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Mode of Action Analysis and Human Relevance of Liver Tumors Induced by PPARα 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 (PPARα). Because of the increased understanding of the relationships between PPARα 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 PPARα, 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 PPARα activators could induce liver tumors in humans because of differences in PPARα 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 PPARα activation does not lead to cell proliferation or liver tumors. This analysis leads to the suggestion that the PPARα 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 (PPARα) in 1990 (Issemann and Green, 1990) followed by extensive work with the PPARα-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 "PPARα activator" to denote the central role PPARα 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 PPARα using biochemical assays. Thus, PPARα activators are those chemicals or their proximate metabolites that interact directly or indirectly with PPARα, initiating events that result in receptor activation. Although most chemicals likely act as classical agonists, there is evidence that other chemicals may activate PPARα secondary to

increases in the availability of natural ligands through perturbation of lipid homeostasis. For example, perfluorooctanoic acid (PFOA) may induce PPARα 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 PPARα 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 PPARα 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 it’s 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 PPARα 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 PPARα Activation

Substantial scientific research on the role of PPARα 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 PPARα activator has not been established, key events for the MOA leading to liver tumors have been identified (Figure 1). These include: activation of PPARα, 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 PPARα activators and liver tumor formation and that appear to be reliable markers a chemical has activated PPARα 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 PPARα activators induce liver tumors in rats and mice, the potential for PPARα 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 PPARα 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 PPARα across species.

There are several plausible explanations for the species-specific effects of PPARα activators. 1) Full-length PPARα 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 PPARα in the liver which inhibits the active PPARα. 3) Even when expressed at levels similar to the mouse PPARα, hPPARα does not induce the cell cycle machinery, cell proliferation or induced liver cancer in mice. 4) The PPARα 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 PPARα 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 PPARα 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 PPARα activators and are required for key events which lead to liver tumors. Although the rodent PPARα 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.

PPARα 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 PPARα activation, the key events in the MOA and liver cancer (see Table 1 for examples of 5 PPARα 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 PPARα 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 PPARα activators, (i.e.,

compounds that minimally induce markers of PPARα 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 PPARα 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).

PPARα 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 PPARα-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 PPARα activators (Klaunig et al., 2003).

Role of Oxidative Stress in PPARα 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 PPARα activators

(Table 1), determined by measuring lipid peroxidation (TBARS, conjugated dienes, lipofuscin, malondialdehyde, F_2-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 weigh of evidence demonstrates that PPARα activators increase oxidative stress.

Possible sources of ROS in the livers of rodents exposed to PPARα activators include enzymes that generate and degrade hydrogen peroxide and other reactive oxygen species. Hydrogen peroxide can oxidize DNA, lipids, and other molecules, and PPARα 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 PPARα 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 PPARα activators including increases in oxidative stress and induction of liver tumors that are dependent on PPARα (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 PPARα 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 PPARα 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 PPARα 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 PPARα-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 PPARα activator-induced oxidative stress. PPARα 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 PPARα 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 PPARα 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, PPARα activators increase the level of oxidative stress through multiple mechanisms. There is little direct evidence that increases in oxidative stress generated after PPARα activator exposure leads to direct or indirect DNA damage. The Ogg1-null mouse studies indicate that PPARα 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 PPARα Activator MOA

Central to the PPARα activator MOA is NF-kB activation. NF-kB transcription factors play critical roles in cancer development and progression (Karin, 2006; Arsura 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 PPARα

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 PPARα 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 PPARα activator in the H4IIEC3 rat hepatoma cell line, responsive to the proliferative effects of PPARα 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 PPARα 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, PPARα activators can promote the growth of spontaneously initiated hepatocytes.

All PPARα 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 PPARα 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 PPARα 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 PPARα (VP16PPARα) was compared to wild-type mice treated with WY (Yang et al., 2007). Expression of VP16PPARα 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 VP16PPARα did not increase liver tumors (Yang et al., 2007). These results indicate that nonparenchymal cell activation is important for hepatocarcinogenesis and that PPARα-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 PPARα activators.

