Organisation for Economic Co-operation and Development

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Adverse Outcome Pathway External Review Report

AOP 6: Antagonist binding to PPARα leading to body-weight loss

Enter any logistical information related to the meeting e.g. meeting date, time and location.

This AOP list seven key events (KEs) leading from the molecular initiating event (MIE) binding and antagonizing PPARα to the adverse outcome (AO), decreased body weight. The proposed AOP states that the MIE subsequently leads to the other key events. Specifically: KE-1, stabilization of PPARα co-repressor, KE2, decreased PPARα transactivation of gene expression, KE3, decreased, peroxisomal fatty acid β-oxidation of fatty acids, C) KE4, decreased, mitochondrial fatty acid β-oxidation, KE5, decreased, ketogenesis (production of ketone bodies), KE6, decrease in serum ketone bodies, KE7, increase in muscle protein catabolism and apically, loss of weight stimulated by lack of PPARα signaling. The complexity of the AOP is revealed in the large number of KEs and the species-specificity of PPARα action. While there are numerous documents describing the PPARα pathway and its physiological/pathological role, few chemicals have been tested for the MIE, any of the KEs or the AO. Thus, there is a disproportional reliance on GW6471-based information. The reviewers state that while the AOP is not perfect, it is important that this AOP be published. There is agreement between the reviews and authors on ways of improving AOP6 and submitting it to OECD for approval and publication.

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1. Introduction and background to specific AOP

OECD AOP6: "Antagonist binding to PPARα leading to body-weight loss" with the short title: PPARα antagonism leading to body-weight loss can be found at https://aopwiki.org/aops/6. AOP6 describes chemical binding and stabilization of a co-repressor to the peroxisome proliferator-activated receptor α (PPARα) signalling complex causing a chain of events that includes: antagonism of PPARα nuclear signalling, decreased transcriptional expression of PPARα-regulated genes that support energy metabolism, and inhibited metabolic energy production culminating with starvation-like weight loss. The MIE for this AOP involves antagonistic PPARα binding. The antagonist-binding to the PPARα regulatory complex causes the KE1, stabilization of co-repressor (SMRT or N-CoR) to PPARα ligand binding domain suppressing PPARα nuclear signalling. PPARα is a transcriptional regulator for a variety of genes that facilitate systemic energy homeostasis. As a result of the MIE and then KE1, the KE2 occurs where PPARα transactivation is inhibited for genes involved in the next 3 key events of the AOP: (KE3) decreased peroxisomal fatty acid β-oxidation, (KE4) decreased mitochondrial fatty acid β-oxidation, and (KE5) decreased ketogenesis. The KE3 results in decreased catabolism of very long chain fatty acids which can reduce substrate availability for energy production. Both KE2 and KE3 can drive KE4 decreasing conversion of short, medium and long chain fatty acids into substrates for use in ATP production. KE2 (and also potentially KE4) can drive KE5 resulting in decreased potential to repackage energy substrates as ketone bodies to support systemic energy demands during periods where the systemic energy budget is negative. The KE6, no change or a decrease in circulating ketone bodies, occurs under cellular energy deficit conditions, a state where ketogenesis is typically induced thus increasing circulating ketone bodies as metabolic fuel to sustain energy homeostasis. Physiological studies of the progression of human starvation have demonstrated the critical importance of ketogenesis, especially production of β-hydroxybutyrate, for meeting systemic energy demands by supplementing glucose to sustain the energy requirements of the brain. Sustained negative energy budgets lead to KE7, an increase in muscle protein catabolism, with glutamine and alanine recycled for gluconeogenesis. Finally, the AO of body-weight loss occurs, which within the context of dynamic energy budget theory decreases energy allocations to organismal maturation and reproduction and has been demonstrated to negatively affect ecological fitness. This AOP (Figure 1) was last updated in June of 2017.

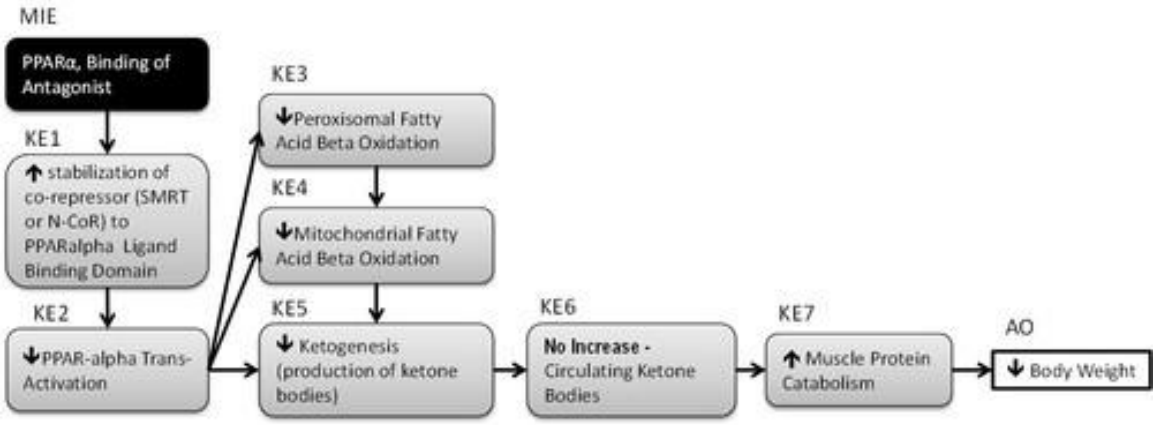

Figure 1: OECD AOP6: Antagonist binding to PPARα leading to body-weight loss.

