**Internal review charge questions - February 2017**

## AOP Information

* AOP title: In utero DNA topoisomerase II inhibition leading to infant leukaemia
* Point of contact author: Andrea Terron
* Associated wiki page: <https://aopwiki.org/aops/202>

## Reviewers

**Primary Reviewer (PR):** Name: Carole Yauk; OECD Country/Org.: Canada; Email:

**Secondary reviewer 1 (SR1)** : didn’t take part in the review

**Secondary reviewer 2 (SR2)** Name: Katy Goyak; OECD Country/Org.: BIAC; Email:

#### Date review completed:

## Review

**Section 1:**

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| **AOP identifier/Title***Does the name of the AOP follow the right convention (MIE or first KE leading to AO)?* *Does the name of the AOP reflect its content/domain?* |

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| **Reviewers' responses and comments** **PR:** Yes.**SR1:****SR2:** Yes, name of the AOP follows the convention. It does accurately reflect the content of the AOP. |

**Section 2:**

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| **Authors***Is it clear who the authors/developers of the AOP are?* *Contact information for one or more corresponding author(s) should be included.* |

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| **Reviewers' responses and comments** **PR:** Yes. An email for the primary contact might be useful**SR1:****SR2:** Yes it is clear who the authors are, but the contact information for the corresponding author is not clearly identified. |

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| olavi.pelkonen@oulu.fi; andrea.terron@efsa.europa.eu**added in wiki** |

**Section 3:**

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| **Date of updating***Reviewer should indicate the date stamp on the PDF snapshot under review.* |

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| **Reviewers' responses and comments** **PR:** 2017-02-09**SR1:****SR2:** The snapshot that I reviewed was created on Feb 9, 2017 at 20:45. |

**Section 4:**

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| **Abstract***Does the abstract concisely describe the main content of the AOP?* |

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| **Reviewers' responses and comments** **PR:** The abstract has relevant background information, but fails to clearly name the MIE, KE1 and AO. The readers should know that there are three events. Also, the overall weight of evidence for the AOP should be stated.**Author response:** The abstract was re-drafted in order to clearly name the MIE, KE and AO**SR1:****SR2:** Yes - the abstract provides a nice overview for the clinical relevance of the AOP, proposed pathway (inhibition of Topo II and chromosomal rearrangements) and also references key knowledge gaps. |

**Section 5:**

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| **Molecular Initiating Event***Is a MIE described? If yes, then:* *Is the MIE description clear and is it biologically plausible?* *Is the MIE described in a way that allows its use in other AOPs?* *Are measurement/prediction methods specified and adequately described/referenced?* *Is the biological context (inc. taxonomic applicability/relevance, level of biological organisation) specified and explained sufficiently?* *Have chemical initiators (prototypical chemicals or chemical features) been identified?* |

