



NUCLEAR RECEPTOR BINDING DOMAIN: FIGURE FROM:
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DOSE-RESPONSE APPROACHES FOR NUCLEAR RECEPTOR- MEDIATED MODES OF ACTION

WORKSHOP

SEPTEMBER 27 - 29, 2010

NATIONAL INSTITUTE FOR ENVIRONMENTAL HEALTH
SCIENCES (NIEHS) RESEARCH TRIANGLE PARK, NC

PPAR Case Study

...EXPLORING THE DEVELOPMENT OF BIOLOGICALLY-BASED DOSE-RESPONSE APPROACHES FOR
NUCLEAR RECEPTOR MEDIATED TOXICITY...

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Tab 1 - Background

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PPAR Panel Members

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**The PPAR α MOA Case Study
and Expert Panel Review Agenda**

Monday, September 27th, 2010

- 1:30 - 2:00** Assembly, opening remarks and charge; PPAR α -mediated liver growth in rodents: review of key events in PPAR α activator-induced rodent liver tumor formation: *Christopher Corton and James Klaunig – Co Chairs*
- 2:00 - 2:45** PPAR α Activation as a Key Event: *Jeffrey Peters, discussion leader*
- 2:45 - 3:00** **BREAK**
- 3:00 - 3:35** PPAR α -Mediated Liver Growth in Rodents: *Michael Cunningham, discussion leader*
- 3:35 - 3:50** PPAR α -Mediated Liver Pathology and Adaptive Changes: *James Popp, discussion leader*
- 3:50 - 4:20** Role of Oxidative Stress and NFKb: *Christopher Corton, discussion leader*
- 4:20 - 4:35** PPAR α -Specific Data on Other Events That May Be Involved in Carcinogenesis: *James Klaunig, discussion leader*
- 4:35 - 4:50** **BREAK**
- 4:50 - 5:05** Species Differences and Other Factors Impacting Risk Assessment: *Timothy Hummer and Phillip Bentley, discussion leaders*
- 5:05 - 5:30** Consideration of objectives for Day 2 including “homework” assignments
- 7:00** **DINNER - Hotel**

Tuesday, September 28th, 2010

Human Relevance Framework Discussions

- 8:00 - 8:30** Review of Day 1, comments and questions: *James Klaunig, discussion leader*

8:30 - 10:00 IPCS Framework Analysis of MOA for PPAR α -induced Rodent Liver Tumors: *Jennifer Seed, discussion leader*

10:00 - 10:15 **BREAK**

10:15 - 10:45 IPCS Framework Analysis of MOA for PPAR α -induced Rodent Liver Tumors, Continued: *Jennifer Seed, discussion leader*

10:45 - 11:30 Biologically Based Dose-Response Modeling for Hepatocarcinogenic Effects of PPAR α activators: *Lorenz Rhomberg, discussion leader*

11:30 - 12:00 Discussion

12:00 - 1:00 **LUNCH**

1:00 - 5:30 Continue Discussion with Breaks as Needed

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

Christopher Corton and James Klaunig

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 mono 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) (Issemann and Green, 1990) followed by extensive work with the PPAR α -null mouse model has provided the molecular underpinnings 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 the following materials, 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 methods. 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.

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).

Because of the increased understanding of the relationships between PPAR α activation and hepatocarcinogenesis, the purpose of this review is to critically reevaluate the state of the science on the rodent mode of action (MOA) of liver tumor induction and

human relevance. 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 we will focus our efforts on the mode of action of PPAR α activator-induced liver tumors.

This case study will review and discuss the PPAR α - mediated mode of action (MOA). The objective of this effort is to use the weight of evidence to define the MOA in terms of the key events derived from in vivo, pathology, molecular, cellular and genomic data for PPAR α activators. In addition, the characterization of the dose-response patterns for the individual key events will be examined to better characterize the dose-response behavior for the apical outcome (i.e., liver tumors) induced through activation of the PPAR α .

The discussion charge questions have been developed building on the IPCS Human Relevance Framework (IPCS 2007) and the modified Hill Criteria for Causality (EPA, 2005) for evaluating the MOA for PPAR α activation. The underlying mechanistic knowledge of PPAR α in pharmacology and toxicology is well characterized. While several previous exercises have characterized the MOA of PPAR α activators in rodent liver tumor induction, data has been published since that provides additional knowledge of biological processes that both impact the key and associated events and may refine our understanding of the overall dose-response behavior. To capture the impact of this degree of mechanistic understanding, refinements to the current IPCS (2007) framework, as being developed by ILSI and others, are being used to characterize the nature of the biological steps involved.

We will utilize two well studied PPAR α activators that induce rodent liver tumors as our model compounds – DEHP and clofibrate.

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Discussion Questions for PPAR α Panel

Introduction and Definitions

This case study reviews a PPAR α -mediated mode-of-action (MOA). The objective of this effort is to use the weight of evidence for the key events (including those derived from molecular, cellular and genomic data) for PPAR α -agonists. In addition, accompanying dose-response data will be used to better characterize the likely dose-response behavior for apical outcomes (e.g., liver tumors) induced through activation of the PPAR α . The goal is to recommend dose-response modeling approaches that most accurately reflect the underlying biology, when the data are available, or identify needed data.

The discussion questions have been developed building on the IPCS Human Relevance Framework (IPCS 2007) and the modified Hill Criteria for Causality (EPA, 2005) for evaluating the MOA for PPAR α activation. Because the underlying mechanistic knowledge of PPAR α is relatively well- characterized, additional knowledge of biological processes beyond the major key events is available to refine our understanding the overall dose-response behavior. To capture the impacts of this degree of mechanistic understanding refinements to the current IPCS (2007) framework, as being developed by ILSI and others, are being used to characterize the nature of the biological steps involved.

Important definitions included in the charge to the expert panel include:

Key Event: An empirically observable causal precursor step to the adverse outcome that is itself a necessary element of the mode of action. Key events are required events for the MOA, but often are not sufficient to induce the adverse outcome in the absence of other key events.

