

Adverse Outcome Pathway External Review Report

AOP 220: Cyp2E1 Activation Leading to Liver Cancer

This document is the final AOP220 review report

This document has been prepared by Mr Jean-Baptiste FINI (fini@mnhn.fr), consultant for the OECD Secretariat

It compiles the views and comments of the reviewers and explains how the authors of the AOP have addressed these comments.

It provides the basis to EAGMST for determining if AOP 220 has been adequately revised by their authors following the review and if it can be released to the Working group of the National Coordinators of the Test Guidelines Programme and to the Working Party on Hazard Assessment for endorsement.

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1. Introduction and background to specific AOP

The subject of this AOP is chronic activation of the Cyp2E1, an enzyme involved in xenobiotic metabolism (MIE) leading to liver cancer (AO). The different intervening key events (KEs) are oxidative stress (KE1), cytotoxicity (KE2), and regenerative proliferation (KE3).

Xenobiotic metabolism typically occurs in three phases: (I) the chemical substrate is enzymatically bio-activated to its primary metabolite; (II) the metabolites produced are made less reactive through conjugation; and (III) the modified chemicals are excreted.

The setting for these events is the liver, which is the body's primary venue for chemical detoxification. Cyp2E1 is a phase I P-450 mono-oxygenase that bio-activates its substrates through the addition of an oxygen, thereby producing an electrophilic metabolite. While this reactive species often undergoes conjugation (phase II metabolism), sometimes it will react with cellular nucleophiles (e.g., proteins or DNA), which results in formation of adducts that produce cytotoxicity in extreme cases. Another feature of Cyp2E1 is that its catalytic cycle is prone to uncoupling, which leads to the production of reactive oxygen species (ROS). ROS are an important source of cytotoxicity (e.g., via lipid peroxidation) and are a source of oxidative lesions to DNA (which may be a source of cancer-causing mutations).

Redox-sensitive proteins are modified by oxidation; importantly, changes in gene expression are carried out by the redox-sensitive transcription factor Nrf2. Nrf2 increases the expression of genes that encode cyto-protective products, such as anti-oxidants and phase II conjugating enzymes. When chronically activated, these molecular signals can produce dysregulated cellular proliferation in which the cyto-protective cellular mechanisms that are intended to promote tissue repair instead may lead to pre-malignant and malignant lesions.

Even if exposure to Cyp2E1 substrates is relatively common, this AOP is an important tool for understanding the adverse health impacts of potentially harmful substances.

Authors focused on evidence gathered from: furan (a group 2B carcinogen), ethanol (group 1), chloroform (group 2B), and carbon tetrachloride (group 2B). These compounds are established Cyp2E1 substrates that are known to be rodent carcinogens and are (group 1) or suspected (group 2B) human carcinogens based on their International Agency for Research on Carcinogens (IARC) evaluations.

1.1. AOP 220 authors

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1.2. External Review

This AOP was reviewed in December 2019/ January 2020 by a panel of four reviewers (see Annex 1). Selection to the panel was driven by the candidates' expertise in carcinogenicity and toxicology. The first selection criterion used, was the skills of the reviewers to assess the AOP. Secondary criteria were balancing gender, academy and industry or origin from different countries.

2. Synthesis of main issues of the review

This section provides an overview of issues raised by the four external reviewers (reviewers' details in Annex 1).

Reviewers, were asked to reply to the following questions regarding different aspects of the AOP:

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?

2. Weight of evidence:

- Are the weight-of-evidence judgement/scoring calls provided by AOP developers for KEs, KERs and the overall AOP, justified, i.e. consistent with the considerations outlined in the Users' Handbook?

3. Additional observations:

- What do you consider to be critical data gaps, if any, and how to fill these gaps?

The version used was the snapshot provided by the OECD Secretariat and accessible at https://aopwiki.org/aopwiki/snapshot/pdf_file/220-2019-12-13T12:46:09+00:00.pdf

A summary of the answers for each point is accessible below, with quotations organised point by point under each question. The complete reviews organised by reviewer are accessible in Annex 2 of this report. The replies made by the authors are accessible in Annex 3.

2.1. Scientific quality

General comments:

The reviewers all agree that the AOP is of high quality. The AOP 220 is clearly written and its description is detailed. The weight of evidence is well balanced. Some issues were discussed, in particular the addition of other indirect key events and other key event relationships. Some major changes in the structure of the AOP were noted and discussed with the authors. The authors modified the AOP, including unequivocal wording, in consultation with the reviewers. Authors fixed also minor revisions, including references missed by the panel. According to the panel, the described mode of action, enriched by non-adjacent events, makes AOP 220 a very relevant tool for human application.

Does the AOP incorporate the appropriate scientific literature?

In general, the AOP incorporates appropriate scientific literature but some missing references have been spotted during the review process.

Reviewer1 suggested including in biological plausibility (and/or to the paragraph on domain of applicability) in the section 'Overall Assessment of the AOP', the publication by Meek et al (2003) on the WHO/IPCS framework, which includes a case study on chloroform for the AOP under study.

Reviewer4 also suggested to review recent articles and incorporate them within the AOP.

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

In general, the scientific content was judged to reflect current knowledge even though some missing references were pointed out. According to reviewers, KEs are intelligibly and well described and the essentiality of KEs clearly presented. Importantly, the biological plausibility of KERs and weight of evidence were objectively evaluated.

However some suggestions were listed in order to improve the AOP:

R1-R2-R4: a **critical part of the AOP was the chronic induction of CYP2E1**. Indeed, CYP2E1 simple, short-term induction is not necessarily leading to liver cancer. The distinction between all these events needs to be described and referenced.

R2: Time- and dose-response concordance: The assumption that “KEs can be measured with equal precision and sensitivity” was not defensible. In reality, the precision and sensitivity of different methods for even the same KE could vary, not to mention for different KEs and particularly in different test systems (e.g. *in vitro* vs *in vivo*).

The panel suggested modifying the discussion in order to focus on the fact that upstream KEs could usually be observed at earlier time points than downstream KEs. Moreover, authors were advised to **emphasise that** downstream KEs (including the AO) can only be observed following exposures at doses equal or higher than those capable of inducing upstream KEs. Finally three reviewers (R1, R2 and R3) suggested to integrate and discuss human data.

R4 made a crucial point on **the regenerative proliferation Key Event** that was also approved by the others reviewers. *In addition to the term “regenerative proliferation” some thought could be considered in relation to the term “frustrated repair.” Regenerative proliferation implies that the tissue and cellular architecture returns to normal or to a near normal state. This is not the case with this carcinogenic pathway – the key pathology term is frustrated repair i.e. normal tissue architecture is not restored or cannot be restored because the events that caused the chronic damage continue to occur.*

It is suggested that a critical part of the AOP is the sustained (possibly inappropriate) induction of CYP2E1. Simple, short-term, induction of CYP2E1 is not necessarily adverse and in many cases is protective;

In addition to the main comments above, further information was requested by the reviewers on the MIE and KE2:

MIE:

- Although regulation of CYP2E1 expression is not done at the transcriptional level, and that protein stability is enhanced by binding to the substances, the ubiquitin-proteasome pathway and the involvement of hsp-based chaperone mechanism should be briefly mentioned.

KE2 hepatotoxicity:

- in the description of WoE, it might be better to clarify either KE2 is cytotoxicity of hepatocyte by oxidative stress or inflammation by other cells such as lymphocyte.

2.2. Weight of evidence

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

For the most part, the weight of evidence was appropriate, clear and precise. All authors agreed that the vast majority of KE and KER were properly weighted. However, some precisions and modifications were needed especially on **KE2, KE3, KER 1516 1517 and KER1518**.

- KEs:

KE2 hepatotoxicity

In the description of WoE, it might be better to clarify if KE2 is cytotoxicity of hepatocyte by oxidative stress or inflammation by other cells such as lymphocyte.

