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Mode of Action Analysis and Human Relevance of Liver Tumors Induced by PPAR α Activation

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Abstract

There are a number of therapeutic hypolipidemic agents and industrial chemicals that cause peroxisome proliferation and induce liver tumors in rodents via activation of the nuclear receptor peroxisome proliferator-activated receptor α (PPAR α). Because of the increased understanding of the relationships between PPAR α activation and hepatocarcinogenesis, the purpose of this review is to describe the state of the science on the rodent mode of action (MOA) of liver tumor induction and human relevance. A wealth of data supports the key events in the MOA which lead to liver tumors. These include activation of PPAR α , increases in oxidative stress, increases in NF-kB activation, perturbation of hepatocyte growth, and selective clonal expansion. While these key events in the rodent MOA are biologically plausible in humans, there is no evidence that suggests that PPAR α activators could induce liver tumors in humans because of differences in PPAR α expression and function between rodents and humans. Lines of evidence supporting this presumption include minimal or no effects on peroxisome proliferation, peroxisomal enzyme activity, increases in oxidative stress, NF-kB activation, hepatocellular proliferation and liver tumors in humans and/or in species that are better human surrogates than mice and rats. Even when over-expressed in the mouse liver (humanized mice), human PPAR α activation does not lead to cell proliferation or liver tumors. This analysis leads to the suggestion that the PPAR α activator-induced rodent liver tumors are not relevant to humans.

Introduction

Peroxisomes are subcellular organelles found in the cytoplasm of mammalian cells and carry out important metabolic functions (deDuve, 1996; Hashimoto, 1996; Mannaerts and vanVeldhoven, 1996). Under a variety of altered physiological and metabolic states, peroxisomes are known to proliferate, most notably with increased concentrations of unsaturated and polyunsaturated fatty acids. Interest in the toxicology community was piqued when peroxisome proliferation was noted in rodent hepatocytes in response to the administration of certain xenobiotics (e.g., Hess et al., 1965; Reddy and Rao, 1977; Reddy and Chu, 1996). Based on the association between exposure and peroxisome proliferation, the chemical and pharmaceutical agents that induce this response have been collectively referred to as “peroxisome proliferators”.

Due to the structural heterogeneity of these compounds, the mechanism of peroxisome proliferation was an enigma for many years. The seminal discovery of the nuclear receptor peroxisome proliferator-activated receptor (PPAR α) in 1990 (Issemann and Green, 1990) followed by extensive work with the PPAR α -null mouse model has provided a molecular underpinning of the numerous biochemical, physiological, and molecular consequences of exposure to these compounds. The term “peroxisome proliferator” remains in broad use today primarily for historical reasons. In this review, the term “peroxisome proliferator” has been replaced with “PPAR α activator” to denote the central role PPAR α plays in mediating the pleiotropic effects of exposure. “Activator” is used in place of “agonist” as very few compounds have been assayed for direct binding to PPAR α using biochemical assays. Thus, PPAR α activators are those chemicals or their proximate metabolites that interact directly or indirectly with PPAR α , initiating events that result in receptor activation. Although most chemicals likely act as classical agonists, there is evidence that other chemicals may activate PPAR α secondary to

increases in the availability of natural ligands through perturbation of lipid homeostasis. For example, perfluorooctanoic acid (PFOA) may induce PPAR α activation indirectly through displacement of fatty acids from fatty acid binding protein (Luebker et al., 2002).

PPAR α activators are a unique class of chemical carcinogens that induce peroxisome proliferation and increase the incidence of liver tumors in rats and/or mice. These include several hypolipidemic drugs (e.g., WY-14,643, gemfibrozil, fenofibrate, bezafibrate, and ciprofibrate) and environmentally-relevant compounds such as phthalates or their metabolites (e.g., di-(2-ethylhexyl) phthalate (DEHP), di-(2-ethylhexyl) adipate (DEHA), diisononyl phthalate (DINP), or 2-ethylhexanol (2-EH)), pesticides (e.g., 2,4-dichlorophenoxyacetic acid, diclofopmethyl, haloxyfop, lactofen, oxidiazon), solvents (e.g., perchloroethylene, trichloroethylene), and other industrial chemicals (e.g., HCFC-123, PFOA) (summarized in Klaunig et al., 2003).

In addition to liver tumors, many PPAR α activators also induce testicular Leydig cell tumors as well as pancreatic acinar cell tumors in rats but not mice (also known as the “tumor triad”). Little progress has been made to refine the proposed modes of action for the pancreatic and testicular rat tumors as detailed in Klaunig et al. (2003). As such the present review will focus on the mode of action of PPAR α activator-induced liver tumors.

Mode of Action Analysis in the EPA Risk Assessment Framework

The U.S. Environmental Protection Agency (U.S. EPA) conducts risk assessments on chemical carcinogens under the guidance provided in its cancer risk assessment guidelines (U.S. EPA, 2005). EPA’s new cancer guidelines highlight the use of mode of action (MOA) data in the assessment of potential human carcinogens and provide a framework for critical analysis of MOA information to address the extent to which the available information supports a

hypothesized MOA, whether alternative MOAs are also plausible, and whether there is confidence that the same inferences can be extended to human populations and lifestages that are not represented among the experimental data. In addition the guidelines conclude that significant information should be developed to ensure that a scientifically justifiable MOA underlies the process leading to cancer at a given site. This approach has been further refined through IPCS guidance (Boobis et al., 2006) and has been extended to noncancer effects (Boobis et al., 2008).

The definition of the term MOA is important in making the determination of the adequacy of information to support it and to test whether a database for a particular chemical is consistent with that MOA. In the guidelines, the MOA is defined as “a sequence of key events and processes, starting with interaction of an agent with a cell, proceeding through operational and anatomical changes, and resulting in cancer formation”. A key event is defined as “an empirically observable precursor step that is itself a necessary element of the mode of action or is a biologically based marker for such an element.” The MOA is contrasted with “mechanism of action,” which implies a more detailed understanding of the events, often at the molecular level, than is meant by the MOA (Boobis et al., 2006).

The framework for analyzing MOA begins with a summary description of the postulated MOA. The judgment of whether a postulated MOA is supported by available data takes into account all of the data in a weight of evidence (WOE) approach. MOA analysis must determine the links between the postulated key events and tumor induction including (i) strength, consistency, specificity of association, (ii) dose-response relationships between the key events and tumor induction, (iii) temporal relationships including the key events preceding tumor induction, (iv) biological plausibility and coherence of the key event and its relationship with the mode of action, and (v) take into account alternative modes of action (Boobis et al., 2006;

2008). The robustness of the proposed MOA for PPAR α activator-induced rodent liver tumors and relevance to humans are examined using this framework.

Summary of the Mode of Action and Human Relevance of Liver Tumors Induced by PPAR α Activation

Substantial scientific research on the role of PPAR α in rodent hepatocarcinogenesis forms the basis for the cascade of key events that describes the MOA. Although the precise mechanism for the formation of liver tumors by a PPAR α activator has not been established, key events for the MOA leading to liver tumors have been identified (Figure 1). These include: activation of PPAR α , increases in oxidative stress, increases in NF-kB activation, perturbation of hepatocyte growth, and selective clonal expansion. This MOA is similar to one proposed earlier by an ILSI workgroup (Klaunig et al., 2003) except for the addition of the NF-kB activation event based on more recent findings. Associated events that are observed with PPAR α activators and liver tumor formation and that appear to be reliable markers a chemical has activated PPAR α include increased expression or activity of some peroxisomal genes (e.g., acyl-CoA oxidase encoding palmitoyl-CoA oxidase (PCO)) and peroxisome proliferation (i.e., an increase in the number and size of peroxisomes). PCO activity levels are correlated with cancer potency (Klaunig et al., 2003).

The EPA cancer guidelines state, “If a hypothesized rodent mode of action is sufficiently supported, the sequence of key precursor events should be reviewed to identify critical similarities and differences between the test animals and humans.” (U.S. EPA, 2005). Despite the fact that PPAR α activators induce liver tumors in rats and mice, the potential for PPAR α activators to induce liver tumors in other species, including humans, is low. Tumor induction is unlikely because evidence obtained from *in vivo* and *in vitro* studies with hamsters, guinea pigs,

non-human primates, and humans (*i.e.*, cells in culture or liver biopsies) shows that, quantitatively, these other species are less likely to exhibit the key events upon PPAR α activator exposure. Increases in liver to body weights and peroxisome proliferation are not evident in humans, although therapeutic hypolipidemic compounds lower triglyceride levels mediated by PPAR α across species.

There are several plausible explanations for the species-specific effects of PPAR α activators. 1) Full-length PPAR α protein is expressed at levels at least a ten-fold greater in rodent liver than in human liver. 2) Humans but not rodents express an inactive form of PPAR α in the liver which inhibits the active PPAR α . 3) Even when expressed at levels similar to the mouse PPAR α , hPPAR α does not induce the cell cycle machinery, cell proliferation or induced liver cancer in mice. 4) The PPAR α responsive elements (PPREs) of some target genes including acyl-CoA oxidase, have been shown to differ between rodents and humans. 5) Human epidemiological studies, although limited in duration, have not provided evidence of increased incidence of any type of cancer including liver neoplasms in humans. These data support the conclusion that induction of rodent liver tumors by this MOA may not be relevant to humans.

Detailed Evaluation of the Rodent Mode of Action

The following sections provide an in-depth analysis of the proposed MOA for rat and mouse liver tumors induced by PPAR α activators. This analysis is not intended to reflect an exhaustive review of the literature but rather a summation of key evidence. This review of the data did not consider alternative effects of PPAR α activators including mitochondrial effects, gap junction intercellular communication (GJIC), or methylation of DNA because the data are weak or a direct causal link to liver tumor induction is lacking.

The liver consists of the hepatic parenchyma (hepatocytes) and nonparenchymal cells

(NPCs) including sinusoidal endothelial cells, Ito cells, and the dedicated hepatic macrophages known as the Kupffer cells. Kupffer cells can be “activated” by some liver toxicants leading to release of signaling molecules such as reactive oxygen species and cytokines (Roberts et al., 2007). Kupffer cells play important roles in responses to PPAR α activators and are required for key events which lead to liver tumors. Although the rodent PPAR α activator mode of action is presented as a linear set of key events (Figure 1), some of the key events are likely dependent on interactions between hepatocytes and Kupffer cells. These relationships are described below. The evaluation of the key events in the MOA is discussed below followed by a discussion of mechanistic studies which provide linkages between the key events.

PPAR α Activation

PPAR α is activated by many environmentally-relevant chemicals 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). Chemical-specific data show excellent correlations between PPAR α activation, the key events in the MOA and liver cancer (see Table 1 for examples of 5 PPAR α activators). PPAR α activation is a causative key event in the PPAR α activator MOA for liver tumor induction. 1) PPAR α activation is consistently associated with exposure to PPAR α activators in trans-activation assays (summarized in Corton et al., 2000 and Klaunig et al., 2003). 2) The level of activation of PPAR α in such assays is roughly proportional to the potency of the chemical as an inducer of liver tumor response (summarized in Klaunig et al., 2003). 3) Importantly, the majority of studies using PPAR α -null mice do not show hepatocyte-specific changes associated with hepatocarcinogenesis (discussed below).