2. Synthesis of main issues of the review

1. Scientific quality:

The reviewers wish to congratulate the authors on their efforts in developing AOP6. The reviewers recognize the complexity of the issues as reflected in the large number of KEs and the species-specificity of PPARα action. The reviewers further realize there are many papers described the PPARα pathway and its physiological/pathological role but few chemicals have been tested for the MIE, any of the KEs or the AO. The reviewers note that this data void has place a disproportional reliance on GW6471-based information.

Does the AOP incorporate the appropriate scientific literature?

There is agreement among the reviewers that there are opportunities for inclusion of additional or updated literature that would refine and strengthen the AOP with additional details from the more current/expanded knowledge. Specifics are highlighted in Section 2- weight-of-evidence. It was also noted that there may be additional and subsequent studies that should be evaluated which may strengthen the KERs.

Does the scientific content of the AOP reflect current scientific knowledge on this specific topic?

More than one reviewer commented that the current AOP does not adequately address the species-specificity of PPARα action. For example, the majority of the scientific supports for the AOP come from rodent studies. However, human and rodent PPARα are distinct in their ability to induce peroxisome proliferation and peroxisomal fatty acid β-oxidation. The AOP largely relies on a single review paper for information on the function of human PPARα (Kersten et al., 2014). A more thorough incorporation of analyses and results

from primary studies of human PPARα and of the effects of GW6471 on human PPARα is needed.

Suggested additional literature includes:

Chan et al., 2006. The V227A polymorphism at the PPARA locus is associated with serum lipid concentrations and modulates the association between dietary polyunsaturated fatty acid intake and serum high density lipoprotein concentrations in Chinese women. Atherosclerosis. 187(2):309-15.

Cheung et al., 2004. Diminished hepatocellular proliferation in mice humanized for the nuclear receptor peroxisome proliferator-activated receptor α. Cancer Res. 64: 3849-3854.

Feige et al., 2010. The pollutant diethylhexyl phthalate regulates hepatic energy metabolism via species-specific PPARα-dependent mechanisms. Environ. Health Perspect. 118(2): 234-241.

Liu et al., 2008. A natural polymorphism in PPAR-alpha hinge region attenuates transcription due to defective release of nuclear receptor corepressor from chromatin. Mol. Endocrinol. 22(5): 1078-1092.

Rakhshandehroo et al., 2009. Comparative analysis of gene regulation by the transcription factor PPARalpha between mouse and human. PLoS One. 4(8): e6796.

McMullen et al., 2014. A map of the PPARα transcription regulatory network for primary human hepatocytes. Chemico-Biological Interactions (209)

Janssen et al., 2015. The impact of PPARα activation on whole genome gene expression in human precision cut liver slices BMC Genomics. 16:760.

2. Weight of evidence:

Are the weight-of-evidence judgement/scoring calls provided by AOP developers for KEs, KERs and the overall AOP justified?

The AOP relies heavily on experimental results for GW6471. WoE provided by 2,4-dinitrotoluene (2,4-DNT) was not received well by the reviewers.

MIE: The reviewers agree that for GW6471, the literature and data reported in the AOP supports the MIE, binding and antagonizing PPARα are excellent. However, as noted by one reviewer, the data supporting 2,4-DNT binding and antagonizing PPARα are not convincing. Particularly, it was noted that 2,4- DNT does not reduce PPARα ligand-induced transcriptional activity, even when 2,4-DNT was included at a concentration 3 orders of magnitude greater than a known PPARα agonist (see Wilbanks et al., 2014).

KE1: The reviewers agree that the molecular consequences of GW6471 binding to human and mouse PPARα (e.g., coregulator recruitment and release) are well supported by the literature and data reported in the AOP. It was noted that Wilbanks et al. (2014) do not show that PPARα activity (positively or negatively) is influenced by 2,4- DNT. It was further noted that while Gust et al. (2015) provides stronger evidence that 2,4-DNT is a PPARα ligand. They did not analyze coregulator recruitment to PPARα.

KE2: The reviewers agree that the decrease in expression of PPARα gene targets by GW6471 in human and mouse models is well supported by the AOP. However, it was recognized by more than one reviewer that the gene expression profile induced by activation of PPARα is species-specific, with distinct differences between mouse and human (See Kersten et al., 2014). There are no data provided about potential species-specific effects of PPARα antagonism in human liver. It is recommended that these data be included in the AOP.

KE3: The reviewers generally agree that the processes and genes involved in peroxisomal fatty acid β-oxidation are well-described and appropriated supported by the cited literature. However, several concerns were raised by different reviewers. It was noted that no evidence of the effect of PPARα antagonism or lack of expression are provided. It is recommended that data from studies in PPARα knockout mice be included. It was further noted that the regulation of peroxisome proliferation and peroxisome-related gene expression is distinct in mouse and human. This KE would be improved by some mention of this distinction. Moreover, there is no mention of the relative importance of peroxisome- vs mitochondria-dependent fatty acid oxidation in mouse and human (see next KE). Lastly, it was questioned whether there is enough evidence to support the proposed mitochondrial fatty acid β-oxidation effects (KE3)

KE4: The evidence from Aoyama et al. (1998) that PPARα knockout in the mouse significantly reduces constitutive and inducible mitochondrial fatty acid oxidation-related gene expression is strong. However, no evidence of the effect of GW6471 or PPARα-knockdown on mitochondrial fatty acid β-oxidation in human cells is provided. Given that peroxisomal fatty acid oxidation is regulated distinctly by PPARα in humans versus mouse (Blaauboer et al., 1990; Rakhshandehroo et al., 2009) and would presumably remain functional in the presence of PPARα antagonists, it is unclear what the physiological significance of antagonism of mitochondrial fatty oxidation alone would be.