The terms “hepatotoxicity”, “hepatocytotoxicity” have been used interchangeably throughout this AOP. Since “hepatotoxicity” has a separate KE number (#1291) in the AOPWiki, the authors should use the term hepatocytotoxicity consistently or explain the differences.

Hepatic Kupffer cells also contain considerable amounts of CYP2E1, and Kupffer cell CYP2E1 is inducible. Kupffer cell CYP2E1 appears to play a role in hepatocarcinogenesis as well as hepatocellular CYP2E1. The term hepatotoxicity covers this perhaps better than hepatocellular toxicity.

KE3 regenerative proliferation

The essentiality of this KE was classified as “weak”, because “rodents lacking AP-1 or NF-kappaB display impaired liver regeneration, often leading to death” (Behrens et al 2020). However, there are available studies either supporting or disapproving the essentiality of this KE (ex Mice lacking TNF receptor type 1 with a decreased liver carcinogenesis).

After a harmonisation TC, all authors agreed that the “weak” classification could be conserved but with the reasons mentioned above. The authors were however advised to discuss a publication by Melnick and Huff (1993).

- KERs:

1) KER 1516 (oxidative stress to liver cancer):

The authors’ classification of Empirical Evidence for this KER was “weak”. Yet much of the literature cited (e.g., evidence of oxidative adducts or other DNA damage associated with hepatocarcinogenesis) suggested a stronger relationship. The reviewers, who had agreed on this point at a harmonisation conference, therefore asked the authors either to provide evidence of inconsistency regarding literature cited for the obviousness of this KER, or to change the designation from “low” to “moderate”.

Reviewers also suggested to add a new KE: either ‘DNA Damage’ between ‘Oxidative stress’ and ‘liver cancer’ (KER 1516) or ‘Increases DNA’ adducts between ‘Oxidative stress’ and ‘liver cancer’.

2) KER 1517 (hepatotoxicity to liver cancer): The panel considered this KER to be less explicit than the others KERs regarding the evidence from the literature and thus questioned the authors’ classification.

i) the authors’ determination of the Biological Plausibility was “weak” and the Empirical Evidence was “moderate”. However, given the hepatocytotoxicity-triggered DAMPs leading to inflammation the reviewers suggested that Biological Plausibility should probably be reconsidered as “moderate”.

ii) Direct evidence for this KER seemed to be weak and limited. First, enzymes indicative of liver damage are not good indicators for liver cancer in humans. Second, the status of inflammation is not mentioned in the cited literature. Regenerative, non-cancerous proliferation cannot be ruled out.

Reviewers also suggested to add a new, separate KE: as ‘inflammation’ between ‘hepatotoxicity’ and ‘liver cancer’ (KER 1517)

3) **KER 1518 (regenerative proliferation to liver cancer):** It is widely accepted that highly dividing cells are at greater risk of obtaining and fixing a mutation. If appropriately placed in the genome, such a mutation can facilitate the malignant transformation of the cell. According to the panel, and based on the criteria of **Biological Plausibility**, this KER may be changed to “**strong**”, rather than “**moderate**”.

As for the **weight of Empirical Evidence**, it may be more suitable to classify it as “**moderate**”, instead of “**strong**”, because evidence showed 1) that some proliferative events are not followed by liver cancer; and 2) some liver carcinogenesis occurs with unchanged proliferation but impaired apoptosis.

Reviewers suggested to change the wording of the Key Event in case of the impossibility of creating a new KE.

2.3. Other considerations:

What do you consider to be critical data gaps, if any, and how to fill these gaps?

As it was presented initially, there were no critical gaps identified in the AOP. Some suggestions were made to improve the readability of the AOP:

- *The AOP description would greatly benefit from more detailed information on how inflammation relates to oxidative stress and the carcinogenesis process.*
- *The AOP should be used and interpreted in a known context.*
- *The disparities of the cancer evidence in rodents and in humans should be considered: Many of the CYP2E1 substrates are clear rodent hepatocarcinogens, yet only probable or possible human carcinogens due to inadequate epidemiological evidence. Further mechanistic studies are needed to fill these gaps.*

2.4. Conclusion:

This AOP was well written and documented. Nevertheless, additional work was required before finalising the process and submission at the OECD. Authors were expected to improve the description of few spotted Key Events and Key Event Relationships and possibly add one or a few Key Events or reword the KE1394 “regenerative proliferation” which was unclear. The panel also suggested to add missing relevant references.

These modifications were discussed at the final TC when both authors and reviewers attended and a consensus was reached.

3. Summary record of the teleconference

3.1. TC agenda

Three teleconferences were organised during the period January to February 2020.

Only Reviewers and review manager attended the first two teleconferences.

The first one took place mid-January and aimed at defining the role of the reviewers. In particular, we discussed the main issues that the reviewers should focus on and what was expected from them in terms of the initial AOP review.

The second took place early February, in order to harmonise the issues to be discussed with the authors at the end-of-review teleconference. All reviewers attended the TC and reached consensus on the issues to discuss at the end of review TC.

The end-of-review teleconference was organised on February 18th 2020 at 1.00 pm CET.

Francina Webster (author), Carole Yauk (author), Rhian Cope (Reviewer), Kumiko Ogawa (Reviewer) Janet Yu Zang (reviewer) and Jean-Baptiste Fini (Review manager) attended. Mirjam Luijten (Reviewer) was excused.

The review manager and the authors thanked the reviewers who devoted significant amount of their time to provide constructive comments, editorial changes and additional literature. All the materials cited have been made available to the authors before the TC. Due to the very short delay between sending comments and the conference, the authors had been unable to respond before the TC.

The agenda of the TC was as follows:

- Brief reminders of what we can expect from an AOP and what an AOP is not.
- Brief reminders on AOP 220, the different Key Events (KE) and Key Event Relationship (KER), the review process and questions asked to the reviewers.
- Discussion of issues raised by the reviewers and answers provided by the authors.
- Other Issues
- Conclusions and elements on the upcoming events in the AOP process.

3.2. Main issues and responses during the call

- The main point of discussion focused on the need to add one or a few new Key Events.

Some new KEs were suggested:

- Inflammation between hepatotoxicity and liver cancer (KER 1517) - A question has arisen as to whether KER 1517 is adjacent or not and if a new KE was necessary
- DNA Damage between Oxidative stress and liver cancer (KER 1516)
- Increases Etheno DNA adducts between Oxidative stress and liver cancer.

The Review manager also suggested to use, whenever possible, existing KEs accessible on the AOPwiki. He gave examples on increased inflammation and DNA repair (see below):

Key Events Title Search Results

Id	Title ▲	Short name	Biological organization
1225	Immune system inflammation	Leukocyte infiltration	Tissue
1633	Increase in inflammation	Increase in inflammation	Tissue
149	Increase, Inflammation	Increase, Inflammation	Cellular
777	Increase, Inflammation (corneal cells)	Increase, Inflammation (corneal cells)	Cellular
1087	Increased, inflammation	Increased, inflammation	Cellular
902	Inflammation, Liver	Inflammation, Liver	Organ
188	N/A, Neuroinflammation	N/A, Neuroinflammation	Tissue
1436	neurogenic inflammation of sensory nerves	neurogenic inflammation	Cellular
486	systemic inflammation leading to hepatic steatosis	inflammation	Organ

Key Events Title Search Results

Id	Title ▲	Short name	Biological organization
1130	Failure in vascular repair mechanisms, Unresolved blood loss (hemorrhage)	Failure in vascular repair mechanisms, Unresolved blood loss (hemorrhage)	Organ
1552	Inadequate DNA repair (deprecated, please use https://aopwiki.org/events/155)	DNA repair	Molecular
1281	Increased, DNA Damage-Repair	Increased, DNA Damage-Repair	Cellular
493	Increased, Insufficient repair or mis-repair of pro-mutagenic DNA adducts	Increased, Insufficient repair or mis-repair of pro-mutagenic DNA adducts	Molecular
155	N/A, Inadequate DNA repair	N/A, Inadequate DNA repair	Cellular
664	Overwhelmed, Mitochondrial DNA repair mechanisms	Overwhelmed, Mitochondrial DNA repair mechanisms	Cellular
1129	Unresolved blood loss (hemorrhage), Failure in vascular repair mechanisms	Unresolved blood loss (hemorrhage), Failure in vascular repair mechanisms	
1126	Unresolved blood loss, Failure in vascular repair mechanisms	Unresolved blood loss, Failure in vascular repair mechanisms	
1128	Unresolved hemorrhage, Failure in vascular repair mechanisms	Unresolved hemorrhage, Failure in vascular repair mechanisms	

- Issue related to KE 3 (Regenerative proliferation):

The whole review team agreed that it was preferable to change the name of the KE “regenerative proliferation”, which appears physiological rather than pathological.