There are examples where PPAR α activation does not consistently lead to liver cancer, and these have been summarized in Klaunig et al. (2003). Weak PPAR α activators, (i.e.,

compounds that minimally induce markers of PPAR α activation) would not necessarily increase liver tumor incidence, as a sufficient level of receptor activation is needed for induction of key events (Klaunig et al., 2003). Pharmacokinetic differences between susceptible and nonsusceptible rodents that lead to differences in tissue chemical concentration could also contribute to discrepancies between the ability of chemicals to activate PPAR α in trans-activation assays and tumor induction. For example, trichloroacetate (TCA) exposure in mice leads to increases in PCO activity at doses similar to or below those that induce liver tumors whereas in rats TCA, even at high doses, only marginally increases PCO in the absence of increases in liver tumors (Corton, 2008).

PPAR α regulates lipid homeostasis and peroxisome proliferation through the modulation of genes involved in fatty acid uptake, activation and oxidation as well as peroxisome assembly (the *Pex* genes) (Desvergne et al., 1998; Desvergne and Wahli, 1999; Schoonjans et al., 1996; Wahli et al., 1995). Collectively, these changes result in increased ability to metabolize fatty acids leading to the therapeutic lowering of lipid levels in mice, rats, Syrian hamsters, guinea pigs, monkeys, and humans. These changes have been shown to be PPAR α -dependent in mice (summarized in Peters et al., 2005). Alteration in lipid metabolism and peroxisome proliferation genes are not thought to be involved in the hepatocarcinogenic effects of PPAR α activators (Klaunig et al., 2003).

Role of Oxidative Stress in PPAR α Activator-Induced Hepatocarcinogenesis

Linkages exist between increases in reactive oxygen species (ROS) and increased incidence of liver cancer by PPAR α activators. Overproduction of oxidants might cause DNA damage leading to mutations and cancer (Reddy and Rao, 1989; Yeldandi et al., 2000). In whole liver of both rats and mice, markers of oxidative stress were increased by PPAR α activators

(Table 1), determined by measuring lipid peroxidation (TBARS, conjugated dienes, lipofuscin, malondialdehyde, F₂-isoprostanes), oxidized glutathione or hydrogen peroxide. A few studies failed to detect increases in markers of oxidative stress, but these are difficult to interpret because other key events were not simultaneously analyzed (e.g., Huber et al., 1991, 1997). There were other studies in which one assay for oxidative stress was positive but another negative (e.g., Conway et al., 1989; Fischer et al., 2002). In spite of these minor discrepancies, the weight of evidence demonstrates that PPAR α activators increase oxidative stress.

Possible sources of ROS in the livers of rodents exposed to PPAR α activators include enzymes that generate and degrade hydrogen peroxide and other reactive oxygen species. Hydrogen peroxide can oxidize DNA, lipids, and other molecules, and PPAR α activators regulate the expression of many enzymes that produce hydrogen peroxide as a byproduct of metabolism including the peroxisomal, mitochondrial, and microsomal oxidases in hepatocytes such as fatty acyl-CoA oxidase (ACO) (Becuwe and Dauca, 2005). Administration of PPAR α activators can also lead to decreased levels of some enzymes which degrade ROS that may contribute to the increases in oxidative stress upon exposure (Glauert et al., 1992; O'Brien et al., 2001a,b). The individual contributions of these enzymes to increases in oxidative stress and downstream key events leading to liver tumor induction has not been comprehensively addressed but will likely be complex. In one example, Reddy and coworkers originally proposed that peroxisomal ACO (*Acox1*) is the enzyme responsible for oxidative stress by PPAR α activators (Nemali et al., 1988). However, ACO was later found to be dispensable for increases in oxidative stress. Control ACO-null mice exhibited the phenotype of wild-type mice exposed to PPAR α activators including increases in oxidative stress and induction of liver tumors that are dependent on PPAR α (Fan et al., 1998; Hashimoto et al., 1999). The role of other ACO family members (*Acox2*, *Acox3*) has not been determined in this *Acox1*-independent induction of

oxidative stress and liver tumors.

Extensive testing of PPAR α activators has shown that these compounds do not consistently induce direct DNA damage. However, indirect DNA damage from oxidative stress has been hypothesized to be a common pathway for many non-genotoxic chemical carcinogens including PPAR α activators (Klaunig et al., 1998). Relationships exist between chemical exposure, DNA damage, and cancer based on measurement of 8-hydroxy-deoxyguanosine (8-OH-dG), a highly mutagenic lesion, in DNA isolated from livers of animals treated with PPAR α activators (Kasai, 1997; Takagi et al., 1990; Qu et al., 2001). However, subsequent studies showed that the increases in oxidative DNA damage may have originated in the way in which the genomic DNA was prepared (Cattley and Glover, 1993; Sausen et al., 1995). Experiments measuring other indicators of DNA damage, i.e., 8-oxoguanine, abasic sites, or single strand breaks in genomic DNA from rats and mice treated with WY for one month failed to show increases over controls (Rusyn et al., 2004). Only in the livers of wild-type but not PPAR α -null mice treated with WY for 5 months were there increases in abasic sites in genomic DNA (Woods et al., 2007b), indicating that exposure times longer than 1 month were necessary to observe increases in DNA damage. The relationship between the increases in abasic sites and subsequent tumor yield has not been determined.

DNA repair mechanisms might compensate for increases in DNA damage and may explain the lack of consistent evidence for DNA damage from PPAR α activator-induced oxidative stress. PPAR α activators increased the expression of liver genes involved in the long-patch base excision DNA repair pathway in a time-dependent manner; the degree of induction roughly correlated with the dose and carcinogenic potency of the PPAR α activators tested (Rusyn et al., 2000a). Additionally, expression of enzymes that do not repair oxidative DNA damage was not changed. This induction of DNA base excision repair genes may be an indicator

that DNA damage is occurring.

Evidence that DNA damage caused by PPAR α activator-induced oxidative stress is not involved in hepatocarcinogenesis comes from recent work with Ogg1-null mice. Ogg1 encodes an 8-oxoguanine DNA glycosylase which repairs one of the major DNA lesions generated by ROS. Control Ogg1-null mice show elevated levels of oxidative DNA damage and exhibit increased spontaneous mutation rates in the absence of chemical exposure (Klungland et al., 1999). Ogg1-null mice when exposed to WY in the diet, did not show additional oxidative DNA damage but exhibited increased numbers and total volumes of preneoplastic lesions in the liver compared to similarly treated wild-type mice (Trapp et al., 2007). The authors concluded that the increase in preneoplastic lesions associated with WY exposure did not arise from induced oxidative damage, but rather from the promotion of spontaneous mutations generated by endogenous oxidative DNA damage.

Overall, PPAR α activators increase the level of oxidative stress through multiple mechanisms. There is little direct evidence that increases in oxidative stress generated after PPAR α activator exposure leads to direct or indirect DNA damage. The Ogg1-null mouse studies indicate that PPAR α activators promote hepatocytes that have been spontaneously initiated. The weight of evidence suggests that direct or oxidatively-induced DNA damage is not part of the MOA.

Role of NF-kB in the PPAR α Activator MOA

Central to the PPAR α activator MOA is NF-kB activation. NF-kB transcription factors play critical roles in cancer development and progression (Karin, 2006; Arsuru and Cavin, 2005). A wealth of data demonstrates that NF-kB is activated under conditions of inflammation and oxidative stress (Czaja, 2007; Gloire et al., 2006). Consistent with this, studies with PPAR α

activators demonstrates linkages between oxidative stress and NF-kB activation. Activation is usually assessed by the ability of nuclear NF-kB (usually a heterodimer composed to p50 and p65 subunits) to bind to a NF-kB response element in an electrophoretic mobility shift assay (EMSA). In whole liver of both rats and mice, activity of NF-kB was increased by PPAR α activators including WY, ciprofibrate and gemfibrozil but not nafenopin (Table 1). The fact that nafenopin did not induce NF-kB may be due to differences in the EMSA procedures carried out by that lab (Ohmura et al., 1996; Menegazzi et al., 1997).

NF-kB is activated in Kupffer cells and in hepatocytes different times after exposure. After a single in vivo dose of WY, NF-kB activity was increased first in Kupffer cells (at 2 hours) and only ~6 hours later, was NF-kB activity increased in hepatocytes. Activation in hepatocytes never achieved the level observed in Kupffer cells (Rusyn et al., 1998). The increase in NF-kB activation in hepatocytes could be due to increases in mitogenic cytokines produced by Kupffer cells that activate signal transduction pathways ultimately impinging on NF-kB. Alternatively, NF-kB can be activated directly by a PPAR α activator in the H4IIEC3 rat hepatoma cell line, responsive to the proliferative effects of PPAR α activators (Li et al., 2000a). Increased NF-kB activity may be secondary to the action of hydrogen peroxide-generating enzymes, such as ACO, since over-expression of ACO in COS-1 cells, in the presence of a hydrogen peroxide-generating substrate, can activate a NF-kB-regulated reporter gene (Li et al., 2000b).

Alteration of Cell Proliferation/Apoptosis Balance by PPAR α Activators.

PPAR α activators produce multiple tumor precursor effects including liver hyperplasia, and altered growth in preneoplastic foci. Increased cell replication induced by PPAR α activators may increase the frequency of spontaneous mutations by increasing the frequency of errors in

DNA repair or replication and can lead to silencing of tumor suppressor genes or increased expression of oncogenes (Cattley et al., 1998; Huber et al., 1991). Alternatively, PPAR α activators can promote the growth of spontaneously initiated hepatocytes.

All PPAR α activators at a sufficient dose produce a strong, albeit transient, increase in replicative DNA synthesis during the first few days of exposure (Table 1). After this initial burst in replication, baseline levels of hepatocyte replication are approached while the liver remains enlarged. Many PPAR α activators exhibit measurable sustained or chronic increases in cell proliferation, although the levels are much lower than that observed after acute exposures (Table 1). There are some PPAR α activators that do not induce chronic cell proliferation; this may be due to the dose used in the experiment and because weak increases above variable background levels of cell proliferation are difficult to detect.

PPAR α activators promote the growth of chemically- and spontaneously-induced lesions through enhanced cell replication (Cattley and Popp, 1987; Cattley et al., 1991; Isenberg et al., 1997; Marsman et al., 1988). Once early lesions are formed, continued exposure to PPAR α activators causes a selective increase in DNA replication of up to ~40% in these liver foci, while replication of hepatocytes in the normal surrounding liver is increased only slightly (Grasl-Kraupp et al., 1993). Furthermore, preneoplastic foci respond to the cell replicative rather than the peroxisome proliferation effects of PPAR α activators, suggesting that the growth stimulus but not the peroxisome proliferation effect is of particular significance for the carcinogenic action of this class of compounds (Grasl-Kraupp et al., 1993).

