March 5, 2004

MEMORANDUM

SUBJECT: Transmittal of Meeting Minutes of the FIFRA Scientific Advisory Panel Meeting Held December 9, 2003

TO: James J. Jones, Director
Office of Pesticide Programs

Charles M. Auer, Director
Office of Pollution Prevention and Toxics

FROM: Steven M. Knott, Designated Federal Official
FIFRA Scientific Advisory Panel
Office of Science Coordination and Policy

THRU: Larry C. Dorsey, Executive Secretary
FIFRA Scientific Advisory Panel
Office of Science Coordination and Policy

Joseph J. Merenda, Jr., Director
Office of Science Coordination and Policy

Attached, please find the meeting minutes of the FIFRA Scientific Advisory Panel open meeting held in Arlington, Virginia on December 9, 2003. This report addresses a set of scientific issues being considered by the Environmental Protection Agency regarding the proposed science policy: PPAR-α agonist-mediated hepatocarcinogenesis in rodents and relevance to human health risk assessment.

Attachment

cc:

Susan Hazen
Adam Sharp
Anne Lindsay
Janet Andersen
Steven Bradbury
cc:

William Diamond
Debbie Edwards
Arnold Layne
Tina Levine
Lois Rossi
Frank Sanders
Margaret Stasikowski
Randolph Perfetti
Karl Baetcke
Vicki Dellarco
Oscar Hernandez
David Lai
Elizabeth Mendez
Esther Rinde
Jennifer Seed
William Jordan
Douglas Parsons
Daniel Rosenblatt
David Deegan
Vanessa Vu (SAB)
OPP Docket

FIFRA Scientific Advisory Panel Members
Gary E. Isom, Ph.D. (Session Chair)
Stephen M. Roberts, Ph.D. (FIFRA SAP Chair)
Christopher J. Portier, Ph.D.

FQPA Science Review Board Members
George B. Corcoran, Ph.D.
Yvonne P. Dragan, Ph.D.
Ronald N. Hines, Ph.D.
Randy L. Jirtle, Ph.D.
Lisa M. Kamendulis, Ph.D.
James P. Kehrer, Ph.D.
Lois D. Lehman-Mckeeman, Ph.D.
David E. Moody, Ph.D.
Daniel J. Noonan, Ph.D.
Carmen E. Perrone, Ph.D.
Martha S. Sandy, Ph.D.
Michael D. Wheeler, Ph.D.

SAP Minutes No. 2003-05

A Set of Scientific Issues Being Considered by the Environmental Protection Agency Regarding:

Proposed Science Policy: PPAR-α Agonist-Mediated Hepatocarcinogenesis in Rodents and Relevance to Human Health Risk Assessment

December 9, 2003
FIFRA Scientific Advisory Panel Meeting,
Held at the Holiday Inn National Airport Hotel,
Arlington, Virginia

NOTICE

These meeting minutes have been written as part of the activities of the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA), Scientific Advisory Panel (SAP). These meeting minutes represent the views and recommendations of the FIFRA SAP, not the United States Environmental Protection Agency (Agency). The content of these meeting minutes does not represent information approved or disseminated by the Agency. These meeting minutes have not been reviewed for approval by the Agency and, hence, the contents of this report do not necessarily represent the views and policies of the Agency, nor of other agencies in the Executive Branch of the Federal government, nor does mention of trade names or commercial products constitute a recommendation for use.

The FIFRA SAP is a Federal advisory committee operating in accordance with the Federal Advisory Committee Act and was established under the provisions of FIFRA, as amended by the Food Quality Protection Act FQPA of 1996. The FIFRA SAP provides advice, information, and recommendations to the Agency Administrator on pesticides and pesticide-related issues regarding the impact of regulatory actions on health and the environment. The Panel serves as the primary scientific peer review mechanism of the EPA, Office of Pesticide Programs (OPP) and is structured to provide balanced expert assessment of pesticide and pesticide-related matters facing the Agency. Food Quality Protection Act Science Review Board members serve the FIFRA SAP on an ad hoc basis to assist in reviews conducted by the FIFRA SAP. Further information about FIFRA SAP reports and activities can be obtained from its website at http://www.epa.gov/scipoly/sap/ or the OPP Docket at (703) 305-5805. Interested persons are invited to contact Steven Knott, SAP Designated Federal Official, via e-mail at knott.steven@.epa.gov.

In preparing these meeting minutes, the Panel carefully considered all information provided and presented by the Agency presenters, as well as information presented by public commenters. This document addresses the information provided and presented within the structure of the charge by the Agency.

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A Set of Scientific Issues Being Considered by the Environmental Protection Agency Regarding:

Proposed Science Policy: PPAR-α Agonist-Mediated Hepatocarcinogenesis in Rodents and Relevance to Human Health Risk Assessment

December 9, 2003
FIFRA Scientific Advisory Panel Meeting, Held at the Holiday Inn National Airport Hotel, Arlington, Virginia

Steven M. Knott, M.S.
Designated Federal Official
FIFRA Scientific Advisory Panel
Panel
Date: March 5, 2004

Gary E. Isom, Ph.D.
FIFRA SAP, Session Chair
FIFRA Scientific Advisory

Date: March 5, 2004

Federal Insecticide, Fungicide, and Rodenticide Act
Scientific Advisory Panel Meeting
December 9, 2003

Proposed Science Policy: PPAR-α Agonist Mediated Hepatocarcinogenesis in Rodents and Relevance to Human Health Risk Assessment

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INTRODUCTION

The Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA), Scientific Advisory Panel (SAP) has completed its review of the set of scientific issues being considered by the Agency pertaining to the Proposed Science Policy: Peroxisome Proliferator Activated Receptor-alpha (PPAR-α) Agonist-Mediated Hepatocarcinogenesis in Rodents and Relevance to Human Health Risk Assessment. Advance notice of the meeting was published in the *Federal Register* on October 24, 2003. The review was conducted in an open Panel meeting held in Arlington, Virginia, on December 9, 2003. Dr. Gary Isom chaired the meeting. Mr. Steven Knott served as the Designated Federal Official.

Dr. Elizabeth Mendez (Health Effects Division, Office of Pesticide Programs, EPA) provided the Agency presentation on the proposed science policy regarding PPAR-α agonist-mediated hepatocarcinogenesis in rodents and relevance to human health risk

assessment. Dr. Jeff Peters (Penn State University) provided a presentation on the paper "PPAR-α Agonist-Induced Rodent Tumors: Modes of Action and Human Relevance" (Klaunig et al., 2003). The paper and presentation summarized the evaluation of a working group convened by the International Life Sciences Institute, Risk Science Institute. This evaluation, along with the pertinent scientific literature, was considered by EPA's Office of Prevention, Pesticides, and Toxic Substances in developing its proposed science policy. Ms. Margaret Stasikowski (Director, Health Effects Division, Office of Pesticide Programs, EPA) provided an introduction to the session and also participated in the meeting. In addition, Dr. Karl Baetcke (Health Effects Division, Office of Pesticide Programs, EPA), Dr. Jennifer Seed and Dr. David Lai (both from the Risk Assessment Division, Office of Pollution Prevention and Toxics, EPA) participated in the session.

In preparing these meeting minutes, the Panel carefully considered all information provided and presented by the Agency presenters, as well as information presented by public commenters. These meeting minutes address the information provided and presented at the meeting, especially the response to the charge by the Agency.

PUBLIC COMMENTERS

Oral statements were presented as follows:

Jennifer B. Sass, Ph.D., Natural Resources Defense Council

Written statements were provided as follows:

Robert A. Bilott, Taft, Stettinius, Hollister, LLP

CHARGE

Developments in the area of research on peroxisome proliferating chemicals have led to a reevaluation of the state of the science to characterize the mode(s) of action (*i.e.,* PPAR-α agonism) and the human relevance of rodent tumors induced by PPAR-α agonists. Recently, the ILSI Risk Science Institute (ILSI RSI) convened a large expert technical group to evaluate new information on the association between PPAR-α agonism and the induction of tumors by peroxisome proliferating chemicals. OPPTS considered the 2003 ILSI report as well as the pertinent scientific literature in developing its proposed science policy.

Please provide comment and advice on the following questions. In addressing these questions consider the completeness of the data sets evaluated.

Issue 1: Rodent PPAR-α Mode of Action (MOA) for Hepatocarcinogenesis

OPPTS has concluded that there is sufficient weight of evidence to establish the mode of action (MOA) for PPAR-α agonist-induced rodent hepatocarcinogenesis. It is proposed in the OPPTS document that PPAR-α agonists activate PPAR-α leading to an increase in cell proliferation and a decrease in apoptosis, and eventually further clonal expansion of preneoplastic cells and formation of liver tumors. The key events in PPAR-α agonist-induced hepatocarcinogenesis may be classified as either causal (required for this MOA) or associative (marker of PPAR-α agonism).