Blaauboer, et al., 1990. The effect of beclobric acid and clofibric acid on peroxisomal beta-oxidation and peroxisome proliferation in primary cultures of rat, monkey and human hepatocytes. Biochemical Pharmacology 40:521e528.

Rakhshandehroo et al., 2009. Comparative analysis of gene regulation by the transcription factor PPARalpha between mouse and human. PLoS One. 4(8): e6796.

KE5: The decrease in ketogenesis by PPARα knockout in mice could be strengthen by including reference to the work of Le May et al. (2000).

Le May et al., 2000. Reduced hepatic fatty acid oxidation in fasting PPARK null mice is due to impaired mitochondrial hydroxymethylglutaryl-CoA synthase gene expression. FEBS Lett. 475: 163-166.

Cahill et al. (2006) reviewed the relationship between fasting and the need to generate ketone bodies to support the brain's metabolic demands. However, there are no data presented demonstrating the connection between fasting, PPARα upregulation and ketone body production in humans. Specifically, human 3-hydroxy-3-methylglutaryl-CoA synthase 2 (HMGCS2) is a mitochondrial in enzyme in humans that is encoded by the HMGCS2 gene. HMGCS2 is the rate limiting step in the ketogenic pathway; HMGCS2 expression is increased when HepG2 cells are engineered to express PPARα (Vilà-Brau et al., 2011). At the very least, this citation should be included. Furthermore, there are no data provided demonstrating how PPARα antagonism affects ketone body production in humans.

Vilà-Brau et al., 2011. Human HMGCS2 regulates mitochondrial fatty acid oxidation and FGF21 expression in HepG2 cell line. J. Biol. Chem. 286(23): 20423-20430.

KE6: The reviewers agree that the literature and data showing a decrease in serum ketone bodies in PPARα knockout mice are strong. Since the references provided for the connection between PPARα and humans are reviews, the AOP would be strengthened by adding appropriate primary literature citations.

KE7 & AO: An increase in muscle protein catabolism and loss of weight stimulated by lack of PPARα signaling is not supported by mouse studies. In Badman, et al 2007 and Muoio, et al., 2002, mice lacking PPARα signaling do not show weight loss on a regular chow diet, and there is similar weight loss in WT and KO mice following endurance exercise. While muscle protein catabolism may be an outcome of extended fasting in humans, no evidence is provided that demonstrates muscle protein catabolism is impacted by PPARα status. Similarly, while it is true that there are dramatic changes in glucose and lipid metabolism in mice lacking PPARα signaling during fasting and endurance exercise, there is no evidence that the lack of PPARα enhances weight loss. Evidence from 2,4-DNT exposure studies in rats and birds showing reduced endurance and weight loss do not directly test the contribution of PPARα to these effects. Therefore, the reviewers question using this line of evidence to support the current AOP.

Muoio, et al., 2002. Fatty acid homeostasis and induction of lipid regulatory genes in skeletal muscles of peroxisome proliferator-activated receptor (PPAR) α knock-out mice. J. Biol. Chem. 277: 26089-26097.

Overall: There is general agreement among the reviewers that the assessment of the scientific evidence supporting the linkages in the AOP largely is an accurate representation. However, a number of issues were raised by the reviewers. Specifically, these include:

1) It must be qualified that:
   A) the data supporting the KEs and their linkages are based largely on rodentstudies.
   B) it is well known that rodent and human PPARα act distinctly.
2) The linkage between a reduction in ketone body production via loss of PPARα function and

increased muscle protein catabolism is weak to non-existent.

3) It is not clear in the current AOP if PPARα function is necessary under normal feed/activity levels. For example, it appears that only when fasting, or during enduring physical activity, that the PPARα function is necessary to generate ketone bodies as an alternative energy source. The logical extension of this is that the AO which results from a lack of PPARα function only occurs when alternative energy sources are required.
4) In terms of taxonomic applicability, the only strong evidence that antagonism of PPARα results in KE2-KE6 is for mice. Moreover, while the evidence is moderate for rats, the evidence is weak for birds and humans.

3. Regulatory applicability:

Considering the strength of evidence and current gaps/weaknesses, what would be the regulatory applicability of this AOP, in your opinion?

The reviewers are not in agreement as to the regulatory application of AOP6 as currently written. Options vary for it being deemed "appropriate for general consideration in regulatory decision-making" to "not believing that the evidence in this AOP is strong enough to support its regulatory applicability". There is general agreement among the reviewers that, at this time, the lack of clear relevance to humans clouds the regulatory applicability of AOP6.

4. Conclusion:

What are your overall conclusions of the assessment of this AOP?

The reviewers are not in agreement as to the overall conclusions as to their assessment of AOP6.

