OECD External Review (September 2015)

Adverse Outcome Pathway 38:

Protein Alkylation Leading to Liver Fibrosis

AOP Author: Brigitte Landesmann, MD, MSc

Systems Toxicology Unit and EURL ECVAM, Institute for Health and Consumer Protection, Joint Research Centre, European Commission, Ispra, ITALY
Email: Brigitte.landesmann@ec.europa.eu

Review Manager: Amanda Hayes, PhD

In vitro Toxicologist, Visiting Fellow, University of New South Wales, Sydney, AUSTRALIA
Email: a.hayes@unsw.edu.au

1 TABLE OF CONTENTS

1.1 INTRODUCTION 3
1.1.1 Background to AOP 38 3
1.2 SYNTHESIS OF MAIN ISSUES OF THE REVIEW 5
1.3 SUMMARY RECORD OF THE TELECONFERENCE 6
1.3.1 Teleconference (TC) Agenda 6
1.3.2 Main Issues and Responses 7
1.3.3 Action List 9
1.4 SUMMARY OF REVISIONS 10
1.5 FURTHER DISCUSSION 12
1.6 OUTCOME OF THE EXTERNAL REVIEW 14
1.7 ANNEX 1: Reviewers Table 15
1.8 ANNEX 2: AOP Wiki Comments/Responses 16

1.1 INTRODUCTION

The Author of AOP 38 is Brigitte Landesmann, MD (Joint Research Centre, European Commission, Ispra, Italy). An internal review of AOP 38: Protein Alkylation Leading to Liver Fibrosis was undertaken in March, 2015. The outcome of this internal review indicated that AOP 38 was well aligned to the refinements as per the AOP handbook, well described and well supported.

This current report details the findings of an external review of AOP 38: Protein Alkylation Leading to Liver Fibrosis (September, 2015). For a list of external reviewers please refer to Annex 1.

1.1.1 Background to AOP 38

Hepatotoxicity in general is of special interest for human health risk assessment. Liver fibrosis in particular is an important human health issue associated with chemical exposure and predictive assays are lacking; it is a typical result of chronic or repeated-dose toxic injury and one of the considered endpoints for regulatory purposes. It is a long-term process in which inflammation, tissue destruction, and repair occur simultaneously, together with sustained production of growth factors and fibrogenic cytokines due to a complex interplay between various hepatic cell types, various receptors and signaling pathways which lead to an imbalance between the deposition and degradation of extracellular matrix (ECM) and a change of ECM composition. Due to this complex situation an adequate cell model is not available and an in vitro evaluation of fibrogenic potential is therefore not feasible. A sufficiently detailed description of the AOP to liver fibrosis might support chemical risk assessment by indicating early (upstream) markers for downstream events and facilitate a testing strategy without the need for a sophisticated cell model. This systematic and coherent display of currently available mechanistic-toxicological information can serve as a knowledge-based repository for identification/selection/development of in vitro methods suitable for measuring key events and their relationships along the AOP and to facilitate the use of alternative data for regulatory purposes. Identified uncertainties and knowledge gaps can direct future research by priority setting and targeted testing. The key event descriptions can be used for hazard identification and read-across to assess the toxic potential of an untested substance.

This AOP describes the linkage between hepatic injury caused by protein alkylation and the formation of liver fibrosis. The MIE (Molecular Initiating Event) is protein alkylation, leading to structural and functional cell injury and further to cell death, the first KE (Key Event). Apoptotic hepatocytes undergo genomic DNA fragmentation and formation of apoptotic bodies. Upon engulfment of apoptotic bodies Kupffer cells (KCs) are activated, the next KE along the pathway. Activated KCs are the main source of TGF-β1, the most potent profibrogenic cytokine. TGF-β1 expression therefore is considered a KE that causes the next KE, hepatic stellate cell (HSCs) activation, meaning the transdifferentiation from a quiescent vitamin A–storing cell to a proliferative and contractile myofibroblast, the central effector in hepatic fibrosis. Activated HSCs cause progressive collagen accumulation, which together with changes in ECM composition signifies the KE on tissue level. The excessive accumulation

of extracellular matrix proteins progressively affects the whole organ and alters its normal functioning, which corresponds to liver fibrosis, the adverse outcome.

There are two further events that play an important role in driving fibrogenesis, namely oxidative stress and chronic inflammation. Both are on-going processes being present throughout the pathway and interconnected with most of the KEs. Hence, they are not classified as KEs themselves and described in the individual KE and KER (Key Event Relationship) descriptions. The inflammatory response plays an important role in driving fibrogenesis, since persistent inflammation precedes fibrosis. Inflammatory signaling stems from injured hepatocytes, activated KCs and HSCs. Inflammatory and fibrogenic cells stimulate each other in amplifying fibrosis. Chemokines and their receptors provoke further fibrogenesis, as well as interacting with inflammatory cells to modify the immune response during injury. Oxidative stress, as well, plays a crucial role in liver fibrogenesis by inducing hepatocyte apoptosis, activation of KCs and HSCs and fuelling inflammation. ROS contributing to oxidative stress are generated by hepatocytes, KCs, HSCs and inflammatory cells.