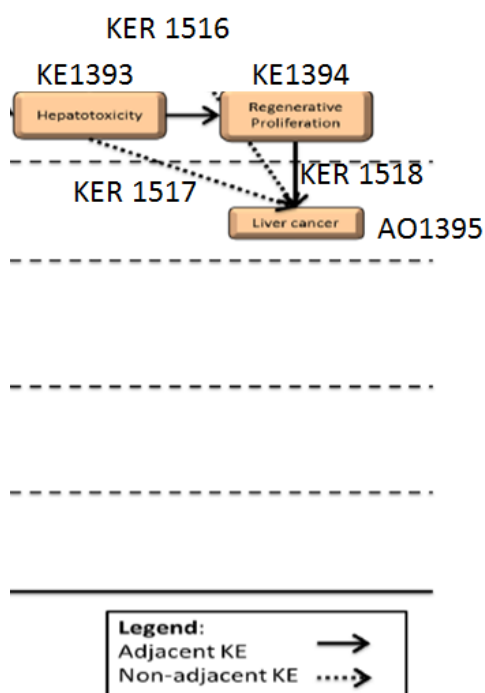
Reviewers found very difficult to see a direct link between regenerative proliferation and liver cancer. They recall that many publications deal with the role of inflammation in tumour formation and this does not appear clearly.

One suggestion was to change the term "regenerative proliferation" to "frustrated repair". Indeed, the term "regenerative proliferation" implies that the tissue and cell architecture returns to normal or near-normal state. This is not the case with this carcinogenic pathway - the key term in pathology is "frustrated repair", meaning that the normal tissue architecture is not restored or cannot be restored because the events that caused the chronic damage continue to occur.

The authors accepted this suggestion, but as described in section "5.further discussion", the name finally accepted was "sustained proliferation".

- Another issue raised by the review manager was the discrepancy between the graphical representation and the description of the KERs in the AOP. Contrary to what appears in the figure, KER 1518 is listed as non-adjacent in the AOP.

The authors explained that KER 1518 is listed as a non-adjacent KER because they consider that current knowledge is insufficient to argue in favour of an adjacent KER. This will be corrected in the figure.



3.3. Conclusion

Overall, the authors agreed to implement the suggested changes by updating and making changes to specific sections of this AOP. Regarding the suggested addition of new KEs though, it was agreed to rely on networks of AOPs in the future, rather than developing new KEs (see Section 4).

Authors were expected to concretise the actions, resulting from both the reviews and the discussions that took place during the teleconference, by adding information to the AOP-Wiki discussion pages (see Section 6).

Once the AOP has been modified according to the reviewers' recommendations, the reviewers would support the AOP to be submitted for approval and publication.

4. Summary of planned revisions

The authors accepted to take on board most of the changes suggested by the reviewers. .

Does the AOP incorporate the appropriate scientific literature?

- Include in biological plausibility (and/or to the paragraph on domain of applicability) in the section ‘Overall Assessment of the AOP’ the publication by Meek et al (2003) on the WHO/IPCS framework, which includes a case study on chloroform for the AOP under study.
- Review recent articles and incorporate them within the AOP. The authors will include the additional relevant references suggested by the reviewers and will use them to enhance aspects related to human response and search the recent relevant literature. Authors also asked reviewers to provide any additional relevant references human responses.

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

- Change the name of the KE “Regenerative proliferation”. Several names were proposed during the discussion such as “frustrated repair” or “sustained proliferation”. The authors and review team finally agreed on “induction, persistent proliferation/sustained proliferation” (See § 5 - Further discussion). This change is mandatory to better reflect the pathological, chronic and abnormal state leading to cancer. The authors appreciated the comments and discussion about the best name for this KE, that is broadly recognizable to the general toxicology community and reflects the effect being measured.
- Rely on networks of AOPs rather than developing new KEs: during the reviewer teleconference, the review group discussed several additional KE(R) that could be involved in this AOP. The authors indicated that there are indeed several AOPs in mature stages of development that will be able to be networked with this AOP to more fully explain the biology of how CYP2E1 activation (sustained) leads to liver cancer. The authors will thus not be building in these additional KEs at present into this AOP and instead will be networking in these AOPs when the timing is right. Regarding the need for sustained or chronic Cyp2E1 activation – this was originally in the title of the KE and was then shifted to the KER, based on EAGMST recommendations. Authors will make the point on the fact that it is the chronic nature of the induction of CYP2E1 that is meaningful to this AOP in all of the relevant KERs and in the overall AOP section.
- Revisit the text associated with inflammation to make its critical role clearer, hoping that future work will bridge this AOP with inflammatory KEs.

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

- Revise the WoE calls: The authors appreciate the feedback on their WoE calls. They will revisit and use the reviewers’ comments in making the final decision for calls on WoE. They will also add the additional references and ideas from the reviewers above.

5. Further discussion

After the TC, Pr Cope sent the literature on the frustrated repair to the whole group. Another reviewer was not comfortable with the change of KE name from “regenerative proliferation” into “frustrated repair”.

Multiple email exchanges between the authors and reviewers made a rich debate happened on the best name to use.

There was finally a poll organised by the review manager with four different choices

- 1) Induction, persistent proliferation/sustained proliferation
- 2) Proliferative lesions (with description of inappropriate repair)
- 3) Cell proliferation, increase (with description of inappropriate repair)
- 4) Inappropriate repair (and adaptation)

Only one reviewer did not vote and all reviewers and the authors agreed on choosing **“Induction, persistent proliferation/sustained proliferation”**

Following this modification. The authors agreed to amend the AOP before submission to the EAGMST.

6. Outcome of the external review

The authors thanked the review panel for a very helpful review process, and for the significant additional literature which they feel would improve the quality of the AOP and its use to the regulatory community.

Following the review, the authors revised the AOP based on the feedback received from the reviewers and review manager. A new version of the AOP220 was available on the AOP-Wiki on May 1st, 2020. The key changes made to the AOP are described below. All changes are visible in red (deletions are not apparent).

The panel had agreed that once the AOP has been modified according to the reviewers' recommendations, the reviewers would support the AOP to be submitted for approval and publication. From a brief overview of the revisions made, the review manager considers these modifications adequately address the comments from the panel. None of the reviewers indicated concerns, although they had not been specifically asked to review the changes.

Revisions to AOP 220 as an outcome of the scientific review

Abstract:

Here we describe the AOP for the **prolonged** activation of Cyp2E1 (MIE) leading to liver cancer (AO). The intervening KEs are oxidative stress (KE1), cytotoxicity (KE2), and **sustained** cellular proliferation (KE3).

Background:

The subject of this AOP is xenobiotic metabolism by Cyp2E1 (MIE) during **prolonged** exposures, leading to liver cancer (AO).