Increases in cell proliferation alone are not sufficient to increase liver tumors. The response of mice transgenic for hepatocyte-specific expression of a constitutively activated form of PPAR α (VP16PPAR α) was compared to wild-type mice treated with WY (Yang et al., 2007). Expression of VP16PPAR α led to increases in hepatocyte proliferation in the absence of

nonparenchymal cell proliferation, in contrast to WY treatment in wild-type livers in which both hepatocytes and nonparenchymal cells exhibited increased replication. Importantly, chronic activation of VP16PPAR α did not increase liver tumors (Yang et al., 2007). These results indicate that nonparenchymal cell activation is important for hepatocarcinogenesis and that PPAR α -mediated hepatocyte proliferation by itself is not sufficient to induce liver cancer. Taken together, the results indicate that it is the combination of events in hepatocytes and NPC that are important for induction of liver tumors by PPAR α activators.

Nongenotoxic carcinogens, in general, and PPAR α activators in particular suppress hepatocyte apoptosis. Suppression of apoptosis could inhibit the ability of the liver to remove DNA-damaged, pre-neoplastic hepatocytes (Bayly et al., 1994; James and Roberts, 1996; Oberhammer and Qin, 1995; Schulte-Hermann et al., 1981). Most of the evidence for apoptosis suppression comes from in vitro studies because of the difficulty in measuring the suppression of already low levels of apoptosis in vivo. Studies conducted in vitro show that the PPAR α activators nafenopin, methylclofenapate, and WY suppress spontaneous hepatocyte apoptosis as well as that induced by a negative regulator of liver growth, transforming growth factor β 1 (TGF β 1) (Bayly et al., 1994; Oberhammer and Qin, 1995) (Table 1). In addition, PPAR α activators can suppress apoptosis in vitro induced by diverse stimuli such as DNA damage or ligation of Fas, a receptor related to the tumor necrosis factor α (TNF α) family of cell surface receptors (Gill et al., 1998). A limited number of in vivo studies also showed suppression of apoptosis after acute dosing with nafenopin, DEHP or WY (Bursch et al., 1984; James et al., 1998; Youssef et al., 2003).

Suppression of apoptosis by PPAR α activators occurs under acute exposure conditions 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 levels or to levels which balance

the low level of cell proliferation that occurs for some PPAR α activators. Consistent with this, two reports suggest that chronic exposure of rats and mice to the PPAR α activator WY results in an increase in apoptosis (Marsman et al., 1992; Burkhardt et al., 2001). Furthermore, PPAR α activators alter the ability of the liver to respond to apoptosis inducers. Sensitivity to two apoptosis inducers (Jo2 antibody and concanavalin A) was dramatically increased in wild-type but not PPAR α -null mice exposed for 1 week to WY (Xiao et al., 2006). Lastly, both cell proliferation and apoptosis increase in parallel in PPAR α activator-induced tumors in the rat compared with normal surrounding tissue, suggesting that cell turnover is increased in tumorigenic lesions (Grasl-Kraupp et al., 1997).

To summarize, alterations in the balance between hepatocyte proliferation and apoptosis have been observed after exposure to multiple PPAR α activators at different stages of carcinogenesis including under acute and chronic exposure conditions and in the preneoplastic and tumorigenic lesions.

Mechanisms of Cell Growth Alterations

Extensive work has been carried out to identify the mechanistic events that lead to alterations in cell growth by PPAR α activators. There are a number of excellent reviews on the subject of signal transduction and downstream events which lead to alterations in cell growth that the reader is referred to (Gonzalez and Shah, 2008; Rusyn et al., 2006; Burns and Vanden Heuvel, 2007). Early studies focused on the regulation of individual growth genes that respond to growth promoting stimuli. More recent studies capitalized on technological advancements in assessing global changes in gene expression or assessing the role of individual genes/pathways in the intact animal using transgenic technologies.

Many studies focused on growth factors derived from the Kupffer cell. Activated NPCs,

particularly Kupffer cells, produce cytokines such as tumor necrosis factor α (TNF α), interleukin-1 α and interleukin-1 β (IL1 α , IL1 β). These cytokines affect the fate of neighboring hepatocytes. TNF α is able to increase hepatocyte proliferation and suppress apoptosis in cultured rodent hepatocytes (Holden et al., 2000; Rolfe et al., 1997). In intact animals hepatocyte growth can be prevented by injection of antibodies to either TNF α (Bojes et al., 1997; Rolfe et al., 1997) or TNF α receptor 1 (West et al., 1999). PPAR α activators increased TNF α mRNA more than two-fold (Bojes et al., 1997; Rolfe et al., 1997). As increases in TNF α expression have not been consistently observed by others (Anderson et al., 2001; Holden et al., 2000), treatment with PPAR α activators may not result in *de novo* TNF α expression, but rather bioactivation or release of preexisting TNF α protein from Kupffer cells (Holden et al., 2000).

Other studies suggest that the cell proliferation response to PPAR α activators is TNF α pathway-independent. Cell proliferation remained intact in TNF α -null and in TNF α receptor-null mice given a PPAR α activator (Lawrence et al., 2001b; Anderson et al., 2001). In addition, IL-1 receptor-null mice retained the ability to respond to the induction of hepatocyte proliferation to WY (Corton et al., unpublished observations). There remains the possibility that loss of TNF α or IL-1 signaling results in compensation by other genes/pathways, including other cytokine-mediated pathways, as multiple growth modulators secreted by Kupffer cells have been suggested to play a role in hepatocyte proliferation after DEN exposure (Maeda et al., 2005). Thus, studies with various nullizygous mice do not necessarily refute the role TNF α or IL-1 may play in PPAR α activator-induced cell proliferation.

MicroRNAs (miRNA) play important roles in complex processes such as development through the regulation of gene expression. Recent global analysis of the miRNA expression pattern after WY exposure has uncovered a signaling pathway which culminates in increased expression of the c-Myc growth regulatory gene (Shah et al., 2007). Expression of let-7C, a

miRNA important in cell growth was down-regulated following acute or chronic treatment with WY in wild-type mice. As let-7C down-regulates the expression of c-Myc, the down-regulation of let-7C by WY resulted in increased expression of c-Myc. These molecular events did not occur in PPAR α -null mice. These studies reveal a let-7C signaling cascade critical for PPAR α activator-induced hepatocyte proliferation.

Other growth signaling pathways may be involved in PPAR α activator growth responses but overall, the data supporting their role is usually confined to gene expression data. Due to the lack of useful genetic models, there is little mechanistic data which shows causal links between specific pathways and modulation of cell fate except for the role of PPAR α and NF-kB activation (discussed below).

Genetic and biochemical inhibition studies support the MOA

Genetic and biochemical inhibition studies have highlighted the relationships between the key events of the PPAR α activator MOA (Table 2). These studies showed that when a key event is inhibited genetically or biochemically, the downstream but not upstream event(s) are inhibited as well.

Genetically-modified mice have been useful to show the relationships between the key events in the PPAR α MOA. PPAR α -null mice provided critical evidence establishing the rodent MOA for PPAR α activator-induced hepatocarcinogenesis. Evidence that a particular compound induces key events in wild-type mice but not in mice lacking PPAR α would be considered strong support for a PPAR α MOA for that particular compound. To date three chronic bioassays have been conducted in these mice (Peters et al., 1997; Hays et al., 2005; Ito et al., 2007). A greater body of data exists in which precursor events for cancer have been assessed in wild-type and PPAR α -null mice after acute or subacute exposures.

Two studies assessed markers of oxidative stress in wild-type and PPAR α -null mice. In the first study, abasic sites (i.e., sites that lack either a purine or a pyrimidine) in genomic DNA were used as a measure of oxidative stress. These sites were increased in wild-type but not PPAR α -null mice after exposure to WY for 5 months (Woods et al., 2007b). In the second study, electron spin resonance (ESR) identified increases in free radicals in the bile of wild-type but not PPAR α -null mice after up to 3 week exposures to WY or DEHP. NF-kB activation was observed in the livers of wild-type but not PPAR α -null mice after exposure to WY (Woods et al., 2007a,b). Using global gene expression profiling, alteration of gene expression by WY, PFOA or ciprofibrate was almost completely abolished in PPAR α -null mice at multiple time points (Anderson et al., 2004a,b; Corton et al., 2004; Woods et al., 2007c; Rosen et al., 2008a,b; Sanderson et al., 2008; Corton et al., unpublished). The up-regulation of the cell cycle components CDK-1, CDK-2, CDK-4 and PCNA proteins and CDK-1, CDK-4 and cyclin D1 mRNA was observed in wild-type but not PPAR α -null mice fed WY (Peters et al., 1998). Wild-type mice exhibited increased hepatocyte proliferation compared to untreated controls while no increases in hepatocyte proliferation were observed in PPAR α -null mice after exposure to WY, diisononyl phthalate, PFOA, or trichloroethylene (Peters et al., 1997, 1998; Valles et al., 2003; Laughter et al., 2004; Corton et al., unpublished; Wolf et al., 2008). The ability of PPAR α activators to suppress apoptosis was lost in hepatocytes isolated from PPAR α -null mouse livers (Hasmall et al., 2000a). Importantly, chronic treatment with WY or bezafibrate resulted in 100% incidence of hepatocellular neoplasia in wild-type mice while the PPAR α -null mice were unaffected (Peters et al., 1997; Hays et al., 2005). An additional bioassay in which DEHP induced liver tumors in PPAR α -null but not wild-type mice (Ito et al., 2007) is discussed below. Although the WY and bezafibrate chronic exposure studies were carried out for relatively short exposure periods (up to a year), the PPAR α -null mice did not exhibit any of the precursor events

associated with carcinogenesis (Peters et al., 1997,1998; Hays et al., 2005), making it unlikely that longer-term exposure would result in liver tumors. These studies demonstrate that all of the key events in the MOA are dependent on PPAR α .

Two transgenic mouse models have been used to determine the relationships between different sources of oxidative stress and downstream events. Catalase converts hydrogen peroxide to water and oxygen. In catalase-transgenic mice which exhibit increased liver expression and activity of catalase, there were decreased levels of NF-kB activation and decreased hepatocyte proliferation upon exposure to ciprofibrate (Nilakantan et al., 1998). NADPH oxidase in Kupffer cells plays an important role in generating superoxide radicals in response to Kupffer cell activators (De Minicis et al., 2006). NADPH oxidase is activated by PPAR α activators and is important in cell proliferation after short-term PPAR α activator exposure. Mice which lack one of the subunits of NADPH oxidase (the p47Phox-null mice) did not exhibit increases in oxidative stress, NF-kB activation, and hepatocyte proliferation after short-term PPAR α activator exposure (Rusyn et al., 2000b,c). However, after exposure of mice to WY for three weeks, there were increases in indicators of oxidative stress (including PCO activity), NF-kB activation and cell proliferation, independent of the status of the p47Phox gene; these key events were dependent on PPAR α (Woods et al., 2007a,b). Longer-term exposure may allow bypass of p47Phox dependence including increases in oxidative stress through activation of enzymes that produce hydrogen peroxide.