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| **Reviewers' responses and comments** **PR:** ***Is the MIE description clear and is it biologically plausible?*** The authors have not named the MIE correctly. Believe the name of the MIE should be something like ‘topoisomerase II inhibition’. The MIE is biologically plausible. I would suggest the authors do not specificy ‘in utero’ in the MIE – then the MIE can be more broadly used. The fact that all of this has to happen in utero can be described in the overall AOP. Also, much of the evidence would not be from in utero testing.**Author response:** T*he in-utero specification, for the MIE, is considered relevant by the authors in order to keep the specificity of the AOP. We agree that some surrogate evidence is not coming from in-utero but the regulatory relevance of this AOP is associated with the developmental condition of the AO. The AO, although a cancer disease, is considered a developmental disease and this is a critical aspect for separating the IFL (a single hit, cancer disease) from childhood leukaemia, (a multiple hits cancer disease).**Menendez et al. (2009) showed that MLL-AF4 fusion gene is present and expressed in bone marrow mesenchymal stem cells in infant patients with t(4;11) B cell-ALL. However, other paediatric B cell-ALL-specifc translocations/gene fusions were never found in this cell population. This suggests that the origin of the fusion gene in infant B cell-ALL is likely prehaematopoietic. Consequently, the target cell for transformation may be an early prehaematopoietic mesodermal precursor, a haematopoietic stem cell or a haematopoietic progenitor cell residing mainly in the liver (Greaves 2015; Sanjuan-Pla et al. 2015). Indeed, the specific link between the MIE and the AO exists only if occurring during the embryo-foetal development. The issue was also discussed at the EAGMST meeting in June 2017 and the an overall consensus was reached that, for the time being, the in-utero specification should remain, as this is a very specific condition for the description of this MIE and for this AOP.*In this section, I was expecting to read more about what are topos, and specifically what is topoII, its structure and function. I’d like to see information about role in normal biology in detail (first), followed by a description of what topoII inhibitors do and how. I think the authors have done a good job in the bottom of the ‘How this KER works’ describing all of the potential outcomes of topoII inhibition and referenced a review article on the topic. Also really like the figure. However, I’d like a more complete description of the various mechanisms by which topoII can be inhibited. Very important for QSAR and should be thoroughly described here. **Author response:** *The text was redrafted to include additional information in line with the comment. The key literature references are now quoted.* ***Is the MIE described in a way that allows its use in other AOPs?*** Within the MIE description categories the authors have described many details about the relationship to the next KE and the entire AOP. For e.g., much of the text in the stressor section makes reference to the entire AOP rather than the MIE. The text should only refer to the evidence that the stressor causes the MIE so that the MIE can be re-used for other AOPs. The text in the ‘Evidence for perturbation of this MIE by stressor’ is the same as the text on ‘stressor’ on the overall summary page for etoposide. This again is written in the context of the overall AOP rather than presenting the evidence and understanding that etoposide is a topoII poison. In general, some of the information written in the MIE stressor text can actually be used in KER support.As above, in the ‘How this KE works’ section, the authors describe how the topoII inhibition leads to KE1. This should all be transferred to KER1, and the text should just be specific to what is the KE, what is its normal biological function (e.g., what is the normal function of topoII) and how do the poisons work to inhibit the enzyme from doing its job. **Author response:** *The text has been redrafted/ reorganized in a way to answer the comment. The stressor part has been widely revisited to be in line with the comments.****Are measurement/prediction methods specified and adequately described/referenced?*** Measuring DNA strand breaks or protein responses supports that there was an effect of the topoII inhibition, rather than telling us that chem is a topoII inhibitor. I don’t suggest delete, but think this needs to be explained. Are there not SARs to predict that something will be a topoII inhibitor? Not sure why the authors are not describing the assays found in Nitiss et al., 2013: <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3397423/>Seems like an excellent source of assays for identifying both topoI and topoII inhibitors, with very detailed protocols within.**Author response:** *The section has been redrafted in order to possibly answer to the comment and the authors thanks the reviewer for the suggested literature.****Is the biological context (inc. taxonomic applicability/relevance, level of biological organisation) specified and explained sufficiently?*** Yes. However, this did not appear to be filled in within the wiki itself (where many of these entries were not made).**Author response:** *Done in the updated version of the wiki*.***Have chemical initiators (prototypical chemicals or chemical features) been identified?*** The authors have only identified etoposide as a stressor for the overall AOP, but in the MIE list many topoII poisons. I think there may be more stressors that can be added to the front page of the AOP? Please see comments above about reference to the rest of the other KE and the AO in this section, which should be removed.**Author response***: More stressors have been added in the new version in order to address the comments made by the reviewers.***SR1:****SR2:** The MIE is described and biologically plausible, but I think that it might need to be more specifically described to allow it to be more accurately measured. Currently the MIE is described as simply in utero exposure (“In utero exposure to DNA topoisomerase II “poisons”), with the measurement assays detect inhibition of DNA topoisomerase II. Given that there are different mechanisms through which topoisomerase II can be ‘poisoned’ (ie, activity diminished), I’d recommend rewording to more specifically characterize the MIE.Further – there are a few references throughout the AOP text indicating that there could be differential sensitivity of topoisomerase II inhibition in different cell types. Should the MIE be rewritten in such a way that this initial event is more cell-type specific? **Author response:** *The text has been redrafted in order to possibly answer to the comments made by the reviewer.* |

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| **Key Events***Are the KE descriptions clear on how the events work and are they biologically plausible?**Are the KEs described in a way that allows their reuse in other AOPs?**Are measurement methods specified and adequately described/referenced?*  |