Associative Event: Biological processes that are themselves not causal necessary key events for the MOA, but are reliable indicators or markers for key events. Associative events can often be used as surrogate markers for a key event in a MOA evaluation or as indicators of exposure to a xenobiotic that has stimulated the molecular initiating event or a key event.

Modulating Factor: There are many factors or biological responses that are not necessary to induce the adverse outcome, but could modulate the dose-response behavior or probability of inducing one or more key events or the adverse outcome. Such biological factors are considered modulating factors. Example: excessive body weight loss at a high dose.

Discussion Questions

A. MOA for PPAR α activators in Rodent Liver Cancer

1. What is the Mode of Action for PPAR α -mediated rodent liver tumors for a model activator (e.g., DEHP and clofibrate), as evaluated by the IPCS Framework and Hill Criteria of causation?
2. Which Events are Key (Causal)?
3. Which Events are Associative?
4. Which Events are Modulating Factors? (Are there Key events that are not mediated via PPAR α activation?)
5. What is the human relevance of this MOA following the ILSI and IPCS Framework?

B. Dose-Response

1. Are the existing data sufficient to determine the dose-response relationship for these potential key events? Is the existing description of mathematical and statistical models for characterizing these key events complete?
2. Is the existing description of concentration/dose-response data for these key events sufficient for dose-response modeling?
3. If not, what are the key data gaps?
4. Does the current understanding of the data best support a threshold or non-threshold dose-response approach for nuclear receptor-mediated secondary key events?
5. On theoretical or practical grounds, is there an amount of ligand that would be insufficient to cause these key events?

C. Outcome

1. Does knowledge of MOA and dose-response determine the appropriate model for either precursor events or apical outcomes? If not, what are the key data gaps?

D. Forward-looking Questions

1. What is the minimum series of assays, tests, experiments, or studies that would specifically confirm this mode of action and rule out others for a compound that has induced liver tumors, or could be reasonably expected to induce liver tumors, based on its likelihood of acting as a PPAR α activator?
2. How to bring multiple receptor interactions into this process? (Work with the other WG to determine if there are assays which can help measure the key events in each MOA.)
3. Does the IPCS Framework need to be modified or updated to encompass this current knowledge and approach?
4. What would be the most appropriate data to generate to do a risk assessment on these molecules in the future?

Tab 2 - Presentations

Tab 2 - Presentations

PPAR Case Study Group Draft Presentation Abstracts and Outlines

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PPAR α Activation as a Key Event: Jeffery Peters

Penn State University

The most definitive evidence to date demonstrating the essential and causal role of PPAR α in PPAR α agonist-induced hepatocarcinogenesis is the observation that *PPAR α* -null mice fed Wy-14,643 for 11 months do not exhibit liver tumors [1]. In contrast, a 100% incidence of liver tumorigenesis was observed in wild-type mice [1]. Another study also found that *PPAR α* -null mice are refractory to the hepatocarcinogenic effects of another PPAR α agonist, bezafibrate fed for one year [2]. However, in this study, one adenoma was found in one bezafibrate-fed *PPAR α* -null mouse. Collectively, these two studies provide solid support to demonstrate the causal role of activating PPAR α in PPAR α agonist-induced hepatocarcinogenesis.

More recently, Ito et al. reported that *PPAR α* -null mice fed DEHP for two years develop liver tumors [3]. Since the monoester metabolite of DEHP (MEHP) can activate PPAR α [4], this has led to speculation that PPAR α is not required to mediate the hepatocarcinogenic effects of all PPAR α agonists [5]. However, the phenotype of the *PPAR α* -null mice may preclude this interpretation. *PPAR α* -null mice exhibit increased lipid accumulation in the liver [6], and hepatic lipid accumulation is known to increase the risk of developing liver cancer. Further, PPAR α also inhibits inflammatory signaling by interfering with NF κ B [7], thus *PPAR α* -null mice also exhibit enhanced inflammation due to the absence of expression of this receptor. These observations suggest that the liver tumorigenesis observed in *PPAR α* -null mice fed DEHP for two years could be influenced by enhanced hepatic lipid accumulation and/or hepatic inflammation. Thus, the MOA for liver tumors in *PPAR α* -null mice could be entirely different than that found with other more potent PPAR α agonists. Further studies using broader dose-dependent analysis in wild-type and *PPAR α* -null mice are needed to examine this idea.

Activation of PPAR α does occur in humans, this is the basis for the use of fibrates as hypolipidemic agents. However, while activating the human PPAR α modulates lipid catabolism by regulating expression of target genes that facilitate this process, the evidence to date suggests that the human PPAR α does not regulate the genes required to increase cell proliferation [8-10]. However, these data are limited to analysis of “humanized” mice; related evidence in humans or non-human primate would provide better support to establish the basis for the observed species difference.

Activation of PPAR α can be measured by a variety of approaches. Reporter assays can be used, but the model has to control for the potential release of endogenous agonists. Quantifying expression of PPAR α target genes is also acceptable, acyl-CoA oxidase and cytochrome P450 4A are two well-characterized examples.

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PPAR α -Mediated Liver Growth in Rodents: Michael Cunningham

NIEHS-NTP

Abstract to be provided

PPAR α -Mediated Liver Pathology and Adaptive Changes: James Popp

Stratoxon LLC

Selective Clonal Expansion of the Preneoplastic Population

Multiple histological effects in the liver of rodents have been described following the administration of molecules that activate PPAR α . These effects extend from relatively acute effects such as peroxisome proliferation noted within days of onset of administration to the formation of hepatocellular cancer that develops after many months to lifetime administration of inciting agent. To understand the relevance of the cancers for human risk assessment, it is important to understand the sequential morphologic steps leading to cancer and to determine if there are unique biological pathways for development of the cancers compared to development of liver cancer by other rodent hepatocarcinogens. Based on available information to date there is no basis to assume that liver cancer formation is different between rats and mice exposed to PPAR α agonists so the following description is applicable to both species.

Early events preceding clonal expansion of altered hepatocytes include the proliferation of peroxisomes in normal hepatocytes either diffusely or at least in specified lobular areas, most frequently central lobular areas. Likewise, enhanced hepatocyte replication has been identified in normal hepatocytes following administration of PPAR α activating molecules and before the appearance of foci of cellular alteration. Such replication is most noticeable within a few days after the initiation of continuous administration of the PPAR α agonist although prolonged or continued enhanced hepatocyte replication may be noted with some molecules. The role of this early wave of cell proliferation to subsequent developments of foci of cellular alteration representing clonal expansion is unclear.