One reviewer recommended the current AOP6 for the adoption by WNT and WPHA groups but at the same time noted that the current version of AOP6 may be too complex and data-poor for regulatory use. One reviewer concluded the current AOP6 was of some use but did not specify that use. Another reviewer felt that there may be other endpoints that could be used as the AO for this AOP (e.g., hepatic steatosis and adiposity). However, the relevance to humans is questionable. The fourth reviewer expressed the opinion that the current version of AOP6 is of little regulatory value. This reviewer base their opinion on: 1) the lack of consideration of species differences in the function of PPARα, which they believe is a significant weakness in this AOP, 2) the lack of primary studies in human models or epidemiological studies in humans, which impairs the usefulness of the AOP, 3) the weak to non-existent evidence for linkage between a reduction in ketone body production via loss of PPARα function and increased muscle protein catabolism, 4) the interpretation that the AOP does not make it apparent adverse outcomes that result from a lack of PPARα function induced during fasting or endurance exercise would be expected to have an adverse effect when feed and activity levels are normal, and 5) the inclusion of studies with DNTs is deemed correlatory, at best, and does not strengthen the AOP.

It was noted that overall, there are many endpoints that could be used as the apical endpoint for this AOP such as hepatic steatosis and adiposity. However, the relevance to humans is questionable.

3. Summary record of the teleconference

3.1. TC agenda

May 2018: Reviewers and Authors Teleconference for AOP6

AGENDA (1 May 2018)
The teleconference starts at 14h00 and closes at 15h30, Paris time.

The aims of this teleconference are to provide the authors an opportunity to respond to the "Synthesis of the Main Issues of the Review" submitted by the review manager and come to agreement with the reviewers on revising AOP6.

The teleconference will be chaired by the review manager. The participants will be the four external reviewers and representatives of the AOP writing team. The teleconference will be recorded. The review manager will draft the summary record of the teleconference.

1) Review of where we stand.
The review manager will review where we stand.

2) Discussion on the reviewer's comments on the question ─ **Does the AOP incorporate the appropriate scientific literature?**

3) Discussion on the reviewer's comments on the question ─ **Does the scientific content of the AOP reflect current scientific knowledge on this specific topic?**

4) Discussion on the reviewer's comments on the question ─ **Are the weight-of-evidence judgement/scoring calls provided by AOP developers for KEs, KERs and the overall AOP justified?**

5) Discussion on the reviewer's comments on the question ─ **Considering the strength of evidence and current gaps/weaknesses, what would be the regulatory applicability of this AOP, in your opinion?**

6) Discussion on the reviewer's comments on the question ─ **What are your overall conclusions of the assessment of this AOP?**

3.2. Main issues and responses during the call

Synthesis of the Main Outcomes of the Reviewers and Authors Teleconference
The aim of the teleconference was to provide the authors an opportunity to respond to the "Synthesis of the Main Issues of the Review" submitted by the review manager and come to agreement on revising AOP6.
The May 2018 teleconference was attended by Scott Belcher, Jennifer Schezinger and Zhicho Dang of

the review team, Kurt Gust and Ed Perkins of the authors and Terry Schultz, the review manager. The teleconference was recorded and from that recording the following synthesis was constructed by the review manager.

Does the AOP incorporate the appropriate scientific literature?
The reviewers and authors agree that the AOP needs both human *in vitro* and rodent *in vivo* information in its formulation. However, the mode of action is primarily based on animal model experimentation. It was further agreed that the AOP should not have a human focus.

Does the scientific content of the AOP reflect current scientific knowledge on this specific topic?
The reviewers and authors agreed that the 2014 review of Kersten and co-workers is an excellent starting point and only appropriate post-2014 knowledge and literature need be added.

2. Weight of evidence:
Are the weight-of-evidence judgement/scoring calls provided by AOP developers for KEs, KERs and the overall AOP justified?
The reviewers and authors agreed on "a way forward" to address this complex question. Specifically, the authors will add a section to the AOP on uncertainties and species differences. In the teleconference what was the appropriate adverse outcome of AOP6 was discussion at length. It was agreed that "decreased energy performance' and "body weight loss" were both adverse outcome linked to "under nutrition". It was further agreed that these outcomes are linked to "hypoketosis" or "decreased ketogenesis" which is most critical under "nutrient constraints" or "starvation conditions". It was further agreed that the apical effect will depend on fat level in the organism, general energy stores and energy budget of the organism, and "physiological state". To provide further clarification, the authors agreed to add a section on what are the modulating factors (i.e., limitations) and assign appropriate condition – "low nutrient availability" and "negative energy budget" to the AOP. The relationships between carbohydrate, fatty acid and ketone body use in energy production mean the key events at the organ level - liver and muscle are integrated. It was agreed that as currently written AOP6 is "liver-centric". The authors agreed to add additional information to make the AOP less liver-oriented.

3. Regulatory applicability:
Considering the strength of evidence and current gaps/weaknesses, what would be the regulatory applicability of this AOP, in your opinion?
The reviewers and authors agree that the AOP should be as widely applicable as possible, but it is up to the regulators to determine its regulatory application. With that said, the additional information to be added by the authors will provide guidance to the users.

4. Conclusion:
What are your overall conclusions of the assessment of this AOP?
The reviewers are in agreement that modification to the AOP6 described above will assure the AOP is a meaningful and useful building block for establishing mechanistic relevance in assessing of PPARα agonists. The reviewers recommended the revised AOP6 to proceed for adoption by WNT and WPHA.

3.3. Action list

The reviewers state that while the AOP is not perfect, it is important that this AOP be published. There is agreement between the reviews and authors on ways of improving AOP6 and submitting it to OECD for approval and publication. Specifically, the authors will:

1) add a section to the AOP on uncertainties and species differences,

2) add a section on what are the modulating factors,

3) further clarify what are the organ level key events and organismal level adverse outcomes associated with of this AOP.