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| **Reviewers' responses and comments** **PR:*****Are the KE descriptions clear on how the events work and are they biologically plausible?***As with MIE, the authors should start by explaining the normal protein structure and function – i.e., 2nd last paragraph of the ‘how the KE event works’. **Author response:** *The text was redrafted in order to answer to the comment made by the reviewer.****Are the KEs described in a way that allows their reuse in other AOPs?***I suggest that the authors not be specific about ‘in utero’ here, because that limits use by other AOPs, and also they can increase the evidence and measurement approaches (they don’t have to be specific to ‘in utero’ tests. Think more stressors could be included. Suggest that the authors define the KER leading from MLL mutation to infant leukemia as requiring occurrence in utero. Would really open applicability up.There is reference to the AO in the descriptions. Should remove and write specifically about the KE and what it involves, rather than what is causes (which can all be transferred to the KER). I think fine to indicate that it is key in infant leukemia, but probably should indicate what other diseases if anything is mentioned. Section should just specifically describe what the fusion protein is and its altered function (i.e., as promoter hyperactivation and re-acquiring stem cell features).**Author response:** *Some text was added to answer the comment. The authors are however not keen, at the moment to not include the in-utero specification in the title for the same reasons explained above. In particular the biological plausibility linking this KE to the AO is strong only if occurring in utero. The comment was discussed during the TC and the reviewer agreed with the authors that this KE should stay as specified. Similar discussion was held at the EGMST meeting in June 2017 and an overall agreement of leaving the in-utero specification was reached.****Are measurement methods specified and adequately described/referenced?***No – references needed to how measurements are made to guide users to relevant protocols. Authors need to insert these here.**Author response:** *Additional test added***SR1:****SR2:** One KE is described: “MLL chromosomal translocation”. The KE is well-described and is biologically plausible. As noted above for the MIE, it might be worth considering whether the KE should be described more specifically, in order to accurately measure the event. The textual description particularly references the MLL-AF4 fusion protein as having a consistent and specific relationship with the AO; however, its cited that at least 120 other partner genes can be fused with MLL. **Author response:** *It is not clear to the authors which additional information should be added here. Indeed there are many translocation and fusion partners for MLL; though MLL can lead to rearrangement with over 120 partner genes the predominance of fusion partner AF4 is extremely important for the AO. The authors think that this part does not need modification. The comment was discussed during the TC and agreed that adding additional KEs is not possible at the current scientific understanding of the disease. In addition, from the regulatory perspective, there would be no benefit in adding additional KEs.* The methods to detect the KE look to be specific to a specific gene (MLL?) based on the reference to PCR methods to identify a fusion gene. Other less-specific methods are cited (eg, comet assay), however the accuracy of the other methods is cited as “cannot be evaluated”. It might be advantageous to specify MLL-AF4 (with appropriately cited level of evidence) and perhaps noting as a gap/inconsistency that other fusion proteins occur and could contribute to the AO incidence.**Author response:** *Additional test added in order to answer the comment*Also as noted above, it might be worth considering whether a specific cell-type should be referenced with appropriately characterized evidence. The textual description says that the affected cell in which the MLL fusion occurs is not definitely known – but is it worthwhile to narrow down the possible genes and charcterize that with weak support?***Author response*** *The authors think that this is clearly defined in the text.* |

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| **Adverse Outcome***Is an AO described?* Yes. *Is the AO description clear and is it biologically plausible?* *Is the AO described in a way that allows its use in other AOPs?* *Are measurement methods specified and adequately described/referenced?* *Is the biological context (inc. taxonomic applicability/relevance, level of biological organisation) specified and explained sufficiently?* *Has the regulatory relevance of the AO been described?*  |

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| **Reviewers' responses and comments** **PR:*****Is an AO described?*** *Y*es ***Is the AO description clear and is it biologically plausible?*** Yes – the AO is described and it is plausible.***Is the AO described in a way that allows its use in other AOPs****?* As described above, the authors refer extensively to the upstream events in the AOP rather than focusing on just the description of the AO. This is particularly true in the information entered under etoposide as a stressor. Here the authors should briefly summarize the evidence that etoposide causes infant leukemia (rather than how it inhibits topoII and causes MLL translocations). Much of the info here would be excellent in the KER sections.**Author response:** *Additional text was added in the section and under the stressor section in order to address the comments made by the reviewer****Are measurement methods specified and adequately described/referenced****?* No references to specific diagnostic parameters are given.**Author response:** *The authors disagree with the comment. Additional information would not add any value and the one mentioned are correct and in line with the clinical standard. Also in line with the comment from reviewer 3.****Has the regulatory relevance of the AO been described?***Yes.**SR1:****SR2:** Yes, the AO is well described, with clear clinical relevance and methods on how the AO is detected/diagnosed. The regulatory relevance is described, which provides nice insight into why the development of this pathway is important (ie, topo II poisoning is a mechanism leading to genotoxicity and carcinogenicity for human medicines but its not clear that existing animal tests done for regulatory purposes would identify chemical stressors leading to the AO). **Author response:** *Additional**text was added to possibly reply to the comment made by the reviewer.* |