Biological plausibility:

There is a strong association, with some defined intervening steps, between liver regeneration and the probability of developing hepatocellular carcinoma, which is likely due to increased probability of incurring cancer-driver mutations with more DNA replication [e.g., tissues undergoing more cellular division have higher incidences of cancer (Tomasetti and Vogelstein 2015, Wu, et al. 2016)]. Moreover, chronic inflammation caused by increased and sustained levels of hepatotoxicity also contributes to increased probability of developing hepatocellular carcinoma. **It is important to emphasize that the adverse effects observed are the product of chronic activation of Cyp2E1, which leads to sustained production of ROS, cytotoxicity and regenerative proliferation. A case study of this mode of action is presented in Meek et al (2003) using chloroform as an example. The case study describes 'sustained cytotoxicity and regenerative cell proliferation' as key events for a range of animal tumors, including for chloroform leading to liver tumours in mice.** Thus, the overall biological plausibility for this AOP, especially in rodent models, is strong.

Essentiality of the Key Events:

Studies in Cyp2E1 knockout mice include: carbon tetrachloride (Wong, et al. 1998), acetone (Bondoc, et al. 1999), benzene (Powley and Carlson 2001), thioacetamide (Chilakapati, et al. 2007), trichloroethylene (Kim and Ghanayem 2006), acrylonitrile (El Hadri, et al. 2005), urethane (Hoffler, et al. 2003, Hoffler and Ghanayem 2005), acetaminophen (Lee, et al. 1996, Zaher, et al. 1998), and ethanol (Bardag-Gorce, et al. 2000).

Cyp2E1 constitutive activation and inhibition in Sprague-Dawley rat liver in the context of diethylnitrosamine-induced hepatocarcinogenesis (DEN) exposure is described by Gao et al. (2018a, 2018b).

The effects of ethanol exposure on the liver are well studied. The role of chronic alcohol exposure leading to inflammation, oxidative stress and DNA damage, and cancer is reviewed by Song et al. (2019). The role of Cyp2E1 in ethanol metabolism leading to the production of ROS, which contribute to carcinogenesis, is explored in Seitz and Mueller (2019). The associated etheno DNA adducts are described in Mueller et al. (2018) and Peccerella et al. (2018).

Non-adjacent KER2	KE1-->AO: Oxidative stress leading to liver cancer	Level of Support: Moderate. Mechanism: ROS-dependent DNA damage causing harmful mutations is known to occur. It is also well known that DNA mutations can lead to cancer. However, the mechanism by which the specific mutations generated in this context promote malignant transformation is incompletely understood.
Non-adjacent KER3	KE2-->AO: Hepatotoxicity leading to liver cancer	Level of Support: Moderate. Mechanism: Cell death by necrosis and necroptosis produces DAMPs that trigger inflammation. Inflammation is widely considered to be an important risk factor that sets the stage for malignant transformation; however, mechanistically, it is unclear how it does so.
Non-adjacent KER4	KE3-->AO: Sustained proliferation leading to liver cancer	Level of Support: Strong. Mechanism: Highly dividing cells are at greater risk of obtaining and fixing a mutation. If appropriately placed in the genome, such a mutation can facilitate the malignant transformation of the cell.

Table 2: Support for essentiality of KEs.

KE3: Regenerative Proliferation Moderate. It is well understood that cellular proliferation is a precursor to cancer; however, a better understanding of the molecular signals involved is required to experimentally demonstrate this using knock-down or knock-out models.

Rodents lacking AP-1 or NF-kappaB display impaired liver regeneration, often leading to death.

In TNF receptor type 1 knockout mice and JNK-1 knockout mice, regenerative proliferation was impaired, accompanying by decreased liver carcinogenesis (Knight et al., 2000; Hui et al., 2008). In the JNK-1 knockout mice, genetic inactivation of p21 restored hepatocyte proliferation and also liver carcinogenesis (Hui et al. 2008). Conversely, the evidence against the essentiality of KE3 also exists, such as Melnick and Huff (1993).

(Behrens, et al. 2002, Schrum, et al. 2000)

(Knight et al., 2000; Hui et al., 2008; Melnick and Huff, 1993).

Table 3: Empirical support for KERs.

Non-adjacent KER4	KE3-- > AO: Sustained proliferation leading to liver cancer	Level of Support: Moderate Defining question 1: That an increase in cellular proliferation precedes tumour formation is a universal occurrence. Defining question 2: Not all cases of regenerative proliferation produce tumours (some simply regenerate the liver to its healthy form). Therefore, it is clear that malignant transformation is accompanied by some sort of abnormal cellular signaling or impaired homeostasis.
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References added:

Gao J, Wang Z, Wang GJ, Zhang HX, Gao N, Wang J, Wang CE, Chang Z, Fang Y, Zhang YF, Zhou J, Jin H, Qiao HL. 2018a. Higher CYP2E1 Activity Correlates with Hepatocarcinogenesis Induced by Diethylnitrosamine. *J Pharmacol Exp Ther.* 365(2):398-407.

Gao J, Wang Z, Wang GJ, Gao N, Li J, Zhang YF, Zhou J, Zhang HX, Wen Q, Jin H, Qiao HL. 2018b. Hepatofibrosis to hepatocarcinogenesis: Higher cytochrome P450 2E1 activity is a potential risk factor. *Mol Carcinog.* 57(10):1371-1382.

Hui L, Zatloukal K, Scheuch H, Stepniak E, Wagner EF. 2008. Proliferation of human HCC cells and chemically induced mouse liver cancers requires JNK1-dependent p21 downregulation. *J Clin Invest.* 118(12):3943-53.

Knight B, Yeoh GC, Husk KL, Ly T, Abraham LJ, Yu C, Rhim JA, Fausto N. 2000. Impaired preneoplastic changes and liver tumor formation in tumor necrosis factor receptor type 1 knockout mice. *J Exp Med.* 192(12):1809-18.

Meek, M.E., Bucher, J.R., Cohen, S.M., Dellarco, V., Hill, R.N., Lehman-McKeeman, L.D., Longfellow, D.G., Pastoor, T., Seed, J., Patton, D.E. 2003, A Framework for Human Relevance Analysis of Information on Carcinogenic Modes of Action, *Critical Reviews in Toxicology*, 33:6, 591-653.

Melnick RL, Huff J. 1993. Liver carcinogenesis is not a predicted outcome of chemically induced hepatocyte proliferation. *Toxicol Ind Health.* 9(3):415-38.

Mueller S, Peccerella T, Qin H, Glassen K, Waldherr R, Flechtenmacher C, Straub BK, Millonig G, Stickel F, Bruckner T, Bartsch H, Seitz HK. 2018. Carcinogenic Etheno DNA Adducts in Alcoholic Liver Disease: Correlation with Cytochrome P-4502E1 and Fibrosis. *Alcohol Clin Exp Res.* 42(2):252-259.

Peccerella T, Arsllic-Schmitt T, Mueller S, Linhart KB, Seth D, Bartsch H, Seitz HK. 2018. Chronic Ethanol Consumption and Generation of Etheno-DNA Adducts in Cancer-Prone Tissues. *Adv Exp Med Biol.* 1032:81-92.

Seitz HK, Mueller S. 2019. [The role of Cytochrom P4502E1 in Alcoholic Liver Disease and alcohol mediated carcinogenesis]. *Z Gastroenterol.* 57(1):37-45.

Song BJ, Abdelmegeed MA, Cho YE, Akbar M, Rhim JS, Song MK, Hardwick JP. 2019. Contributing Roles of CYP2E1 and Other Cytochrome P450 Isoforms in Alcohol-Related Tissue Injury and Carcinogenesis. *Adv Exp Med Biol.* 1164:73-87.