NF-kB activation is involved in modulation of hepatocyte fate in response to inducers of oxidative stress (e.g., Maeda et al., 2005) including PPAR α activators. Wild-type mice and mice deficient in the p50 subunit of NF-kB (p50-null mice) were fed a diet with or without 0.01% ciprofibrate for 10 days. NF-kB DNA binding activity was increased after ciprofibrate treatment in wild-type mice but not p50-null mice. The apoptotic index was low in wild-type mice in the

presence or absence of ciprofibrate. Consistent with NF- κ B acting as a negative regulator of apoptosis (Karin, 2006; Arsurra and Cavin, 2005), apoptosis was higher in untreated p50-null mice compared to wild-type mice (Tharappel et al., 2003). Apoptosis was reduced in p50-null mice after ciprofibrate feeding but was still higher than wild-type levels. The untreated p50-null mice had a higher level of hepatic cell proliferation, as measured by BrdU labeling, than did untreated wild-type mice possibly as a mechanism to compensate for the higher levels of apoptosis. However, ciprofibrate-fed p50-null mice had lower levels of cell proliferation than comparatively treated wild-type mice (Tharappel et al., 2003).

A chronic (38-week) exposure study provides direct evidence that NF- κ B activation is necessary for hepatocarcinogenesis induced by a PPAR α activator (Glauert et al., 2006). Wild-type mice receiving only diethylnitrosamine (DEN) developed a low incidence of tumors (25%). The majority of wild-type mice receiving both DEN + WY developed tumors (63%). However, no tumors were seen in the DEN or DEN + WY treated p50-null mice, demonstrating that the p50 subunit of NF- κ B was required for the promotion of hepatic tumors by WY. Treatment with DEN + WY increased both cell proliferation and apoptosis in wild-type and p50-null mice. Consistent with the tumor levels, cell proliferation and apoptosis were lower in the p50-null mice than in wild-type mice (Glauert et al., 2006). This study shows direct dependence on the p50 subunit of NF- κ B for liver tumor induction by a PPAR α activator.

Biochemical inhibition studies using compounds that inhibit oxidative stress or inflammation also highlight linkages of the key events in the PPAR α MOA. In these studies animals were pretreated with the inhibitor before PPAR α activator exposure or co-treated with a PPAR α activator and the inhibitor. The free radical scavenger and xanthine oxidase inhibitor allopurinol inhibited the activation of NF- κ B in the livers of WY-treated rats (Rusyn et al., 1998). In *in vitro* studies, the anti-oxidants vitamin E or N-acetylcysteine blocked the ability of

NF- κ B to activate a reporter gene in ciprofibrate-treated HIIIE3C cells (Li et al., 2000b). Co-treatment with ciprofibrate and one of two anti-oxidants, 2(3)-tert-butyl-14-hydroxyanisole or ethoxyquin decreased the incidence and size of liver tumors compared to ciprofibrate treatment alone (Rao et al., 1984). Studies using either dimethylthiourea or deferoxamine as antioxidants decreased the incidence of liver tumors in rats fed the PPAR α activator ciprofibrate (Rao and Subbarao, 1997a, 1999). When co-treating rats with the PPAR α activator ciprofibrate and the antioxidant vitamin E, the levels of the antioxidant glutathione were paradoxically depleted, and the animals exhibited increased tumor numbers (Glauert et al., 1990). In other studies vitamin E inhibited clofibrate-induced increases in lipofuscin-like products and ciprofibrate-induced increases in NF- κ B activation in the absence of effects on markers of PPAR α activation (Stanko et al., 1995; Calfee-Mason et al., 2004).

Inhibition of key events by compounds that alter inflammatory states including Kupffer cell activation has been observed in multiple studies. The glucocorticoid receptor agonist dexamethasone is an anti-inflammatory agent that decreases the ability of NF- κ B to be activated under a variety of inflammatory conditions (Ray and Prefontaine, 1994; Widen et al., 2003; Chang et al., 1997; De Bosscher et al., 2006). Dexamethasone decreased PPAR α activator-induced hepatocyte proliferation after acute exposures (Lawrence et al., 2001a; Rao and Subbarao, 1997b; Omura et al., 1996) while having either no effect (Lawrence et al., 2001a; Rao and Subbarao, 1997b) or modest decreases (Omura et al., 1996) on markers of PPAR α activation. Compounds that inhibit Kupffer cell activation (glycine, methylpalmitate) or inhibit NADPH oxidase (diphenyleneiodonium) inhibited increases in oxidative stress and NF- κ B activation after exposure to PPAR α activators but had no effects on markers of PPAR α activation (Rose et al., 1997a,b; Rose et al., 1999a,b; Rusyn et al., 2001; Rusyn et al., 2000b,c). While pretreatment with diphenyleneiodonium, glycine or methylpalmitate decreased acute cell

proliferation (Rose et al., 1997a,b; Rusyn et al., 2000b,c; Rose et al., 1999a), glycine had no effect on chronic cell proliferation but did decrease the size and number of tumors (Rose et al., 1999b). Taken together, these biochemical and genetic inhibition studies demonstrate the linkages of the key events in the PPAR α activator MOA.

Some PPAR α activators exhibit complex MOAs

Before a PPAR α activator MOA can be defined as the primary MOA, alternative MOA(s) must be considered. Comparison of wild-type and PPAR α -null mice have provided opportunities to determine if additional key event sare necessary in addition to PPAR α activation. In one example, PFOA was analyzed for liver effects in wild-type and PPAR α -null mice. At two doses tested (1 and 3 mg/kg/day), PPAR α -null mice lacked increases in cell proliferation but retained increases in liver to body weights. At the highest dose tested (10 mg/kg/day) PPAR α -null mice exhibited increases in cell proliferation (Wolf et al., 2008). Microarray analysis using full-genome gene chips showed that PFOA altered ~85% of the total number of genes in a PPAR α -dependent manner at 3 mg/kg/day. The PPAR α -independent genes exhibited signatures of activation of other nuclear receptors. In particular, the PPAR α -independent genes significantly overlapped with those regulated by the constitutive activated receptor (CAR) which regulates cell growth and xenobiotic metabolism genes including *Cyp2b* family members (Rosen et al., 2008a,b). These CAR signature genes were more robustly regulated in PFOA-treated PPAR α -null mice compared to wild-type mice. These findings indicate that CAR activation may be a key event in the transcriptional and cell proliferation effects in PPAR α -null mice. In wild-type mice, there were relatively minor alterations of CAR signature genes compared to the strong changes in PPAR α -dependent genes indicating that CAR plays a minor role in mediating PFOA effects in wild-type mice (Rosen et al., 2008).

The carcinogenic effects of DEHP were examined in wild-type and PPAR α -null mice for 22 months (Ito et al., 2007). A low level of liver tumors was observed in PPAR α -null but not wild-type mice. These data suggest that an additional biological event may be operating in DEHP-induced rodent liver tumors¹. The tumors in PPAR α -null mice most likely arose through a mechanism that is not dominant in wild-type mice. Wild-type and PPAR α -null mice did not exhibit equivalent levels of tumor induction. There were no statistically significant increases in liver tumors in the wild-type mice under these exposure conditions, indicating that the biological effects of exposure were not equivalent in these two strains. Expression of growth control genes showed responses in PPAR α -null mice but not in wild-type mice at equivalent doses. In follow-up work from the same lab (Takashima et al., 2008), transcript profiling and RT-PCR showed highly dissimilar changes in gene expression in the liver tumors from the two strains. These data indicate that although DEHP can induce marginal increases in liver tumors in PPAR α -null mice, the MOA is different from that in wild-type mice. DEHP is a strong inducer of *Cyp2b* family members in wild-type mice (Currie et al., 2005; Corton et al., unpublished) suggesting that in the absence of PPAR α , DEHP activates CAR, as the rate-limiting key event resulting in increases in liver tumors by a CAR-dependent pathway.

In summary, chemicals may produce similar PPAR α -independent effects defined in part as effects observed in PPAR α -null mice. These effects may suggest additional key events that become the main control points in the absence of PPAR α . A determination of the relative contribution of each proposed key event would require comparison of signature genes and biomarkers representing each key event in the two strains.

The PPAR α activator MOA is chemical-independent

Mode of action is a series of key events that together result in an adverse health effect

such as a liver tumor and as such is chemical-independent (Holsapple et al., 2006; Meek, 2008; Boobis et al., 2008). Consistent with this the MOA for PPAR α activators is an endogenous series of events that can occur independent of chemical exposure. Livers from ACO-null mice exhibit severe steatosis, increases in markers of PPAR α activation (i.e., genes involved in β - and ω -fatty acid oxidation), increases in hydrogen peroxide levels, increases in cell proliferation and liver tumors (Fan et al., 1998). The increases in the markers of PPAR α were shown to be PPAR α -dependent as the changes were abolished in a double ACO-/PPAR α -null mouse (Hashimoto et al., 1999). Microarray analysis of the tumors spontaneously induced in ACO-null mice showed extensive similarity with the liver tumors induced by the PPAR α activator ciprofibrate, indicating the mechanism leading to the induction of the tumors was similar (Meyer et al., 2003). Additional mouse models nullizygous for other genes involved in fatty acid oxidation exhibit phenotypes indicative of constitutive PPAR α activation (Jia et al., 2003). A mouse model of hepatitis C virus (HCV)-induced hepatocellular carcinoma (HCC) which over-expresses the HCV core protein was used to show that induction of oxidative stress, increases in cell proliferation and liver tumor induction were PPAR α -dependent (Tanaka et al., 2008a,b). The authors conclude that there “is the absolute requirement of persistent PPAR α activation for the development of HCV core protein-induced steatosis and HCC”. All of these mouse models exhibit disruption of fatty acid transport and metabolism resulting in increases in endogenous activators of PPAR α including fatty acids (Fan et al., 1998; Tanaka et al., 2008a,b). Taken together, the PPAR α MOA is operational in the absence of chemical exposure. Chemical PPAR α activators will persistently activate this MOA resulting in liver tumors.

Species differences in responsiveness of key events in the PPAR α MOA

Studies conducted in numerous test species indicate that while some rodents (mice and rats) are highly responsive to PPAR α activator-induced hepatocarcinogenicity and associated responses, other species (e.g., Syrian hamsters, dogs, guinea pigs, New and Old World primates, and humans) are less sensitive (Ashby et al., 1994; Bentley et al., 1993; Cattley et al., 1998; Doull et al., 1999). This difference is likely based in large part on differing levels of PPAR α expression among species. In a side-by-side comparison, mice had ~10-fold more PPAR α expression than guinea pigs and ~3-fold more than Syrian hamsters (Choudhury et al., 2004). Humans exhibited \geq 10-fold lower expression than mice and rats (described in greater detail below). Thus, guinea pigs may be the more relevant model for PPAR α activator effects in the human liver based solely on expression levels of the full-length active PPAR α .