This purely qualitative AOP description is plausible, the scientific data supporting the AOP are logic, coherent and consistent and there is temporal agreement between the individual KEs. Quantitative data on dose-response-relationships and temporal sequences between KEs are still lacking; the provision of quantitative data will further strengthen the weight of evidence (WoE) and make the AOP applicable for a wide range of purposes.

1.2 SYNTHESIS OF MAIN ISSUES OF THE REVIEW

The AOP author gave a brief overview to the external reviewers explaining the development of the stated AOP to date. The first stages of the development of the AOP were very complicated given the interaction of so many cell types. The process first started by identifying the MIE. A knowledge gap was identified in relation to the binding site. Quantitative data is lacking as there is no suitable cell model available to represent the complex interactions of all cells involved.

The main issue highlighted in the external review process was the lack of specificity and quantitative data in relation to the MIE "protein alkylation". A number of reviewers questioned whether the MIE would be better described as DNA alkylation or oxidative stress which would constitute a new separate AOP. Oxidative stress is interrelated in all KEs and in individual KER's. A better definition of the protein targets constitutes a knowledge gap.

More data is needed in the discussion of the MIE with particular reference to a review article cited by Fujikawa (2015). Eleven chemicals were referred to as case studies but only some of the references were cited in the AOP. The term "neuronal nuclei" should be removed and substituted with a more general term. This would fit with the AOP key principles of making the AOP generic and reusable for future use.

The WoE of protein alkylation leading to cell injury/death is limited. It is recommended that the empirical support of protein alkylation to cell injury/death should be changed from STRONG to MODERATE as MIE linkage to KE1 lacks specificity. More references are also needed to support the WoE.

Further explanation is needed on cell types involved in the KEs. For example, macrophages are not only Kupffer cells but also monocytes from bone marrow. The relationships between KEs are reasonably described but more information is needed on studies to support the evidence for the KERs.

Some editing is needed in relation to paragraph "KE1 cell injury/death". There is double entry of the paragraph "KE2- hepatic macrophages (Kupffer cells) activation and recruitment". Removal of "Wikipedia" sourced references is recommended.

1.3 SUMMARY RECORD OF THE TELECONFERENCE

Date: Wednesday 21st October, 2015
Start time: 12:00 (Paris GMT+2)
Participants: Akiyoshi Nishikawa, Monica Vaccari, Kumiko Ogawa, Brigitte Landesmann and Amanda Hayes
Apologies: Esther Brandon

1.3.1 Teleconference (TC) Agenda

Welcome and Introductions (5 mins)	Amanda and All
Objective of Today's TC (5 mins)	Amanda
Main Issues for Discussion (40-60 mins) Charge Question 1: • Is the MIE- protein alkylation specific enough? • Is there other mechanisms that need to be discussed? • Why are 11 compounds included in the case study but references cited only for 5? • Editing queries for para KE1-Cell injury/death. • Referencing queries e.g. Oxidative stress; use of Wikipedia. • Query in Section 5, protein alkylation. Wording as highlighted by the internal review was somewhat confusing. Charge Question 2: • Relationships between KEs needs to be discussed. • Discussion needed on the linkage between MIE and KE1 eg. induction of cell death, lipid peroxidation and/or oxidative stress. • How strong is the link between protein alkylation and cell death? Charge Question 3: • Is more quantitative info available on KERs? • Is the MIE protein alkylation specific enough (as per Q1)? Charge Question 4: • Recommended after revision and minor changes?	All Reviewers and Brigitte
Any Other Questions or Concerns with the AOP (15 mins)	All
Next Steps (5 mins)	Amanda

1.3.2 Main Issues and Responses

Charge Q1: Check if the AOP incorporates the critical scientific literature & if the scientific content of the AOP reflects the current scientific knowledge on this specific topic.

AKIYOSHI - With regards to the MIE, protein alkylation is one of the causes of liver fibrosis, however, other mechanisms of binding to other molecules like lipids may exist and hence influence downstream events. Other mechanisms such as DNA alkylation and oxidative stress are also important events in liver damage. Although 11 chemicals were referred to in the case study not all references were cited.

AOP AUTHOR RESPONSE - MIE is only about protein alkylation- DNA alkylation would constitute a separate AOP. Oxidative stress is inter-related in all KEs and also described in the individual KER's. References will be updated for all 11 chemicals.

MONICA - The MIE represents a well-known mechanism of toxicity but is quite unspecific and qualitative. The case study of the 11 chemicals focuses on necrotic neuronal death but other cell types are involved in necrosis. Needs to be more general. Suggest changing Wikipedia as a reference.