> **Question 1** - Please comment on the weight of evidence and key events for the proposed MOA for the PPAR-α agonist-induced rodent hepatocarcinogenesis. Please comment on the adequacy of the data available to identify the key events in the PPAR-α MOA. Discuss whether the uncertainties and limitations of these data have been adequately characterized.

Issue 2: Relative Sensitivity of Fetal, Neonatal, and Adult Rodent

OPPTS has provided a review of the ontogeny of PPAR-α expression and peroxisomal assemblage during fetal and postnatal development in rodents as well as an analysis of the available data evaluating effects on peroxisomal proliferation, peroxisomal enzyme activity, and liver weights following exposure to PPAR-α agonists during fetal and postnatal development in rats and mice (see Section V of the OPPTS Document). Based on this analysis, OPPTS concluded that fetal and neonatal rats do not exhibit an increased sensitivity to PPAR-α agonist-induced hepatocarcinogenicity relative to the adult rodent. Therefore, any conclusions regarding this MOA in adult rodents would also apply to young rodents, and similarly any conclusions regarding the relevance of this MOA for human hepatocarcinogenesis would apply to the young, as well as the adults.

> **Question 2** - Please comment on the weight of the evidence approach and mechanistic data used to support this conclusion.

Issue 3: Human Relevance

OPPTS has provided an analysis of a variety of *in vitro* and *in vivo* studies on the key events pertaining to PPAR-α agonist-induced hepatocarcinogenesis with hamsters, guinea pigs, non-human primates, and humans. Based on the weight of the evidence, OPPTS concludes that although PPAR-α agonists can induce liver tumors in rodents and while PPAR-α is functional in humans, quantitatively, humans and nonhuman primates are refractory to the hepatic effects of PPAR-α agonists.

Therefore, OPPTS is proposing the following scientific policy:

> When liver tumors are observed in long term studies in rats and mice, and 1) the data are sufficient to establish that the liver tumors are a result of a

PPAR-α agonist MOA and 2) other potential MOAs have been evaluated and found not operative, the evidence of liver tumor formation in rodents should not be used to characterize potential human hazard.

Question 3 - Please comment on the data and weight of evidence regarding the hepatic effects of PPAR-α agonists in humans, and please comment on the proposed OPPTS's science policy regarding human relevance.

Issue 4: Data Requirements

OPPTS has proposed a data set that would be sufficient to demonstrate that PPAR-α agonism is the MOA for the induction of rodent liver tumors. The data set includes evidence of PPAR-α agonism (*i.e.,* from an *in vitro* reporter gene assay), *in vivo* evidence of an increase in number and size of peroxisomes, increases in the activity of acyl CoA oxidase, and hepatic cell proliferation. The *in vivo* evidence should be collected from studies designed to provide the data needed to show dose-response and temporal concordance between precursor events and liver tumor formation.

Question 4 - Please comment in general on the proposed data set and particularly on its adequacy to demonstrate that a PPAR-α agonist-mediated MOA is operating in rodent hepatocarcinogenesis.

Issue 5: Other Tumors Induced by PPAR-α Agonists

Some PPAR-α agonists may also induce pancreatic acinar cell and Leydig cell tumors in rats and modes of action involving agonism of PPAR-α have been proposed. An in depth analysis of these tumors is provided in the 2003 ILSI technical panel report. Based on this analysis, OPPTS agrees that the data available to date are insufficient to support the proposed MOAs.

Thus, OPPTS is proposing the following science policy:

> Given the limited evidence available to support that a chemical may induce pancreatic and Leydig cell tumors through a PPAR-α agonist MOA, the evidence is inadequate at this time to support a linkage between PPAR-α agonism and formation of these tumor types. Thus, it is presumed that chemicals that induce pancreatic or Leydig cell tumors may pose a carcinogenic hazard for humans.

Question 5 - Please comment on OPPTS's conclusion that there is limited evidence that a chemical may induce pancreatic and Leydig cell tumors through a PPAR-α agonist MOA, and OPPTS's proposed science policy regarding other tumors induced by PPAR-α agonists.

SUMMARY OF PANEL DISCUSSION AND RECOMMENDATIONS

Rodent PPAR-α Mode of Action (MOA) for Hepatocarcinogenesis

Overall, the majority of the Panel felt the evidence in support of the proposed MOA for PPAR-α agonist induced rodent hepatocarcinogenesis was adequate, though the opinions of individual Panel members ranged from full agreement to complete disagreement. The key event in the MOA is PPAR-α activation. PPAR-α activation triggers multiple events leading to tumorigenesis but the PPAR-α-altered genes in the causal pathway for tumor induction have not been identified. While some of the key events that occur after PPARα activation, such as increased cell proliferation, inhibition of apoptosis, and the clonal expansion of preneoplastic lesions are known, the PPAR-α dependent mechanism for the perturbation of these key events is less well established. Specifically, mechanisms and steps linking key events downstream of PPAR-α activation are not known. The data are sufficient to demonstrate a PPAR-α activation dependence to the MOA, but are inadequate to provide the quantitative linkages associated with a more defined mechanism of action. The Panel members agreed that additional evidence of specific alterations associated with PPAR-α activation would greatly strengthen the proposed MOA.

There was agreement among most, but not all, of the Panel that data from the PPAR-α -/- (null or knockout) mouse indicate the requirement for the activation of PPAR-α in the MOA of the hepatocarcinogenic effect of these agents. That the PPAR-α null mouse fails to exhibit the key and associated events when challenged with 11 months exposure to a potent PPAR-α agonist at a dose that induces 100% incidence of multiple liver adenomas in concurrently exposed control (wildtype) mice demonstrated to most, but not all, Panel members the underlying basis of the MOA statement. A few Panel members expressed concern over the short duration of the studies in the PPAR-α -/- mouse (i.e., 11 months vs. 24 months in standard cancer bioassays), which rendered the studies incapable of assessing the lifetime liver cancer risk of PPAR-α agonists in this knockout mouse model, and thus, inadequate to conclusively demonstrate that PPAR-α activation is required for hepatocarcinogenesis. One Panel member did not find the weight of evidence for the proposed MOA to be sufficient based on the current absence of scientific understanding or identification of any of the intermediate critical events on the causal pathway which link PPAR-α activation with increased proliferation, decreased apoptosis, clonal expansion of preneoplastic lesions, or liver tumor formation. In addition, this Panel member observed that there is a large body of data demonstrating that PPAR-α agonists activate Kupffer cells through a PPAR-α independent mechanism, resulting in the release of cytokines capable of stimulating parenchymal cell mitosis and suppressing apoptosis.

Relative Sensitivity of Fetal, Neonatal, and Adult Rodent

The Panel does not support the OPPTS conclusions that the PPAR-α agonist MOA in adult rodents would also apply to young rodents, and similarly any conclusions regarding the relevance of this MOA for human hepatocarcinogenesis would apply to the young, as well as the adults. Differences in peroxisome biogenesis have been reported during the ontogenic development of rodents and humans. While the assembly of peroxisomes in rats and mice, including the insertion of β-oxidation enzymes into the peroxisomes, occurs near birth, the assembly of human peroxisomes has been observed as early as 8 weeks of gestation (Espeel, et al, 1997). The number and density of peroxisomes plateau by 17 weeks of gestation in humans. Moreover, acyl-CoA oxidase and 3-ketoacyl CoA thiolase are immunodetectable in the peroxisomes by 10 and 9 weeks of gestation, respectively. Thus, this suggests differences in β-oxidation capabilities in developing rodents and humans. It was also considered that differences in cell proliferation, xenobiotic metabolism, and other factors in the developing rodent (or human) could affect sensitivity to PPAR-α hepatocarcinogenesis. Therefore, information on the expression of the PPAR-α during ontogeny as well as responses of embryonic and fetal human hepatocytes to PPAR-α agonists should be evaluated before concluding that the developing human conceptus is unresponsive to PPAR-α agonist exposures.