4. Summary of planned revisions

In the planned revisions, the authors will:

1) add a section to the AOP on uncertainties and species differences,

2) add a section on what are the modulating factors,

3) further clarify what are the organ level key events and organismal level adverse outcomes associated with of this AOP.

5. Further discussion

No further discussions.

6. Outcome of the external review

The reviewers and authors agree that the AOP should be as widely applicable as possible, but it is up to the regulators to determine its regulatory applications. To this end, the reviewers are in agreement that the agreed to modification of AOP6, in particular adding a section on uncertainties and species differences, adding a section on what are the modulating factors and further clarifying what are the organ level key events and organismal level adverse outcomes will assure the AOP is a meaningful and useful building block for establishing mechanistic relevance in assessing of PPARα agonists. The reviewers recommend the revised AOP6 to move for adoption by WNT and WPHA. At the time that this report was prepared the agreed changes were not implemented in the Wiki.

Annex 1: Table with reviewers' name

Dr. Ella Atlas
Environmental Health Science and Research Bureau
University of Ottawa, Canada

Dr. Jennifer Schlezinger
School of Public Health
Boston University, USA

Dr. Scott Belcher
Department of Biological Sciences
North Carolina State University, USA

Dr. ZhiChao Dang
Centre for Safety of Substances and Products
National Institute for Public Health and the Environment (RIVM), The Netherlands

Annex 2: Individual reviewers' comments

Comments by Reviewer 1

1. Scientific quality:
• Does the AOP incorporate the appropriate scientific literature?
For most part the literature incorporated in the AOP is appropriate; however more human relevant literature should also be incorporated. This comment is due to the fact that it has been shown that the gene profiles resulting from mouse hepatocytes treated with PPARα agonists are different than the profile generated by agonists in mouse hepatocytes (Rakhshandehroo M. et al. 2009). Further it has also been shown that several compounds have different affinities to the murine PPARα and the mouse PPARα (Kliewer S. A et al PNAS 1997). Further, in rodents, the relative contribution of peroxisomal –oxidation, as a result of PPARα activation, in lowering tryglicerides levels appears to be far more important than in humans. In addition, treatment with fibrate a PPARα agonist results in peroxisome proliferation and hepatomegaly in rodents, and not observed in humans.

• Does the scientific content of the AOP reflect current scientific knowledge on this specific topic?
The authors of the AOP may want to include some of the literature relevant to human liver such as McMullen P.D. et al 2013, and Janssen et al, 2015.

2. Weight of evidence:
• Are the weight-of-evidence judgement/scoring calls provided by AOP developers for KEs, KERs and the overall AOP justified?
KE1: Stabilization, PPAR alpha co-repressor: GW6471 binding and stabilizing the receptor is appropriately supported by the literature.
KE2: Decreased, PPARα transactivation of gene expression is also supported for GW6471.
KE3: Decreased, Ketogenesis (production of ketone bodies). Well supported in the PPAR KO mouse model and the literature cited.
KE4: Not Increased, Circulating Ketone Bodies. Seems to me that it is redundant with KE4.
KE5: Decreased, Mitochondrial Fatty Acid Beta Oxidation: This section would benefit from the inclusion of additional original papers from the PPARα null mouse model.
KE6: Increased, Catabolism of Muscle Protein: I am not convinced that there is enough evidence for the increase in catabolism of muscle protein in PPARα KO mice.

Overall: The body weight loss is less clearly supported. For example, in obese mice treatment with a PPARα agonist and not antagonist resulted in weight loss (Laurent D, Gounarides JS, Gao J, Boettcher BR 2009 Diabetes Obes Metab 11:632– 636) and PPARα -/- mice similar in weight to the PPARwt mice (Fu J. et al. Nature. 2003).

3. Regulatory applicability:
• Considering the strength of evidence and current gaps / weaknesses, what would be the regulatory applicability of this AOP, in your opinion?
Due to the lack of clear relevance to humans I am not sure that there is regulatory applicability at the moment.

4. Conclusion:
• What are your overall conclusions of the assessment of this AOP?
Overall, there are many endpoints that could be used as the endpoint for this AOP such as hepatic steatosis and adiposity. However, the relevance to humans is questionable.

Comments by Reviewer 2

1. Scientific quality:

• Does the AOP incorporate the appropriate scientific literature?

- The scientific literature is well-incorporated into AOP 6. In general, seminal findings and supporting literature is appropriate for the proposed MIE, KEs and KERs. Over reliance on some reviews weakens the strength of the AOP slightly. As mentioned below there are opportunities for inclusion of additional or updated literature that would refine and strengthen the AOP with additional details from the more current/expanded knowledge base.

• Does the scientific content of the AOP reflect current scientific knowledge on this specific topic?

- There is some limitations in the cited literature which could be strengthened if more current findings where properly referenced. The AOP author's manuscript (below) is listed as in press and should be updated – there may also be additional and subsequent studies that should be evaluated which may strengthen support for the AOP (especially related to some KERs).

Gust KA, Nanduri B, Rawat A, Wilbanks MS, Ang CY, Johnson DR, Pendarvis K, Chen X, Quinn Jr. MJ, Johnson MS, Burgess SC, Perkins EJ (2015) Systems Toxicology Identifies Mechanistic Impacts of 2-amino-4,6-dinitrotoluene (2A-DNT) Exposure in Northern Bobwhite. BMC Genomics. In Press.