Annex 1: Table with reviewers' name

Function	Name	Country	Contacts
Review manager	Jean-Baptiste Fini	CNRS, France	fini@mnhn.fr
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Annex 2: Individual reviewers' comments
Review #1**AOP 220: Cyp2E1 Activation Leading to Liver Cancer****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?

The authors have provided a detailed description of the AOP that is well-written and certainly of added value for regulatory purposes. For application of this AOP for regulatory decision-making, knowledge on human relevance is highly relevant. Therefore, I suggest to include in the section 'Overall Assessment of the AOP' the publication by Meek et al (2003) on the WHO/IPCS framework, which includes a case study (#6) on chloroform for the AOP under study. This reference could be added to the paragraph on biological plausibility and/or to the paragraph on domain of applicability.

p.13, Evidence for perturbation by stressor:

Please include Meek et al (2003; PMID 14727733) as reference for chloroform.

p.13, MIE, Domain of applicability:

Martignoni et al (2006; PMID 17125407) have reported an overview on species differences (or lack thereof) regarding CYPs, which is a valuable reference to include here.

p.16, oxidative stress, How it is measured or detected:

The authors state correctly that oxidative stress results from an imbalance between ROS and antioxidant defense mechanisms. Therefore, it would be preferable to rephrase the sentence 'Oxidative Stress. Direct measurement of ROS is difficult because ROS are unstable' into 'Direct measurement of an excessive level of ROS as marker of oxidative stress is difficult because ROS are unstable.'

Moreover, the authors should include some text here about the difficulties regarding accurate measurement of oxidative stress. It is well-known that handling of the samples to be measured may seriously impact the results. Furthermore, they should better highlight the differences between the assays proposed. Which one is most suited for this particular AOP?

The rationale for the upper paragraph and the lower one is not clear.

p.18, hepatotoxicity, How it is measured or detected:

LDH release is also a useful marker for in vitro studies. Please move this assay to the lower paragraph.

p.20, regenerative proliferation, How it is measured or detected:

It is well accepted that regenerative proliferation is the KE of importance for this AOP. Still, the authors should acknowledge the fact that the methods listed cannot discriminate between proliferation and regenerative proliferation.

p.22, liver cancer, Domain of applicability:

Hepatocellular carcinoma is a specification of liver cancer; suggest to change into 'hepatocellular adenoma/carcinoma' or 'liver cancer'.

Given the amount of evidence available for rodents, mice and rats should also be listed here.

p.22, liver cancer, Key event description:

The second part of the sentence ‘Liver cancer is among the most common forms of cancer and the second leading cause of cancer death.’ is incorrect. See e.g. GLOBOCAN 2018: <https://gco.iarc.fr/today/home>. A reference for a more global perspective should also be added for the gender difference.

The sentence on the risk factors is confusing, as it triggers the question why inflammation is not included in this AOP.

p.22, Regulatory Significance of the AO

The fact that NTP conducts 2-year carcinogenicity studies is not of relevance here. This should be moved to the preceding paragraph, where it should be listed as example because it is not the only organization conducting the bioassay.

p.31, KER 1517:

The authors introduce here inflammation and state that the biological plausibility for this KER is weak. However, there is quite some literature on the role of inflammation in tumor formation, so the KER description needs refinement.

Furthermore, the description should reflect possible interactions with other KERs, because of the cross-talk between Nrf2 and NFKappaB.

Publications that may be considered helpful are Colotta et al. 2009 (19468060); Schetter et al. 2010 (PMID 19955394); Taniguchi et al. 2018 (PMID 29379212); Furman et al. 2019 (PMID 31806905); Kohler et al 2016 (PMID 26348541).

2. Weight of evidence:

-Are the weight-of-evidence judgement/scoring calls provided by AOP developers for KEs, KERs and the overall AOP, justified, i.e. consistent with the considerations outlined in the Users’ Handbook?

Overall the authors have done a nice job; however, a few sections could be further improved.

Both KER 1516 and 1517 are evaluated as ‘moderate’, although the weight of evidence provided for biological plausibility and empirical evidence is the opposite for the two KERs.

According to the handbook, biological plausibility has more weight compared to empirical evidence. The authors should reconsider these overall calls, after having possibly modified KER 1517.

KER 1518 is listed as non-adjacent KER because the authors consider the current knowledge insufficient to argue for an adjacent KER. Not all cases of regenerative proliferation lead to tumor formation. The authors may consider to mention that this might be an issue of severity. In other words, quantification of this AOP may elucidate whether this KER should be considered as adjacent or non-adjacent.

Furthermore, the authors should reflect on all KERs in a consistent manner. Is their rationale not also applicable to some of the other KERs?

3. Additional observations:

-What do you consider to be critical data gaps, if any, and how to fill these gaps?

Although the focus of this AOP is on oxidative stress leading ultimately to cancer, the AOP description would greatly benefit from more detailed information on how inflammation relates to oxidative stress and the carcinogenesis process.

Minor comments

Abstract:

It is stated that hepatotoxicity is often leading to liver cancer. This is really dependent on the circumstances. Suggest to rephrase to 'may lead to'.

'These events occur in the liver, which is the primary site of xenobiotic metabolism in the body.' Please modify into 'human body'.

p.17: At the bottom of the page, the arrows have dropped out from '(ASK1MKK4JNK)'. Please correct.

p.19, A causal network for regenerative proliferation: please change into 'regenerative'.

Review #2

General Comments

This AOP is related to a well-established mode of action (MOA) for liver cancer that is considered to be relevant to humans (Holsapple et al., 2006; Felter et al., 2018). The overall evidence of biological plausibility for this AOP is strong. The MIE and several critical KEs and KERs are widely recognized and supported by a substantial amount of mechanistic studies demonstrating dose-dependent and temporal concurrence. The AOP is well-written and well-organized, with data gaps clearly identified. A thorough evaluation of KEs and KERs is useful for carcinogenic risk assessments and has the potential to be applied on the screenings for non-genotoxic carcinogens under regulatory settings.

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? Anything missing in this AOP?

This AOP incorporates a large body of relevant scientific literature that covers each KE and KER. The KEs are intelligibly described, the essentiality of the KEs are clearly presented, and the biological plausibility of KERs and weight of evidence are evaluated objectively.

In order to capture the most current scientific knowledge that supports the evaluation, the following areas can be extended with more details.

- Time- and dose-response concordance: The assumption that “KEs can be measured with equal precision and sensitivity” is not defensible. In reality, the precision and sensitivity of different methods for even the same KE can vary, not to mention for different KEs and particularly in different test systems (e.g. *in vitro* vs *in vivo*). The discussion may focus on the fact that upstream KEs can usually be observed at earlier time points than downstream KEs, and that downstream KEs (including the AO) can only be observed following exposures at doses equal or higher than those capable of inducing upstream KEs.
- Quantitative Consideration (the furan table): Please provide further descriptions and a summary of the content. What are the conclusions from these data? Do data generated using other CYP2E1 substrates support the same conclusions?
- Are there any time-course studies (for the KEs) supporting the necessity of chronic exposure?
- MIE:
 - Although it has been explained that CYP2E1 is not regulated at the transcriptional level and that the protein stability is enhanced by binding to the substances, the ubiquitin-proteasome pathway and the involvement of hsp-based chaperone mechanism should be briefly mentioned.
 - There are known CYP2E1 inhibitors used in many mechanistic studies. They are as important as the biomarkers and animal models. Suggest list specific inhibitors as a separate bullet point, with several examples.
- KE2 (hepatocytotoxicity)
 - The terms “hepatotoxicity”, “hepatocytotoxicity” have been used interchangeably throughout this AOP. Since “hepatotoxicity” has a separate event number (#1291) in AOPWiki, the authors should use the term hepatocytotoxicity consistently or explain the differences.

--Similarly, what is the difference between “hepatocytotoxicity”, event 1393 and “increase, Cytotoxicity (hepatocytes)”, event 786? Any KER related to the event 786 should be considered for AOP220?