Table 3 summarizes PPAR α MOA key events in responsive species (rats and mice summarized from Table 1) compared to Syrian hamsters, guinea pigs, Cynomolgus monkeys, and humans. Due to the relative paucity of data for key events, other endpoints associated with exposure to PPAR α activators are included (i.e., liver weight to body weight, hypolipidemic effects).

Syrian hamsters and guinea pigs exhibit a partial PPAR α activator response even though they are considered “non-responsive species” compared to rats and mice. Fatty acid metabolism genes/proteins are only weakly activated after PPAR α activator exposure in the livers of these species. Diminished responsiveness in guinea pigs is not due to a defective PPAR α because when over-expressed in cell lines, PPAR α from guinea pigs activates reporter genes to levels comparable to rats and mice (Bell et al., 1998; Tugwood et al., 1998; MacDonald et al., 1999). PPAR α activators WY or methyleclofenapate decrease triglycerides or VLDL-triglycerides in Syrian hamsters and guinea pigs. Five out of the six PPAR α activators examined increase liver

to body weights in Syrian hamsters, but only one chemical out of seven examined increased liver to body weights in guinea pigs, and for that chemical perfluorodecanoic acid, there was conflicting evidence of increases. WY does not activate NF- κ B in hamsters, indicating that this response is species-specific. Differences were also seen between species in relationship to cell proliferation. Studies measuring changes in cell proliferation in Syrian hamsters showed either no response, a weak response, or inconsistent results. Multiple studies showed guinea pigs did not exhibit increases in cell proliferation to four chemicals. Syrian hamsters exhibited suppression of apoptosis after exposure to nafenopin, and guinea pigs exhibited suppression of apoptosis with nafenopin but no change with methylclofenapate. Cancer bioassays performed in Syrian hamsters with nafenopin, WY and DEHP were negative (Lake et al., 1993; Schmezer et al., 1988). In summary, although Syrian hamsters and to a lesser extent guinea pigs exhibit changes in endpoints associated with PPAR α activation (hypolipidemic effects and changes in fatty acid metabolizing enzymes), they do not exhibit consistent changes in the key events associated with the PPAR α activator MOA for liver cancer in rats and mice.

In vitro and in vivo data on *Cynomolgus* monkeys (Table 3) and other species of monkeys (marmoset, Rhesus) indicate that the key events in the PPAR α activator MOA are relatively nonresponsive in monkeys. Palmitoyl-CoA oxidase activity was evaluated in monkeys after in vivo exposure to a variety of PPAR α activators (e.g., bezafibrate, clofibrate, DEHP, MEHP, fenofibrate, nafenopin, and LY171883) and changes were minimal or non-existent relative to controls (Klaunig et al., 2003). Moreover, *Cynomolgus* monkeys exposed to DEHP, di-isononyl phthalate (DINP), or clofibrate failed to exhibit an increase in cell proliferation (Doull et al., 1999; Pugh et al., 2000). *Cynomolgus* monkeys treated for two weeks with clinically relevant doses of the PPAR α activators fenofibrate or ciprofibrate exhibited increases in the number of hepatic peroxisomes (Hoivik et al., 2004). In this study ciprofibrate but not

fenofibrate increased liver to body weights in the absence of hepatocyte proliferation. In a follow-up to this study, transcript profiling was used to characterize the genes altered by ciprofibrate exposure (Cariello et al., 2005). Many genes involved in fatty acid metabolism and mitochondrial oxidative phosphorylation were up-regulated, reflecting the known hypolipidemic effects of exposure. However, the magnitude of induction in the β -oxidation pathway was substantially less in monkeys compared to mice and rats. Consistent with the lack of hepatocyte proliferation, there were a number of key regulatory genes that were down-regulated, including members of the JUN, MYC, and NF- κ B families. In contrast, JUN and MYC gene expression were up-regulated after PPAR α activator treatment in rats (Hsieh et al., 1991). No transcriptional signal for DNA damage or oxidative stress was observed. Lastly, marmosets exposed for 6.5 years to clofibrate at relatively high doses (94 mg/kg/day or higher) did not develop liver tumors over the duration of this study (Tucker and Orton, 1995)². Taken together, the key events after PPAR α activation in the rodent MOA for liver tumors were not observed in primates treated with PPAR α activators.

Humans are generally nonresponsive to the effects of PPAR α activators. Liver weights were not increased in patients treated with fenofibrate (Gariot et al., 1987). Liver biopsies from humans treated with hypolipidemic drugs or primary human hepatocytes treated with PPAR α activators were almost uniformly negative for peroxisome proliferation (reviewed in Bentley et al., 1993). In only one out of five studies was there a statistically significant increase in peroxisome number (~50%), but there was no corresponding increase in volume of peroxisomes (Hanefeld et al., 1980; De La Iglesia et al., 1982; Blumcke et al., 1983; Gariot et al., 1983; Hanefeld et al., 1983).

Exposure to PPAR α activators alters different PPAR α gene targets in rodents and

humans, including the ACO gene. Unlike the large increases in the expression of marker mRNAs and proteins that are found in rodent primary hepatocytes treated with PPAR α activators in vitro, very minor increases, if any, are observed in human primary hepatocytes (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). ACO mRNA in liver samples from 48 patients treated with one of several fibrates (bezafibrate, fenofibrate or gemfibrozil) was not induced despite significant induction of hepatic apolipoprotein A-I mRNA and lowering of serum lipids following treatment (Roglans et al., 2002). The relatively weak increases in ACO observed in human primary hepatocytes are in stark contrast to the robust inductions observed in the livers of mice and rats exposed to PPAR α activators (summarized in Klaunig et al., 2003). In summary, there is no evidence that the ACO gene exhibits more than minor inductions in humans.

Species differences in sensitivity to PPAR α activators may be explained in part by differences in the structure of the promoter regions that regulate the expression of target genes. The lack of ACO induction in human livers and primary human hepatocytes may be attributable to an inactive PPRE. Evidence that a functional PPRE exists in the human ACO gene promoter (Varanasi et al., 1996), was challenged by subsequent studies which showed that the PPRE is inactive in in vitro trans-activation assays and that the sequence differs from that originally reported at 3 positions (Woodyatt et al., 1999). Little heterogeneity exists within the human ACO PPRE as the same altered PPRE sequence was found in all 22 unrelated humans that were investigated as well as in the human hepatoblastoma cell line HepG2 (Woodyatt et al., 1999). A nonfunctional PPRE in the ACO promoter would be consistent with studies showing little, if any induction of the ACO gene/protein expression upon exposure to PPAR α activators in human primary hepatocytes.

PPAR α 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., 1999, 2000b; Williams and Perrone, 1995). Many of these studies included a positive control to ensure that human hepatocytes were of sufficient quality to mount a positive growth response. In comparison, rat or mouse primary hepatocytes exposed to PPAR α activators exhibit up to 8-fold induction in cell proliferation (summarized in Klaunig et al., 2003). There are no data on human hepatocyte proliferation in vivo, although in vivo and in vitro data from nonhuman primates show cell proliferation is not induced by PPAR α activators (Table 3 and reviewed in Doull et al., 1999). In summary, available data suggest that PPAR α activators are unlikely to alter apoptosis and proliferation in human hepatocytes.

Molecular basis of species differences

In the following section, the properties of PPAR α and associated responses in the livers of rodents and primates are compared with an emphasis on human data, if available. The weight of evidence demonstrates that humans respond to PPAR α activators differently than rodents in that many of the typical markers of PPAR α activator exposure associated with hepatocarcinogenesis in rodents are absent in humans. Differences in the properties of PPAR α , including structure, function and expression, determine the underlying basis for human-rodent differences in the biological effects of PPAR α activators. The properties of mouse and rat PPAR α versus human PPAR α in liver are summarized in Table 4.

Allelic variants of human PPAR α . The human PPAR α (hPPAR α) is indistinguishable from the rodent PPAR α in overall structure (Sher et al., 1993; Tugwood et al., 1996; Mukherjee et al., 1994), but a number of allelic variants of hPPAR α have been isolated which possess properties different from the original cloned hPPAR α . The L162V variant containing an amino

acid change in the DNA-binding domain is found at an allelic frequency of ~0.025-0.073 in ethnically diverse populations (Flavell et al., 2000; Lacquemant et al., 2000; Tai et al., 2002). In North Indians, this allele is found at high frequencies (0.745) (Sapone et al., 2000). The hPPAR α L162V variant exhibits no response to low doses of WY but greater ligand-induced activity (up to ~4-fold) at higher doses compared to the wild-type receptor (Flavell et al., 2000; Sapone et al., 2000). Humans carrying this variant exhibit greater decreases in total serum cholesterol to the hypolipidemic, bezafibrate (Flavell et al., 2000). Three different Asian populations carry a hPPAR α variant (V227A) within the hinge region between the DNA binding and ligand binding domains at frequencies of 0.003-0.051 (Yamakawa-Kobayashi et al., 2002; Chan et al., 2006). This allele has been associated with decreases in serum cholesterol and triglycerides in a Japanese population (Yamakawa-Kobayashi et al., 2002) and in Chinese women (Chan et al., 2006). Because of increased interactions with a co-repressor, Nuclear Receptor Corepressor (NCoR), this variant exhibits decreased responsiveness to PPAR α activators (Liu et al., 2008). The hPPAR α -6/29 variant containing four amino acid substitutions is a dominant negative that binds to a PPARE but cannot be activated by PPAR α activators (James et al., 1998b). The hPPAR α -6/29 variant is likely very rare, as it was not detected in any of the 173 human subjects from two studies (Roberts, 1999; Sapone et al., 2000). Overall, some PPAR α allelic heterogeneity exists in human populations, but no variants have been identified that are more sensitive to low, environmentally-relevant doses of PPAR α activators than the “wild-type” human receptor. The field would benefit from a side-by-side comparison of wild type and hPPAR α variants in trans-activation assays to determine dose-response relationships of PPAR α activators.