- For KER 879, 880 and 881, the "quantitative understanding" for linkage scoring was modified to "Not Specified" in this lasted version of the AOP. This change maybe due to the reliance of information for the "Quantitative understanding of linkage" coming from data presented in the referenced reviews that was from studies using "gene knockout" models and a resulting lack of concentration/dose response relationship. This lack of appropriate literature references is considered a weakness that could likely be avoided with additional evaluation of the literature (including clinical and preclinical studies) specifically looking for applicable studies that analyzed impacts of PPARα antagonists (e.g. GW6471, MK886 or NXT629) on each specific KE in a variety of experimental systems including in vitro analysis.

2. Weight of evidence:

• Are the weight-of-evidence judgement/scoring calls provided by AOP developers for KEs, KERs and the overall AOP justified?

- With the exception of the points made above, which if addressed would serve to increase overall justification for the KER's and the AOP overall, the WOE/judgment calls are appropriate and justified. The majority of MIE and related physiologic evidence for the AOP is well established and generally well understood.

3. Regulatory applicability:

• Considering the strength of evidence and current gaps / weaknesses, what would be the regulatory applicability of this AOP, in your opinion?

- AOP6 is appropriate for general consideration in regulatory decision-making. Significantly, the impaired energy metabolism and resulting decreased aerobic exercise performance demonstrated by exposures to 2,4 dinitrotoluene, which mimic defects observed as a result of PPARα genetic defects (e Wilbanks eta al, 2014), strongly support plausible negative impacts on individuals and populations that will have wide applicability for both human and environmental risk assessment and regulatory decision making.

4. Conclusion:

• What are your overall conclusions of the assessment of this AOP?

- Overall, this is strong and well-supported AOP. There are only minor concerns, that when addressed will only strengthen confidence in the value and utility

Comments by Reviewer 3

- **Does the AOP correctly incorporate the critical scientific literature and does the scientific content of the AOP reflect the current scientific knowledge on this specific topic?**

The current AOP is well described and included the critical literature. To increase understanding and application of this AOP in the regulatory field, following points need to be clarified and further elucidated.
A few chemicals have been included. Except the chemicals developed by pharmaceutical companies, one major point here is a lack of experimental evidence on the binding of nitrotoluenes to PPARα. Binding can be indicated via x-ray crystallography and also via a competitive binding assay. No such evidence is available in this AOP for nitrotoluenes. The binding of chemicals to PPARα and its stabilization with co-repressors is showed in GW6471 but not in nitrotoluenes. These lead to a question whether nitrotoluenes works in a same way to GW6471. In addition, the KEs are largely dependent on the results of GW6471. It would be of help if data/literature on nitrotoluenes are included in these KEs.

- **Are the weight-of-evidence judgement/scoring calls provided by AOP developers for KEs, KERs and the overall AOP justified?**

There are many papers described the PPARα pathway and its physiological/pathological role. The AOP developers have incorporated the essential information and the overall AOP is justified. There may be, however, some difference in understanding the weight-of-evidence approach. Especially, the papers used in this AOP have not been evaluated according to the reliability scoring often used in the toxicological field.

- **What would be the regulatory applicability of this AOP in your opinion?**

There are several possible applications of this AOP in the regulatory context.

1. Identification of endocrine disrupting chemicals (EDCs)

Identification of Endocrine Disrupting Chemicals (EDCs) is needed under several pieces of European Union (EU) legislation, including the Regulation on industrial chemicals (Registration, Evaluation, Authorization and restriction of Chemicals, EC 1907/2006, REACH), the Plant Protection Products Regulation (EC 1107/2009, PPPR), and the Biocides Products Regulation (528/2012, BPR). Identification of EDCs is based on three essential elements, i.e. chemical-induced adverse effects (adversity), chemical specific endocrine modes/mechanisms of action (MOAs) and the causal relationship (causality) between adverse effects and endocrine MOAs. AOPs cover all essential elements for identification of EDCs and show the complex biology of adversity and MOAs. These will help regulators understand the complexity of identification of EDCs and may help regulators fill in data gaps by using some results of KEs. The AOP concept has been included in the draft ECHA/EFSA Guidance for identification of EDCs, which will be publically available in the middle of 2018. According to this Guidance, the current focus is on EATS pathways. However, the PPAR pathway and other pathways are not excluded

from the scope of the regulating EDCs in the EU legal frameworks. The current AOP could be used for identification of EDCs.

2. AOP for prioritizing chemicals

The current AOP includes different targets at molecular, cellular, organ/tissue and individual levels. If the critical KEs leading to adversity are identified, information on KEs would be of help for prioritizing chemicals, for grouping chemicals and for developing an integrated testing strategy.

3. KEs for adversity (e.g. classification) and alternative animal testing

The classification system (GHS) is mainly developed on the basis of the animal testing results. There is a pressure for using alternative animal testing results. As some KEs can be performed in vitro, the KEs, critical to leading to adversity, can be considered for classification instead of animal testing.

- **What is your overall assessment of the AOP?**

Body weight is an important endpoint for testing in the fields of both human health and the Environment. The current AOP introduced one important pathway leading to the loss of body weight. It is noted that this AOP may be too complex and difficult for the regulators to catch up the essential and overall picture. It is therefore suggested that the AOP should be made clearer and more easily understandable (if it is possible). I would like to recommend this AOP for the adoption by WNT and TFHA groups.