Nongenotoxic carcinogens, in general, and PPARα 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 PPARα activators nafenopin, methylclofenapate, and WY suppress spontaneous hepatocyte apoptosis as well as that induced by a negative regulator of liver growth, transforming growth factor β1 (TGFβ1) (Bayly et al., 1994; Oberhammer and Qin, 1995) (Table 1). In addition, PPARα 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 (TNFα) 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 PPARα 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 PPARα activators. Consistent with this, two reports suggest that chronic exposure of rats and mice to the PPARα activator WY results in an increase in apoptosis (Marsman et al., 1992; Burkhardt et al., 2001). Furthermore, PPARα activators alter the ability of the liver to respond to apoptosis inducers. Sensitivity to two apoptosis inducers (Jo2 antibody and conconavalin A) was dramatically increased in wild-type but not PPARα-null mice exposed for 1 week to WY (Xiao et al., 2006). Lastly, both cell proliferation and apoptosis increase in parallel in PPARα 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 PPARα 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 PPARα 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-1α and interleukin-1β (IL1α, IL1β). These cytokines affect the fate of neighboring hepatocytes. TNFα 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 TNFα 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 PPARα activators is TNFα pathway-independent. Cell proliferation remained intact in TNF*α*-null and in TNF*α* receptor-null mice given a PPARα 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 TNFα 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 TNFα or IL-1 may play in PPARα 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 PPARα-null mice. These studies reveal a let-7C signaling cascade critical for PPARα activator-induced hepatocyte proliferation.

Other growth signaling pathways may be involved in PPARα 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 PPARα 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 PPARα 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 PPARα MOA. PPARα-null mice provided critical evidence establishing the rodent MOA for PPARα activator-induced hepatocarcinogenesis. Evidence that a particular compound induces key events in wild-type mice but not in mice lacking PPARα would be considered strong support for a PPARα 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 PPARα-null mice after acute or subacute exposures.

Two studies assessed markers of oxidative stress in wild-type and PPARα-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 PPARα-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 PPARα-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 PPARα-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 PPARα-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 PPARα-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 PPARα-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 PPARα.

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 PPARα activators and is important in cell proliferation after short-term PPARα 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 PPARα 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 PPARα (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 PPARα 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-kB acting as a negative regulator of apoptosis (Karin, 2006; Arsura 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-kB activation is necessary for hepatocarcinogenesis induced by a PPARα 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-kB 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-kB for liver tumor induction by a PPARα activator.

Biochemical inhibition studies using compounds that inhibit oxidative stress or inflammation also highlight linkages of the key events in the PPARα MOA. In these studies animals were pretreated with the inhibitor before PPARα activator exposure or co-treated with a PPARα activator and the inhibitor. The free radical scavenger and xanthine oxidase inhibitor allopurinol inhibited the activation of NF-kB 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-kB to activate a reporter gene in ciprofibrate-treated HIIE3C 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 PPARα 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-kB activation in the absence of effects on markers of PPARα 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-kB 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 PPARα 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 PPARα activation. Compounds that inhibit Kupffer cell activation (glycine, methylpalmitate) or inhibit NADPH oxidase (diphenyleneiodonium) inhibited increases in oxidative stress and NF-kB activation after exposure to PPARα activators but had no effects on markers of PPARα 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 PPARα activator MOA.

Some PPARα activators exhibit complex MOAs

Before a PPARα activator MOA can be defined as the primary MOA, alternative MOA(s) must be considered. Comparison of wild-type and PPARα-null mice have provided opportunities to determine if additional key event sare necessary in addition to PPARα activation. In one example, PFOA was analyzed for liver effects in wild-type and PPARα-null mice. At two doses tested (1 and 3 mg/kg/day), PPARα-null mice lacked increases in cell proliferation but retained increases in liver to body weights. At the highest dose tested (10 mg/kg/day) PPARα-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 PPARα-dependent manner at 3 mg/kg/day. The PPARα-independent genes exhibited signatures of activation of other nuclear receptors. In particular, the PPARα-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 PPARα-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 PPARα-null mice. In wild-type mice, there were relatively minor alterations of CAR signature genes compared to the strong changes in PPARα-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 PPARα-null mice for 22 months (Ito et al., 2007). A low level of liver tumors was observed in PPARα-null but not wild-type mice. These data suggest that an additional biological event may be operating in DEHP-induced rodent liver tumors[1]. The tumors in PPARα-null mice most likely arose through a mechanism that is not dominant in wild-type mice. Wild-type and PPARα-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 PPARα-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 PPARα-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 PPARα, 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 PPARα-independent effects defined in part as effects observed in PPARα-null mice. These effects may suggest additional key events that become the main control points in the absence of PPARα. 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 PPARα 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 PPARα 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 PPARα 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 PPARα were shown to be PPARα-dependent as the changes were abolished in a double ACO-/PPARα-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 PPARα 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 PPARα 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 PPARα-dependent (Tanaka et al., 2008a,b). The authors conclude that there "is the absolute requirement of persistent PPARα 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 PPARα including fatty acids (Fan et al., 1998; Tanaka et al., 2008a,b). Taken together, the PPARα MOA is operational in the absence of chemical exposure. Chemical PPARα activators will persistently activate this MOA resulting in liver tumors.