The appearance of foci of hepatocellular alteration is observed generally after several months of treatment with PPAR α agonists. Such foci are frequently basophilic compared to the surrounding hepatocytes but may have other staining properties. These foci tend to increase in number and size with continued administration. However, these foci are apparently dependent on the continued administration of the inciting agent since they may regress on cessation of the inciting agent. However, it should be noted that the fate of the focal hepatocytes during regression is uncertain since it is not known whether the cells totally disappear or alternatively if some cells remain but lose their defining histological staining properties.

As would be expected, the foci have an enhanced hepatocyte replication rate compared to the surrounding tissues. The difference in replicative rate may be dependent on agent and/or dose although this point has been incompletely characterized. In addition, a reduced rate of hepatocyte apoptosis has been noted in foci with some PPAR α agonists. The enhanced cell proliferation and the reduced apoptosis in hepatocytes in foci account for the expansion of the lesion.

Hepatocellular tumors frequently develop after continued administration of PPAR α agonists. Both benign and malignant tumors are observed. There is a morphologic continuity for the foci, benign and malignant tumors strongly suggesting that the tumors are the direct result of expanding hepatocytes originating in foci of cellular alteration.

The molecular basis for the clonal expansion to tumor formation is not clearly established. However, the evaluation of hepatocellular tumors from PPAR α null mice compared to the tumors in wild type mice each exposed to DEHP suggest a different molecular mechanism for tumor formation between DEHP induced and spontaneous tumors (J Occup Health 50: 169-180, 2008). The molecular basis for the DEHP tumors compared to tumors related to treatment with other nongenotoxic agents is unclear.

The entire series of morphologic events occurring in the development of neoplasms following administration of PPAR α agonists is similar to the events that occur with nongenotoxic carcinogens. In other words there are no discernible morphologic events that distinguish the development of hepatocellular neoplasms related to PPAR α agonists from the development of hepatic neoplasms related to administration of other nongenotoxic agents. Although the formation of tumors related to PPAR α agonists may have similar or different biological mechanisms, the morphology does not provide any assistance in making this determination.

Points for Consideration: Selective Clonal Expansion of Preneoplastic Cell Population

- Is early hepatocyte proliferation essential for the formation of clones of preneoplastic hepatocytes (i.e. foci of cellular alteration) related to PPAR α agonist administration?
- Are there any unique morphologic characteristics or formative stages of PPAR α agonist induced hepatic tumors?
- Is the role of cell proliferation or reduced apoptosis in developing tumors unique or in any way different in PPAR α induced tumors compared to hepatic tumors produced by nongenotoxic agents?
- What points of cell replication/apoptosis control are altered in foci of cellular alteration/tumors with PPAR α agonists?
- Is the mechanism of enhanced cell replication in foci of cellular alteration related to PPAR α agonists the same or different than the mechanism of altered cell replication in foci of cellular alteration occurring with other genotoxic agents?

Role of Oxidative Stress and NFKb: Christopher Corton

U.S. EPA

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 is likely 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, *Acox1* was later found to be dispensable for increases in oxidative stress. Control *Acox1*-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 *Acox* 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.

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-kB 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.

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.

Thus, there is evidence that increases in oxidative stress is a key event in the PPAR α MOA.

Role of NF-kB in the PPAR α Activator MOA

There is evidence that NF-kB activation is a key event in the PPAR α activator MOA. 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 PPAR α activators demonstrate 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- κ B may be due to differences in the EMSA procedures carried out by that lab (Ohmura et al., 1996; Menegazzi et al., 1997). NF- κ B is activated in Kupffer cells and in hepatocytes at different times after exposure. After a single *in vivo* dose of WY, NF- κ B activity was increased first in Kupffer cells (at 2 hours) and only ~6 hours later, was NF- κ B activity increased in hepatocytes. Activation in hepatocytes never achieved the level observed in Kupffer cells (Rusyn et al., 1998). The increase in NF- κ B activation in hepatocytes could be due to increases in mitogenic cytokines produced by Kupffer cells that activate signal transduction pathways ultimately impinging on NF- κ B. Alternatively, NF- κ B 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- κ B 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- κ B-regulated reporter gene (Li et al., 2000b).

NF- κ B 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- κ B (p50-null mice) were fed a diet with or without 0.01% ciprofibrate for 10 days. NF- κ B 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; Arsuru 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 showed direct dependence on the p50 subunit of NF- κ B for liver tumor induction by a PPAR α activator.

Thus, there is evidence that NF- κ B activation is a key event in the PPAR α MOA.

PPAR α -Specific Data on Other Events that May be Involved in Carcinogenesis: James Klaunig

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Role of Gap Junctional Intercellular Communication in PPAR α Rodent Carcinogenesis

Gap junctional intercellular communication (GJIC) is the means by which multicellular organisms can exchange low molecular weight signals directly from within one cell to the interior of neighboring cells. GJIC is altered by many tumor-promoting agents and in many human and rodent tumors. Liver tumor-promoting agents inhibit GJIC in the rat liver *in vivo*. Molecular mechanisms which could lead to aberrant GJIC include: (1) mutation of connexin genes; (2) reduced and/or aberrant expression of connexin mRNA; (3) aberrant localization of connexin proteins, i.e., intracytoplasmic rather than in the cytoplasmic membrane; and (4) modulation of connexin functions by other proteins, such as those involved in extracellular matrix and cell adhesion. Whilst mutations of the cx 32 gene appear to be rare in tumors, cx 37 gene mutations have been reported in a mouse lung tumor cell line. Our results suggest that aberrant connexin localization is rather common in cancer cells and that possible molecular mechanisms include aberrant phosphorylation of connexin proteins and lack of cell adhesion molecules. Studies on transfection of connexin genes into tumor cells suggest that certain connexin genes (e.g., cx 26, cx 43 and cx 32) act as tumor-suppressor genes.