Human Relevance

Overall, the majority of the Panel agreed that there are relevant data indicating that humans are less sensitive than rodents to the hepatic effects of PPAR-α agonists. However, the opinions of individual Panel members ranged from full agreement with the proposed OPPTS policy statement, as currently written, to complete disagreement. The majority of the Panel recognized weaknesses in the data that supported the policy noting in particular that the case for lack of human relevance was deficient in the human data. In addition, the Panel members agreed that the MOA and its application to addressing human relevance would be greatly strengthened by additional evidence of the specific alterations associated with PPAR-α activation that lead to the more general steps of hepatocellular proliferation, clonal expansion of initiated hepatocytes and tumor development. However, the Panel was divided regarding whether such additional evidence is necessary before accepting the MOA and its application to human relevance. Some Panel members believed that the data failed to demonstrate that the effect could only occur in liver and that, therefore, the policy statement should be limited to hepatocarcinogenic effects (see number 2 below). Other Panel members believed that the overall data limitations were significant enough to disagree with the MOA and its application to addressing human relevance.

As noted previously, there was agreement among most, but not all of the Panel that data from PPAR-α null mice showing that, in the absence of the receptor, there were no ensuing changes in cell proliferation and hepatic tumor formation, was strong evidence that activation of PPAR-α is necessary for all subsequent steps in the MOA. It also was noted previously that a few Panel members expressed concern over the short duration of the studies in the PPAR-α null mice (i.e., 11 months vs. 24 months in

standard cancer bioassays), which rendered the studies incapable of assessing the lifetime liver cancer risk of PPAR-α agonists in this knockout mouse model, and thus, inadequate to conclusively demonstrate that PPAR-α activation is required for hepatocarcinogenesis. Considering the proposed MOA, there was agreement that PPAR-α is present in humans and that the receptor is activated in human liver following exposure to known agonists. Accordingly, the proposed MOA for PPAR-α agonist-induced hepatocellular carcinogenesis in rodents is plausible for humans. There was also agreement that the nature of gene expression associated with hepatocellular PPAR-α activation is qualitatively different between humans and rodents. This difference may result from species differences in peroxisome proliferator response elements (PPREs), but there are few data available that identify these potentially important differences, particularly in humans. Humans are at least as sensitive to activation end-points that lead to hypolipidemia but are much less sensitive to other end-points normally associated with peroxisome proliferation. This qualitative difference will be what is referred to in subsequent references as human sensitivity.

One overall concern with the proposed MOA and the application of the MOA to addressing human relevance was that, whereas PPAR-α activation is a very specific component of the MOA, the other steps deemed to be causally-related, namely increased hepatocellular proliferation and clonal expansion of initiated hepatocytes leading to tumor development, were very general and non-specific. Overall, the Panel members agreed that additional evidence of specific alterations associated with PPAR-α activation in primates and especially humans would greatly strengthen the proposed MOA.

The Panel discussed three other issues relative to assessing the weight of evidence regarding the hepatic effects of PPAR-α agonists in humans, and the proposed science policy regarding human relevance. These included:

1. The use of the word "refractory" to describe the human response to PPAR-α activation is too absolute. The Panel agreed that "less sensitive" is a more appropriate description of the nature of the human response relative to that observed in rats and mice.

2. The policy statement drafted by OPPTS concludes with the phrase "evidence of liver tumor formation in rodents should not be used to characterize potential human hazard." After some discussion, it was suggested by one member of the Panel, and supported by several other Panel members, that this phrase should be modified to read, "evidence of liver tumor formation in rodents should not be used to characterize potential human hepatocarcinogenic hazard."

3. One member of the Panel expressed a concern, which was shared by some other Panel members, that the MOA and evaluation of human relevance was lacking in its assessment of altered gene expression that could be associated with altered methylation of DNA. There is evidence that DNA methylation is modified in rodents following exposure to PPAR-α agonists (Ge et al., 2001, Ge et al., 2002, and Pereira, et al., 2004).

Given the accepted role for DNA methylation in gene imprinting and the loss of imprinting in cancer etiology (see for example McClachlan et al., 2001), such a role for PPAR-α agonists in causing similar alterations in humans should be explored before human relevance can be appropriately evaluated, particularly for exposure during early life stages and for questions regarding site concordance.

Data Requirements

There was general consensus among the Panel that the proposed data set was adequate and provided a straight forward approach to classify a chemical as a PPAR-α agonist. The Panel also concurred that the use of PPAR-α knockout mice would provide definitive evidence to classify a chemical as a PPAR-α agonist, but that the proposed data set would be sufficient in lieu of the use of this rather costly tool.

In the course of the Panel's discussion, questions for clarification were posed to the Agency as to when (i.e., before or after a positive liver tumor finding in rodents) this set of assays testing for PPAR-α agonist activity would be conducted. The Agency indicated that data demonstrating PPAR-α agonist activity could be submitted in the absence of testing in long-term carcinogenesis studies. In response to this, a Panel member observed that in the absence of testing in standard long-term rodent carcinogenicity studies, it is not possible to determine whether the chemical would operate through a PPAR-α agonist MOA producing rodent liver tumors. A chemical with PPAR-α agonist activity may either: 1) not cause cancer in rodents, 2) cause liver cancer in rodents by the proposed PPAR-α agonist MOA, 3) cause liver cancer by a MOA other than the proposed PPAR-α agonist MOA (e.g., cytotoxicity), or 4) cause cancer at sites other than the liver (with or without liver cancer). The Panel concurred that an overriding requirement is that other MOAs have been excluded. For example, rigorous tests must be performed to exclude mutagenicity, other forms of DNA damage (clastogenicity), or overt cytotoxicity directly produced by the test compound, or its metabolic products.

Other Tumors Induced by PPAR-α Agonists

In addition to the hepatic tumors that appear to be a general occurrence in rats and mice, nine PPAR-α agonists have been reported to induce Leydig cell tumors (LCTs) and pancreatic acinar cell tumors (PACTs) in rats. Together with the hepatic tumors, this is referred to as the tumor triad. The Panel was in agreement with the OPPTS conclusion that chemicals that induce pancreatic or Leydig cell tumors may pose a carcinogenic hazard for humans.

Given the limited amount of data available on the true MOA for LCTs or PACTs, including the possibility raised by some Panel members that epigenetic effects of the PPAR-α agonists may occur, it is not possible to determine whether PPAR-α agonists pose a carcinogenic hazard to humans. Thus, the conclusion by the OPPTS that the

available data for the induction of rat LCTs and PACTs by PPAR-α agonists are insufficient to conclude that the sole MOA involves the PPAR-α receptor is considered by the Panel to be appropriate. Further, the Panel concurs that it should be presumed that chemicals that induce pancreatic or Leydig cell tumors may pose a carcinogenic hazard for humans.

PANEL DELIBERATIONS AND RESPONSE TO CHARGE

The specific issues addressed by the Panel are keyed to the Agency's background documents, and the Agency's charge questions.

Response to Charge

Question 1 - Rodent PPAR-α Mode of Action (MOA) for Hepatocarcinogenesis

OPPTS has concluded that there is sufficient weight of evidence to establish the MOA for PPAR-α agonist-induced rodent hepatocarcinogenesis. It is proposed in the OPPTS document that PPAR-α agonists activate PPAR-α leading to an increase in cell proliferation and a decrease in apoptosis, and eventually further clonal expansion of preneoplastic cells and formation of liver tumors. The key events in PPAR-α agonist-induced hepatocarcinogenesis may be classified as either causal (required for this MOA) or associative (marker of PPAR-α agonism).

Please comment on the weight of evidence and key events for the proposed MOA for the PPAR-α agonist-induced rodent hepatocarcinogenesis. Please comment on the adequacy of the data available to identify the key events in the PPAR-α MOA. Discuss whether the uncertainties and limitations of these data have been adequately characterized.

Response

Weight of the Evidence for Proposed MOA

Overall, the majority of the Panel felt the evidence in support of the proposed MOA for PPAR-α agonist induced rodent hepatocarcinogenesis was adequate, though the opinions of individual Panel members ranged from full agreement to complete disagreement. The majority of the Panel felt the weight of evidence in support of the proposed MOA in rodents is adequate for PPAR-α agonists in which hepatic activation of PPAR-α results in the key downstream events of increased proliferation, decreased apoptosis, and clonal expansion of preneoplastic lesions resulting in hepatocarcinogenesis. Associated events (indicators of PPAR-α activation) include induction of peroxisome proliferation and altered expression of related genes. One Panel member did not find the weight of evidence for the proposed MOA to be sufficient, based

on the current absence of scientific understanding or identification of any of the intermediate critical events on the causal pathway which link PPAR-α activation with increased proliferation, decreased apoptosis, clonal expansion of preneoplastic lesions, or liver tumor formation. In addition, this Panel member observed that there is a large body of data demonstrating that PPAR-α agonists activate Kupffer cells through a PPAR-α independent mechanism, resulting in the release of cytokines capable of stimulating parenchymal cell mitosis and suppressing apoptosis (Rolfe et al., 1997; Rusyn et al., 2001; Parzefall et al., 2001; Hasmall et al., 2001).