**Section 6:**

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| **Key Event Relationships***Are the KERs well described and in a way that allows their use in other AOPs?* *Are the KERs biologically plausible and is there sufficient evidence presented?* *Is the level of empirical support adequately described in accordance with the OECD AOP Handbook?* *Are inconsistencies, uncertainties and level of confidence adequately described?* *Is the quantitative understanding of the KER described?"* *[refer to Tables 2 & 3 in the handbook]* |

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| **Reviewers' responses and comments** **PR:*****Are the KERs well described and in a way that allows their use in other AOPs?*** **KER1 (topoII inhibition leading to MLL-r)**: Description refers to the AO in numerous places. For e.g., first paragraph of empirical evidence is about the model for leukemia.**KER2:** The information on cell type specificity is about topoII inhibition rather than MLL-rearrangement leading to leukemia. Here there should discussion relating to whether MLL-r tends to happen in some cell types and at some developmental stages over others. The rest of the description of how this KER works is excellent.**Author response:** *The authors do understand the first part of the comments. However, the authors rather prefer to not change the text as the cell population and the in utero exposure condition for this AOP are relevant for the specificity of the pathway. See the response to the comments above for potential resolution of the issue on definition of specific cell-type in the MIE.* ***Are the KERs biologically plausible and is there sufficient evidence presented?*****KER1**:Think that the authors call of ‘strong’ for this KER is based on biological plausibility (as empirical evidence is fairly weak), but this is not clearly explained.**Author response:** *Additional**text added under the WoE section to clarify this point.***KER2:** Yes, although there are many steps between the fusion and leukemia (e.g., epigenetic effects, hyper-promotion of downstream genes, loss of control of differentiation, etc.)? The authors may consider a ‘gap’ KE in the middle and have these as non-adjacent (i.e., indirect KER) KEs as in the future more essential and measurable elements may be fleshed out – e.g., alterations in DOT1L activity sites, differential expression of key marker genes, epigenetic modifications, etc. Many of these are well described in the KER. Note, there is even evidence of the essentiality of DOT1L in the KER description. Authors seem to have a fair bit of evidence here – and even a therapeutic target. Is there any reason it was not included as a KE?I really like the section on ‘possible facilitating mutations’.**Author response:** *as commented above, the authors do understand this comment but rather prefer not to add additional KE, at least at the actual state of the scientific knowledge. The working group widely discussed if additional Kes would be beneficial. The discussion was held with worldwide recognized scientists in the field of IFL and they were reluctant in adding KEs when is likely that multiple (epi)genetic factors triggered by the fusion patterns will be act (simultaneously) as a modulator factors. The WG also widely discussed what would be the benefit from the regulatory application of this AOP, in adding additional Kes, coming to the conclusion that the actual AOP is representing well the unicity of the disease ie single hit event and is pragmatically recapitulating all the necessary and measurable KE s of regulatory relevance. The precise molecular and cellular processes behind KER2 remain incompletely understood, but changes in gene activation and repression as well as in epigenetic regulation in a hypothetical “permissible” cellular environment, restricted in time and space, likely play a decisive role (Greaves 2015; Sanjuan-Pla et al. 2015). In summary, current scientific evidence, including the stable genome of the patients, suggests that infant leukaemia originates from one “big-hit” occurring during a critical developmental window of stem cell vulnerability (Andersson et al. 2015; Greaves 2015). The item was discussed at the TC and it was agreed that no further KEs are necessary between KE1 and the AO.****Is the level of empirical support adequately described in accordance with the OECD AOP Handbook?*** **KER1**: The empirical evidence section is not strong (one stressor only, limited evidence in vivo except in DNA repair deficient mice). If there is more than this the authors should describe or reference. Also, would suggest that the in vitro models come first in the order of presentation (is there more evidence here? Or just the two studies?), then support by evidence in vivo. If the authors did not make the KE so specific to ‘in utero’ then a better case could be built here. If the authors choose to do this, the next KER (MLL-r leading to infant leukemia) could specify that the translocations have to occur during in utero developmental stages.**Author response:** *The authors think that the empirical support is strong for etoposide though we agree that it is only referring to one stressor. The authors think the relevant evidence are reported and clearly described.***KER2:** Empirical evidence is well presented. Doesn’t look like there are any stressor-related measures (which I think is okay, but authors should clearly state), but there appears to be clear temporal concordance (authors should note this). Authors have not said what their overall call is for the empirical evidence.***Are inconsistencies, uncertainties and level of confidence adequately described?*** **KER1**: Some of the uncertainties listed for KER1 would be more appropriate in the overall AOP assessment uncertainties section, as they reference entire AOP (or the AO) rather than just this KER. The question is whether there are uncertainties that topoII inhibition leads to MLL-r.**KER2:** Think that the uncertainties point to the need for a ‘gap’ KE.**Author response:** *See answers above for this point**Is the quantitative understanding of the KER described?"* **No for KER1 and KER1.****SR1:****SR2:** The KER are well described, with very thoughtful discussion on the weight of evidence considerations. The steps in this pathway are very broad and not many are included (one MIE, one KE, one AO), and after reading through the textual descriptions in the KER section, I questioned whether there are more steps that should be highlighted in the pathway. Particuarly because both KER described are described as have strong supporting evidence; yet there’s not a consistent association between the MIE and AO (pg 16: “dose-response relationships between etoposide and treatment0related leukaemia are difficult to unravel”). This suggests to me that the pathway overall isn’t specific enough. The KER section highlights points to events that might be further considered as to whether they are actually KE in and of themselves, such as:* Pg 18: MLL-rearranged fusion genes and their protein products are intimately involved in both the blocked differentiation of HSPCs and the expansion of the fusion gene-carrying clone.
	+ Where cell differentiation block is described as recruitment of repressor molecules like histone deacetylase
	+ And clonal expansion measured by activation of “key target genes”
* Page 19: activation of the RAS pathways is associated with rapid progression of the infant leukemia..may aid disease onset b accelerating the initial expansion of cells