Species differences in responsiveness of key events in the PPARα MOA

Studies conducted in numerous test species indicate that while some rodents (mice and rats) are highly responsive to PPARα 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 PPARα expression among species. In a side-by-side comparison, mice had ~10-fold more PPARα expression than guinea pigs and ~3-fold more than Syrian hamsters (Choudhury et al., 2004). Humans exhibited ≥ 10-fold lower expression than mice and rats (described in greater detail below). Thus, guinea pigs may be the more relevant model for PPARα activator effects in the human liver based solely on expression levels of the full-length active PPARα.

Table 3 summarizes PPARα 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 PPARα activators are included (i.e., liver weight to body weight, hypolipidemic effects).

Syrian hamsters and guinea pigs exhibit a partial PPARα 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 PPARα activator exposure in the livers of these species. Diminished responsiveness in guinea pigs is not due to a defective PPARα because when over-expressed in cell lines, PPARα 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). PPARα activators WY or methylclofenapate decrease triglycerides or VLDL-triglycerides in Syrian hamsters and guinea pigs. Five out of the six PPARα 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-kB 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 PPARα activation (hypolipidemic effects and changes in fatty acid metabolizing enzymes), they do not exhibit consistent changes in the key events associated with the PPARα 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 PPARα activator MOA are relatively nonresponsive in monkeys. Palmitoyl-CoA oxidase activity was evaluated in monkeys after in vivo exposure to a variety of PPARα 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 PPARα 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-kB families. In contrast, JUN and MYC gene expression were up-regulated after PPARα 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 PPARα activation in the rodent MOA for liver tumors were not observed in primates treated with PPARα activators.

Humans are generally nonresponsive to the effects of PPARα 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 PPARα 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 PPARα activators alters different PPARα 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 PPARα 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 PPARα 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.

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., 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 PPARα 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 PPARα activators (Table 3 and reviewed in Doull et al., 1999). In summary, available data suggest that PPARα activators are unlikely to alter apoptosis and proliferation in human hepatocytes.

Molecular basis of species differences

In the following section, the properties of PPARα 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 PPARα activators differently than rodents in that many of the typical markers of PPARα activator exposure associated with hepatocarcinogenesis in rodents are absent in humans. Differences in the properties of PPARα, including structure, function and expression, determine the underlying basis for human-rodent differences in the biological effects of PPARα activators. The properties of mouse and rat PPARα versus human PPARα in liver are summarized in Table 4.

Allelic variants of human PPARα. The human PPARα (hPPARα) is indistinguishable from the rodent PPARα in overall structure (Sher et al., 1993; Tugwood et al., 1996; Mukherjee et al., 1994), but a number of allelic variants of hPPARα have been isolated which possess properties different from the original cloned hPPARα. 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 hPPARα 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 hPPARα 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 PPARα activators (Liu et al., 2008). The hPPARα-6/29 variant containing four amino acid substitutions is a dominant negative that binds to a PPRE but cannot be activated by PPARα activators (James et al., 1998b). The hPPARα-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 PPARα allelic heterogeneity exists in human populations, but no variants have been identified that are more sensitive to low, environmentally-relevant doses of PPARα activators than the "wild-type" human receptor. The field would benefit from a side-by-side comparison of wild type and hPPARα variants in trans-activation assays to determine dose-response relationships of PPARα activators.

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

PPARα 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 PPARα at lower concentrations or to higher absolute levels than hPPARα in side-by-side trans-activation studies. These PPARα 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 PPARα activators show no differences between activation of the mouse and human PPARα, 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 PPARα at one lower dose (25 uM vs. 34 uM in human vs. mouse, respectively). Overall, the data indicate that hPPARα is no more sensitive than the mouse or rat PPARα to significant activation by environmentally-relevant PPARα activators.