AOP AUTHOR RESPONSE – Qualitative data on the MIE represents a knowledge gap. Will make it more generic to fit in with the description.

KUMIKO - The MIE is only about protein alkylation however in the AOP it indicates that it is an MIE also for DNA alkylation, hence it needs to be more specific. The WoE of protein alkylation leading to cell death was quite limited especially the empirical support for linkage is unclear.

AOP AUTHOR RESPONSE - DNA alkylation was removed from the main page in the internal review- if there is any other notes remaining in the AOP these will be removed. More references can be added to increase the WoE.

ESTHER - Some double entry of information. Would like more info on which compounds lead to protein alkylation but understand that AOPs are not chemical specific.

AOP AUTHOR RESPONSE - Binding to other molecules may occur but this current AOP is limited to protein alkylation only.

Charge Q2: Verify the weight of evidence (WOE) judgement/scoring provided by the AOP developers for KEs, KERs and the overall AOP.

AKIYOSHI - Notes that the pathway is very clear but more information is needed in relation to the Key Events (KEs). With regards to cell types, macrophages are not only Kupffer cells but also monocytes from the bone marrow. In particular shouldn't the next step after stellate cell activation be myofibroblast activation which produces collagen? Doesn't the AOP only target post necrotic liver fibrosis?

AOP AUTHOR REPSONSE - There is mention in the KE description of the Kupffer cells and the bone marrow-derived macrophages. Yes both macrophages and myofibroblasts are important contributors to liver fibrosis as mentioned under collagen accumulation but it is

not a KE. If there are too many KEs this is not good from a regulatory point of view. Brigitte emphasised that the AOP targets apoptosis or cell injury not necrosis.

MONICA - Linkage between the MIE – KE1 lacks specificity and literature support is limited. Possibility of other MIEs? The knowledge of quantitative understanding is not available hence this limits the empirical support to almost all KERs.

AOP AUTHOR REPSONSE - Process itself causes liver fibrosis but the linkage between MEI-KE presents a knowledge gap and more research is needed in this area. This is purely a qualitative pathway description and the lack of quantitative data is noted as a knowledge gap due to the fact that currently there is no cell model for liver fibrosis available.

KUMIKO - AOP ok, but quite general in that all alkylating agents lead to liver fibrosis. However, only a critical subset of protein alkylation events contributes to injury- therefore it is difficult to score as strong.

AOP AUTHOR REPSONSE - Agreed WoE to be changed from strong to moderate.

ESTHER - AOP ok.

Charge Q3: What would be the regulatory applicability of this AOP in your opinion?

AKIYOSHI - Suggests that the MIE needs more data in the discussion as highlighted in previous charge comments. The regulatory applicability depends on the clarifications and outcomes of Q1 and 2.

AOP AUTHOR REPSONSE - Agreed

MONICA - Noted that there are some gaps in the knowledge. Monica stated that this AOP is a qualitative description of the process triggered by protein alkylation and leading to liver fibrosis. In spite of the limitations the AOP can be useful in providing a framework to identify research needs and knowledge gaps. A deeper mechanistic knowledge of the KEs and KERs might support the development and validation of alternative methods for measuring KEs which could lead to the development of prediction models.

AOP AUTHOR REPSONSE - Explains that the AOP is a work in progress which identifies knowledge gaps that can be used to determine what to look at and to drive future research in this area.

KUMIKO - Each step of the KEs is reasonably described. Continuous studies to evaluate the importance of these WoEs are necessary.

AOP AUTHOR REPSONSE - Agreed

ESTHER - The regulatory applicability would be high.

Charge Q4: Overall Assessment of the AOP- Would you recommend this AOP to be submitted to the Working group of the National Coordinators for the Test Guidelines Programme (WNT) and the Task Force on Hazard Assessment (TFHA) for endorsement?

A general discussion was had around Charge Q4. All participants suggested that it depends on the suggested changes made to the AOP based on their review of Charge questions (1 and 2).

AKIYOSHI - Depends on the outcomes of Q1's and 2.
MONICA - Yes, after revision and minor editorial changes.
KUMIKO - After revision it may be possible.
ESTHER - Yes after suggested changes.

1.3.3 Action List

1. As stated - This AOP is only about the MIE "protein alkylation". There is text throughout the AOP that indicate that DNA alkylation/oxidative stress may also be misconstrued as a MIE. This text needs to be removed. MIE needs to be more specific. There is a lack of quantitative data in relation to the MIE.
2. Include more information on the 11 chemicals in the case study by Fujikawa (2015). This study should be described more generally rather than focusing on only necrotic "neuronal" cells but all cells involved.
3. More data is needed in the discussion of the MIE.
4. The WoE of protein alkylation leading to cell death is limited. The empirical support is unclear. More references are needed to support the WoE.
5. The empirical support of protein alkylation to cell death should be changed from strong to moderate as MIE-KE1 lacks specificity and only a critical subset of protein alkylation events contribute to injury.
6. More information is needed on KEs. More explanation needed around the KEs and cell types.
7. Remove Wikipedia references as highlighted in the AOP Wiki.
8. Double entry of information to be removed as highlighted in the AOP Wiki.