Comments by Reviewer 4

1. Scientific quality:
Does the AOP incorporate the appropriate scientific literature?
Does the scientific content of the AOP reflect current scientific knowledge on this specific topic?
This AOP does not adequately address the species-specificity of PPARa action. The majority of the scientific support for the AOP come from rodent studies. However, human and rodent PPARa are distinct in their ability to induce peroxisome proliferation and peroxisomal fatty acid beta oxidation.
Only rodent PPARa stimulates peroxisome proliferation and efficiently upregulates expression of genes involved in peroxisomal fatty acid beta oxidation. According to Rakhshandehroo et al., 2009, and based on comparative analysis of PPARa-agonist induced gene expression in primary mouse and human hepatocytes, "[T]he role of PPARa as master regulator of hepatic lipid metabolism is generally well-conserved between mouse and human. Overall, however, PPARa regulates a mostly divergent set of genes in mouse and human hepatocytes."
The AOP largely relies on a single review paper for information on the function of human PPARa (Kersten et al., 2014). A more thorough incorporation of analyses and results from primary studies of human PPARa and of the effects of GW6471 on human PPARa is needed. Furthermore, there is no mention of a natural human polymorphism of PPARa, which favors N-Cor recruitment similarly to GW6471 (Liu et al., 2008). Epidemiological analyses of humans carrying the polymorphism did not note a difference in weight in those individuals (Chan et al., 2006). There is no mention of the humanized PPARa mouse model (Cheung et al., 2004). This mouse has been shown to respond distinctly to PPARa agonists, compared to the mouse expressing mouse PPARa, in both fatty acid oxidation and body weight gain (Feige et al., 2010). Last, the overlapping and potentially compensatory actions of PPARa and PPARb/d are not considered.

- Chan et al., 2006. The V227A polymorphism at the PPARA locus is associated with serum lipid concentrations and modulates the association between dietary polyunsaturated fatty acid intake and serum high density lipoprotein concentrations in Chinese women. Atherosclerosis. 187(2):309-15.
- Cheung et al., 2004. Diminished Hepatocellular Proliferation in Mice Humanized for the Nuclear Receptor Peroxisome Proliferator-Activated Receptor a. Cancer Res. 64, 3849–3854.
- Feige et al., 2010. The Pollutant Diethylhexyl Phthalate Regulates Hepatic Energy Metabolism via Species-Specific PPARα-Dependent Mechanisms. Environ. Health Perspect. 118(2): 234–241.
- Liu et al., 2008. A natural polymorphism in PPAR-alpha hinge region attenuates transcription due to defective release of nuclear receptor corepressor from chromatin. Mol. Endocrinol. 22(5):1078-92.
- Rakhshandehroo et al., 2009. Comparative analysis of gene regulation by the transcription factor PPARalpha between mouse and human. PLoS One. 4(8):e6796

2. Weight of evidence:
Are the weight-of-evidence judgement/scoring calls provided by AOP developers for KEs, KERs and the overall AOP justified?
MIE - Binding of antagonist, PPAR alpha
For GW6471, the data supporting it binding and antagonizing PPARa are excellent. For nitrotoluenes, the data supporting 2,4-DNT binding and antagonizing PPARa are not convincing. 2,4-DNT does not reduce PPARa ligand-induced transcriptional activity, even when 2,4-DNT was included at a

concentration 3 orders of magnitude greater than the PPARa agonist (100 nM GW590753 vs 55 uM 2,4-DNT) (Wilbanks et al., 2014).

Comments from this point on are based solely on the data from studies with GW6471, with PPARalpha knockout mice or using PPARalpha knockdown.

KE1 – Stabilization, PPAR alpha co-repressor

The molecular consequences of GW6471 binding to human and mouse PPARa (e.g. coregulator recruitment and release) are well supported by the data. Wilbanks et al., 2014 does NOT show that PPARa activity (positively or negatively) is influenced by 2,4-DNT. The antagonism of PPARa by 2,4-DNT is implied by the authors, but I do not believe that this can be concluded based on the data. This study should not be used in support of this KE.

Gust et al., 2015 provides stronger evidence that 2A-DNT is a PPARa ligand. However, there is no analysis of coregulator recruitment to PPARa. This study should not be used in support of this KE.

KE2 – Decreased, PPARalpha transactivation of gene expression

The decrease in expression of PPARalpha gene targets by GW6471 in human and mouse models is well supported. However, the gene expression profile induced by activation of PPARa is species-specific, with distinct differences between mouse and human (See Kersten et al., 2014). There are no data provided about potential species-specific effects of PPARa antagonism in human liver.

KE3 – Decreased, Peroxisomal Fatty Acid Beta Oxidation of Fatty Acids

The processes and genes involved in peroxisomal fatty acid beta oxidation are well described. However, no evidence of the effect of PPARa antagonism or lack of expression are provided. At the very least, data from studies in PPARa knockout mice should be included. Regulation of peroxisome proliferation and peroxisome-related gene expression is distinct in mouse and human. No mention of this, nor the relative importance of peroxisome- vs mitochondria-dependent fatty acid oxidation in mouse and human, are made.

KE4 – Decreased, Mitochondrial Fatty Acid Beta Oxidation

The evidence from Aoyama et al., 1998 that PPARa knockout in the mouse significantly reduces constitutive and inducible mitochondrial fatty acid oxidation-related gene expression is strong. The evidence discussed is largely gene expression analyses rather than functional analyses. The Aoyama et al., 1998 paper also shows that inducible beta oxidation is functionally reduced in PPARa knockout mice. No evidence of the effect of GW6471 or PPARa-knockdown on mitochondrial fatty acid beta oxidation in human cells is provided. Given that peroxisomal fatty acid oxidation is regulated distinctly by PPARa in humans versus mouse (Blaauboer et al., 1990; Rakhshandehroo et al., 2009) and would presumably remain functional in the presence of PPARa antagonists, it is unclear what the physiological significance of antagonism of mitochondrial fatty oxidation alone would be.