- The AOP focuses on rodent and in-vitro data. It would be helpful to include some literature on the MIE, KEs and KERs in humans. An evaluation of the weight of evidence of this AOP in humans is extremely helpful for the evaluation of human relevance for rodent hepatocarcinogens.
- Is it appropriate/necessary to place additional KEs between the current KERs? For example: **Inflammation** between hepatotoxicity and liver cancer (KER 1517), **DNA Damage** between Oxidative stress and liver cancer (KER 1516).
- For KERs with moderate or weak evidence, it is important to address the possible reasons for the inconsistency, such as the differences in the testing system and testing dose. The mechanisms for any reversible and thresholdable KEs (Phase II reactions, antioxidant capacity, DNA repair, etc) should also be discussed.

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 classification for the KEs and KERs are appropriate and adequately justified in general, with a few exceptions mentioned below:

- Essentiality of KE3 (regenerative proliferation): The essentiality of this KE was classified as “weak”, because “rodents lacking AP-1 or NF-kappaB display impaired liver regeneration, often leading to death” (Behrens et al 2020). However, there are available studies either supporting or disapproving the essentiality of this KE. For example, it has been reported that in TNF receptor type 1 knockout mice and JNK-1 knockout mice, regenerative proliferation was impaired, accompanying by decreased liver carcinogenesis (Knight et al., 2000; Hui et al., 2008). In the JNK-1 knockout mice, genetic inactivation of p21 restored hepatocyte proliferation and also liver carcinogenesis (Hui et al. 2008). On the other hand, the evidence against the essentiality of KE3 also exists, such as Melnick and Huff (1993).
- KERs:
 - 1) KER 1516: Due to the controversial/complicated role of ROS and Nrf2 pathways in the initiation, development and progress of cancer, it is fair that the Biological Plausibility is determined to be moderate. However, although the authors’ classification of Empirical Evidence for this KER is “weak”, all the literature cited (e.g. evidence of oxidative adducts and other DNA damage associating with hepatocarcinogenesis) have supported this KER. Please consider either providing evidence showing the inconsistent evidence for this KER, or changing the determination from “weak” to “moderate”.
 - 2) KER 1517: This KER is not described as explicitly as others. The authors’ determination of the Biological Plausibility is “weak” and the Empirical Evidence is “moderate”. However, given the hepatocytotoxicity-triggered DAMPs leading to inflammation (may be added as a separate KE), the Biological Plausibility should probably be “moderate”. On the other hand, direct evidence for this KER seems to be weak and limited. First, enzymes indicative of liver damage are not good indicators for liver cancer in humans. Second, the status of inflammation is not mentioned in the cited literature. The possibility of the involvement of regenerative proliferation (KER 1514) cannot be excluded.

- 3) KER 1518: It is widely accepted that highly dividing cells are at greater risk of obtaining and fixing a mutation. If appropriately placed in the genome, such a mutation can facilitate the malignant transformation of the cell. Based on the criteria of Biological Plausibility, this KER may be placed “strong”, rather than “moderate”. As for the weight of Empirical Evidence, it may be more suitable to be classified as “moderate”, instead of “strong”, because there are inconsistent evidence showing 1) some proliferative events are not followed by liver cancer; and 2) some liver carcinogenesis occurs with unchanged proliferation but impaired apoptosis.

3. Additional observations: What do you consider to be critical data gaps, if any, and how to fill these gaps?

- The disparities of the cancer evidence in rodents and in humans. Many of the CYP2E1 substrates are clear rodent hepatocarcinogens, yet only probable or possible human carcinogens due to inadequate epidemiological evidence. Further mechanistic studies are needed to fill these gaps.
- A quantitative relationship between the KEs. It is generally accepted that the indirect carcinogenic MOAs are thresholdable. However, it is not clear what is the in vivo threshold for the upstream event in order to activate the of downstream events. This information can be derived from quality high-throughput testing and in vitro-in vivo extrapolation, with adequate validation.

References:

Hui L, Zatloukal K, Scheuch H, Stepniak E, Wagner EF. 2008. Proliferation of human HCC cells and chemically induced mouse liver cancers requires JNK1-dependent p21 downregulation. *J Clin Invest.*;118(12):3943–3953.

Knight B, Yeoh GC, Husk KL, Ly T, Abraham LJ, Yu C, Rhim JA, Fausto N. 2000. Impaired preneoplastic changes and liver tumor formation in tumor necrosis factor receptor type 1 knockout mice. *J Exp Med.*192(12):1809-18.

Melnick, R. L., & Huff, J. 1993. Liver Carcinogenesis is Not a Predicted Outcome of Chemically Induced Hepatocyte Proliferation. *Toxicology and Industrial Health*, 9(3), 415–438.

Michael P. Holsapple, Henri C. Pitot, Samuel H. Cohen, Alan R. Boobis, James E. Klaunig, Timothy Pastoor, Vicki L. Dellarco, Yvonne P. Dragan. 2006. Mode of Action in Relevance of Rodent Liver Tumors to Human Cancer Risk, *Toxicological Sciences*, 89(1): 51–56.

Susan P. Felter, Jennifer E. Foreman, Alan Boobis, J. Christopher Corton, Adriana M. Doi, Lynn Flowers, Jay Goodman, Lynne T. Haber, Abigail Jacobs, James E. Klaunig, Angela M. Lynch, Jonathan Moggs, Arun Pandiri. 2018. Human relevance of rodent liver tumors: Key insights from a Toxicology Forum workshop on nongenotoxic modes of action, *Regulatory Toxicology and Pharmacology*, 92: 1-7.

Review #3

AOP 220: Cyp2E1 Activation Leading to Liver Cancer

General:

This AOP (SNAPSHOT created at 2019-12-13 12:41) is very clearly described based on scientific data with appropriate references. As an AOP, there is no critical comment to be changed.

1. Scientific quality:

-Does the AOP incorporate the appropriate scientific literature?

Generally, appropriate scientific literatures are incorporated. There were a few points to be clarified as follows,

- 1) Page 4, the last line of Domain of Applicability; for the human HCC case, it had better to use chemical induced case. Please explain whether virus-related HCC case (Poungpaioj, et al . 2015) which should have severe inflammation is appropriate?
- 2) Page 5, the first line of Essentiality of the Key Event; There is a reference of Wong, et al, 1998, which is not included in the reference in page 12.

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

- 1) MIE; Please explain whether there is no necessity to describe as Activation of Cyp2E1 “in hepatocyte”.
- 2) KE2 hepatotoxicity; In the description of WoE, it might be better to clarify either KE2 is cytotoxicity of hepatocyte by oxidative stress or inflammation by other cells such as lymphocyte.

2. Weight of evidence:

-Are the weight-of-evidence judgement/scoring calls provided by AOP developers for KEs, KERs and the overall AOP, justified, i.e. consistent with the considerations outlined in the Users' Handbook? Except for the points described above, there is no further comment for WoE.

3. Additional observations:

-What do you consider to be critical data gaps, if any, and how to fill these gaps?

As an AOP, there may not be critical gaps.

For the detection of MIE, clear evidence was described based on the research using freshly isolated hepatocyte from mouse, rat and human (Kedderis G.L., and Held S.D., 1996). It is not clear there is evidence if HepG2-E47 cells or other easily available hepatocyte can be used for detection of this MIE. If it is possible, the information regarding example of cells for analysis might be helpful.