1.4 SUMMARY OF REVISIONS

The external review process identified two main knowledge gaps of the AOP:

1) The lack of specificity of the MIE, and
2) The lack of quantitative data.

These knowledge gaps are due to the lack of understanding of the mechanisms and consequences of protein modifications by reactive intermediates. To date there is no suitable cell model available that accounts for the complex interaction of all cells involved in liver fibrosis. However, there are research groups continually working on the development of cell culture models to reflect this complex mixed interaction of cell types and mechanisms of liver fibrosis.

Critical scientific literature of the AOP

MIE is only about protein alkylation in this AOP. Protein alkylation has been identified as a key triggering event in chemical toxicity. DNA alkylation may play a role- but there is insufficient data to substantiate this claim. Notes on DNA alkylation were removed following the internal review from the main page. Remaining references on DNA alkylation in the text passage have now also been removed. Oxidative stress remains interrelated to all KEs and KERs but it is not the “mechanism of action” for the stated AOP (as per Handbook, Section 5). Oxidative stress had been removed as an individual event description in a previous internal review. Methods or measurement and detection have also been removed in line with the outcomes of the internal review.

The case study of the investigated chemicals (11) has been updated to include references for all 11 chemicals and the review paper by Fujikawa (2015). The paragraph on structural analysis of the 11 chemicals has been removed as it was an in house study and had low informative value for the AOP. The term “neuronal nuclei” was removed and substituted with “cell nuclei”. This is in line with the AOP key principles where KEs and KERs should be generic, reusable and they do not need to be regenerated independently for every new AOP.

The description for KERs has been updated with more detail and references for empirical support were added and explained.

KE1 cell injury/death has been edited. Reference to Wikipedia has been deleted, double entry of paragraph “KE2…recruitment” has been deleted.

WoE judgement for KEs, KERs and overall AOP

In the KE description Hepatic macrophages (Kupffer cells), the fact that bone marrow-derived macrophages originating from circulating monocytes are being recruited is mentioned. In the KE description of Collagen, Accumulation the additional recruitment of myofibroblasts is also mentioned. These cells are noted as important contributors to fibrogenesis they are not labelled as a KE as this event is not measureable and its essentiality for the AO is not proven.

To support the WoE for the KER – “Protein, alkylation directly leading to cell death” more detail and references for experimental studies have been added and outcomes of these

studies is included in more detail in the text. The WoE was originally scored as “strong” this has now been has been changed to “moderate” (after consultation with the reviewers) due to the fact that only a critical subset of protein alkylation events contribute to injury.

There are other possible MIEs which may lead to cell injury and liver fibrosis by the same downstream KEs however, this would constitute another AOP. Hepatocyte injury is an early convergent KE for several AOPs and may be a crucial event for the pathway to fibrosis.

This is a purely qualitative pathway description. The lack of quantitative data is definitely an identified knowledge gap and also due to the fact that there is currently no suitable cell model for liver fibrosis available.

1.5 FURTHER DISCUSSION

AKIYOSHI - It is true that protein alkylation can be measured in detail by recent technologies. There is still knowledge gaps as to what type of protein alkylation really causes liver cell apoptosis/necrosis and subsequently leads to liver fibrosis. It is also possible that almost all the protein alkylation may be just the consequence of chemical exposure. In fact, no references cited clearly state direct relationship of protein alkylation with liver cell apoptosis/ necrosis in terms of the mechanism of action. In conclusion, protein alkylation seems not so much strong as a molecular initiating event leading to liver fibrosis. Only if liver cell apoptosis or necrosis is the starting key event, this AOP may be accepted. Even if so, because the process from necrosis/apoptosis to fibrosis could be multifaceted including modulation of immune system, the regulatory applicability may be largely limited.

AOP AUTHOR RESPONSE - It is undisputed that protein alkylation leads to cell injury and that the main mechanism is via disturbance of the cellular redox balance, oxidative stress and mitochondrial injury, which finally ends up in apoptosis or necrosis. It is still unknown though, whether there are fibrosis-specific features of this process. This knowledge gap is repeatedly mentioned in the AOP description. I also think that hepatocyte injury is the crucial event with more predictive power than the MIE; but this is only a hypothesis that still needs to be confirmed. Besides, following AOP theory a specific MIE is needed as one of the anchors of the AOP.