The proposed MOA for PPAR-α agonist induced rodent hepatocarcinogenesis is based on a considerable body of evidence that has accrued over the past 3 decades, and particularly on the more recent demonstration of a lack of a tumorigenic response in the PPAR-α -/- mouse after 11 months of PPAR-α agonist administration at a dose that induces 100% incidence of liver adenomas in concurrent studies in the PPAR-α +/+ mouse with the same genetic background. This PPAR-α null mouse is devoid of responses indicative of PPAR-α agonism. There was agreement among most, but not all, of the Panel that data from the PPAR-α -/- mouse indicate the requirement for the activation of PPAR-α in the MOA of the hepatocarcinogenic effect of these agents. A few Panel members expressed concern over the short duration of the studies in the PPAR-α -/- mouse (i.e., 11 months vs. 24 months in standard cancer bioassays), which rendered the studies incapable of assessing the lifetime liver cancer risk of PPAR-α agonists in this knockout mouse model, and thus, inadequate to conclusively demonstrate that PPAR-α activation is required for hepatocarcinogenesis.

Additional supporting evidence for the MOA, as discussed in the review by Klaunig et al. (2003) comes from the concordance of this MOA for several PPAR-α agonists, dose dependence of the effect, with both consistency and biological plausibility for the key events. One Panel member noted several inconsistencies in the supporting data however. These include observations from long-term carcinogenicity studies of the PPAR-α agonist gemfibrozil, where a dose-related increase in liver tumors was observed in male rats, while in females, a dose-dependent decrease in liver tumors was seen (IARC, 1996). In another example, studies in rats with two PPAR-α agonists, WY-14,463 and DEHP, demonstrated that doses that produced equivalent levels of hepatic peroxisome proliferation, measured as peroxisome number and peroxisomal enzyme activity, produced markedly different liver tumor incidences (Marsman et al., 1988). Another Panel member noted that these differences may be due to sex, species, and strain differences in pharmacokinetics.

In addition to the above, a Panel member expressed concern with the lack of understanding of key causal events in the proposed MOA intermediate between PPAR-α activation and cell proliferation, suppression of apoptosis and clonal expansion, given that activation of PPAR-α results in regulation of a multitude of genes involved in a variety of cellular functions, including lipid metabolism and transport, amino acid

metabolism, signaling molecules, transcription factors, and cell cycle and growth regulatory proteins.

The Panel agreed that data in the wild type and the PPAR-α knockout mouse would be strengthened if it were determined that the null mice generated on a 129 genetic background are not resistant to liver tumorigenesis in general, as opposed to specifically resistant to PPAR-α agonists (see Drinkwater and Bennett, 1991). In addition, the PPAR-α knockout mouse data would be strengthened by a demonstration of gene dose sensitivity. The Panel members also agreed that additional evidence of specific alterations associated with PPAR-α activation would greatly strengthen the proposed MOA.

Adequacy of the Data

Though the opinions of individual Panel members ranged from full agreement to complete disagreement, overall, the majority of the Panel felt the data supporting the key events associated with the proposed MOA in rats and mice are adequate, but recognized areas where the data could be strengthened. One overall concern with the proposed MOA was that, whereas PPAR-α activation is a very specific component of the MOA, the other steps deemed to be causally related, namely increased hepatocellular proliferation and clonal expansion of the initiated hepatocytes leading to tumor development, were very general and non-specific.

In support of the adequacy of the data, the key events and the associated events have been demonstrated to occur following administration of PPAR-α agonists. These data have been derived from many laboratories over the course of the last 30 years. Many of the associative events are highly correlated markers of PPAR-α agonist exposure and potential contributors to the causal events in the proposed MOA. The mechanistic linkage between the required step of PPAR-α activation and the key events (increased cell proliferation, decreased apoptosis, and clonal expansion of preneoplastic hepatic lesions) has not been determined. Although having these steps in the mechanism of PPAR-α induced rat and mouse hepatocarcinogenesis would strengthen the MOA, the majority of the Panel agreed that the current dataset is adequate to support the MOA. That the PPAR-α null mouse fails to exhibit the key and associated events when challenged with 11 months exposure to a potent PPAR-α agonist at a dose that induces 100% incidence of multiple liver adenomas in concurrently exposed control (wildtype) mice demonstrated to most, but not all, Panel members the underlying basis of the MOA statement.

Several concerns regarding the adequacy of the data also were discussed. As previously noted, a few Panel members expressed concern over the short duration of the studies in PPAR-α null mice which rendered the studies inadequate to conclusively demonstrate that PPAR-α activation is required for hepatocarcinogenesis. One member of

the Panel was concerned that the data were not adequate to identify the key events in the MOA for PPAR-α agonist induced rodent hepatocarcinogenesis, stating that although PPAR-α activation is believed to be the earliest key event, none of the many genes whose expression is regulated by PPAR-α has been identified as being in the causal pathway for liver tumorigenesis. More data are needed to establish and link the events that have been proposed as key causal events in the proposed MOA. In addition, a number of studies provide compelling data that suggest that a PPAR-α independent event, namely Kupffer cell activation, is required for increased hepatocyte proliferation by PPAR-α agonists. The Panel member felt that more data characterizing the relationship between Kupffer cell activation, and the cytokines that are released upon activation in hepatocarcinogenesis, and PPAR-α activation were needed before the identification of key events in the MOA could be properly evaluated. Another member of the Panel expressed concern, which was shared by some other Panel members, that data were lacking on the potential roles alterations in DNA methylation and chromatin structure play in the hepatocarcinogenic MOA of PPAR-α agonists.

Uncertainties and Inadequacies of the Data

Limitations of the available data have been detailed in the Klaunig et al. (2003) review. As noted above, the mechanism for the induction of cell proliferation and apoptosis suppression induced by PPAR-α agonists is not known. One significant factor to consider is the role of nonparenchymal hepatic cells in these processes. For example, Kupffer cells release cytokines, some of which are mitogenic to parenchymal cells and some that affect parenchymal cell apoptosis. In addition, many of the enzymes used as indicators of PPAR-α activation are regulated through a well defined mechanism of action that involves altered transcription of PPRE containing genes. Because this pathway of PPAR-α–dependent alteration of gene regulation is only associated with PPAR-α activation and not with the regulation of key events in the MOA, other mechanisms for induction of the key events need to be considered. Specific uncertainties may include whether agents must be metabolized from a pro-form to an active-form to be able to modulate the PPAR-α pathway, the induction of PPREs, or other indirect events.

Many, but not all, agents that demonstrate an ability to induce peroxisomes in rats and mice also induce a neoplastic response in the liver of rats and mice. Morphologic and biochemical evidence of peroxisome proliferation in rat and mouse liver is supportive evidence of the proposed MOA. It should be noted that these remain associated key events that are not proposed at this time to be causally related to tumor formation. The Panel agreed that there were considerable uncertainties as to the significance of associated key events, such as hepatic acyl CoA oxidase induction, with regard to the tumor forming potential of PPAR-α agonists in rats and mice. PPAR-α agonists can bind directly to PPAR-α, but may also perturb interactions with the RXR binding partner, the binding of co-activators and co-repressors to the receptor, or the availability and action of endogenous ligands or inhibitors.

Question 2 - Relative Sensitivity of Fetal, Neonatal, and Adult Rodents

OPPTS has provided a review of the ontogeny of PPAR-α expression and peroxisomal assemblage during fetal and postnatal development in rodents as well as an analysis of the available data evaluating effects on peroxisomal proliferation, peroxisomal enzyme activity, and liver weights following exposure to PPAR-α agonists during fetal and postnatal development in rats and mice (see Section V of the OPPTS Document). Based on this analysis, OPPTS concluded that fetal and neonatal rats do not exhibit an increased sensitivity to PPAR-α agonist-induced hepatocarcinogenicity relative to the adult rodent. Therefore, any conclusions regarding this MOA in adult rodents would also apply to young rodents, and similarly any conclusions regarding the relevance of this MOA for human hepatocarcinogenesis would apply to the young, as well as the adults.

Please comment on the weight of the evidence approach and mechanistic data used to support this conclusion.

Response

The Panel does not support the OPPTS conclusions. Although fetal and embryonic rats and mice respond to PPAR-α agonists as demonstrated by changes in peroxisomal enzyme activities, strong evidence demonstrating that fetal and neonatal rats do not exhibit an increased sensitivity to PPAR-α agonist-induced hepatocarcinogenesis is lacking. Moreover, conclusions regarding this MOA for human hepatocarcinogenesis should not be applied to developing humans.

