al. (1999)
	PP:	—	—	—	4×	7.5×	
	Males:						
	Liver:	5/80 (7%)	5/50 (10%)	4/55 (7%)	11/65 (17%)	34/80 (43%)	
	LCT:	59/64 (92%)	45/50 (90%)	50/55 (91%)	60/65 (92%)	20/64 (31%)	
	PACT:	0/60 (0%)	0/17 (0%)	0/14 (0%)	0/18 (0%)	5/59 (8%)	
	Females:						
	Liver:	0/80 (0%)	4/50 (8%)	1/55 (2%)	3/65 (5%)	22/80 (31%)	
	PACT:	0/60 (0%)	0/7 (0%)	0/10 (0%)	0/14 (0%)	2/60 (3%)	

DINP–dietary (F344)	Dose levels:	0	15	150	300 mg/kg		Lington et al. (1997)
	PP:	—	—	—	3×		
	Males:						
	Liver:	0/81 (0%)	0/80 (0%)	0/80 (0%)	3/80 (4%)		
	LCT:	58/61 (95%)	51/55 (93%)	47/50 (94%)	49/51 (96%)		
	PACT:	1/61 (2%)	0/55 (0%)	0/50 (0%)	0/51 (0%)		
	Females:						
	Liver:	1/81 (1%)	0/81 (0%)	0/80 (0%)	1/80 (1%)		
	PACT:	0/65 (0%)	0/57 (0%)	0/49 (0%)	0/51 (0%)		
DINP–dietary (F344)	Dose levels:	0	29	88	359	733 mg/kg	Butala et al. (1996)
	Males:						
	PP:	—	—	—	4×	8×	
	Liver:	1/65 (2%)	0/50 (0%)	0/50 (0%)	1/65(2%)	12/65 (18%)	
	LCT:	59/85 (70%)	48/70 (69%)	46/70 (66%)	60/85 (70%)	65/85 (76%)	
	PACT:	0/85 (0%)	0/70 (0%)	0/70 (0%)	0/85 (0%)	1/85 (1%)	
	Females:						
	Dose levels:	0	36	109	442	885 mg/kg	
	PP:	—	—	—	4×	8×	
	Liver:	1/65 (2%)	0/49 (0%)	0/50 (0%)	1/65 (2%)	5/65 (8%)	
	PACT:	0/85 (0%)	0/70 (0%)	0/70 (0%)	0/85 (0%)	1/85 (1%)	
DINP–dietary (Sprague Dawley)	Dose level:	0	500	5000	10,000 ppm		Bio/Dynamics (1986)
	PP:	—	—	2×	5×		
	Males:						
	Liver:	4/70 (6%)	7/69 (10%)	12/69 (17%)	9/70 (13%)		
	LCT:	2/69 (3%)	0/70 (0%)	0/70 (0%)	7/70 (10%)		
	PACT:	Not reported—assumed no dose response					
Fenofibrate–NS (NS)	Dose level	0	10	45	200 mg/kg/d		PDR (2002)
	Males:						
	PP:	NS	NS	NS	NS		
	Liver:	—	—	—	+		
	LCT:	—	—	—	+		
	PACT:	—	—	—	+		

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TABLE 35
Summary of PPARα Agonists Which Induce Extrahepatic and Hepatic Tumors *(Continued)*

Compound–route (strain)	Tumor incidence (tumor percent)					Reference
	Females:					
	Liver:	—	—	—	+	
	PACT:	—	—	—	—	
Gemfibrozil–gavage (Sprague-Dawley)	Dose levels:	0	30	300 mg/kg		Fitzgerald et al. (1981)
	PP:	—	2.5×	5×		
	Males only:					
	Liver:	1/50 (2%)	6/50 (12%)	23/50 (46%)		
	LCT:	1/50 (2%)	8/50 (16%)	17/50 (34%)		
	PACT:	0/50 (0%)	6/50 (12%)	2/50 (4%)		
HCFC-123–inhalation (CD)	Dose level:	0	300	1000	5000 ppm	Malley et al. (1995)
	Males:					
	PP:	—	2×	3×	4×	
	Liver:	3/67 (4%)	2/66 (3%)	2/66 (3%)	8/66 (12%)	
	LCT:	4/67 (6%)	12/66 (18%)	9/66 (14%)	14/66 (21%)	
	PACT:	2/67 (3%)	4/66 (6%)	12/64 (19%)	16/66 (24%)	
	Females:					
	PP:	—	1.2×	1.7×	3×	
	Liver:	0/65 (0%)	5/67 (8%)	2/67 (3%)	7/69 (10%)	
	PACT:	0/65 (0%)	2/66 (3%)	0/67 (0%)	2/69 (3%)	
Methylclofenapate–dietary (Wistar)	Dose level:	0	10	50	250 ppm	Tucker and Orton (1995)
	PP:	—	3×	12.5×	>12.5×	
	Males:					
	Liver[a]:	0/24 (0%)	0/24 (0%)	9/25 (36%)	22/23 (96%)	
	LCT:	1/24 (4%)	3/24 (12%)	10/25 (40%)	9/23 (39%)	
	PACT:	2/24 (8%)	5/24 (21%)	6/25 (24%)	9/23 (39%)	
	Females:					
	Liver[a]:	0/24 (0%)	1/24 (4%)	4/25 (16%)	20/24 (83%)	
	PACT:	0/24 (0%)	0/24 (0%)	1/25 (4%)	2/20 (10%)	
Nafenopin–dietary (F344)	Dose levels:	0	0.1%			Reddy and Rao (1997a, 1997b)
	Males					
	PP:	—	8×			
	Liver:	0/10 (0%)	11/15 (73%)			
	LCT:	6/10 (60%)	10/15 (66%)			
	PACT:	0/10 (0%)	3/15 (20%)			