MONICA - I have read through all Brigitte's changes and revised sections on the AOP Wiki. All issues have been addressed and the descriptions of the MIE and the MIE-K1 KER have been deeply improved.

AOP AUTHOR RESPONSE - No further comment.

KUMIKO - The document seems to be well revised. However, there are two points to discuss:

1. With regards to the data and information of the 11 sample chemicals summarised in the data matrix, 6 compounds were not indicated for "Protein binding", as author described.

AOP AUTHOR RESPONSE - Yes the only chemicals with protein binding as MIE were included in the list of initiating chemicals in the AOP. Initially the matrix has not been included in the Wiki, because it does not show all the steps of the pathway for all the investigated chemicals. As the reviewers asked for more information on these further chemicals the whole matrix was then uploaded to the Wiki, as it shows that hepatocyte injury/death has been described for all these chemicals on the way to fibrosis. For the rest it must be noted that these studies have not been done to explore the pathway and its KEs. If a KE has not been described it does not necessarily mean that it does not exist, but that it has not been investigated; but there is a considerable overlap of KEs. These studies are a mixture of *in vivo* animal studies, human case reports and *in vitro* experiments; for the first two kinds of studies it is difficult to investigate and observe the whole sequence of events and for the *in vitro* testing a suitable co-culture cell model is still lacking.

2) The relationship between “Protein binding” and “Protein alkylation” is not clear. Please explain the example target proteins and also the example alkylated protein product (I am not clear, “protein binding” might be a MIE instead of “protein alkylation”?).

AOP AUTHOR RESPONSE - Protein alkylation is a kind of covalent protein binding and therefore the MIE could be called protein binding but this would make the MIE even broader and more unspecific. The authors of the studies for the fibrogenic chemicals have described the molecular event as protein binding, because this obviously was sufficient for their purpose and as they were not thinking in terms of an AOP or MIE, they did not aim for a more detailed description of this interaction. Protein binding is not contradictory to protein alkylation, just a broader category.

ESTHER - Changes have been made, AOP is now acceptable.

AOP AUTHOR RESPONSE - No further comment.

Other general comments:
MONICA - Found the referencing system difficult to understand in the AOP snapshot. The version on the Wiki has many layers that can be confusing when navigating through the AOP.

1.6 OUTCOME OF THE EXTERNAL REVIEW

All comments/recommendations captured as actions in the summary record of the Teleconference (Section 1.3.3) have now been satisfactorily addressed by the author. The AOP Wiki has been updated based on these recommendations.

It has been noted openly that there are knowledge gaps associated with this AOP. These "knowledge gaps" are due to scientific data that is currently unavailable rather than data which is intentionally not included. Until a suitable cell model that incorporates the complex cellular components for liver fibrosis is available there will be scientific calculated assumptions made. In particular the main discussions have focused on the MIE – "protein alkylation". It is agreed by all review participants that the MIE is somewhat unspecific and there is a lack of quantitative associated data. Protein alkylation may lead to cell injury whether there are fibrosis specific features of this process involved, this is unknown. The author and all reviewers recognise that this is a "purely qualitative" pathway. AOPs are designed to be constantly evolving and updated when new data/research becomes available. As noted by reviewers and authors, despite the identified knowledge gaps this AOP is useful as a knowledge- based repository of available mechanistic- toxicological information. It may also be helpful for KEs along the pathway and identification of novel biomarkers for liver fibrosis and facilitate a testing strategy for chemical risk assessment based on KEs and KERs. The KEs can also be used for hazard identification and to assess the toxic potential of untested substances.

1.7 ANNEX 1: REVIEWERS TABLE

Participants		
Reviewers	**Affiliation**	**Regulatory/Academia**
Akiyoshi Nishikawa (MD, PhD)	Division of Pathology Director, Biological Safety Research Center National Institute of Health Sciences (NIHS) Tokyo, JAPAN Email: nishikaw@nihs.go.jp	Pathologist, partly contributing to regulatory field
Monica Vaccari (PhD)	Area Tossicologia Sperimentale CTR Tossicologia Ambientale ARPA Emilia-Romagna Bologna, ITALY Email: monica.vaccari4@unibo	Experimental toxicologist, partly contributing to regulatory field.
Kumiko Ogawa (MD, PhD)	Division of Pathology Biological Safety Research Center NIHS Tokyo, JAPAN Email: ogawa93@nihs.go.jp	Pathologist working in the field of toxicologic pathology.
Esther Brandon (PhD)	National Institute for Public Health and the Environment Bilthoven, THE NETHERLANDS Email: esther.brandon@rivm.nl	Pharmacokinetics/Toxicologist (metabolism, transporters, and interactions)

AOP Author: Brigitte Landesmann, MD, MSc

Systems Toxicology Unit and EURL ECVAM, Institute for Health and Consumer Protection, Joint Research Centre, European Commission, Ispra, ITALY
Email: Brigitte.landesmann@ec.europa.eu

Review Manager: Amanda Hayes, PhD

In vitro Toxicologist, Visiting Fellow, University of New South Wales, Sydney, AUSTRALIA
Email: a.hayes@unsw.edu.au

1.8 ANNEX 2: AOP WIKI COMMENTS/RESPONSES

Charge question 1: Check if the AOP incorporates the critical scientific literature and if the scientific content of the AOP reflects the current scientific knowledge on this specific topic.