Expression of the PPARα gene and protein. PPARα expression is the factor most often cited for determining species-specific differences in PPARα activator responsiveness. Palmer et al. (1998) used EMSA to determine the level of PPARα that binds to a PPRE from the CYP4A6 gene. In 7 lysates from individual human livers in which PPARα 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 PPARα mRNA between human samples was noted. The data demonstrates that hPPARα

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

Truncated PPARα. A truncated PPARα variant has been identified in a number of labs and is called hPPARα-8/14 (Tugwood et al., 1996), $hPPAR_{SV}$ (Palmer et al., 1998), $PPAR\alpha_{tr}$ (Gervois et al., 1999), and PPARα2 (Hanselman et al., 2001). This truncated form lacks exon 6 due to alternative splicing, resulting in a hPPARα 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 hPPARα 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 hPPARα forms are simultaneously measured with well-characterized hPPARα target genes in primary human hepatocytes exposed to PPARα activators.

Differences in transcriptional networks controlled by human and rodent PPARα. There is overwhelming evidence that the transcriptional networks controlled by PPARα are different between humans and rodents and underlie species-specific differences in key events in the PPARα MOA. Humans and rodents do share hypolipidemic effects of PPARα 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 PPARα activators in humans.

Many of these genes have functional PPREs that are transcriptionally regulated by human PPARα, 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 PPARα 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 PPARα 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, PPARα activation may lower lipid levels in humans and rodents through regulation of different sets of genes.

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

PPARα mouse corresponds to the location of mPPARα expression in wild-type mice, i.e., in hepatocytes but not Kupffer cells (Peters et al., 2000). The hPPARα^{PAC} mouse contains a 211 kilobase region encoding the regulatory and structural regions of the human PPARα gene. The hPPARα is expressed in the same tissues as those of the mouse PPARα (Yang et al., 2008). The humanized PPARα mouse strains do not respond to a PPARα activator (WY) in the same manner as wild-type mice even though both strains express hPPARα to levels comparable to mPPARα 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 PPARα 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 PPARα activator WY, the TRE-hPPARα mice were also resistant to PPARα 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 hPPARα 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 PPARα 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 PPARα ligand binding domains (LBD) do possess amino acid differences with human PPARα 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 PPARα 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 PPARα MOA describes the sequence of events beginning with PPARα 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 PPARα activators. The overall weight of evidence supports a MOA that involves five key events. First, PPARα activators activate PPARα. Second, PPARα 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 PPARα 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). PPARα 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 PPARα.Table 1 provides examples of chemical-specific data evaluating whether the key events occur after exposure to 5 different PPARα 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 PPARα 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 PPARα ACTIVATOR-INDUCED RODENT LIVER TUMOR RESPONSE TO HUMANS

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

up to 13 years (summarized in Klaunig et al., 2003). Species comparisons of key events and other endpoints relevant to the PPARα 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 PPARα target genes, sensitivity of PPARα to activation, the expression level of full-length and dominant negative forms of PPARα and species differences in the ability of PPARα 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 PPARα activators.