PCE–inhalation (F344)	Dose levels: 0	200	400 ppm			Mennear (1986)
	Males:					
	PP:	—	1.25×	1.25×		
	Liver:	4/50 (8%)	8/50 (16%)	5/50 (10%)		
	LCT:	35/50 (70%)	39/49 (80%)	41/50 (82%)		
	PACT:	0/50 (0%)	0/50 (0%)	0/50 (0%)		
PFOA–dietary (Sprague Dawley)	Dose levels:	0	30	300 ppm		Sibinski et al. (1987)
	PP:	—	3×	6×		
	Males:					
	Liver[a]:	3/50 (6%)	1/50 (2%)	5/50 (10%)		
	LCT:	0/50 (0%)	3/50 (6%)	7/50 (14%)		
	PACT:	Not reported—assumed no dose response				
	Females:					
	Liver[a]:	0/50 (0%)	0/50 (0%)	3/50 (6%)		
	Mammary:	10/50 (20%)	19/50 (38%)	21/50 (42%)		
PFOA–dietary (CD)	Dose levels:	0	0 (Pair-fed)	300 ppm		Biegel et al. (2001)
	PP:	—	—	6×		
	Liver:	2/80 (3%)	3/79 (4%)	10/76 (13%)		
	LCT:	0/80 (0%)	2/78 (3%)	8/76 (11%)		
	PACT:	0/80 (0%)	1/79 (1%)	8/76 (11%)		
TCE–gavage (Marshall)	Dose levels:	0	0	500	1000 mg/kg	Mennear (1988)
	PP:	—	—	2×	2×	
	Liver:	Not reported—assumed no dose response				
	LCT:	16/46 (35%)	17/46 (35%)	21/48 (44%)	32/48 (66%)	
	PACT:	Not reported—assumed no dose response				
TCE–inhalation (Sprague-Dawley)	Dose levels:	0	100	300	600 ppm	Maltoni et al. (1988)
	PP:	—	2×	2×	2×	
	Liver:	Not reported—assumed no dose response				
	LCT:	6/135 (4.4%)	16/130 (12%)	30/130 (23%)	31/130 (24%)	

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TABLE 35
Summary of PPARα Agonists Which Induce Extrahepatic and Hepatic Tumors *(Continued)*

Compound–route (strain)	Tumor incidence (tumor percent)				Reference
Tibric acid–gavage (SD)	Dose levels:	0	30	100 mg/kg	Faccini et al. (1979)
	PP[d]:	—	2×	4×	
	Male:				
	Liver:	0/49 (0%)	23/48 (48%)	18/48 (38%)	
	LCT:	1/49 (2%)	0/47 (0%)	4/47 (9%)	
	PACT:	0/49 (0%)	3/47 (6%)	5/45 (55%)	
	Female:				
	Liver:	0/47 (0%)	9/47 (19%)	8/48 (11%)	
	PACT:	0/46 (0%)	1/47 (2%)	0/47 (0%)	
Wyeth 14,643–dietary (CD)	Dose levels:	0	50(25) ppm		Biegel et al. (2001)
	PP:	—	>6×		
	Liver:	2/80 (3%)	17/67 (25%)		
	LCT:	0/80 (0%)	16/67 (24%)		
	PACT:	0/80 (0%)	25/67 (37%)		

Note. NS, not specified; +, positive tumor finding.
[a]Hepatocellular carcinoma.
[b]Different studies.
[c]Abstract did not list tumor incidence but stated study was positive for LCTs.
[d]The fold peroxisome proliferation (PP) was derived from either the cited reference (HCFC-123, PFOA—Biegel et al. [2001], WY 14,643), other related papers (PFOA—Sibinski et al. [1987] used data from Liu et al. [1996b]), or estimated from Ashby et al. (1994). For the following compounds, estimation of PP induction was from Appendix 1 of Ashby and coworkers (1994): BBP (Figure 1), cinnamyl anthranilate (Figure 5), clofibrate (Figure 9), DEHP (Figure 13), DINP (Figure 16), gemfibrozil (Figure 17), methylclofenapate (Figure 20), nafenopin (Figure 22), PCE (Figure 23), TCE (Figure 26), and tibric acid (Figure 24).

expression. In the rat, PFOA produces minimal peroxisome proliferation at 2 mg/kg/day and no peroxisome proliferation at 0.2 mg/kg/day (Liu et al., 1996b). Hence, the NOEL for β-oxidation induction is somewhere between 0.2 and 2 mg/kg/day. Because of the significant toxicodynamic differences between the rat and the human, it is unlikely that a carcinogenic response induced *via* the proposed MOAs for liver, Leydig-cell, and pancreatic acinar-cell tumorigenesis in rodents would occur in humans following exposure to PFOA, assuming that the proposed MOAs for the Leydig-cell and pancreatic acinar-cell tumors are affirmed with additional investigation.

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