**Author response:S***see answer above for this point* |

**Section 7:**

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| **Overall Assessment of the AOP** *Is the domain of applicability of the AOP defined appropriately?* *Is the level of support for essentiality of the KEs adequately described and assessed?* *Has the degree of quantitative understanding of KERs been assessed properly?* *Has consideration been given to the overall weight of evidence for the AOP?* *Are the calls on Overall WoE and Quantitative Understanding supported?* |

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| **Reviewers' responses and comments** **PR:*****Is the domain of applicability of the AOP defined appropriately?*** Yes.***Is the level of support for essentiality of the KEs adequately described and assessed?*** The authors should refer to the Users’ Handbook instructions on how to assess essentiality. What they have described in essentiality is empirical evidence in support of the relationship or biological plausibility. I do think there are some data that they can use. For e.g.., if you block DOT1L you reduce the impact of the promoter activity of the fusion gene, and reduce probability of leukemia (correct?). This is evidence of essentiality. I would also think that infusing animals with cells containing the MLL-r leading to enhanced probability of leukemia is evidence in support of essentiality. They should carefully review the description of how essentiality should be tested and revise. Consider also, if DSBs are made a KE, then you could report on stop-start experiments (remove exposure to opoi inhibitors and see if DNA DSBs decline). Those are the sorts of experiments that would support essentiality. I think they may still get their ‘moderate’ call if they carefully consider the questions and find the appropriate experiments. However, ***as written I would say the evidence for essentiality is weak***.At present, if there are no essentiality tests conducted, the authors can simply state no clear tests done to date (and possible no inconsistent data). Also, if something can NOT be tested the authors should state this. (e.g., if you can’t inhibit a opoi inhibitor?).**Author response:** *The authors would like to discuss more with the reviewers on this point, also in light of the different approach taken by the third reviewer. The authors do understand that essentiality of the KE and MIE in the context of this AOP is rather complex, difficult to prove and likely different from a more standard definition of essentiality. The authors agreed with the suggestion made by the reviewer to add additional experimental evidence for essentiality although the authors still think that the valuable, although indirect/circumstantial, evidences are properly quoted in the section. Because of the indirect or circumstantial nature of the evidences, the authors think that the essentiality is moderate but not weak. Indeed there is no IFL without MLL translocation for the large majority of clinical cases. More text was added and agreed at the TC with the reviewers.****Has the degree of quantitative understanding of KERs been assessed properly?*** No.***Has consideration been given to the overall weight of evidence for the AOP?*** Nicely described for etoposide as the stressor. Not clear if there is evidence from other stressors or not (note in the Strength, Consistency and of the experimental evidence section the authors indicate other stressors but appears that they have not completed this section, which has the statement ‘Mention!’ in the text). **Author response** *More text added under the stressors section****Are the calls on Overall WoE and Quantitative Understanding supported?*** I am not sure what the OVERALL call on the AOP is – where is this found in the AOP? I think a ‘strong’ call would be too strong given that there are gaps and there is only one stressor described. I would argue that with the uncertainties and the limited empirical evidence, a moderate call is more appropriate.