As discussed in the response to question 1, the proposed MOA involves activation of PPAR-α, which regulates the expression of numerous genes, including several that encode for peroxisomal enzymes, and identifies as key causal events increases in cell proliferation, inhibition of apoptosis, and clonal expansion of preneoplastic lesions, which result in the formation of liver tumors. Published reports have shown that both the expression of PPAR-α and the assembly of peroxisomes occur late in the development of rats and mice. Furthermore, it has been shown that, as in adult livers, embryonic, fetal and neonatal livers of rats and mice respond to PPAR-α agonists by increasing peroxisome number, peroxisome volume density, liver weight, and the expression of the peroxisomal enzyme palmitoyl CoA oxidase. This suggests that at least some of the cellular macromolecules involved in the proposed PPAR-α agonist MOA are functional and responsive to PPAR-α agonists in rat and mouse embryonic, fetal, and neonatal livers. However, data on the hepatocarcinogenic response of rat and mouse embryonic, fetal, and neonatal livers to PPAR-α agonists are lacking and, therefore, no conclusions can be made at this time as to the relative sensitivity of these early life stages to PPAR-α agonist induced hepatocarcinogenicity.

Although the exposure of pregnant rats and mice led to increases in peroxisomal enzyme activities and increases in liver weight in embryonic, fetal, and neonatal liver tissues, other parameters involved in the proposed MOA, such as cell proliferation, inhibition of apoptosis and clonal expansion of preneoplastic cells, were not examined in these studies. In addition, responses to PPAR-α agonists in the fetal and neonatal rat and mouse, as measured by the peroxisomal enzyme expression levels, suggest that there are differences in young animals relative to adults. It is unclear how these differences in enzyme expression levels might translate into differences in sensitivity to hepatocarcinogenesis. Regarding the comparison of changes in liver weights across early and later life stages, it is inappropriate to assume that a given proliferative response seen at one stage of life is equivalent to a similar proliferative response at another stage of life. For example, an increase in liver weight during the neonatal period might result in a much greater lifetime risk of cancer than an equivalent increase occurring during adulthood, because a larger number of cells in the neonatal liver will undergo multiple cell divisions than in the adult. Finally, none of the studies examining the response of the rodent in utero or during early life stages were carried out with the late onset of tumors as a specific endpoint. A two-generation study conducted in mice was designed as a reproductive study and not as a cancer study. Thus, no liver pathology was documented from F1 male and female mice after approximately 4 and 6 months of exposure, respectively (one Panel member noted that complete pathology was not evaluated in this study). The available data pertain to effects that have not been demonstrated as causally linked to the carcinogenic MOA of these agents. The relevance of the induction of peroxisomes or peroxisomal enzymes to the carcinogenic process has not been established. As stated above, there is the possibility that developing organs and tissues may respond differently to peroxisome proliferators compared to adult organs and tissues. There may also be PPAR-α independent effects occurring in the young animal that result in an increased cancer risk. In the absence of this information, conclusions regarding the sensitivity of developing rodents to PPAR-α agonists cannot be formulated. Chemical exposures early in development could increase the sensitivity to cancer risk. It is known that PPAR-α modulates metabolic pathways other than β-oxidation of fatty acids, such as glucose and amino acid metabolism. Moreover, PPAR-α is a transcription factor involved in the modulation of gene expression. PPAR-α agonists not only modulate the expression of genes with PPREs but they may also regulate gene expression by altering levels of gene methylation (Ge, et al., 2001). Such DNA methylation is known to be involved in imprinting and alterations or loss of imprinting can directly or indirectly impact disease risk at later life stages (Cui, H. et al., 2003).

Conclusions regarding the relevance of the PPAR-α agonist MOA for human hepatocarcinogenesis applied to adults may not apply to the young. In contrast to adult human liver, there are no data establishing PPAR-α expression levels in embryonic, fetal and neonatal human liver. To date, there is only one publication reporting the effects of one PPAR-α agonist in lactating non-human primates (Cappon et al. 2002). In this report, the exposure of four Rhesus monkey females to HCFC-123 for short periods of time decreased the activities of cytochrome P450 enzymes and acyl CoA oxidase in

maternal monkey liver, as well as induced centrilobular hepatocyte vacuolation, necrosis and mild to moderate inflammation; however, no histological or biochemical data were reported from the infant monkeys. Non-human primate studies investigating preneoplastic and neoplastic effects of fetal or neonatal exposure to PPAR-α agonists would be desirable.

In contrast to embryonic and fetal rodent liver in which cytochrome P450 enzymes are expressed near, during and after birth (Ring et al. 1999), embryonic and fetal human livers possess metabolic activation capabilities resulting from the early developmental expression of cytochrome P450 enzymes. Moreover, the expression profiles of xenobiotic metabolizing enzymes and isozymes are different in embryonic, fetal, neonatal and adult human livers. Like the gene expression profile of xenobiotic metabolizing enzymes, it is difficult to disregard the possibility that there could be differences between the expression of PPAR-α and its transcriptional co-factors in the human conceptus and adult human liver. In addition, metabolic differences in rats and mice play an important role in determining the degree of response to some PPAR-α agonists (Lake, 1995) and that could also apply to the human conceptus.

Differences in peroxisome biogenesis have been reported during the ontogenic development of rodents and humans. While the assembly of peroxisomes in rats and mice, including the insertion of β-oxidation enzymes into the peroxisomes, occurs near birth, the assembly of human peroxisomes has been observed as early as 8 weeks of gestation (Espeel, et al, 1997). The number and density of peroxisomes plateau by 17 weeks of gestation in humans. Moreover, acyl-CoA oxidase and 3-ketoacyl CoA thiolase are immunodetectable in the peroxisomes by 10 and 9 weeks of gestation, respectively. These observations suggest differences in β-oxidation capabilities in developing rodents and humans and therefore information on the expression of the PPAR-α during ontogeny, as well as responses to PPAR-α agonists in embryonic and fetal human hepatocytes should be evaluated before concluding that the developing human conceptus is unresponsive to PPAR-α agonist exposures.

There are numerous uncertainties concerning the relevance of the PPAR-α agonist MOA for human hepatocarcinogenesis in the young. These uncertainties stem largely from our incomplete understanding of the species-specific differences in sensitivity. Although numerous mechanisms have been posited (see Klauning et al., 2003), none have adequate data supporting their validity. Some of these include differences in the PPREs in specific critical genes, species-specific co-factors that suppress transactivation ability of the ligand activated PPAR-α, sequence differences that result in the prevalence of inactive, splice variants and/or dominant negative PPAR-α gene products, perturbation of RXR binding partner interactions with other nuclear receptors, and polymorphisms that result in a less efficient transcription factor. Most importantly, there is no reason to eliminate the possibility that one or more of these scenarios would function differently in the human fetus, neonate or infant relative to the adult, impacting both MOA and sensitivity at these different life stages.

Question 3 – Human Relevance

OPPTS has provided an analysis of a variety of *in vitro* and *in vivo* studies on the key events pertaining to PPAR-α agonist-induced hepatocarcinogenesis with hamsters, guinea pigs, non-human primates, and humans. Based on the weight of the evidence, OPPTS concludes that although PPAR-α agonists can induce liver tumors in rodents and while PPAR-α is functional in humans, quantitatively, humans and nonhuman primates are refractory to the hepatic effects of PPAR-α agonists.

Therefore, OPPTS is proposing the following scientific policy:

> When liver tumors are observed in long term studies in rats and mice, and 1) the data are sufficient to establish that the liver tumors are a result of a PPAR-α agonist MOA and 2) other potential MOAs have been evaluated and found not operative, the evidence of liver tumor formation in rodents should not be used to characterize potential human hazard.

Please comment on the data and weight of evidence regarding the hepatic effects of PPAR-α agonists in humans, and please comment on the proposed OPPTS's science policy regarding human relevance.

Response

Overall, the majority of the Panel agreed that there are relevant data indicating that humans are less sensitive than rodents to the hepatic effects of PPAR-α agonists. However, the opinions of individual Panel members ranged from full agreement with the proposed OPPTS policy statement, as currently written, to complete disagreement. The majority of the Panel recognized weaknesses in the data that supported the policy noting in particular that the case for lack of human relevance was deficient in the human data. In addition, the Panel members agreed that the MOA and its application to addressing human relevance would be greatly strengthened by additional evidence of the specific alterations associated with PPAR-α activation that lead to the more general steps of hepatocellular proliferation, clonal expansion of initiated hepatocytes and tumor development. However, the Panel was divided regarding whether such additional evidence is necessary before accepting the MOA and its application to human relevance. Some Panel members believed that the data failed to demonstrate that the effect could only occur in liver and that, therefore, the policy statement should be limited to hepatocarcinogenic effects (see number 2 below). Other Panel members believed that the overall data limitations were significant enough to disagree with the MOA and its application to addressing human relevance.