Chemical	PPARα 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. PPARα 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 PPARα 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 PPARα 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: [1]Calfee-Mason et al., 2004 (R); [2]Fischer et al., 2002 (increase in TBARS but not conjugated dienes) (R); [3]Rusyn et al., 2000b,c (M,R); [4]Nilakantan et al., 1998 (M); [5]Rusyn et al., 1998 (R); [6]Wada et al., 1992 (lipofuscin) (R); [7]Marsman et al., 1992 (lipofuscin; trend for increase in cell proliferation by clofibrate) (R); [8]Conway et al., 1989 (lipofuscin – positive for both WY and DEHP but only WY positive for conjugated dienes) (R); [9]Reddy et al., 1982 (lipofuscin) (R); [10]Cattley et al., 1987 (lipofuscin) (R); [11]Tharappel et al., 2003 (M); [12]Tharappel et al., 2001 (consistent changes with WY but only one condition resulted in increases in NF-kB activation after gemfibrozil treatment) (R); [13]Menegazzi et al., 1997 (R); [14]Rao et al., 1987a (lipofuscin) (R); [15]Lake et al., 1987 (lipofuscin) (R); [16]Rao et al., 1982 (lipofuscin) (R); [17]Rao et al., 1991 (lipofuscin) (R); [18]Goel et al., 1986 (lipid peroxidation and hydrogen peroxide) (R); [19]Li et al., (1996) (R); [20]Hinton et al., 1986 (lipofuscin) (R); [21]Stanko et al., 1995 (lipofuscin) (R); [22]Lake et al., 1989a (increases in oxidized glutathione and decreases in Vit E) (R); [23]Tomaszewski et al., 1990 (in vitro cultures; oxidized dienes) (R); [24]Cai et al., 1995 (lipid peroxidation – trend increases for PFOA, nafenopin and clofibrate) (M); [26]Bility et al., 2004 (in vitro trans-activation assays;(M); [27]Corton and Lapinskas, 2005 (review of in vitro trans-activation data) (M,R); [28]Isseman and Green, 1990 (in vitro trans-activation assays) (M); [29]Gottlicher et al., 1992 (in vitro trans-activation assays) (R); [30]Corton et al., 2000 (review) (M,R); [31]Marsman et al., 1988 (R); [32]Smith-Oliver and Butterworth, 1987 (R); [33]Tanaka et al., 1992 (R); [34]Yeldandi et al., 1989 (chronic increases in cell proliferation) (R); [35]Lake et al., 1993 (R); [36]Schulte-Hermann et al., 1981 (R); [37]Chen et al., 1994 (R); [38]Dwivedi et al., 1989 (M); [39]Barrass et al., 1993 (R); [40]Seo et al., 2004 (malondialdehyde) (R); [41]Isenberg et al., 2001 (M,R); [42]Isenberg et al., 2000 (M,R); [43]Hasmall et al., 2000a (R, in vivo (DEHP) and in vitro (MEHP)); [44]Soames et al., 1999 (R); [45]Busser and Lutz, 1987 (R); [47]Reddy and Qureshi, 1979 (R); [48]Svoboda and Arzarnoff, 1979 (R); [49]Reddy and Rao, 1977 (R); [50]Rao et al., 1986a (R); [51]Elliott and Elcombe, 1987 (malondialdehyde – significant change for DEHP and clofibrate but trend increase for methyl clofenapate) (R); [54]James and Roberts, 1996 (in vitro) (M,R); [55]Youssef et al., 2003 (R); [59]Thottassery et al., 1992 (R); [62]Abdellatif et al., 1990 (initiated with DEN, 2-AAF and carbon tetrachloride) (R); [66]Nicholls-Grzemski et al., 2000 (TBARS) (M); [71]Soliman et al., 1997 (F_2-isoprostanes) (R); [72]Fischer et al., 2002

(TBARS increase with treatment but conjugated dienes do not) (R); [73]Marsman and Popp, 1994 (R); [74]Rose et al., 1999a (R); [75]O'Brien et al., 2001b (decreases in vitamin E) (R); [76]Qu et al., 2000; [80]Price et al., 1992 (R); [83]Bursch et al., 1984 (R, in vivo); [84]Dostalek et al., 2008 (M, increases in hydrogen peroxide, malondialdehyde, and urine F_2-isoprostanes but not liver F_2-isoprostanes); [87]Ohmura et al., 1996 (R).

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

	Key event					
Mechanism of inhibition	PPARα activation	Oxidative stress	NF-kB activation	Alteration in hepatocyte growth	Clonal expansion	Liver tumors
Genetic inhibition						
PPARα-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]		
Diphenyleneiodonium	NC[29]	↓[29]	↓[29]	↓[29]		

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

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

Table 3. Species differences in responses to PPARα activators.

			Response							
			PPARα 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
Species	Relative PPARα expression	Chemical								
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: PPARα 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 PPARα activation and are dependent on level of PPARα 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.

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

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

Table 4. Properties of Rodent (Rat and Mouse) PPARα Versus Human PPARα 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 PPARα (deleted exon 6)	Below 10% of total PPARα	10-50% of total PPARα	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 PPARα	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; hPPARα does not regulate cell proliferation in mice.

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

Causal Key Event	Specificity	Evidence
1. Activation of PPARα	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 PPARα MOA, based on empirical evidence
Specificity of each key event to PPARα induced rodent hepatic tumors is considered high if it is unique to this MOA and low if not. The key events other than PPARα 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 PPARα.

Evidence was determined to be strong if several studies support that key event as part of the MOA, preferably with multiple PPARα activators from multiple laboratories, with limited evidence of contradiction. Evidence is considered weak if only a single study with a single PPARα 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 PPARα	Yes	Yes	PPARα 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 PPARα 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 PPARα activators; no tumors in hamsters with expression of PPARα intermediate between mice/rats and humans

Figure Legend

FIGURE 1. Proposed mode of action (MOA) of rodent liver tumor induction by PPAR*α* activators. PPARα activators including endogenous fatty acids activate the nuclear receptor PPARα, which then regulates the transcription of different classes of genes including lipid metabolizing enzymes involved in the therapeutic hypolipidemic effects of PPARα 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 TNFα, 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. PPARα 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.

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

Figure 1.