- Blaauboer, et al., 1990. The effect of beclobric acid and clofibric acid on peroxisomal betaoxidation and peroxisome proliferation in primary cultures of rat, monkey and human hepatocytes. Biochemical Pharmacology 40:521e528.

KE5 – Decreased, Ketogenesis (production of ketone bodies)

The decrease in ketogenesis by PPARalpha knockout in mice could be strengthen by including le May et al., 2000. Cahill et al., 2006 reviews the relationship between fasting and the need to generate ketone bodies to support the brain's metabolic demands. However, there are no data presented demonstrating the connection between fasting, PPARa upregulation and ketone body production in humans. Human HMGCS2 is the rate limiting step in the ketogenic pathway. HMGCS2 expression is increased when HepG2 cells are engineered to express PPARa (Vilà-Brau et al., 2011). At the very least, this citation should be included. Furthermore, there are no data provided demonstrating how PPARa antagonism affects ketone body production in humans.

- Le May et al., 2000. Reduced hepatic fatty acid oxidation in fasting PPARK null mice is due to impaired mitochondrial hydroxymethylglutaryl-CoA synthase gene expression. FEBS Lett. 475: 163-166.

- Vilà-Brau et al., 2011. Human HMGCS2 regulates mitochondrial fatty acid oxidation and FGF21 expression in HepG2 cell line. J. Biol. Chem. 286(23):20423-30.

KE6 – Not Increased, Circulating Ketone Bodies

The data showing a decrease in serum ketone bodies in PPARa knockout mice is strong. The references provided for the connection between PPARalpha and humans are reviews. Primary literature is needed.

KE7 – Muscle protein catabolism

An increase in muscle protein catabolism and loss of weight stimulated by lack of PPARa signaling is not supported by mouse studies. In Badman, et al 2007 and Muoio, et al., 2002, mice lacking PPARg signaling do not show weight loss on a regular chow diet, and there is similar weight loss in WT and KO mice following endurance exercise. While muscle protein catabolism may be an outcome of extended fasting in humans, no evidence is provided that muscle protein catabolism is impacted by PPARa status.

- Muoio, et al., 2002. Fatty Acid Homeostasis and Induction of Lipid Regulatory Genes in Skeletal Muscles of Peroxisome Proliferator-activated Receptor (PPAR) a Knock-out Mice. J. Biol. Chem. 277: pp. 26089–26097

AO – Decreased, Body Weight

An increase in muscle protein catabolism and loss of weight stimulated by lack of PPARa signaling is not supported by mouse studies. In Badman, et al 2007 and Muoio, et al., 2002, mice lacking PPARg signaling do not show weight loss on a regular chow diet and similar weight loss following endurance exercise. It is true that there are dramatic changes in glucose and lipid metabolism in mice lacking PPARa
signaling during fasting and endurance exercise, but there is no evidence that lack of PPARa enhances weight loss. Evidence from nitrotoluene exposure studies in rats and birds showing reduced endurance and weight loss do not directly test the contribution of PPARa to these effects. Therefore, I do not believe that they should be used to support the AOP.

Overall

The assessment of the scientific evidence supporting the linkages in the AOP largely are accurately represented. However, I have three caveats to this: 1) The linkage between a reduction in ketone body production via loss of PPARa function and increased muscle protein catabolism is weak to non-existent. 2) It is not clear in the AOP that PPARa function is not strictly necessary under normal feed/activity levels. It is only when fasting or during enduring physical activity that PPARa function is necessary to generate ketone bodies as an alternative energy source. Thus, the adverse outcomes the result from a lack of PPARa function will only occur when alternative energy sources are necessary. 3) It must be qualified that the data supporting these linkages are based largely on rodent studies. And, it is well known that rodent and human PPARa act distinctly. In terms of taxonomic applicability, the only strong evidence that antagonism of PPARalpha results in KE2-KE6 is for mice. I agree that the evidence is moderate for rats. The evidence is weak for birds and humans is weak.

Please note: I am not questioning that DNTs have adverse health effects on birds and rodents, rather I am questioning if PPARa has a role to play.

3. Regulatory applicability:

Considering the strength of evidence and current gaps/weaknesses, what would be the regulatory applicability of this AOP, in your opinion?

At this point, I do not believe that the evidence in this AOP is strong enough to support its regulatory applicability.

4. Conclusion:

What are your overall conclusions of the assessment of this AOP?

As noted above, the lack of consideration of species differences in the function of PPARa is a significant weakness in this AOP. Lack of primary studies in human models or epidemiological studies in humans impairs the usefulness of the AOP. Further, the evidence for linkage between a reduction in ketone body production via loss of PPARa function and increased muscle protein catabolism is weak to non-existent. The AOP does not make it apparent adverse outcomes that result from a lack of PPARa function would

only be induced during fasting or endurance exercise. Lack of PPARa function would not be expected to have an adverse effect when feed and activity levels are normal. Last, the inclusion of studies with DNTs is correlatory, at best, and does not strengthen the AOP.

Annex 3: Written response from the authors in preparation for the end of review Teleconference

Due to their request for clarifications, the authors provided no written responses prior to the teleconference. All issues were resolved during the teleconference.