Over the past 30 years, a variety of data have been accumulated that demonstrate species-specific sensitivities to agonist activation of PPAR-α, PPAR-α agonist-induced

liver peroxisome proliferation and PPAR-α agonist-induced hepatocarcinogenesis. As noted in the response to question 1, there was agreement among most, but not all of the Panel that data from PPAR-α null mice, showing that in the absence of the receptor, there were no ensuing changes in cell proliferation and hepatic tumor formation, was strong evidence that activation of PPAR-α is necessary for all subsequent steps in the MOA. It also was noted in the response to question 1 that a few Panel members expressed concern over the short duration of the studies in the PPAR-α null mice (i.e., 11 months vs. 24 months in standard cancer bioassays), which rendered the studies incapable of assessing the lifetime liver cancer risk of PPAR-α agonists in this knockout mouse model, and thus, inadequate to conclusively demonstrate that PPAR-α activation is required for hepatocarcinogenesis. Considering the proposed MOA, there was agreement that PPAR-α is present in humans and that the receptor is activated in human liver following exposure to known agonists. Accordingly, the proposed MOA for PPAR-α agonist-induced hepatocellular carcinogenesis in rodents is plausible for humans. There was also agreement that the nature of gene expression associated with hepatocellular PPAR-α activation is qualitatively different between humans and rodents. This difference may result from species differences in PPREs, but there are few data available that identify these potentially important differences, particularly in humans. Humans are at least as sensitive to activation end-points that lead to hypolipidemia but are much less sensitive to other end-points normally associated with peroxisome proliferation. This qualitative difference will be what is referred to in subsequent references as human sensitivity.

One overall concern with the proposed MOA was noted in the response to question 1 and is also a concern regarding the application of the MOA to addressing human relevance. Whereas PPAR-α activation is a very specific component of the MOA, the other steps deemed to be causally-related, namely increased hepatocellular proliferation and clonal expansion of initiated hepatocytes leading to tumor development were very general and non-specific. Overall, the Panel members agreed that additional evidence of specific alterations associated with PPAR-α activation in primates and especially humans would greatly strengthen the proposed MOA.

Although much of the data cumulatively support the hypothesis that agonist-induced human PPAR-α (hPPAR-α) activation fails to follow the MOA seen in rodent livers, namely, increased liver cell proliferation, decreased apoptosis, formation of preneoplastic foci and clonal expansion of these foci into liver tumors, the weight of evidence for this MOA and consequences of agonist-induced PPAR-α activation events in humans is less well defined than in rodents. Human liver biopsy data, while limited, indicate that clinical administration of PPAR-α agonists results in increases in the number and volume density of hepatic peroxisomes. The Panel agreed that the available cancer epidemiological data on pharmacologic PPAR-α agonists are too limited in study size and duration to provide any relevant information to evaluate human relevance. As such, data from other animals, including non-human primates, along with in vitro studies in human hepatocytes, or cell lines, provide the basis for evaluating the relevance of the proposed MOA in humans.

The available data from other animals includes guinea pigs, hamsters, dogs and non-human primates. In all cases, these animals demonstrate reduced liver sensitivities to PPAR-α agonists. Hamsters have a functional PPAR-α receptor but are intermediate in response between rats (and mice) and humans, and no increased cell proliferation or liver tumors have been observed in hamsters (Lake et al., 1993). Similarly, PPAR-α is constitutively present in guinea pigs, albeit at lower levels than rats or mice, and guinea pigs are also less sensitive than rats and mice to PPAR-α activation (Roberts et al., 2000). Data from non-human primates are limited, but generally indicate that PPAR-α agonists do not elicit the typical pattern of histopathological and biochemical changes associated with peroxisome proliferation in rats and mice, as the non-human primate responses to PPAR-α agonists have involved changes of lesser magnitude in fewer of the histopathological and biochemical markers (Reddy et al., 1984; Lalwani et al., 1985; Lake et al., 1989; Graham et al., 1994; and Kurata et al., 1998). Collectively, the Panel was split on the applicability of data from other animals to contribute to a weight of evidence regarding the hepatocarcinogenic effects of PPAR-α agonists in humans. All Panel members recognized that the data on non-rodent, non-human species provided relevant information on the reduced activity of PPAR-α agonists and contributed to the MOA. Also, while all Panel members recognized the limitations of these data (number of compounds studied, study sizes, and study durations), some believed that the data were sufficient to conclude the MOA was working, whereas others were concerned that the limitations were significant enough to disagree with the MOA.

There was a general consensus that the data linking PPAR-α activation to increased cell proliferation in all species was relatively weak. The strongest evidence in support of the importance of this step in subsequent tumor development is derived from the PPAR-α knockout mouse studies in which no increase in hepatic cell proliferation and no tumors are observed after 11 months of treatment (Peters et al., 1997). The Panel was again divided on the conclusions that can be reached from studies in the knockout mouse, as some were convinced by such data, whereas others felt that the overall susceptibility of this mouse model to hepatocarcinogenesis in 11 months had not been defined.

The strength of the hypothesis that humans are less sensitive to agonist-induced PPAR-α-mediated hepatocarcinogenesis lies in the human primary hepatocyte data. The Panel was again divided on the interpretation and utility of these data. First, there was a difference of opinion on the applicability of the in vitro studies used to assess the ability of human hepatocytes to proliferate in response to treatment with a PPAR-α agonist. Although limited in total sample size, these studies have shown that in vitro cultured human hepatocytes respond differently to PPAR-α agonists when compared to in vitro cultured rodent hepatocytes. As discussed in more detail below, whether these differences are attributable to true interspecies differences or reflect differences in human and rodent hepatocyte culture preparations remains an open question. In parallel experiments with in vitro cultured rodent hepatocytes, in vitro cultured human

hepatocytes fail to display several of the key responses deemed essential for the MOA in agonist-induced PPAR-α-mediated rodent hepatocarcinogenesis, those being increased cell proliferation and decreased apoptosis. Furthermore, in vitro cultured human hepatocytes appear to be less responsive to upregulation of peroxisomal genes and proliferation of peroxisomes, two key associative events of agonist-induced PPAR-α-mediated rodent hepatocarcinogenesis. Several Panel members suggested that further experiments in human primary hepatocytes (co-cultured with and without Kupffer cells; see comments below) would be useful if they provide additional biochemical data that demonstrate reduced levels of PPAR-α expression in human liver and an inability for agonist-induced PPAR-α to modulate the gene expression for several key peroxisomal enzymes. Such experiments would strongly support the hypothesis that human liver cells are less sensitive to agonist-induced PPAR-α-mediated hepatocarcinogenesis. Positive controls for known hPPAR-α responsive gene products should be included in such experiments (see, for example, Lawrence et al. 2001).

Those who disagreed with the conclusions noted above based their opinion largely on data that suggest that Kupffer cells are required to elicit a proliferative response in cultured hepatocytes. Specifically, evidence is emerging that supports a role for Kupffer cell activation on the induction of DNA synthesis, and subsequent neoplastic development following PPAR-α agonist treatment. In vivo studies have shown that depletion of Kupffer cells or inhibition of Kupffer cell activation prevents the induction of DNA synthesis by several PPAR-α agonists. These findings suggest that the lack of response from PPAR-α agonist exposure in human hepatocytes in vitro, may be due to the lack of nonparenchymal cells in the hepatocyte preparations. For example, the growth permissive factors released from activated Kupffer cells following PPAR-α agonist exposure are absent and may explain the lack of induction of DNA synthesis seen in cultured human hepatocytes. Support for this possibility has been demonstrated in rodent cultures in vitro (Rose, et al., 1999). In these studies, PPAR-α agonists were unable to induce DNA synthesis in purified preparations of rodent hepatocytes (devoid of nonparenchymal cells), while PPAR-α agonist-induced DNA synthesis was restored upon the addition of nonparenchymal cells, or medium derived from activated Kupffer cells, to the purified hepatocyte cultures.

It was noted that arguments against the involvement of the Kupffer cells comes from studies in the PPAR-α null mice. In these mice, agonists failed to elicit a DNA synthetic response. Since this model is replete with Kupffer cells, the lack of DNA synthesis has been interpreted as indicating that the Kupffer cell is not required. On the other hand, some members of the Panel felt that the communication and/or interplay between PPAR-α agonism and Kupffer cells has not been fully characterized and as such, the null mouse, lacking PPAR-α, is not directly applicable to the human situation in which PPAR-α is present and can be activated.