**Author response** *This point was discussed during the TC***SR1:****SR2:** The overall assessment of the AOP is very well done. The evidence tables supporting describing the essentiality of the key events make the WoE decision for each KE very transparent. Also, the textual description for the dose-temporal concordance and stength/consistency for the overall AOP is thoughtful and presents clear, relevant information. Also a thoughtful discussion of the uncertainties and inconsistencies. With all that being said, I’m still left questioning whether the MIE and KEs are adequately described/covered, based on the inconsistency between the moderate-to-strong WoE for each KE/KER and the overall not clear quantitative association between the MIE and AO. I agree with the moderate-to-strong WoE decision, and perhaps this pathway will remain as a qualitative AOP. Nonetheless – I think perhaps adding more specificity or events may make a more consistent, coherent pathway between MIE and AO, especially given the breadth of information provided in the textual descriptions on specificity of cell type and additional tissue/cellular level events associated with MLL fusions (such as cell differentiation blockage and/or high clonogenic potential). **Author response:** *The authors think that quantification of this AOP is at the moment unlikely considering the specificity of the early in-utero exposure. The authors think that a qualitative approach is anyway not downgrading the regulatory and scientific relevance of this AOP dealing with a genotoxic associated MIE* |

**Section 8:**

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| **Potential application of the AOP (optional):** *Is any context provided as regards the reason for development or the intended use?* |

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| **Reviewers' responses and comments** **PR:****SR1:****SR2:** Clear relevance was provided for why the AOP was developed. However, given the inconsistent relationship provided between MIE and AO together with the relatively non-specific MIE/KE, I’m not sure that the current form of the AOP improves the decision-making process when faced with the finding that a chemical acts as a topo II poison, given that not all topo II poisons result in infant leukemia (which is my understanding purely solely on the background/biological context information provided in this AOP snapshot). The potential for regulatory/clinical relevance for improved understanding of this AO seems high, so I’d recommend further consideration of whether an expansion of the specificity/nature of the MIE and KE could bring more consistency to the overall pathway. |

**General Observations and Conclusions of the Reviewer**

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| **Reviewers' responses and comments** **PR: Overall a very solid AOP and excellent review of the literature by the authors. General considerations are given below.****General considerations for authors:**1. Authors might consider adding a KE between the MIE and KE – induction of double strand breaks. These are required for the translocation to occur, and there are many excellent in vitro and in vivo assays (some of which the authors refer to) that could be used to support the case that topoII inhibition leads to DSBs, translocations in MLL…. Infant leukemia. Just thinking of testing strategies and since the authors frequently reference DSBs as endpoints that are associated with the formation of the MLL fusion genes, it seems logical to include (the DSB is both measurable and essential- and the KER could easily integrate the information that the DSBs have to occur in the correct location for the translocation to occur). They’d also be able to clearly present some ‘strong’ evidence with a lot of supporting data here, especially if the MIE and KE are NOT specific to in utero (and I don’t see why they should be until the end of the AOP).
2. Similarly, there is a lot going on between KE1 and the AO. The authors should consider (a) additional KEs (e.g., .alterations in DOT1L activity) or a ‘gap’ KE. The relationship between MLL-r and infant leukemia would then be a non-adjacent relationship. This would also mean that they now would be able to present some data on essentiality.
3. The authors should revisit the WoE calls, which I think are stronger than they should be. They have posed a very interesting question that the EAGMST maybe should discuss at the face to face meeting (authors may have meant to delete) - **What would be consequences if we say that the AOP is biologically possible, feasible, even probable, and then say that most of the evidence is impossible to get directly and has to be based on surrogates?** I would say it should be explicitly stated if this is the case and the calls reduced but with this caveat. Should be discussed in detail.