Blockage of cell-to-cell communication has particularly been associated with the tumor promotion stage of chemical carcinogenesis and has been suggested as a mechanism of action for the tumor promotion process. Additionally, the inhibition of gap junctional intercellular communication observed following treatment with tumor promoting compounds correlates with species and strain sensitivity of the chemical. Studies with di(2-ethylhexyl) (DEHP) and diisononyl (DINP) phthalates demonstrated that inhibition of GJIC in rats and mice was well correlated with induction of both liver tumors and markers for peroxisomal proliferation. Inhibition of GJIC continues as long as DEHP treatment was present but returned to normal control levels upon the removal of the DEHP. Blockage of GJIC correlated with the dose- and species-specific tumorigenicity of DEHP.

DEHP (MEHP) inhibited GJIC in rodent hepatocytes in a dose dependent manner. The inhibition of GJIC in mouse and rat hepatocytes was reversed within 24 hrs of MEHP removal. GJIC was not inhibited by MEHP in similarly treated hamster, cynomolgus monkey, or human primary cultured hepatocytes. Similarly in *in vivo* treated non human primates (cynomolgus monkey) no effect on GJIC was observed.

Summary

Inhibition of GJIC by PPAR agonists showed a concentration dependent pattern in rats and mice. The inhibition correlated with species sensitivity to tumor induction by the PPAR α agonists. This inhibition was reversed by removal of exposure to the compound.

Humans, nonhuman primates and hamster primary cultured hepatocytes did not exhibit inhibition of GJIC following exposure to PPAR α agonists. Blockage of GJIC by PPAR α agonists appears to be an associated event in the MOA, and may be a marker of tumor promotion in general but not specific for PPAR α agonists

Kupffer cells in PPAR α Mediated Liver Carcinogenesis

Kupffer cells are the resident macrophage of the liver. Activated Kupffer cells are involved in many chronic liver responses, including neoplasia. While historically, most research on genotoxic and nongenotoxic hepatic carcinogenesis has focused on the hepatocyte as the target cell recent studies have emerged that suggest a role for non parenchymal cells, specifically Kupffer cells, as important mediators of cell proliferation by tumor promoters. Activated Kupffer cells release a wide array of biologically active products including reactive oxygen species (ROS), interleukins, and cytokines which may be capable of modulating hepatocellular growth. TNF- α has been linked to the stimulation of hepatocellular growth by tumor promoting compounds. TNF α is essential for liver regeneration and blocking TNF α activity with an anti-TNF α antibody prevents liver regeneration.

A number of studies have examined a role for Kupffer cell activation in the PPAR α agonist induced liver cancer. Kupffer cell phagocytosis is activated by peroxisome proliferators *in vivo*; however, the mechanisms by which peroxisome proliferators activate Kupffer cells and increase TNF α and ROS production are not well established. Wy-14,643-stimulated increases in the hepatocyte mitogen TNF α were also blocked by inactivating Kupffer cells. Antibodies to TNF α prevented increases in cell proliferation caused by Wy-14,643. Furthermore, the transcription factor NF κ B, which is central in TNF α production, was shown to be activated predominantly in Kupffer cells following treatment with Wy-14,643. Inhibition of Kupffer cells with dietary glycine largely prevented TNF α production and blocked mortality in rats treated with lethal doses of lipopolysaccharide (LPS). Similarly, dietary glycine (which inhibits Kupffer function) prevented TNF α production and blocked increased rates of cell proliferation in rats treated with Wy-14,643. These data suggest a role for the Kupffer cell as the key mitogen responsible for Wy-14,643-induced hepatocyte proliferation. However there have been questions if the levels of TNF α in response to PPAR α agonists are sufficient to produce the growth response.

A link between Kupffer cell production of cytokines and chronic effects of PPAR α agonists has not been established yet. Kupffer cell activation may persist for longer than a few days with WY-14,643 but not DEHP. Thus raises concerns about the sustainability of the Kupffer cell-mediated effects suggest an early response that is not translated to longer exposure effects. In addition, hepatic proliferation was not induced in PPAR α -null mice fed the Wy-14,643 diet for 1 or 5 weeks which suggests that effects of peroxisome proliferators on cell proliferation in mouse liver are mediated by PPAR α but not via Kupffer cells (kupffer cells do not contain PPAR α).

Co treatment *in vivo* with a PPAR α agonist and glycine and methyl palmitate (nonselective agents that inactivate but do not eliminate Kupffer cells) have been shown to decrease hepatocellular growth. However, GdCl₃ depletion of Kupffer cells has been shown to increase liver cell proliferation in rodent liver. This conflict may be due in part to the fact that GdCl₃ inactivation of Kupffer cells initially provokes a burst of growth mediators from the Kupffer cells that in turn cause a temporal increase in DNA synthesis. Depletion of Kupffer cells by clodronate liposomes (which kills the cells by apoptosis without release of factors) showed a growth permissive role of the Kupffer cell in the induction of cell proliferation by PPAR α inducers. In a separate study, the role of Kupffer cells in the modulation of preneoplastic lesion growth was evaluated in hepatic focal lesions, LPS increased the relative volume of hepatic focal lesions after 30 days (~4-fold increase over control) while Kupffer cell depletion with clodronate liposomes significantly reduced the LPS-induced increase in focal lesion volume. In addition, LPS increased DNA synthesis within focal lesions (~3-fold increase over control) after 30 days but this was inhibited by depletion of Kupffer cells. These data collectively provide support for the involvement of the Kupffer cell in hepatic carcinogenesis, and suggest that activation of this cell type may function at the promotion stage of the cancer process.

Other studies show that Kupffer cell activation by PPAR α agents is independent of the PPAR α receptor since Kupffer cells have been reported not to contain the receptor. Kupffer cells isolated from wild-type or PPAR α -null mice treated with WY-14,643 *in vitro* showed similar superoxide production. In addition, non-parenchymal cells isolated from PPAR α -null mice, like those isolated from the wild-type mice, restored the proliferative hepatocyte response to nafenopin that was lost in purified liver parenchymal cells. However, as expected, PPAR α -null hepatocytes remained non-responsive to PPs, irrespective of the type of the added non-parenchymal cells. In another study the effect of TNF α and a peroxisome proliferator WY-14,643 on cell proliferation in purified cultured rat hepatocytes was examined and concluded that an increase in mitogenic cytokine production by Kupffer cells is necessary for stimulation of DNA synthesis in purified rat parenchymal cells since the presence of both the PP and TNF α was required for a maximal proliferative response similar to that in whole liver.