REVIEWER 1:

I have a question about the molecular initiating event. As mentioned in the text, it is unclear whether protein alkylation per se is sufficient to initiate the pathway or alkylation to specific proteins need to be affected and whether various biding sites influence the further downstream process. This means that protein alkylation may not be certainly solid as an initiating event. Besides protein alkylation, some other mechanisms such as DNA alkylation and oxidative stress had better be discussed more in detail. In addition, 11 chemicals were referred to as case studies, but only some of references were cited.

Response from AOP author:

Though covalent protein alkylation by reactive electrophiles was identified as a key triggering event in chemical toxicity already over 40 years ago and despite intense efforts to get better understanding of the mechanism and consequences of protein modification by reactive intermediates – both oxidizing and alkylating agents - this knowledge is still quite limited. A better definition of the protein targets constitutes a knowledge gap.

The MIE is only about protein alkylation; DNA alkylation (methylation) could play a role as well, but for the time being there is no sufficient data to substantiate this claim. Besides, that would constitute another AOP.

Oxidative stress is inter-related to all KEs and described in the individual KERs. To explain why the role of oxidative stress in cell injury is not described in detail, please see Handbook Section 5: "In describing KEs within an AOP, it is important to recognise their distinction with “mechanism of action”. AOPs provide a description of a limited number of critical, measurable events leading to induction of the relevant end-point of toxicity. They do not necessarily provide a comprehensive molecular description of every aspect of the biology involved."

The references for the 11 investigated chemicals were added.

REVIEWER 2:

I think that the Authors did an excellent work in describing the critical scientific literature related to the identified MIE, the KEs and KERs. Data gaps and research needs are clearly identified. The MIE “Protein alkylation” represents a well-known mechanism of toxicity, but it is quite unspecific and qualitative. Based upon existing knowledge, the extent of protein damage does not correlate well with the adverse outcome of the AOP.

The Authors reported that an extensive literature search looking for information from in vivo repeated dose toxicity studies was performed on 11 compounds selected as known inducers of liver fibrosis. Moreover, they reported also that a structural analysis has been performed to assess structural similarities and possibly "correlate" structure with toxicological potential. The reported references about chemical initiators are focused only on 5 chemicals (carbon

tetrachloride, Acrolein, retinol, dimethylnitrosamine and thioacetamide) out of 11 and the references about the structural analysis are not cited. The review paper by Fujikawa is focused on necrotic neuronal death as the result of Excitotoxicity. The pathways and processes associated with necrosis have been described in other cell types or tissues, not only in neurons. For instance, several reported studies were conducted in mouse embryonic fibroblasts.

The paragraph “KE1 cell injury/death” needs some editing (Paragraph 1 line 1-5: not clear, missing bracket; Paragraph 2 line 8: replace kinesis with Kinases; Paragraph 2 line 16: The exit from nuclei of PAR is true for neurons only?; Paragraph 3: the reference Fujikawa, 2015 is not reported in the document). I suggest not citing “Wikipedia” as a reference for assay description (see TUNEL assay).In “KE2 - Hepatic macrophages (Kupffer cells), activation and recruitment”, the following paragraph is repeated twice in the text: “Expressed TNF-α, TRAIL (TNF-related apoptosis inducing ligand), and FasL (Fas Ligand) are not only pro-inflammatory active but also capable of inducing death receptor mediated apoptosis in hepatocytes”.

Response from AOP author:

For the unspecific MIE please see the response above.

The references for the 11 chemicals, as well as the review paper by Fujikawa were added to the list of references.

In order to investigate the structural similarity between the 11 fibrogenic chemicals, a list of 661 sub-structures from the FDA Redbook was cross checked against the 11 chemicals by using the Chemotyper tool. Since structural similarity was not found, in silico analysis was conducted in order to investigate if the 11 chemicals had structural features indicative of potential protein binding. For this analysis, QSAR Toolbox was used to predict the protein binding of the 11 chemicals by using via 2 profilers called "Protein binding by OECD"(Author: School of Pharmacy and Chemistry, Liverpool John Moores University, UK) and "OASIS v1.2. Protein binding alerts for skin sensitization" (Author: Laboratory of Mathematical Chemistry (LMC), Bourgas, Bulgaria). References cannot be given, because this was an in-house activity that has not been published. Considering the low informative value for the AOP description and the confusion that it might cause, the paragraph on structural analysis has been removed.