With regard to the human data, the Panel noted deficiencies arising from studies in which the duration of exposures to PPAR-α agonists were significantly less than

lifetime, the exposure levels were at therapeutic doses, and the populations of exposed individuals were fairly small. As stated previously, the Panel agreed that the available cancer epidemiological data on pharmacologic PPAR-α agonists are too limited in study size and duration to provide sufficient information to evaluate human risk potential. Although the human data are limited, the existing data do provide some important information for consideration. Human liver contains functional PPAR-α receptors and the fibrate class of drugs is able to activate this receptor to alter the expression of genes involved in lipid metabolism that induce hypolipidemia. Chronic exposure data reported in humans for two different PPAR-α agonists suggest that humans do not respond to PPAR-α agonists by an increase of the associated key events (such as cell proliferation, suppressed apoptosis, and clonal expansion of preneoplastic hepatic lesions) observed during PPAR-α activation in rats and mice exposed to these agonists. In addition to the short duration of exposure and the use of therapeutic doses (lower than the doses used in studies with rats and mice), the limitations of these studies include the use of weak agonists. The human epidemiology data from short duration follow up (5 year time period) indicated an early increase in GI tract tumors, although liver cancer was not reported independently. However, no differences were noted after 8 years of follow up. Evidence for peroxisome proliferation and increased cell proliferation was lacking in human liver biopsies. Problems with these observations include the high variability in assessing peroxisome increases in biopsy material that are not representative of all zones of the liver, and whether the timing of biopsy sample acquisition was appropriate for detecting an increase in cell proliferation. A slight increase in the number and density of peroxisomes is observed in humans with chronic exposure to therapeutic levels of a PPAR-α agonist. This level is indicative of normal physiologic or metabolic changes and is lower than the approximately three fold level defined by Ashby et al. (1994) as the threshold level of peroxisome induction associated with liver cancer risk in rats and mice. These observations in humans are strengthened by the studies of chronic exposure of non-human primates to PPAR-α agonists for 5 or more years. Again, the number of non-human primates exposed was limited and the duration of exposure was less than lifetime. Assessment of the presence or absence of PPAR-α regulated gene expression and of preneoplastic lesions needs to be detailed in primates compared to rats and mice following exposure to PPAR-α agonists. The non-human primate appears to have a markedly attenuated response to fairly potent PPAR-α agonists (e.g., ciprofibrate) compared with rats and mice, although, as with the human data, the PPAR-α agonist challenge has been at lower doses of shorter duration. Studies by Pugh et al., (2000) wherein numerous PPARα agonists were administered to nonhuman primates support this contention in that a lack of increase in liver weights indicates a lack of cell proliferation as verified by replicative DNA synthesis.

The Panel discussed three other issues relative to assessing weight of evidence regarding the hepatic effects of PPAR-α agonists in humans, and the proposed science policy regarding human relevance. These included:

1. The use of the word "refractory" to describe the human response to PPAR-α activation is too absolute. The Panel agreed that "less sensitive" is a more appropriate description of the nature of the human response relative to that observed in rats and mice.

2. The policy statement drafted by OPPTS concludes with the phrase "evidence of liver tumor formation in rodents should not be used to characterize potential human hazard." After some discussion, it was suggested by one member of the Panel, and supported by several other Panel members, that this phrase should be modified to read, "evidence of liver tumor formation in rodents should not be used to characterize potential human hepatocarcinogenic hazard."

3. One member of the Panel expressed concern, which was shared by some other Panel members, that the MOA and evaluation of human relevance was lacking in its assessment of altered gene expression that could be associated with altered methylation of DNA. There is evidence that DNA methylation is modified in rodents following exposure to PPAR-α agonists (Ge et al., 2001, Ge et al., 2002, and Pereira, et al., 2004). Given the accepted role for DNA methylation in gene imprinting and the loss of imprinting in cancer etiology (see for example McClachlan et al., 2001), such a role for PPAR-α agonists in causing similar alterations in humans should be explored before human relevance can be appropriately evaluated, particularly for exposure during early life stages and for questions regarding site concordance.

Question 4 – Data Requirements

OPPTS has proposed a data set that would be sufficient to demonstrate that PPAR-α agonism is the MOA for the induction of rodent liver tumors. The data set includes evidence of PPAR-α agonism (*i.e.,* from an *in vitro* reporter gene assay), *in vivo* evidence of an increase in number and size of peroxisomes, increases in the activity of acyl CoA oxidase, and hepatic cell proliferation. The *in vivo* evidence should be collected from studies designed to provide the data needed to show dose-response and temporal concordance between precursor events and liver tumor formation.

Please comment in general on the proposed data set and particularly on its adequacy to demonstrate that a PPAR-α agonist-mediated MOA is operating in rodent hepatocarcinogenesis.

Response

Data requirements refer to the experimental data needed to demonstrate that a compound acts through a PPAR-α agonist MOA. These data may be used subsequent to a bioassay that finds induction of hepatic tumors to demonstrate such tumors arose from a PPAR-α agonist MOA, or subsequent to initial (sub)acute experiments to assist in the subsequent experimental design of long-term experiments for submission to the Agency. This use of the data may dictate some differences in the data requirements needed. The

following discussion focuses on requirements after a positive bioassay, with suggestions provided for the converse situation.

There was general consensus among the Panel that the proposed data set was adequate and provided a straight forward approach to classifying a chemical as a PPAR-α agonist. The Panel also concurred that the use of PPAR-α knockout mice would be definitive evidence to ascribe a chemical as a PPAR-α agonist, but that the proposed data set would be sufficient in lieu of the use of this rather costly tool. While the Panel agreed with these data needs, they suggested some clarifications and additional supportive approaches.

The clarifications indicated were as follows: the term 'direct DNA reactivity' may need to be clarified as 'direct' may be interpreted by some to mean "without metabolism"; in keeping with the ILSI document (Klaunig et al., 2003), rather than using the term 'mutagenicity' alone, the terms 'mutagenicity and/or clastogenicity' may be more appropriate; palmitoyl CoA activity is simply a substrate-specific name for acyl CoA oxidase activity; and microsomal fatty acid oxidation (as opposed to microsomal fatty acid omega-oxidation) is not specific enough to designate CYP4A activity.

In the course of the Panel's discussion, questions for clarification were posed to the Agency as to when (i.e., before or after a positive liver tumor finding in rodents) this set of assays testing for PPAR-α agonist activity would be conducted. The Agency indicated that data demonstrating PPAR-α agonist activity could be submitted in the absence of testing in long-term carcinogenesis studies. In response to this, a Panel member observed that in the absence of testing in standard long-term rodent carcinogenicity studies, it is not possible to determine whether the chemical would operate through a PPAR-α agonist MOA producing rodent liver tumors. A chemical with PPAR-α agonist activity may either: 1) not cause cancer in rodents, 2) cause liver cancer in rodents by the proposed PPAR-α agonist MOA, 3) cause liver cancer by a MOA other than the proposed PPAR-α agonist MOA (e.g., cytotoxicity), or 4) cause cancer at sites other than the liver (with or without liver cancer). The Panel concurred that an overriding requirement is that other MOAs have been excluded. For example, rigorous tests must be performed to exclude mutagenicity, other forms of DNA damage (clastogenicity), or overt cytotoxicity directly produced by the test compound, or its metabolic products.

The Panel also concurred that direct evidence of the activation of PPAR-α is required to show that complementary in vivo results do not result from activation of other PPARs or from an unknown mechanism as exemplified by dehydroepiandrosterone (DHEA) (Isseman and Green, 1990, Peters, et al., 1996 and Waxman, 1996). The activation of PPAR-α is often demonstrated using chimeric systems that include an expression system for the PPAR-α receptor and a reporting system that includes the PPRE in the promoting region. It was recommended by one Panel member that this study could be supplemented by gene-dosage experiments in knockout mice or transgenic mice

overexpressing the receptor with respective loss or exacerbation of responsiveness. These experiments would demonstrate a direct effect of the receptor on a true genomic PPRE, rather than a construct. It was also recommended that it be acknowledged that in some cases a metabolite of the test compound may be a more suitable substrate to use in these experiments. Direct involvement of PPAR-α can alternatively be assessed using in vivo experiments with wild type (PPARα +/+) and knockout mice (PPARα -/-); end-points for these in vivo experiments are discussed below. Compounds with positive bioassays in rats but not mice would not be suitable for this alternative approach.