It appears that activation of Kupffer cells in the liver occurs rapidly. In rat liver, activity of the transcription factor NF- κ B, a major regulator of TNF α transcription, is transiently increased in 2- to 8 hrs followed by a steady decline to near control levels after 36 hrs following treatment with WY-14,643. DEHP activates Kupffer cells within hours of treatment and before induction of peroxisomal enzymes.

Studies with cultured rodent hepatocytes, Kupffer cells, or mixed cell populations suggest a role for Kupffer cells in carcinogenesis. Both WY-14,643 and MEHP directly activate rat Kupffer cells *in vitro* to produce ROS. Addition of non-parenchymal cells to hepatocyte cultures increased DNA synthesis 2-3 fold and suppressed TGF β 1-induced apoptosis by 50-70%. Removal of non-parenchymal cells from normal hepatocyte cultures prevented both the nafenopin- and TNF α -induced increases in DNA synthesis and suppression of hepatocyte apoptosis. Kupffer cells isolated from rats fed WY-14,643 generated ROS significantly greater than cells from controls for up to 3 weeks of

treatment; however, superoxide production was not stimulated by feeding DEHP for the same period of time

Treatment with WY-14,643 and MEHP, were able to increase superoxide production by isolated rat Kupffer cells in a dose-dependent manner, indicating that they can affect Kupffer cells directly. Kupffer cells isolated from *p47^{phox}*-null mice showed no increase in superoxide production. WY-14,643-induced activation of NF- κ B, increase in TNF α mRNA, and acute increases in liver weight and cell proliferation did not occur in *p47^{phox}*-null mice. Since apparently PPAR α is not involved in activation of Kupffer cells by PPs it remains unresolved.

Species Differences and Other Factors Impacting Risk Assessment: Timothy Hummer¹ and Phillip Bentley²

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

In rodents, PPAR α treatment causes hepatocyte proliferation, decreased apoptosis, and hypertrophy resulting in increased liver weight. Limited human data are available that evaluate PPAR α -induced hepatocellular hyperplasia. Results from one clinical study indicated that hepatomegaly did not occur in 12 patients receiving fenofibrate for 4 to 86 months (Gariot et al., 1987). Similar findings have been observed in nonhuman primates; the administration of therapeutic doses of clofibrate, ciprofibrate, or fenofibrate for either 2 or 13 weeks did not result in an increase in relative liver weight (Hoivik et al., 2004; Hall et al., 1999). When administered ciprofibrate for 15 days at doses 5 to 9 times higher than the therapeutic dose, a significant increase in liver weight was observed in monkeys; however, in contrast to rodents, livers from nonhuman primates treated with either fenofibrate or ciprofibrate showed no increase in mitotic figures, Ki-67-positive cells, or mRNA expression of genes that are upregulated during cell proliferation (Hoivik et al., 2004). Additionally, *in vitro* experiments investigating the ability of PPAR α agonists to induce proliferation of primary hepatocytes have collectively showed that rodent hepatocytes exhibited markers of cell proliferation whereas human hepatocytes did not (reviewed in Klaunig et al., 2003; Doull et al., 1999).

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.

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.

Differences in peroxisome proliferation

Marked hepatic peroxisome proliferation is observed in rats and mice treated with PPAR α agonists, whereas the available data indicate that peroxisome proliferation either does not occur or occurs only to a minimal extent in humans. A 3- to 5-fold increase in hepatic peroxisome number was observed in rats receiving 200 mg/kg clofibrate BID, which represents approximately 14X the clinical dose (Anthony et al., 1978). Humans treated with clofibrate for up to 7 years had only a 50% increase in liver peroxisome number with an insignificant change in peroxisome volume (Hanefeld et al., 1983). Other researchers have reported seeing no hepatic peroxisome proliferation in humans treated with clofibrate, gemfibrozil, or fenofibrate for 1 to 7 years (Hanefeld et al., 1980; De La Iglesia et al., 1982; Blumcke et al., 1983; Gariot et al., 1987). Varying reports have been published regarding peroxisome proliferation in nonhuman primates. Peroxisome proliferation was not observed in monkeys treated with bezafibrate, ciprofibrate, nafenopin, or DEHP (Watanabe et al., 1989; Makowska et al., 1992; Lake et al., 1989; Graham et al., 1994), however, other reports have described the presence of peroxisome proliferation in monkeys treated with ciprofibrate or DL-040 (Lalwani et al., 1985; Reddy et al., 1984).

Differences in oxidative stress

Hepatic oxidative stress is thought to occur through the PPAR α -mediated induction of hepatic enzymes involved in fatty acid oxidation. One enzyme in particular, acyl-CoA oxidase (ACO), has been implicated in inducing oxidative stress because the enzyme catabolizes long chain fatty acids resulting in the production of hydrogen peroxide. A large increase (~15 fold) in ACO expression has been observed in rat liver within 24 hours of treatment with 250 mg/kg ciprofibrate or 500 mg/kg clofibrate (Reddy et al., 1996); this effect is sustained with prolonged exposure to these or related PPAR α agonists (Peters et al., 1998). However, in humans treated with fenofibrate, bezafibrate, or gemfibrozil for 8 weeks, increases in ACO expression were not observed even though markers of pharmacodynamic activity were observed (e.g., reductions in plasma LDL cholesterol and triglycerides and increased hepatic Apo AI expression) (Roglans et al., 2002). It has also been noted that nonhuman primates treated with PPAR α agonists have no or a marginal increase in hepatic ACO expression (reviewed in Klaunig et al., 2003; Doull et al., 1999). Similar differences in PPAR α agonist induced ACO expression have been observed between rodent-derived cells or cell lines and primary human hepatocytes (reviewed in Klaunig et al., 2003; Doull et al., 1999).