The term neuronal nuclei was removed and substituted by cell nuclei, making the description generic again. The reference to Fujikawa was kept,though it is focused on necrotic neuronal death. One of AOP's key principles is their modularity; KEs and KERs, the two fundamental building blocks of an AOP, when described in a generic way, are reusable and do not need to be regenerated independently for every new AOP. Therefore, KE descriptions should be kept as broadly applicable as possible while including the necessary level of detail. Other developers using the same KE may add any missing information in a generic way to keep reproducibility, but they may include specific references (to support the applicability for various cells or species). Here, this principle in KE description has erroneously not been followed and this error was corrected.

The paragraph “KE1 cell injury/death” has been edited and the reference to Wikipedia has been deleted.

The double entry in the paragraph "KE2 - Hepatic macrophages (Kupffer cells), activation and recruitment” has been deleted.

REVIEWER 3:

REVIEWER 4:

1) In Page 11, regarding oxidative stress, the methods of measurement and detection are very important. I would like to request more precise references. I wonder whether web site provided by company (Ref. 4 in page 13) is appropriate and enough as a scientific reference, since the contents of web site may not be permanent and can be changed.

2) In the same reason, references cited from “Wikipedia” may not be suitable for OECD document. e.g.; page 3, page 5

3) Page 15, regarding Protein, alkylation directly lead to cell death, N/A, this is one of the most important section for this AOP. However, the explanation for the Weight of evidence was quite limited, especially “Empirical Support for Linkage” was not clear and the listed references were not explained in detail.

4) In the section in the latter half, references were listed but not explained.

5) One of the internal reviewer suggested in Section 5, “The description of alkylating agent effects on DNA is related to protein alkylation, however I would suggest that it be removed since this MIE is only about protein alkylation. This would avoid confusion of this MIE also being an MIE for DNA alkylation”. Then, authors' responded as “The mentioning of DNA alkylation has been removed.” I would like to support the suggestion of the internal reviewer. However, the descriptions regarding “DNA alkylation” remain in page 2 and 3.

Response from AOP author:

1.) Oxidate stress description and measurement was included in a prior version of the AOP (snapshot March 2015); following the comments of the internal reviewers, oxidative stress has been removed from the individual event descriptions and remained only within the KER–descriptions; the description of measurement methods has therefore also been removed. Both are not present any more in the latest snapshot (September 2015).

2.) All references cited from “Wikipedia” have been removed.

3.) The description of this KER has been explicated in more detail and references for empirical support were added and explained.

4.) The content of the listed references was summarised in the text.

5.) Following the internal reviewer's comments the notes concerning DNA Alkylation have been removed from the main page. Unfortunately this text passage in the MIE description has been overlooked, but now it has been deleted, as well.

REVIEWER 5: The AOP start with protein alkylation, but in the figure information on parent compound and metabolite is presented leading to the protein alkylation. It is advised that a paragraph is added with information on which compound (and especially metabolites) lead to protein alkylation. The rest of the AOP is easy to read, but sometimes information is double.

Response from AOP author:

More information on chemicals and their metabolites would be undoubtedly useful. But, by definition, AOPs are not chemical-specific and the pathway description is independent from any specific chemical initiator. Experimental data derived from exposure to prototypic chemicals for the outcome of interest are useful for understanding the pattern of biological response and building the AOP. The more detailed information on chemicals and their specific properties and metabolites are not part of an AOP. The first box of the AOP graph should only indicate the link to the chemical initiator without being part of the AOP itself.

Charge question 2: Verify the weight of evidence judgement/scoring provided by AOP developers for KEs, KERs and the overall AOP.

REVIEWER 1: The relationships between key events are reasonably described, but some other factors had better be discussed. For example, macrophages are not only Kupffer cells but also monocytes from the bone marrow. Nest step after stellate cell activation is “myfibroblast formation” which actually produces collagen. It is known that myofibroblasts are derived from stem cells in the bone marrow or fibrobalsts in the periportal area as well as stellate cells.

Response from AOP author:

Macrophages: In the KE description of Hepatic macrophages (Kupffer Cells), the fact that bone marrow-derived macrophages, originating from circulating monocytes are being recruited, is mentioned: "Besides Kupffer cells, the resident hepatic macrophages, infiltrating bone marrow-derived macrophages, originating from circulating monocytes are recruited to the injured liver via chemokine signals. Kupffer cells appear essential for sensing tissue injury and initiating inflammatory responses, while infiltrating Ly-6C+ monocyte-derived macrophages are linked to chronic inflammation and fibrogenesis. The profibrotic functions of Kupffer cells (HSC activation via paracrine mechanisms) during chronic hepatic injury remain functionally relevant, even if the infiltration of additional inflammatory monocytes is blocked via pharmacological inhibition of the chemokine CCL2 KC activation and macrophage recruitment are two separate events and both are necessary for fibrogenesis, but as they occur in parallel, they can be summarised as one key event."