1. Scientific quality:**-Does the AOP incorporate the appropriate scientific literature?**

It is recommended that the following should be reviewed:

- 1: Song BJ, Abdelmegeed MA, Cho YE, Akbar M, Rhim JS, Song MK, Hardwick JP. Contributing Roles of CYP2E1 and Other Cytochrome P450 Isoforms in Alcohol-Related Tissue Injury and Carcinogenesis. *Adv Exp Med Biol.* 2019;1164:73-87. doi: 10.1007/978-3-030-22254-3_6. Review. PubMed PMID: 31576541.
- 2: Gaviria-Calle M, Duque-Jaramillo A, Aranzazu M, Di Filippo D, Montoya M, Roldán I, Palacio N, Jaramillo S, Restrepo JC, Hoyos S, Navas MC. Polymorphisms in alcohol dehydrogenase (ADH1) and cytochrome p450 2E1 (CYP2E1) genes in patients with cirrhosis and/or hepatocellular carcinoma. *Biomedica.* 2018 Dec 1;38(4):555-568. doi: 10.7705/biomedica.v38i4.3897. English, Spanish. PubMed PMID: 30653870.
- 3: Seitz HK, Mueller S. [The role of Cytochrom P4502E1 in Alcoholic Liver Disease and alcohol mediated carcinogenesis]. *Z Gastroenterol.* 2019 Jan;57(1):37-45. doi: 10.1055/a-0784-8815. Epub 2019 Jan 14. German. PubMed PMID: 30641601.
- 4: Peccerella T, Arsllic-Schmitt T, Mueller S, Linhart KB, Seth D, Bartsch H, Seitz HK. Chronic Ethanol Consumption and Generation of Etheno-DNA Adducts in Cancer-Prone Tissues. *Adv Exp Med Biol.* 2018;1032:81-92. doi: 10.1007/978-3-319-98788-0_6. Review. PubMed PMID: 30362092.
- 5: Peter Guengerich F, Avadhani NG. Roles of Cytochrome P450 in Metabolism of Ethanol and Carcinogens. *Adv Exp Med Biol.* 2018;1032:15-35. doi: 10.1007/978-3-319-98788-0_2. Review. PubMed PMID: 30362088; PubMed Central PMCID: PMC6371814.
- 6: Gao J, Wang Z, Wang GJ, Zhang HX, Gao N, Wang J, Wang CE, Chang Z, Fang Y, Zhang YF, Zhou J, Jin H, Qiao HL. Higher CYP2E1 Activity Correlates with Hepatocarcinogenesis Induced by Diethylnitrosamine. *J Pharmacol Exp Ther.* 2018 May;365(2):398-407. doi: 10.1124/jpet.117.245555. Epub 2018 Feb 21. PubMed PMID: 29467309.
- 7: Gao J, Wang Z, Wang GJ, Gao N, Li J, Zhang YF, Zhou J, Zhang HX, Wen Q, Jin H, Qiao HL. From hepatofibrosis to hepatocarcinogenesis: Higher cytochrome P450 2E1 activity is a potential risk factor. *Mol Carcinog.* 2018 Oct;57(10):1371-1382. doi: 10.1002/mc.22851. Epub 2018 Aug 20. PubMed PMID: 29917271.
- 8: Mueller S, Peccerella T, Qin H, Glassen K, Waldherr R, Flechtenmacher C, Straub BK, Millonig G, Stickel F, Bruckner T, Bartsch H, Seitz HK. Carcinogenic Etheno DNA Adducts in Alcoholic Liver Disease: Correlation with Cytochrome P-4502E1 and Fibrosis. *Alcohol Clin Exp Res.* 2018 Feb;42(2):252-259. doi: 10.1111/acer.13546. Epub 2017 Dec 19. PubMed PMID: 29120493.

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

- In addition to the term “regenerative proliferation” some thought could be considered in relation to the term “frustrated repair.” Regenerative proliferation implies that the tissue and cellular architecture returns to normal or to a near normal state. This is not the case with this carcinogenic pathway – the key pathology term is frustrated repair i.e. normal tissue architecture is not restored or cannot be restored because the events that caused the chronic damage continue to occur.
- It is suggested that a critical part of the AOP is the sustained (possibly inappropriate) induction of CYP2E1. Simple, short-term, induction of CYP2E1 is not necessarily adverse and in many cases is protective;
- The fact that the signalling events that trigger CYP2E1 induction in the liver are complex and are not fully elucidated should be included in the discussion;

- Hepatic Kupffer cells also contain considerable amounts of CYP2E1 and Kupffer cell CYP2E1 is inducible.¹ Kupffer cell CYP2E1 appears to play a role in hepatocarcinogenesis as well as hepatocellular CYP2E1. The term hepatotoxicity covers this perhaps better than hepatocellular toxicity;
- It should be noted that in addition to regenerative proliferation, ROS production associated with CYP2E1 induction results in lipidperoxidation which forms 4-hydroxynonenal (4-HNE) or malondialdehyde which then result in Etheno-DNA adducts. Thus CYP2E1 induction associated ROS production produces both stochastic DNA damage due to frustrated repair as well as DNA adducts.² 1,N⁶-etheno-2'-deoxyadenosine (εdA) adducts appear to be particularly important;³

2. Weight of evidence:

-Are the weight-of-evidence judgement/scoring calls provided by AOP developers for KEs, KERs and the overall AOP, justified, i.e. consistent with the considerations outlined in the Users' Handbook?

- At the high level the KE and KERs are plausible. An additional key event regarding the formation of Etheno-DNA adducts which do not undergo repair resulting in mutation could be considered.
- Critically hepatic neoplasia is considered a complex, multistep and multifactorial disease. This AOP is one in a number of other putative AOPs that could be developed for this effect.
- It should be noted that the basic high level key mode of action of oxidative damage → cell damage → cell proliferation + frustrated repair is well established in pathology.
- It is important to make clear that chronic cell proliferation + frustrated repair may not necessarily be sufficient for hepatocarcinogenesis. Other pathways and events also have to occur e.g. events surrounding hepatic stellate cells, cancer-associated fibroblasts, tumour microenvironment and Kupffer cells also appear to be critical.⁴ In particular, the chronic inflammatory cascade (triggered by ROS production as well as by other events e.g. increased portal LPS levels) is important in the formation of the pre-malignant environment. The formation of fibrosis in the liver lobule is important in that it is an important factor in ongoing frustrated repair and creating a tissue environment that fosters further frustrated repair. In other words, it is not as simple as the stochastic accumulation of mutations associated with chronically proliferating cells: there are many factors in addition to sustained CYP2E1 induction, sustained increased ROS and regenerative proliferation.

¹ Koop DR, Chernosky A, Brass EP Identification and induction of cytochrome P450 2E1 in rat Kupffer cells

J Pharmacol Exp Ther. 1991 Sep; 258(3):1072-6.

² Peccerella T, Arslic-Schmitt T, Mueller S, Linhart KB, Seth D, Bartsch H, Seitz HK. Chronic Ethanol Consumption and Generation of Etheno-DNA Adducts in Cancer-Prone Tissues. Adv Exp Med Biol. 2018;1032:81-92. doi:

10.1007/978-3-319-98788-0_6. Review. PubMed PMID: 30362092.

³ Mueller S, Peccerella T, Qin H, Glassen K, Waldherr R, Flechtenmacher C, Straub BK, Millonig G, Stickel F, Bruckner T, Bartsch H, Seitz HK. Carcinogenic Etheno DNA Adducts in Alcoholic Liver Disease: Correlation with Cytochrome P-4502E1 and Fibrosis. Alcohol Clin Exp Res. 2018 Feb;42(2):252-259. doi: 10.1111/acer.13546. Epub 2017 Dec 19. PubMed PMID: 29120493.

⁴ <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6479943/>

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- Of important note is that hepatic neoplasia occurs with a background of hepatic fibrosis and a pro-fibrotic and pro-inflammatory background. Some investigators have presented data that the pro-fibrotic environment (which can be created via ROS-triggered inflammation) is a prerequisite for hepatic neoplasia.
 - The antioxidant response element and the keap1-Nrf2 pathway, while important, is not covered by this AOP (but could be included in the discussion). As discussed by other reviewers the dysregulation of Nrf2

3. Additional observations:

-What do you consider to be critical data gaps, if any, and how to fill these gaps?