In contrast, PPAR α -humanized mice treated with WY-14,643 showed increased ACO expression as well as other target genes involved in lipid homeostasis (Cheung et al., 2004). However, this contradiction may derive from the difference in coactivator expression patterns or PPREs of target genes found in murine hepatocytes versus human hepatocytes. It has been reported that the human ACO PPRE is nonresponsive to PPAR α agonism (Woodyatt et al., 1999; Lambe et al., 1999). Therefore, it is likely that the human PPAR α /murine coactivator complex is able to induce ACO gene expression via the murine ACO PPRE but is unable to do so in human hepatocytes because of differences in binding affinity at the PPRE due to either a difference in the human ACO PPRE sequence compared with mouse or a difference in binding of the human PPAR α /human coactivator complex.

Species differences have also been noted for other proteins that can impact oxidative stress. Studies in dogs and monkeys have shown little or slight (twofold) changes in beta-oxidation, catalase, or CYP4504A activities after treatment with PPAR α agonists (Meyer et al., 1999; Hall et al., 1999; Foxworthy et al., 1990; Gibson, 1992). In hamsters, treatment with PPAR α agonists result in increased peroxisomal beta-oxidation and slightly decreased catalase activity, which would be expected to increase oxidative stress. However, unlike mice and rats, hamsters do not exhibit increased hepatocellular proliferation. It was noted that the basal level of glutathione-S-transferase, glutathione reductase, glutathione peroxidase, and DT-diaphorase were much higher in livers from hamsters compared with rats (O'Brien et al., 2001a; O'Brien et al., 2001b). Additionally glutathione peroxidase activity was increased in hamsters after treatment with WY-14,643 but was decreased in rats (O'Brien et al., 2001a; O'Brien et al., 2001b; Lake et al., 1989). Therefore, not only differences in the induction of enzymes that produce oxidative stress but differences in basal and inducible levels of enzymes that protect cells from oxidative stress have been observed between species.

Overall assessment of human relevance

Although several key events believed to be involved in the MOA for PPAR α -induced hepatocarcinogenesis in rodents are theoretically plausible in humans, the collective database suggests that humans are considerably less sensitive to the tumorigenic effects of PPAR α agonists observed in rats and mice. Most noteworthy are the data that show human and nonhuman primate hepatocytes to be refractory to the induction of cell proliferation *in vivo* and *in vitro*. These data include markers of cell proliferation such as proliferation-related gene or protein expression, assessment of liver hyperplasia/hepatomegaly, and human epidemiological findings.

However, there are currently gaps in the literature that limit the ability to definitively conclude that the MOA for PPAR α agonist-induced hepatocarcinogenesis is not relevant to humans. Although epidemiology data indicate that the clinical use of gemfibrozil or clofibrate has not resulted in an increase in liver cancer in patients receiving these drugs (Huttunen et al., 1994; Frick et al., 1987; WHO investigator cooperative, 1978, 1980, and 1984), there are some limitations in these data.

First, these observations are based on patients receiving relatively low potency compounds that have similar or less affinity for human PPAR α compared with rodent PPAR α . Newer generation PPAR α agonists are currently being developed by the pharmaceutical industry. Not only do these compounds have a much greater potency for PPAR α compared with the fibrates (up to 1000 times), they also tend to have a greater potency for the human receptor compared with the rodent receptor (generally between 10 and 100 fold). Much of the research described above has been conducted with the lower potency compounds. Therefore, it is uncertain whether the species differences observed with the lower potency compounds will also be observed with the high potency agonists. A demonstration of a lack of increased cell proliferation in “nonresponsive” species in response to very potent compounds would provide stronger evidence that the MOA for PPAR α agonist-induced hepatocarcinogenesis is not relevant to humans, regardless of the agonist potency (Peters et al., 2005).

Second, the epidemiology data are based on a limited duration of human exposure. In carcinogenicity studies, rodents are exposed for nearly their entire life of around 2 years. In contrast, the induction of human tumors is generally thought to require 10 to 20 years. Therefore, epidemiology studies based on clinical exposures less than 20 years would likely not be able to detect increases in liver cancer rates above background unless the compound was a very potent human carcinogen. Additionally, humans are being treated with therapeutic doses compared with higher doses generally used in nonclinical toxicology studies. Given the differences in receptor number, binding affinity, and induction of gene expression between humans and rodents, there is the possibility that the relatively low clinical exposures are not sufficient to induce liver tumors in humans. This scenario could indicate a threshold effect of the tumorigenic process rather than the irrelevance of the rodent MOA. However, the hepatocyte proliferation data from nonhuman primates treated with doses up to 9 fold higher than clinical doses suggest that the lack of human liver tumors likely involves differences in the key steps of the rodent MOA rather than only a threshold effect.

Third, limited epidemiology data are unable to determine whether there are human populations with greater susceptibility to the proliferative effects of PPAR α agonists compared with the general population. A variant of the human PPAR α gene (L162V) has been identified and shown to have significantly greater *in vitro* ligand-inducing activity compared with the wild-type receptor (Sapone et al., 2000; Flavell et al., 2000; Vohl et al., 2000). Furthermore, humans who have this receptor variant are reported to be more responsive to the lipid lowering effects of bezafibrate (Flavell et al., 2000). It has also been hypothesized that polymorphisms in the promoters of PPAR α target genes could result in variable responses in humans (reviewed in Peters et al., 2005). Additionally, polymorphisms in anti-oxidant genes, such as glutathione-S-transferase, could enhance an individual’s risk for developing cancer (reviewed in Eaton and Bammler, 1999). Therefore, heterogeneity in the human population could result in differences in susceptibility to the tumorigenic effects of PPAR α agonists.

Molecular differences in PPAR α between species

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 PPRE 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 (Shiple 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 (Shiple 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.

However, a newer generation of PPAR α agonists, which often have dual or pan PPAR receptor activity, are being developed for pharmaceutical use that have *in vitro* receptor alpha activity up to 1000 times higher than fibrates (Brown et al., 2001; Benardeau et al., 2009; Ye et al., 2010; FDA, unpublished information). Additionally, these more potent compounds tend to activate the human receptor 10 to nearly 100 times more than the mouse receptor (Lawrence et al., 2001; FDA, unpublished information). It is currently uncertain whether these compounds with a greater potency for human PPAR α have an enhanced carcinogenic potential for humans compared with the weaker agonists.