Myofibroblasts: In the KE description of Collagen, Accumulation this additional recruitment of myofibroblasts is mentioned:

"Upon activation of HSCs and other myofibroblast precursors, there is a > 50-fold increase in α 1(I) procollagen mRNA levels."and "Besides the transition of quiescent HSCs into activated HSCs and then further into contractile myofibroblasts, other cells may transdifferentiate into fibrogenic myofibroblasts in liver injury. Additional sources of ECM include bone marrow

(which probably gives rise to circulating fibrocytes), portal fibroblasts, EMT (epithelial–mesenchymal cell transition) from hepatocytes and cholangiocytes."

Though these cells are important contributors to fibrogenesis, their appearance was not labelled KE, because this event is not measurable and its essentiality for the AO is not proven.

REVIEWER 2: The WoE provided for the linkage between the MIE and the KE1 (induction of cell death) is not adequately supported by the reported studies. The description of the biological plausibility of the first KER suggests the existence of other possible MIEs (lipid peroxidation and/or oxidative stress). Knowledge on quantitative understanding is not available and the lack of quantitative studies limits the extent of the empirical support to almost all the KERs.

Response from AOP author:

WoE: More experimental studies to support the evidence for this KER have been added and the content of each study has been summarised in the text.

Existence of other possible MIEs: Absolutely there are other possible MIEs. By definition, an AOP has only one MIE and one final AO, the two anchor points. Any other MIE that leads to cell injury and further to liver fibrosis via the same downstream KEs constitutes another AOP. Different agents cause various types of hepatocyte injury by various MIEs that finally lead to fibrosis via the same described downstream KEs (as also can be seen in the data matrix with the mechanistic-toxicological information on the 11 chemicals). Hepatocyte injury, therefore, is an early convergent KE for several AOPs and might be the crucial event for the pathway to fibrosis.

Lack of quantitative studies: This is a purely qualitative pathway description. The lack of quantitative data is definitely a knowledge gap and also due to the fact that currently there is no suitable cell model for liver fibrosis available.

REVIEWER 3:

REVIEWER 4: Although all the weight of evidences were judged as “strong” by the authors, some of the linkage seems to be not “strong”. Most of all, as authors mentioned in page 2, “only a critical subset of protein alkylation events contributes to injury.” If they can’t specify critical subsets, it might be difficult to score as ”strong” at least between protein alkylation and cell death. Related to the comment 3) in the Charge question 1, more precise explanation regarding the linkage between protein alkylation and cell death might be required.

Response from AOP author:

WoE has been changed to "moderate".

More experimental studies to support the evidence for this KER have been added and the content of each study has been summarised in the text.

REVIEWER 5:

Charge question 3: What would be the regulatory applicability of this AOP in your opinion?

REVIEWER 1: Before clarification of the questions 1 and 2, it is difficult to mention about the regulatory applicability.

REVIEWER 2: As the Authors clearly highlighted in the text, this AOP is a qualitative description of the process triggered by protein alkylation and leading to liver fibrosis. Quantitative information on KERs is limited or lacking. The identified MIE is quite unspecific and the structural analysis that has been performed to assess structural similarities did not identify features that are clearly associated with toxicological potential and fibrosis induction. These gaps in the knowledge about the MIE "Protein alkylation" represent a possible limitation for the development of specific assays that could be validated and included in the OECD TG program, as well as for the applicability of the AOP for regulatory purposes. In spite of the limitations described above, this AOP can be useful in providing a framework which helps in identifying research needs. Currently, the biological plausibility of each of the KERs in the AOP is "strong", whereas the extent of empirical support is scored as "moderate". A deeper mechanistic knowledge of KEs and KERs might support the development and validation of alternative test methods for measuring the KEs, leading to the development of prediction models. The AOP might be further developed to gather sufficient scientific confidence to be applied for utilization for priority-setting, chemical category formation or hazard identification.

REVIEWER 3:

REVIEWER 4: Each step of KEs is reasonably described. Continuous studies to evaluate the importance of these weight of evidences are certainly necessary.

REVIEWER 5: High

Charge question 4: Overall Assessment of the AOP - Would you recommend this AOP to be submitted to the Working group of the National Coordinators for the Test Guidelines Programme (WNT) and the Task Force on Hazard Assessment (TFHA) for endorsement?

Responses

REVIEWER 1: Similarly to Charge question 3, I am not sure of the recommendation.

REVIEWER 2: I recommend this AOP to be submitted for endorsement after revision and minor editorial changes.

REVIEWER 3:

REVIEWER 4: This is a good challenge, however, I can't recommend this AOP in the present form. (After revision, it is possible.)

REVIEWER 5: After suggested changes, yes