**SR1:****SR2:** Overall, very well written with thoughtful consideration of the level of evidence supporting individual events and relationships between events. I particularly liked the WoE tables provided in the overall assessment of the AOP section – made the call as weak/moderate/strong very transparent. My biggest recommendation is to try to work more specificity into the MIE/KE so that the events are specifically and accurately measured, and consider further whether additional KE might be useful.  |

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| **Author response:***The authors understand the comments and thank the reviewers for the thorough evaluation done. The authors agreed that incorporation of an additional KE (ie DSB) would increase the use and the value of this AOP, and this is now incorporated in the AOP in line with the comment made by the reviewer. Additional KEs, following KE1 are not supported by the authors for the reason explained above. We also think that the specificity of the pathway is rather strong. More text was added under the uncertainties sections for the overall empirical support and the second KER in order to justify why additional Kes is an unlikely option.* *This AOP condenses molecular, pathological, regulatory and clinical knowledge in a pragmatic, transparent and weight of evidence-based framework. This facilitates the interpretation and integration of epidemiological studies in the process of risk assessment by defning the biologically plausible causative mechanism(s). The AOP identified important gaps in the knowledge relevant to aetiology and risk assessment, including the specific embryonic target cell during the short and spatially restricted period of susceptibility, and the role of (epi) genetic features modifying the initiation and progression of the disease. Furthermore, the suggested AOP informs on a potential Integrated Approach to Testing and Assessment to address the risk caused by environmental chemicals in the future.*  |

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| **Internal review final comments – following post EAGMST meeting revision of the AOP****PR:** The authors have put some significant effort into this revision to improve it. I appreciate this significant amount of work. They have removed many references to other KEs, KERs and the entire AOP within the various modules. They have added DSBs as a KE. This has improved the weight of evidence presentation of the KERs and overall AOP. The overall assessment of the AOP is done really well.  I still think that the front-end of this AOP, for which the KEs are all primarily measured and studied in vitro or at least not in the relevant cell types in utero, would be better if not all specific to ‘in utero’. When someone wants to test if an agent is a topo poison they’re not going to go to an in utero test to begin with – they’d do an in vitro assay. By broadening it and making the MIE and KE1 (and KER1) not-specific to in utero, this would mean that other developers could borrow the MIE leading to the first KE (topo inhibition leading to DSBs would be used in other AOPs I’m sure). This is the reason that AOPs are intended to be modular – so that KEs and KERs and can re-used. This is also how AOP networks are developed – in this case this AOP will not make it into a network that captures other health effects of topo poisons. Overall, an AOP is only as broad as its narrowest KE or KER. Thus, if the authors make the MLL translocations specific to occurring in their specific cell type in utero, this would then mean that the entire AOP is only relevant to that life stage and cell type. The authors don’t provide a very good rationale why this is not possible. At this stage, I’m happy for the authors to send to external review, and see what the reviewers think. However, I’d be happy if the authors could, over this review process, reconsider the above. I recall there being support for this at the EAGMST. In particular, I recall Dan Villeneuve, the lead of the OECD Users’ Handbook team, emphasizing this as an important point.I am very glad that the authors have incorporated DSBs as a new KE. The authors may take one last look at their ‘How to measure this KE’ section for this new KE. I am surprised not to see some OECD test guidelines there – comet assay or micronucleus assays for example. Also, there seem to be a lot of blank entries on that KE page (stressors, cell term, organ term, taxonomic applicability, life stages).I like the new KER on topo poison leading to DSBs. In the biological plausibility section, it would be useful to reference a good review article or text book chapter. In the KER of DSBs leading to MLL translocation, the authors should delete the statement ‘for the scope of this AOP’, which precedes ‘this KER should occur in utero’. This statement should refer to the KER only. I think this statement ‘However, for example the MLL-AF4 knock-in mice develop leukaemia only after a prolonged latency (Chen et al 2006), thus not recapitulating the ‘pathognomonic’ feature of infant leukaemia.’ Should be moved to the MLL-translocations leading to leukemia KER and not retained in this KER. |