In vivo experiments should be conducted using doses that produce positive bioassays; as they are normally (sub)acute they will meet temporal requirements that they occur prior to tumor formation. Of highest priority, they must demonstrate an increase in hepatocyte cell replication/reduced apoptosis, induction of peroxisomal acylCoA oxidase and an increase in number and volume percent of peroxisomes. Demonstration of induction of other enzymes with PPRE sequences in the promoter region (CYP4A, carnitine acetyl transferase, fatty acid binding protein, etc.) or catalase provides supportive evidence. It was recommended that at least one 'supportive example of enzyme induction' be included. Induction of enzymes can be demonstrated from increased enzyme activity and/or increased expression of mRNA. It was also noted that the need to show both increases in peroxisome volume percent and density would require morphometric analysis of liver sections examined by electron microscopy (demonstration of increased density, but not volume percent, could be approached using light microscopic methods).

One Panel member suggested that when acute evidence of a PPAR-α agonist MOA has been found prior to long-term dosing studies, the evidence of the MOA can be further enhanced by inclusion of an initiation/promotion test system where the test compound is administered as the promoter after suitable initiation. These experiments demonstrate the key event of clonal expansion. In addition, there are some (immuno)histochemical stains that can be used to show a greater degree of specificity for this MOA. It was acknowledged that while such experiments would further support the MOA, they were fairly time- and cost-inefficient with regard to the main objective of demonstrating that the compound is a PPAR-α agonist.

Question 5 – Other Tumors Induced by PPAR-α Agonists

Some PPAR-α agonists may also induce pancreatic acinar cell and Leydig cell tumors in rats and modes of action involving agonism of PPAR-α have been proposed. An in depth analysis of these tumors is provided in the 2003 ILSI technical panel report. Based on this analysis, OPPTS agrees that the data available to date are insufficient to support the proposed MOAs.

Thus, OPPTS is proposing the following science policy:

> Given the limited evidence available to support that a chemical may induce pancreatic and Leydig cell tumors through a PPAR-α agonist MOA, the evidence is inadequate at this time to support a linkage between PPAR-α agonism and formation of these tumor types. Thus, it is presumed that chemicals that induce pancreatic or Leydig cell tumors may pose a carcinogenic hazard for humans.

Please comment on OPPTS's conclusion that there is limited evidence that a chemical may induce pancreatic and Leydig cell tumors through a PPAR-α agonist MOA, and OPPTS's proposed science policy regarding other tumors induced by PPAR-α agonists.

Response

In addition to the hepatic tumors that appear to be a general occurrence in rats and mice, nine PPAR-α agonists have been reported to induce Leydig cell tumors (LCTs) and pancreatic acinar cell tumors (PACTs) in rats. Together with the hepatic tumors, this is referred to as the tumor triad. The Panel was in agreement with the OPPTS conclusion that chemicals that induce pancreatic or Leydig cell tumors may pose a carcinogenic hazard for humans.

LCTs were most apparent when PPAR-α agonists were tested in non-F344 male rats, likely because by 2 years of age, the F344 rat has virtually a 100% incidence of spontaneously occurring LCTs. This will obscure any ability to detect a xenobiotic-induced testicular tumor in this strain. The finding that a relationship appears to exist between PPAR-α agonists and LCT formation has led to speculation that many, if not all, such agonists would induce this tumor if tested adequately in a rat strain other than F344. This speculation has been supported by limited studies in other strains (Biegel, et al., 2001, Maltoni, et al., 1988 and Mennear, 1988).

It was originally hypothesized that PPAR-α agonists cause LCTs by a PPAR-α-dependent mechanism similar to that of the liver. However, evidence exists using PPAR-α null mice (Ward et al., 1998) to suggest that the PPAR-α agonist DEHP can induce toxic lesions in kidney and testis independently of this receptor. In addition, although Leydig and pancreatic acinar cells contain PPAR-α, agonists do not appear to induce peroxisome proliferation in these cells. This suggests that tumors developing in these tissues in rats do so via different mechanisms than in the liver where peroxisome proliferation is always observed. A prevailing hypothesis is that PPAR-α agonists cause an increase in estradiol that promotes the secretion of transforming growth factor (TGF-α). Evidence in support of this hypothesis is that PPAR-α agonists increase the expression of aromatase, an enzyme that under normal conditions maintains serum estradiol concentrations by converting testosterone to estradiol (Biegel, et al., 1995). Estradiol stimulates TGF-α production which induces Leydig cell proliferation (Khan, Teerds, and Dorrington, 1992).

Another proposed MOA of PPAR-α agonist-induced LCTs is that they cause testicular hypertrophy by decreasing testosterone biosynthesis, leading to an imbalance of androgen/estrogen levels. This leads to an increase in leutinizing hormone (LH) which promotes LCTs. However, it is not known whether steroid synthesis pathways in testis are regulated by PPAR-α, and in Cook et al. (2001) no changes in LH were observed.

The Panel agreed that although some data suggest LCTs may involve PPAR-α, additional research will be required to confirm this role. In addition, the link to PPAR-α activation is considered tenuous because limited studies of PPAR-α agonists in other animal species, such as the mouse, hamster and nonhuman primates, did not show extrahepatic carcinogenic responses, including PACTs and LCTs. As noted previously, the Panel agreed that the available cancer epidemiological data for pharmaceutical PPAR-α agonists are too limited in study size and duration to be informative as to cancer risk at any site. While LCT data in mice remain limited, this species difference from rats is certainly indicative of some unique feature either in rats which causes the tumors, or in mice which are resistant. Further data are needed to determine which is the case. It is also noteworthy that the spontaneous rate of LCTs is much lower in humans than in rats suggesting innate resistance to this type of cancer, and that rat and human Leydig cells respond differently to human chorionic gonadotropin (human cells undergo hypertrophy while rat cells proliferate). Finally, a human condition with constant LH receptor activation does not lead to LCTs, even though this is one of the major proposed MOAs in rats.

Key events in the postulated MOA for PACTs in rats are considered to begin with PPAR-α activation in the liver, followed by changes in bile composition and a decrease in its synthesis. This results in cholestasis and a sustained increase in cholecystokinin. This stimulates acinar cell proliferation and promotes the development of PACTs. If this is true, then the rat PACTs are secondary to the liver effects of PPAR-α agonists. Some data indicate that many of the non-hepatocarcinogenic parameters and symptoms manifested in rodents upon long-term administration of PPAR-α agonists are also manifested in humans. This is particularly true since it has been observed in rodents that long-term administration of PPAR-α agonists results in marked changes in bile acid secretion and composition. In human studies it is also established, by multiple investigators, that fibric acid drug treatment increases biliary cholesterol and induces supersaturation of bile. Studies demonstrating that hPPAR-α is functional in the regulation of a variety of enzymes associated with bile acid metabolism in human liver cells would suggest that the risks of PACTs in humans exposed to PPAR-α agonists could involve a PPAR-α mechanism. However, the data are not sufficient to firmly conclude that this MOA is operative. Furthermore, the difference between rodents and humans in the cellular origin of pancreatic tumors (acinar in rat, ductal in humans) suggests that these animal data are of limited relevance to humans. Again, although data in other species are limited, only rats have shown these tumors.

Finally, in addition to PPAR-α agonism as a potential MOA of extrahepatic tumors, as noted previously, one member of the Panel expressed concern, which was shared by some other Panel members, that consideration needs to be given to epigenetic phenomena that may be activated by these chemicals. DNA methylation and chromatin structure alterations are significant nongenotoxic mechanisms involved in deregulating gene function. Furthermore, PPAR-α agonists inhibit methylation during DNA replication (Ge et al., 2001), thereby altering the cellular epigenome. This is important since the earliest change identified in tumor cells compared to their normal counterpart is genome-wide hypomethylation (Feinberg and Vogelstein, 1983). These changes can be particularly critical during development, including puberty, but even adults vary dramatically in their susceptibility to cancer because of marked differences in the epigenome. For example, there is now evidence that approximately 10% of the human population is at high risk, at least for colon cancer, because of either an inability to maintain imprinting at the IGF2 locus or exposure early in development to some environmental factor resulted in IGF2 loss of imprinting (Cui et al., 2003). It is conceivable that these "preneoplastic" individuals are more susceptible to PPAR-α agonists than the general population.

In summary, given the limited amount of data available on the true MOA for LCTs or PACTs, including the possibility raised by some Panel members that epigenetic effects of the PPAR-α agonists may occur, it is not possible to determine whether PPAR-α agonists pose a carcinogenic hazard to humans. Thus, the conclusion by the OPPTS that the available data for the induction of rat LCTs and PACTs by PPAR-α agonists are insufficient to conclude that the sole MOA involves the PPAR-α receptor is considered by the Panel to be appropriate. Further, the Panel concurs that it should be presumed that chemicals that induce pancreatic or Leydig cell tumors may pose a carcinogenic hazard for humans.

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