My primary concern is that the AOP must be interpreted in context.

- It should be made clear that this AOP in and of itself is important, but is not necessarily absolutely sufficient for hepatic neoplasia – it part of a complex, multifactorial series of events;
- That the web of events leading to hepatic neoplasia has not been fully elucidated or established;
- That the pathway show in the AOP has to be sustained and high level. A critical aspect of it is that the rate of damage exceeds the rate of tissue repair which results in chronic frustrated regenerative hyperplasia. Low level induction of CYP2E1 and ROS production that does not exceed the cellular antioxidant defence mechanisms and/or capacity of the liver to repair in a manner that retains normal architecture is not likely to result in neoplasia.
- Critically, neoplasia is a disease of stem cells, not of terminally differentiated hepatocytes. This should be clearly noted in the AOP discussion.

Annex 3: Written response from the authors after the end of review Teleconference

Author comment: We are so pleased to hear that the reviewers were generally supportive of our AOP. We have indicated how we intent to address their comments below.

Does the AOP incorporate the appropriate scientific literature?

R1 suggests to include in biological plausibility (and/or to the paragraph on domain of applicability) in the section ‘Overall Assessment of the AOP’ the publication by Meek et al (2003) on the WHO/IPCS framework, which includes a case study on chloroform for the AOP under study.

R4 also suggest to review recent articles and incorporate them within the AOP .

Author response: These are excellent suggestions and we intend to add these additional references to the “overall assessment of the AOP” section. We will also add the references suggested by R4.

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

R2, R1 and R3 suggest to integrate human data.

Author response: As indicated above, we will include the additional suggested references. We will use the additional references provided to enhance aspects related to human response and search the literature to see if there is anything further more recently. We appreciate any references sent out way on human responses relevant to this AOP.

R4 makes a crucial point on the regenerative proliferation Key event which is also approved by the others reviewers. *In addition to the term “regenerative proliferation” some thought could be considered in relation to the term “frustrated repair.” Regenerative proliferation implies that the tissue and cellular architecture returns to normal or to a near normal state. This is not the case with this carcinogenic pathway – the key pathology term is frustrated repair i.e. normal tissue architecture is not restored or cannot be restored because the events that caused the chronic damage continue to occur. It is suggested that a critical part of the AOP is the sustained (possibly inappropriate) induction of CYP2E1. Simple, short-term, induction of CYP2E1 is not necessarily adverse and in many cases is protective;*

Author response: We appreciate the comments and discussion about the best name for this KE, that is broadly recognizable to the general toxicology community and reflects the effect being measured. The review manager was very helpful in helping to arrive at a consensus on this via a poll post-meeting. The authors and review team have settled on “induction, persistent proliferation/sustained proliferation” based on this poll. We will change the KE name accordingly.

Regarding the need for sustained or chronic Cyp2E1 activation – this was originally in the title of the KE and was then shifted to the KER, based on EAGMST recommendations. We will make the fact that it is the chronic nature of the induction of CYP2E1 that is meaningful to this AOP in all of the relevant KERs and in the overall AOP section.

(R2)MIE:

-- Although it has been explained that CYP2E1 is not regulated at the transcriptional level and that the protein stability is enhanced by binding to the substances, the ubiquitin-proteasome pathway and the involvement of hsp-based chaperone mechanism should be briefly mentioned.

Author response: We will include this information.

(R3)KE2 hepatotoxicity; In the description of WoE, it might be better to clarify either KE2 is cytotoxicity of hepatocyte by oxidative stress or inflammation by other cells such as lymphocyte.

Author response: We will clarify as suggested.

1.2. Weight of evidence

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

Weight of evidence is most of the time appropriate clear and accurate. All authors agree that the vast majority of the KE and KER are correctly weighted.

The classification for the KEs and KERs are appropriate and adequately justified in general, with a few exceptions mentioned below:

- Essentiality of KE3 (regenerative proliferation): The essentiality of this KE was classified as “weak”, because “rodents lacking AP-1 or NF-kappaB display impaired liver regeneration, often leading to death” (Behrens et al 2020). However, there are available studies either supporting or disapproving the essentiality of this KE. For example, it has been reported that in TNF receptor type 1 knockout mice and JNK-1 knockout mice, regenerative proliferation was impaired, accompanying by decreased liver carcinogenesis (Knight et al., 2000; Hui et al., 2008). In the JNK-1 knockout mice, genetic inactivation of p21 restored hepatocyte proliferation and also liver carcinogenesis (Hui et al. 2008). On the other hand, the evidence against the essentiality of KE3 also exists, such as Melnick and Huff (1993).

- KERs:

- 1) KER 1516: Due to the controversial/complicated role of ROS and Nrf2 pathways in the initiation, development and progress of cancer, it is fair that the Biological Plausibility is determined to be moderate. However, although the authors’ classification of Empirical Evidence for this KER is “weak”, all the literature cited (e.g. evidence of oxidative adducts and other DNA damage associating with hepatocarcinogenesis) have supported this KER. Please consider either providing evidence showing the inconsistent evidence for this KER, or changing the determination from “weak” to “moderate”.

- 2) KER 1517: This KER is not described as explicitly as others. The authors’ determination of the Biological Plausibility is “weak” and the Empirical Evidence is “moderate”. However, given the hepatocytotoxicity-triggered DAMPs leading to inflammation (may be added as a separate KE), the Biological Plausibility should probably be “moderate”. On the other hand, direct evidence for this KER seems to be weak and limited. First, enzymes indicative of liver damage are not good indicators for liver cancer in humans. Second, the status of inflammation is not mentioned in the cited literature. The possibility of the involvement of regenerative proliferation (KER 1514) cannot be excluded.

- 3) KER 1518: It is widely accepted that highly dividing cells are at greater risk of obtaining and fixing a mutation. If appropriately placed in the genome, such a mutation can facilitate the malignant transformation of the cell. Based on the criteria of Biological Plausibility, this KER may be placed “strong”, rather than “moderate”. As for the weight of Empirical Evidence, it may be more suitable to be classified as “moderate”, instead of “strong”, because there are inconsistent evidence showing 1) some proliferative events are not followed by liver cancer; and 2) some liver carcinogenesis occurs with unchanged proliferation but impaired apoptosis.

Author response: We appreciate the feedback on our WoE calls. We will revisit and use the reviewers’ comments in making the final decision for calls on WoE. We will also add the additional references and ideas from the reviewers above.

1.3. Regulatory applicability:

What do you consider to be critical data gaps, if any, and how to fill these gaps

As it currently stands, divergent opinions arise for the critical gaps identified in the AOP.

R#1 suggests that the AOP description would greatly benefit from more detailed information on how inflammation relates to oxidative stress and the carcinogenesis process.

Author response: During the reviewer teleconference we had discussion on several additional KE(R) that could be involved in this AOP. Indeed, we are aware of several AOPs in mature stages of development that will be able to be networked with this AOP to more fully explain the biology of how CYP2E1 activation (sustained) leads to liver cancer. Thus, based on this and based on the teleconference with the review committee, we will not be building in these additional KEs at present into this AOP and instead will be networking in to these AOPs when the timing is right. We will revisit the text associated with inflammation to make its critical role clear, and we hope that future work will bridge this AOP with inflammatory KEs. The same holds true for the role of mutagenicity in this pathway (parallel AOPs that are in development will be networked in).

1.4. Conclusion:

This AOP is well written but a substantial work is needed before finalisation. Authors are needed to implement the Key event and key events relationships. This will need to be discussed at the TC with both authors and reviewers planned to be February 17th

Author response: We appreciate the review and, as described, will be implementing most of the suggestions provided (note that on the TC there was agreement relating to which comments were mandatory and these will all be addressed).