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 electrophoretic mobility shift assays (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 α_{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.

There is evidence that 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.

IPCS Framework Analysis of MOA for PPAR α -Induced Rodent Liver Tumors: Jennifer Seed

U.S. EPA-OCSPP

Mode of Action Analysis Using the Human Relevancy 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 the animal 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 lifestyles 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 ILSI/IPCS (Meek et al., 2003; Boobis et al., 2006) to include an analysis of human relevance; the ILSI/IPCS MOA/human relevance framework has also been extended to noncancer effects (Seed et al., 2005; 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 MOA/human relevance framework consists of a series of three questions. The first question is: "Is the weight of evidence sufficient to establish a mode of action (MOA) of a toxicological response in animals?" The judgment of whether a postulated animal MOA is supported by available data takes into account all of the data in a weight of evidence (WOE) approach utilizing the Bradford-Hill criteria. 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).

If the animal MOA has been established, then the analysis moves to the second and third questions which address human relevance. The second question of the MOA/human

relevance framework is: “Can human relevance of the MOA be reasonably excluded on the basis of fundamental, qualitative differences in key events between experimental animals and humans?” This analysis involves a qualitative analysis of whether the key events are biologically plausible in humans, and may rely on knowledge of biological processes, disease states, etc, rather than chemical specific information. The final question is: Can human relevance of the MOA be reasonably excluded on the basis of quantitative differences in either kinetic or dynamic factors between experimental animals and humans?” This analysis involves consideration of potential quantitative differences between the key events in the animal model and humans such as receptor number, substantial pharmacokinetic differences, etc. The robustness of the proposed MOA for PPAR α activator-induced rodent liver tumors and relevance to humans are examined using this framework.

**Biologically Based Dose-Response Modeling for
Hepatocarcinogenic Effects of PPAR α Activators: Lorenz Rhomberg**

Gradient Corporation

Abstract to be provided

Tab 3 – Figure and Tables

Tab 3 - Figure and Tables

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STRAWMAN - Mode of Action of PPAR α Agonists in the Induction of Rodent Liver Tumors

Key events:

1. Activation of the PPAR α receptor
2. Increases in oxidative stress
3. NF-kB activation
- 4 Induction of cell growth genes
5. Perturbation of cell growth and survival
6. Selective proliferation of preneoplastic cells in the liver
7. Formation of neoplasms in the liver

Definitions:

The Workshop Steering Committee and case study teams have agreed on the following definitions for use in evaluating key steps in a proposed MOA:

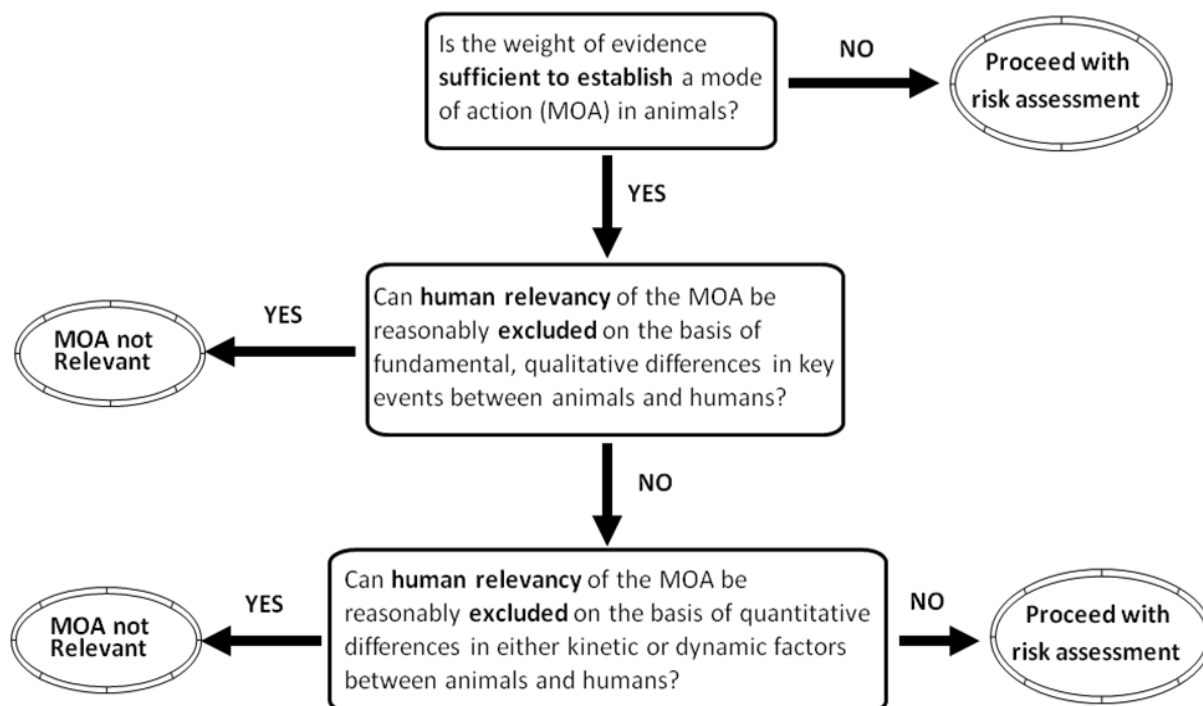
Key Event: An empirically observable causal precursor step to the adverse outcome that is itself a necessary element of the mode of action. Key events are required events for the MOA, but often are not sufficient to induce the adverse outcome in the absence of other key events.

Associative Event: Biological processes that are themselves not causal necessary key events for the MOA, but are reliable indicators or markers for key events. Associative events can often be used as surrogate markers for a key event in a MOA evaluation or as indicators of exposure to a xenobiotic that has stimulated the molecular initiating event or a key event.

Modulating Factor: There are many factors or biological responses that are not necessary to induce the adverse outcome, but could modulate the dose-response behavior or probability of inducing one or more key events or the adverse outcome. Such biological factors are considered modulating factors. Example: excessive body weight loss at a high dose.