Differences in ligand inducibility. Human PPAR α is not more sensitive than rodent

PPAR α to chemical activation. Most compounds activate the rodent receptor better or exhibit no differences between species. A number of environmentally-relevant chemicals and hypolipidemic agents were able to activate rat or mouse PPAR α at lower concentrations or to higher absolute levels than hPPAR α in side-by-side trans-activation studies. These PPAR α activators include WY (Keller et al., 1997; Maloney and Waxman, 1999; Takacs and Abbott, 2007), PFOA (Maloney and Waxman, 1999), perfluorooctanesulfonate (Shipley et al., 2004; Takacs and Abbott, 2007), and a number of phthalate ester metabolites (Bility et al., 2004 and summarized in Corton and Lapinskas, 2005). Some PPAR α activators show no differences between activation of the mouse and human PPAR α , including TCA, dichloroacetate, 2-ethylhexanoic acid (Maloney and Waxman, 1999), a number of phthalates (Bility et al., 2004), clofibrate (Keller et al., 1993), and PFOA (Vanden Heuvel et al., 2006). Only perfluorooctanesulfonamide (Shipley et al., 2004) was shown to modestly activate the human but not the rodent PPAR α at one lower dose (25 μ M vs. 34 μ M in human vs. mouse, respectively). Overall, the data indicate that hPPAR α is no more sensitive than the mouse or rat PPAR α to significant activation by environmentally-relevant PPAR α activators.

Expression of the PPAR α gene and protein. PPAR α expression is the factor most often cited for determining species-specific differences in PPAR α activator responsiveness. Palmer et al. (1998) used EMSA to determine the level of PPAR α that binds to a PPRE from the CYP4A6 gene. In 7 lysates from individual human livers in which PPAR α could be detected by the assay, the amounts were ~10-fold lower than those detected in the livers of CD-1 or BALB/cByJ mice and for the remainder of the 13 individual human livers, the amounts were below detection (>20-fold less than mouse liver). A 3-fold variation in the expression of the full-length PPAR α mRNA between human samples was noted. The data demonstrates that hPPAR α

in liver is expressed at levels far below that expressed in rodent liver. Additional studies evaluating expression and function of PPAR α in human liver are needed to more definitively determine the relative expression of PPAR α in rodents and humans. Such studies would benefit from better assessment of the degree of protein and mRNA degradation in the samples.

Truncated PPAR α . A truncated PPAR α variant has been identified in a number of labs and is called hPPAR α -8/14 (Tugwood et al., 1996), hPPAR_{SV} (Palmer et al., 1998), PPAR α _{tr} (Gervois et al., 1999), and PPAR α 2 (Hanselman et al., 2001). This truncated form lacks exon 6 due to alternative splicing, resulting in a hPPAR α lacking the hinge region and ligand binding domain. This form acts as a dominant negative, inhibiting the ability of the wild-type receptor to activate transcription, possibly by titrating out limiting amounts of co-activators (Gervois et al., 1999). The level of the mRNA of this form ranges from 10-50% of full-length hPPAR α mRNA (Palmer et al., 1998; Gervois et al., 1999; Roberts et al., 2000; Hanselman et al., 2001) similar to *Cynomolgus* monkeys (Hanselman et al., 2001). In comparison, this level is below 10% in mice and rats (Hanselman et al., 2001). A more definitive role for this truncated form awaits studies in which the levels of full-length and truncated hPPAR α forms are simultaneously measured with well-characterized hPPAR α target genes in primary human hepatocytes exposed to PPAR α activators.

Differences in transcriptional networks controlled by human and rodent PPAR α .

There is overwhelming evidence that the transcriptional networks controlled by PPAR α are different between humans and rodents and underlie species-specific differences in key events in the PPAR α MOA. Humans and rodents do share hypolipidemic effects of PPAR α activators but may achieve this beneficial effect through regulation of different gene sets. A number of genes are likely responsible for the therapeutic hypolipidemic effects of PPAR α activators in humans.

Many of these genes have functional PPREs that are transcriptionally regulated by human PPAR α , including apolipoprotein (apo) C-III (Hertz et al., 1995), lipoprotein lipase (Schoonjans et al., 1996), apo A-I (Vu-Dac et al., 1994), apo A-II (Vu-Dac et al., 1995), and carnitine palmitoyl transferase-I (Mascaro et al., 1998). Human PPAR α activation of apolipoprotein A-II and lipoprotein lipase transcription and suppression of apolipoprotein C-III expression are key to lowering serum triglycerides (Auwerx et al., 1996; Staels et al., 1997; Vu-Dac et al., 1995). Human apolipoprotein C-III can be down-regulated by fibrates in cultured human hepatocytes in the absence of changes in PPAR α target genes encoding peroxisomal enzymes including ACO, bifunctional enzyme, and thiolase (Lawrence et al., 2001c). Further, stably transfected HepG2 cells expressing either human or murine PPAR α at levels similar to rodent liver, respond to fibrates by increased expression of HMG-CoA synthase and carnitine palmitoyl transferase-I (CPT-I) but lack the typical robust induction of typical PPAR α targets, i.e., ACO, bifunctional enzyme, or thiolase (Hsu et al., 2001; Lawrence et al., 2001c; Tachibana et al., 2005). In a global analysis of gene expression, genes of the cytosolic, microsomal, and mitochondrial pathways involved in fatty acid transport and metabolism were up-regulated by clofibrate in both rodent and human hepatocyte cultures, whereas genes of the peroxisomal pathway of lipid metabolism were up-regulated only in rodents (Richert et al., 2003). Thus, PPAR α activation may lower lipid levels in humans and rodents through regulation of different sets of genes.

The human PPAR α does not possess all of the functions of the rodent PPAR α including the ability to regulate cell proliferation. Two mouse strains have been created which express the hPPAR α in the absence of mPPAR α (“humanized” PPAR α mice). In the TRE-hPPAR α mouse, PPAR α is under the control of a liver-specific promoter and is preferentially expressed in hepatocytes (Cheung et al., 2004); the cellular location of hPPAR α expression in the humanized

PPAR α mouse corresponds to the location of mPPAR α expression in wild-type mice, i.e., in hepatocytes but not Kupffer cells (Peters et al., 2000). The hPPAR α ^{PAC} mouse contains a 211 kilobase region encoding the regulatory and structural regions of the human PPAR α gene. The hPPAR α is expressed in the same tissues as those of the mouse PPAR α (Yang et al., 2008). The humanized PPAR α mouse strains do not respond to a PPAR α activator (WY) in the same manner as wild-type mice even though both strains express hPPAR α to levels comparable to mPPAR α in wild-type mice. The humanized mice exhibit increases in peroxisome proliferation, decreases in serum total triglycerides and normal activation of lipid metabolism genes including those involved in peroxisome proliferation. However, these mice do not exhibit increased expression of cell cycle genes or increased hepatocyte proliferation in response to a PPAR α activator as do wild-type mice (Cheung et al., 2004; Morimura et al., 2006; Yang et al., 2008). In a 38-44 week exposure study with the PPAR α activator WY, the TRE-hPPAR α mice were also resistant to PPAR α activator-induced liver cancer. Wild-type mice but not humanized mice exhibited a significant increase in liver tumors despite the fact that the humanized mice were exposed 6 weeks longer than the wild-type mice to the compound (Morimura et al., 2006). These studies show that hPPAR α is pharmacologically-active but does not regulate the full spectrum of responses necessary for hepatocarcinogenesis in rodents.

The molecular basis for differences between mouse and human PPAR α may be differences in the ability of the receptors to interact with transcriptional co-activators or to regulate miRNA cascades. Co-activators convey the transcriptional activation of the ligand-induced nuclear receptor to the transcriptional machinery. Elegant biochemical and crystallographic analyses have shown key interactions between co-activators and the ligand binding domains of nuclear receptors including PPAR family members (Xu and Li, 2008; Li et

al., 2008). The mouse and rat PPAR α ligand binding domains (LBD) do possess amino acid differences with human PPAR α LBD (Sher et al., 1993; Mukherjee et al., 1994; Tugwood et al., 1996). Amino acid differences in the LBD between mice and humans may uncouple receptor co-activator interactions in humans required for cell proliferation gene regulation while retaining those important in lipid metabolism gene regulation. Alternatively, differences in miRNA regulation may contribute to species differences, as the ability to regulate the let-7c cascade is lost in humanized mice in response to a PPAR α activator (Yang et al., 2008). Further studies are needed to define the specific mechanistic basis for species differences.

SUMMARY OF KEY DATA THAT SUPPORTS THE MODE OF ACTION

The PPAR α MOA describes the sequence of events beginning with PPAR α activation and leading to an increased incidence of liver tumors in rats and mice. This MOA exists independent of exposure to any particular chemical but has been shown to be triggered by chemicals collectively referred to as PPAR α activators. The overall weight of evidence supports a MOA that involves five key events. First, PPAR α activators activate PPAR α . Second, PPAR α activation leads to alterations in the expression of genes that regulate oxidative stress and increases in oxidative stress. Third, oxidative stress activates the transcription factor NF-kB. Fourth, NF-kB activation leads to increased cell proliferation and decreased apoptosis in the liver. Fifth, sustained growth signaling upon chronic exposure causes clonal expansion of initiated cells leading to preneoplastic foci and tumors, i.e., hepatocellular adenomas and carcinomas.

Table 5 summarizes the specificity and weight of evidence of the PPAR α activator MOA. The weight of evidence strongly supports the MOA due to the large number of studies

that have been carried out since the discovery of peroxisome proliferation by these chemicals in 1965 (Hess et al., 1965). PPAR α activation is by definition specific, because this key event is distinct from other initiating events such as CAR activation or increases in cytotoxicity. The other key events by themselves are considered to have low specificity, because these events are observed with other carcinogens. However, the key events when linked are considered to have high specificity because they are dependent on PPAR α . Table 1 provides examples of chemical-specific data evaluating whether the key events occur after exposure to 5 different PPAR α activators.

Evidence showing the mechanistic linkages between the key events of the MOA is summarized in Table 2. Studies that inhibit key events by genetic or biochemical means reveal such relationships because inhibition of one event blocks downstream events.

Additional support for the PPAR α MOA comes from a comparison of responses in rats and mice to “non-responsive” species such as Syrian hamsters, guinea pigs, and monkeys. These data is summarized in Table 3. Overall, these data shows that while all species exhibit a hypolipidemic response and alterations in lipid metabolism and transport genes, Syrian hamsters, guinea pigs and monkeys exhibit little if any changes in oxidative stress markers, NF-kB activation, and alterations of hepatocyte growth or tumor response (Klaunig et al., 2003).

RELEVANCE OF PPAR α ACTIVATOR-INDUCED RODENT LIVER TUMOR RESPONSE TO HUMANS

Although humans have been regularly exposed to PPAR α activators through administration of hypolipidemic pharmaceuticals, epidemiological studies have not provided evidence of increased incidence of liver neoplasms in humans exposed to PPAR α activators for

up to 13 years (summarized in Klaunig et al., 2003). Species comparisons of key events and other endpoints relevant to the PPAR α MOA show that mice and rats are much more responsive than humans (Table 6) and other species (e.g., hamsters, guinea pigs, and primates) (Table 3). Experimental evidence suggests the differences in responsiveness among species may be due to differences in promoter structure and/or function of PPAR α target genes, sensitivity of PPAR α to activation, the expression level of full-length and dominant negative forms of PPAR α and species differences in the ability of PPAR α to alter expression of genes involved in cell fate (Table 4). Overall, the weight of evidence suggests that although the rodent MOA is plausible in humans, humans would not be expected to respond with an hepatocarcinogenic effect from chronic exposure consistent with the original conclusion by an ILSI workgroup (Klaunig et al., 2003).

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Table 1. Occurrence of key events in the MOA after exposure to PPAR α activators.

Chemical	PPAR α activation	Oxidative stress	NF-kB activation	Increases in transient acute cell proliferation	Decreases in acute apoptosis	Increases in chronic cell proliferation	Increases in cell proliferation in preneoplastic foci	Liver tumors
WY-14,643	+ ^{26,27,29}	+ ^{2,6,7,8,9,16,18, 72,75} - ^{71,72}	+ ^{2,3,5,12,72}	+ ^{6,7,31,35}	+ ⁵⁵	+ ^{6,7,35}	+ ^{73,74}	+ ³⁵
DEHP	+ ^{26,27,28}	+ ^{8,10,14,15,20,40,41,59} - ^{8,23,40}		+ ^{31,32,42-45}	+ ⁴³	- ¹⁰		+ ¹⁰
Clofibrate	+ ^{28,29}	+ ^{9,15,21,24,51,76, 84} - ^{7,23,66}		+ ^{7,33,39,45}		+ ⁷ - ³⁹		+ ^{47,48}
Nafenopin	+ ^{28,30}	+ ^{9,22-24} - ^{25,65}	- ^{13,87}	+ ³⁵	+ ^{54,83}	+ ⁸⁰ +/- ³⁵	+ ³⁶	+ ^{35,49,62}
Ciprofibrate	+ ³⁰	+ ^{17,18}	+ ^{1,4,11,19}	+ ^{34,37,38}		+ ³⁴	+ ³⁷	+ ⁵⁰

Comments: In the table, (+) indicates the chemical was found to lead to the key event; (-) indicates the chemical was found not to lead to the key event; (+/-) indicates mixed results in the same study. PPAR α activation was measured using trans-activation assays. NF-kB activation refers to binding of NF-kB (p65:p50 heterodimer) to the NF-kB response element in electrophoretic mobility shift assays. Acute cell proliferation was measured in the livers of treated mice or rats usually with 7 days or less of exposure. Apoptosis was mostly measured in primary hepatocytes given the low background in intact livers. However, three studies have measured apoptosis in rodent livers after exposure to a PPAR α activator (Bursch et al., 1984; James et al., 1998; Youssef et al., 2003). Chronic cell proliferation was measured in the livers of mice or rats exposed to PPAR α activators, usually for > 3 weeks.

Noted in the references below: Studies were carried out with rats (R) or mice (M) or both species (M,R). The endpoint is indicated for studies that measured oxidative stress. If there are inconsistent effects, the possible origin of the inconsistency is indicated. In vitro studies are also noted.

References: ¹Calfee-Mason et al., 2004 (R); ²Fischer et al., 2002 (increase in TBARS but not conjugated dienes) (R); ³Rusyn et al., 2000b,c (M,R); ⁴Nilakantan et al., 1998 (M); ⁵Rusyn et al., 1998 (R); ⁶Wada et al., 1992 (lipofuscin) (R); ⁷Marsman et al., 1992 (lipofuscin; trend for increase in cell proliferation by clofibrate) (R); ⁸Conway et al., 1989 (lipofuscin – positive for both WY and DEHP but only WY positive for conjugated dienes) (R); ⁹Reddy et al., 1982 (lipofuscin) (R); ¹⁰Cattley et al., 1987 (lipofuscin) (R); ¹¹Tharappel et al., 2003 (M); ¹²Tharappel et al., 2001 (consistent changes with WY but only one condition resulted in increases in NF-kB activation after gemfibrozil treatment) (R); ¹³Menegazzi et al., 1997 (R); ¹⁴Rao et al., 1987a (lipofuscin) (R); ¹⁵Lake et al., 1987 (lipofuscin) (R); ¹⁶Rao et al., 1982 (lipofuscin) (R); ¹⁷Rao et al., 1991 (lipofuscin) (R); ¹⁸Goel et al., 1986 (lipid peroxidation and hydrogen peroxide) (R); ¹⁹Li et al., (1996) (R); ²⁰Hinton et al., 1986 (lipofuscin) (R); ²¹Stanko et al., 1995 (lipofuscin) (R); ²²Lake et al., 1989a (increases in oxidized glutathione and decreases in Vit E) (R); ²³Tomaszewski et al., 1990 (in vitro cultures; oxidized dienes) (R); ²⁴Cai et al., 1995 (lipid peroxidation – trend increases for PFOA, nafenopin and clofibrate) (M); ²⁶Bility et al., 2004 (in vitro trans-activation assays;(M); ²⁷Corton and Lapinskas, 2005 (review of in vitro trans-activation data) (M,R); ²⁸Isseman and Green, 1990 (in vitro trans-activation assays) (M); ²⁹Gottlicher et al., 1992 (in vitro trans-activation assays) (R); ³⁰Corton et al., 2000 (review) (M,R); ³¹Marsman et al., 1988 (R); ³²Smith-Oliver and Butterworth, 1987 (R); ³³Tanaka et al., 1992 (R); ³⁴Yeldandi et al., 1989 (chronic increases in cell proliferation) (R); ³⁵Lake et al., 1993 (R); ³⁶Schulte-Hermann et al., 1981 (R); ³⁷Chen et al., 1994 (R); ³⁸Dwivedi et al., 1989 (M); ³⁹Barrass et al., 1993 (R); ⁴⁰Seo et al., 2004 (malondialdehyde) (R); ⁴¹Isenberg et al., 2001 (M,R); ⁴²Isenberg et al., 2000 (M,R); ⁴³Hasmall et al., 2000a (R, in vivo (DEHP) and in vitro (MEHP)); ⁴⁴Soames et al., 1999 (R); ⁴⁵Busser and Lutz, 1987 (R); ⁴⁷Reddy and Qureshi, 1979 (R); ⁴⁸Svoboda and Arzarnoff, 1979 (R); ⁴⁹Reddy and Rao, 1977 (R); ⁵⁰Rao et al., 1986a (R); ⁵¹Elliott and Elcombe, 1987 (malondialdehyde – significant change for DEHP and clofibrate but trend increase for methyl clofenapate) (R); ⁵⁴James and Roberts, 1996 (in vitro) (M,R); ⁵⁵Youssef et al., 2003 (R); ⁵⁹Thottassery et al., 1992 (R); ⁶²Abdellatif et al., 1990 (initiated with DEN, 2-AAF and carbon tetrachloride) (R); ⁶⁶Nicholls-Grzemeski et al., 2000 (TBARS) (M); ⁷¹Soliman et al., 1997 (F₂-isoprostanes) (R); ⁷²Fischer et al., 2002

(TBARS increase with treatment but conjugated dienes do not) (R); ⁷³Marsman and Popp, 1994 (R); ⁷⁴Rose et al., 1999a (R); ⁷⁵O'Brien et al., 2001b (decreases in vitamin E) (R); ⁷⁶Qu et al., 2000; ⁸⁰Price et al., 1992 (R); ⁸³Bursch et al., 1984 (R, in vivo); ⁸⁴Dostalek et al., 2008 (M, increases in hydrogen peroxide, malondialdehyde, and urine F₂-isoprostanes but not liver F₂-isoprostanes); ⁸⁷Ohmura et al., 1996 (R).

Table 2. Effects of inhibition of key events in the PPAR α activator MOA.

Mechanism of inhibition	Key event					
	PPAR α activation	Oxidative stress	NF-kB activation	Alteration in hepatocyte growth	Clonal expansion	Liver tumors
Genetic inhibition						
PPAR α -null	↓ (by definition)	↓ ^{1,27} ↑ ³²	↓ ¹	↓ ^{2,3,4}	↓ ^{2,4}	↓ ^{2,4} ↑ ³²
Catalase transgenic	NC ¹¹		↓ ¹¹	↓ ¹¹		
P47Phox-null	NC ^{1,27,29}	↓ ²⁹ , NC ¹	↓ ²⁹ , NC ²⁷	↓ ²⁹ , NC ²⁷		
P50-null	NC ^{8,9}		↓ ⁸	↓ ^{8,9}	↓ ⁹	↓ ⁹
Biochemical inhibition						
Antioxidants in diet	NC ^{6,10}	↓ ^{7,16} ↑ ¹⁰	↓ ^{6,7,26}		↑ ¹⁰	↓ ^{5,12,13} ↑ ¹⁰
Dexamethasone	NC ^{14,15} ↓ ²³		↓ ^{17,18,24,25}	↓ ^{14,15,23}		
Glycine	NC ^{19,21}	↓ ^{20,22}		↓ ¹⁹ , NC ²¹		↓ ²¹
Methylpalmitate	NC ³⁰			↓ ³⁰		
Diphenylethylidenehydrazide	NC ²⁹	↓ ²⁹	↓ ²⁹	↓ ²⁹		

↓, inhibited; NC, no change; ↑, indicates increases in the parameters measured. For studies in which antioxidants were co-treated with PPAR α activators, the antioxidant is indicated in parentheses.

References: ¹Woods et al., 2007a; ²Peters et al., 1997; ³Peters et al., 1998; ⁴Hays et al., 2005; ⁵Rao et al., 1984 (ethoxyquin, 2(3)-tertbutyl-14-hydroxyanisole); ⁶Calfee-Mason et al., 2004 (Vit E); ⁷Li et al., 2000a (in vitro studies with Vit E treated H4IIE3C cells); ⁸Tharappel et al., 2003; ⁹Glauert et al., 2006; ¹⁰Glauert et al., 1990 (Vit E increases the number of tumors while depleting glutathione reserves); ¹¹Nilakantan et al., 1998; ¹²Rao and Subbarao, 1999 (dimethylthiourea); ¹³Rao and Subbarao, 1997a (deferoxamine – iron chelator); ¹⁴Lawrence et al., 2001a; ¹⁵Rao and Subbarao, 1997b (dexamethasone); ¹⁶Stanko et al., 1995 (Vit E); ¹⁷Ray and Prefontaine, 1994; ¹⁸Widen et al., 2003; ¹⁹Rose et al., 1997a,b; ²⁰Rose et al., 1999a (superoxide production in Kupffer cells); ²¹Rose et al., 1999b; ²²Rusyn et al., 2001 (free radicals in bile); ²³Ohmura et al., 1996 (measured peroxisomal bifunctional enzyme as PPAR α marker); ²⁴Chang et al., 1997; ²⁵De Bosscher et al., 2006 (review); ²⁶Rusyn et al., 1998 (allopurinol); ²⁷Woods et al., 2007b; ²⁹Rusyn et al., 2000b,c; ³⁰Rose et al., 1997b; ³²Ito et al., 2007.

Table 3. Species differences in responses to PPAR α activators.