Figure 1. IPCS Framework Analysis of MOA for PPAR α -induced Rodent Liver Tumors

Mode of Action/Human Relevance Framework



Adapted from Boobis et al.(2006)¹¹

Table 1. Occurrence of Key Events in the Mode of Action After Exposure to PPAR α Agonists (From Corton, 2010 review)

Chemical	PPAR α activation	Oxidative stress	NF- κ B 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
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		+ ^{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}	+ ^{34,37,38}		+ ³⁴	+ ³⁷	+ ⁵⁰

Comments: In the table, (+) indicates that the chemical was found to lead to the key event; (-) indicates that the chemical was found not to lead to the key event; (+/-) indicates mixed results. PPAR α activation was measured using transactivation assays. NF- κ B activation refers to binding of NF- κ B (p65:p50 heterodimer) to the NF- κ B response element in electrophoretic mobility shift assays. Acute cell proliferation was measured in the livers of treated mice or rats, usually with seven 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 agonist (Bursch et al., 1984; James et al., 1998a; Youssef et al., 2003). Chronic cell proliferation was measured in the livers of mice or rats exposed to PPAR α agonists, usually for more than three 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 (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 and DBP but only one condition resulted in increases in NF- κ B activation after gemfibrozil treatment) (R); ¹³Menegazzi et al., 1997 (R); ¹⁴Rao et al., 1987 (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); ¹⁹Marsman, 1995 (lipofuscin) (M,R); ²⁰Hinton et al., 1986 (lipofuscin) (R); ²¹Stanko et al., 1995 (lipofuscin) (R); ²²Lake et al., 1989a (increases in oxidized glutathione and decreases in vitamin 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); ²⁵Huber et al., 1991 (malondialdehyde and TBARS) (R); ²⁶Bility et al., 2004 (in vitro transactivation assays; monobutyl phthalate does not activate either) (M); ²⁷Corton and Lapinskas, 2005 (review of in vitro transactivation data; DBP may need to be metabolized to the MBP-glucuronide to be an activator) (M,R); ²⁸Issemann and Green, 1990 (in vitro transactivation assays) (M); ²⁹Gottlicher et al., 1992 (in vitro transactivation 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 (periportal proliferation; sustained chronic cell proliferation) (R); ⁴⁰Seo et al., 2004 (malondialdehyde) (R); ⁴¹Isenberg et al., 2001 (M,R); ⁴²Isenberg et al., 2000 (M,R); ⁴³Hasmall et al., 2000b (R, in vivo [DEHP] and in vitro [MEHP]); ⁴⁴Soames et al., 1999 (R); ⁴⁵Busser and Lutz, 1987 (R); ⁴⁶Kim et al., 2004 (M); ⁴⁷Reddy and Qureshi, 1979 (R); ⁴⁸Svoboda and Arzarnoff, 1979 (R); ⁴⁹Reddy and Rao, 1977 (R); ⁵⁰Rao et al., 1986 (R); ⁵¹Elliott and Elcombe, 1987 (malondialdehyde—significant change for DEHP and clofibrate but trend increase for methyl clofenapate) (R); ⁵²Fitzgerald et al., 1981 (positive in male rats and mice but not female rats and mice) (R,M); ⁵³Plant et al., 1998 (in vitro) (R); ⁵⁴James and Roberts, 1996 (in vitro) (M,R); ⁵⁵Youssef et al., 2003 (R); ⁵⁶Bull et al., 1990 (lipofuscin) (M); ⁵⁷Stauber and Bull, 1997 (M); ⁵⁸Dees and Travis, 1994 (M); ⁵⁹Thottassery et al., 1992 (R); ⁶⁰Handler et al., 1992 (looked for hydrogen peroxide production after only one dose five days prior to analysis) (R); ⁶¹Permadi et al., 1993 (analyzed TBARS only in mitochondria under conditions of increased numbers of mitochondria) (M); ⁶²Abdellatif et al., 1990 (initiated with DEN, 2-AAF, and carbon tetrachloride) (R); ⁶³Abdellatif et al., 1991 (R); ⁶⁴Vanden Heuvel et al., 2006 (in vitro transactivation assay) (M); ⁶⁵Huber et al., 1997 (TBARS) (R); ⁶⁶Nicholls-Grzemski et al., 2000 (TBARS) (M); ⁶⁷Austin et al., 1995 (TBARS) (M); ⁶⁸Kawashima et al., 1994 (lipid peroxidation) (R); ⁶⁹Biegel et al., 2001 (R); ⁷⁰Styles et al., 1988 (M,R); 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., 1999b (R); ⁷⁵O'Brien et al., 2001b (decreases in vitamin E) (R); ⁷⁶Qu et al., 2000; ⁷⁷Alsarra et al., 2006 (R); ⁷⁸Takacs and Abbott, 2007 (in vitro transactivation assay) (M); ⁸⁰Price et al., 1992 (R); ⁸¹Bull et al., 2002 (M); ⁸²Herren-Freund et al., 1987 (M); 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); ⁸⁵DeAngelo et al., 1989 (R); ⁸⁶DeAngelo et al., 1997 (R); ⁸⁷Ohmura et al., 1996 (R).

Table 2. Hill Criteria for MOA Analysis

Possible Key Events	Strength	Consistency (Reproducibility)	Specificity	Temporal Relationship	Biological Gradient (Dose-Response)	Biological Plausibility	Coherence	Causal (Key Event)	Associated (Marker?) Neither	Importance?
PPAR α activation										
Lipid metabolism gene expression										
Altered expression of genes involved in oxidative stress										
Increases in oxidative stress										
NF-kB activation										
Altered expression of cell proliferation genes										
Increased cell proliferation										
Decreased apoptosis										

Kupffer cell activation										
Gap Junction intracellular communication inhibition										
Possible Key Events	Strength	Consistency (Reproducibility)	Specificity	Temporal Relationship	Biological Gradient (Dose-Response)	Biological Plausibility	Coherence	Causal (Key Event)	Associated (Marker?) Neither	Importance?
Peroxisome proliferation										
Clonal expansion										
Tumors										



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