Species	Relative PPAR α expression	Chemical	Response								
			PPAR α activation	Hypolipidemic effect (decreases in triglycerides or VLDL-triglycerides)	Increases in liver weight	Oxidative stress	NF-kB activation	Increases in acute cell proliferation	Decreases in apoptosis	Liver tumors	
Rats	Likely similar to mice	See table 1 for chemical and reference	+	+	+	+	+	+	+	+	
Mice	10	See table 1 for chemical and reference	+	+	+	+	+	+	+	+	
Syrian hamster	3	Nafenopin	+ ^{1,2,23}		+ ^{1,2,23}				- ^{1,2,17}	+ ¹⁷	- ¹
		WY-14,643	+ ^{1,7,8}	+ ^{7,9}	+ ^{1,7,8,9}		- ⁵	- ^{1,8}		- ¹	
		DEHP	(+) ^{4,27}		+ ^{4,27}			(+) ⁴		- ⁴⁶	
		Methyl clofenapate	+ ⁸	+ ^{7,9}	+ ^{7,8,9}			(+) ²⁸			
		Ciprofibrate	+ ^{8,22}		+ ^{8,22}			+ ²⁵ - ⁸			
		Bezafibrate	+ ²⁴		- ²⁴			- ⁸			
Guinea pig	1	Methylclofenapate	- ^{8,21}	+ ^{9,11}	- ⁸			- ^{8,25}	- ¹³		
		Ciprofibrate	+ ^{8,18} - ^{15,22}		- ^{8,22}			- ⁸			
		WY-14,643	+ ^{9,11,16} - ⁸	+ ^{9,11}	- ⁸			- ⁸			
		Nafenopin	+ ^{16,23} - ^{10,12}		- ^{12,23}			- ^{12,14,17}	+ ³²		
		Fenofibrate	- ¹⁹								
		Perfluorodecanoic	- ^{20,26}		+ ²⁶ - ²⁰						

		acid					
		Bezafibrate	- ²⁴		- ²⁴		
Cynomolgus monkey	?	DEHP	- ³		- ³		- ³
		DINP			- ³		- ³
		Clofibrate	- ³		- ³		- ³
		Fenofibrate	+ ⁶	- ⁶	- ⁶	- ⁶	- ⁶
		Ciprofibrate	+ ⁶ (+) ³³		+ ⁶	- ^{6,33}	- ⁶
Humans ³⁰	≤ 1	See footnotes for compound used	+ ³⁴ - ^{35-39,45}	+ ⁴⁰	- ³¹		- ^{41-44, 45} - ^{12,42,43,45}

Comments: PPAR α activation is a summary of trans-activation data as well as response of markers such as ACO and CYP4A gene, protein and enzymatic activity, which are indicators of PPAR α activation and are dependent on level of PPAR α expression. The endpoint examined in these studies is indicated below. + indicates a strong response, (+) indicates a weak response, and - indicates no response. Spaces left blank indicate no data available. It should be noted that the table does not include PCO data from monkey species other than Cynomolgus monkeys; other monkey data (which is almost universally negative) is summarized in Klaunig et al., 2003. PCO, palmitoyl-CoA oxidase.

¹Lake et al., 1993 (ACO); ²Price et al., 1992 (ACO); ³Pugh et al., 2000 (peroxisomal fatty acid beta-oxidation); ⁴Isenberg et al., 2000; ⁵Tharappel et al., 2001; ⁶Hoivik et al., 2004 (lipofuscin, peroxisome number, PCO); ⁷Choudhury et al., 2004 (CYP4A increases); ⁸Lake et al., 2000 (peroxisome proliferation, CYP4A and carnitine acetyl transferase); ⁹Choudhury et al., 2000 (trans-activation assay); ¹⁰MacDonald et al., 1999 (trans-activation assay); ¹¹Bell et al., 1998 (trans-activation assay); ¹²Hasmall et al., 1998 (nafenopin); ¹³Plant et al., 1998 (in vitro apoptosis assay); ¹⁴Elcock et al., 1998 (in vitro); ¹⁵Caira et al., 1998 (multifunctional protein, ACO, thiolase); ¹⁶Tugwood et al., 1998 (trans-activation assay); ¹⁷James and Roberts, 1996; ¹⁸Pacot et al., 1996 (ACO increases only 1.6-fold); ¹⁹Cornu-Chagnon et al., 1995 (ACO in vitro); ²⁰Chinje et al., 1994 (CYP4A); ²¹Bell et al., 1993 (CYP4A13); ²²Makowska et al., 1992 (ACO, CYP4A); ²³Lake et al., 1989b (ACO, CYP4A); ²⁴Watanabe et al., 1989 (slight increases in ACO); ²⁵Styles et al., 1988; ²⁶Van Rafelghem et al., 1987 (peroxisome proliferation); ²⁷Lake et al., 1987 (ACO in vivo and in vitro); ²⁸Styles et al., 1990; ³⁰Compounds used to treat humans or

human primary hepatocytes are indicated in the footnotes; ³¹Gariot et al., 1987 (fenofibrate); ³²James and Roberts, 1996; ³³Cariello et al., 2005 (fatty acid β -oxidation genes); ³⁴Hanefeld et al., 1983 (clofibrate); ³⁵Hanefeld et al., 1980 (clofibrate); ³⁶De La Iglesia et al., 1982 (gemfibrozil); ³⁷Blumcke et al., 1983 (fenofibrate); ³⁸Gariot et al., 1983 (fenofibrate); ³⁹Bentley et al., 1993 (review); ⁴⁰Klaunig et al., 2003 (review); ⁴¹Perrone et al., 1998 (clofibric acid, diprofibrate); ⁴²Goll et al., 1999 (clofibrate, ciprofibrate, bezafibrate, nafenopin, DEHP); ⁴³Hasmall et al., 1999 (MEHP, MINP, primary metabolite of DINP); ⁴⁴Hasmall et al., 2000b (MEHP); ⁴⁵Shaw et al., 2002 (MINP); ⁴⁶Schmezer et al., 1988.

Table 4. Properties of Rodent (Rat and Mouse) PPAR α Versus Human PPAR α in Liver

Property	Rodent (rat/mouse)	Human	Impact on responsiveness to PPARα activators in humans compared to mice and rats
Allelic variants	None identified	L162V	Exhibits greater ligand-induced activity at higher doses compared to the wild-type receptor; found at high frequencies in some populations
		V227A	Decreased responsiveness; rare variant
		“6/29”	Decreased responsiveness; acts as a dominant negative; very rare variant
Truncated PPAR α (deleted exon 6)	Below 10% of total PPAR α	10-50% of total PPAR α	Decreased responsiveness
Inducibility by environmentally-relevant ligands	Chemical-specific range of responsiveness	Some differences with rodent activation noted leading to decreased activation	Equal or decreased responsiveness
Basal expression of PPAR α	High in liver	<~10% of mice based on one study	Much lower responsiveness
Regulation of hypolipidemic response	Intact	Intact	No difference in endpoint but different genes may be regulated in the different species
Regulation of liver growth	Intact	No evidence	No response in humans because of fundamental differences in spectrum of genes regulated; hPPAR α does not regulate cell proliferation in mice.

TABLE 5. PPAR α Activators: Mode of Action (MOA) Key Events

Causal Key Event	Specificity	Evidence
1. Activation of PPAR α	High	Strong
2. Increases in oxidative stress	Low	Strong
3. NF-kB activation	Low	Strong
4. Perturbation of cell growth and survival	Low	Strong
5. Clonal expansion of preneoplastic foci	Low	Strong

Causal Key Event is a required step for PPAR α MOA, based on empirical evidence. Specificity of each key event to PPAR α induced rodent hepatic tumors is considered high if it is unique to this MOA and low if not. The key events other than PPAR α activation by themselves are considered to have low specificity, because these events are observed with other carcinogens. However, the key events when linked are considered to have high specificity because they are dependent on PPAR α .

Evidence was determined to be strong if several studies support that key event as part of the MOA, preferably with multiple PPAR α activators from multiple laboratories, with limited evidence of contradiction. Evidence is considered weak if only a single study with a single PPAR α activator from a single laboratory supports that key event or if a significant amount of contradiction appears in the literature.

TABLE 6. Comparative analysis of rodent and human data – liver tumors

Causal Key events	Plausible in humans?	Taking into account kinetic and dynamic factors, is the key event plausible in humans?	Comments
1. Activation of PPAR α	Yes	Yes	PPAR α is a target of human hypolipidemic drugs
2. Increases in oxidative stress	Yes	Unknown	Gene products that produce oxidative stress in rodents exist in humans but are not induced to the same extent in humans or monkeys. More traditional methods of measuring oxidative stress have not been used.
3. NF-kB activation	Yes	Unknown	NF-kB exists in humans but has not been measured in human liver or primary hepatocytes after exposure to PPAR α activators
4. Perturbation of cell growth and survival	Yes	Not likely	Not seen in independent studies of human hepatocytes in vitro; not measured in vivo; not seen in non-human primates in vivo or in vitro; not seen in hamsters or guinea pigs
5. Selective clonal expansion of preneoplastic foci	Yes	Not likely	No response in non-human primates
6. Liver tumors	Yes	Not likely	Not measured in livers of humans exposed to PPAR α activators; no tumors in hamsters with expression of PPAR α intermediate between mice/rats and humans

Figure Legend

FIGURE 1. Proposed mode of action (MOA) of rodent liver tumor induction by PPAR α activators. PPAR α activators including endogenous fatty acids activate the nuclear receptor PPAR α , which then regulates the transcription of different classes of genes including lipid metabolizing enzymes involved in the therapeutic hypolipidemic effects of PPAR α activators. Increased activity of oxidant generating enzymes leads to increases in oxidants and oxidative stress. Oxidative stress activates NF-kB (composed of p65 and p50 subunits) either directly by cell signaling or indirectly by increases in cytokine levels including TNF α , IL-1 and IL-6 released from activated Kupffer cells. NF-kB either directly or indirectly regulates genes involved in cell growth including those involved in cell proliferation and apoptosis. PPAR α activator exposure increases cell proliferation and decreases apoptosis in the liver. Preneoplastic foci that arise either spontaneously or through a mechanism that involves oxidative stress-induced DNA damage exhibit increases in cell proliferation compared to the surrounding parenchyma. Additional mutational or epigenetic changes may occur leading to hepatocellular adenomas and carcinomas. This proposed MOA is an extension of that proposed earlier (Klaunig et al., 2003) incorporating the findings of recent studies. The MOA is an endogenous pathway that can be activated independently of chemical exposure by perturbations in fatty acid levels.

Footnotes

¹ It should be noted that the authors combined different types of liver tumors in their analysis, a nonstandard method of analyzing tumor data leaving open the possibility that the increase in tumor response is actually not statistically significant.

²It should be noted that the duration of this study did not represent a life-time exposure (Tucker and Orton, 1995).

Figure 1.

