

Adverse Outcome Pathway External Review Report

AOP 202: In-utero DNA topoisomerase II poisons leading to infant leukaemia

Short Title: topoisomerase II poisons, infant leukaemia

This document replaces the AOP 202 report that was uploaded on 1 June 2018. In this version, the texts in the Section: *Outcome of the external review* have been revised to add clarity.

Infant leukaemia (IFL) is a rare haematological disease observed soon after birth (<1 year) with characteristics distinct from the more frequently reported childhood leukaemia. This AOP was developed in part to acknowledge European Union requirements for use of epidemiologic data in assessments of pesticides when such data are available. The authors recognized that there are limitations in the human data, but they note that meta-analyses reveal consistent observations between leukemias and exposure to pesticides, including those that interfere with topoisomerase II (Topo II). The authors engaged in AOP development as a means to identify key events (KE) at a molecular level, which would provide some early observable effects that may support application of early diagnostic tools or support for hazard identification. The draft AOP identifies a Molecular Initiating Event (MIE) of interference or poisoning of Topo II, through KE of double strand DNA breaks (DSB), and in utero MLL gene rearrangements, with an adverse outcome (AO) of IFL.

Major issues discussed in the review included specificity of the AO, description of the MIE, and weight of empirical evidence.

1. Introduction and background to AOP 202

[The text below is adapted from the abstract of the external review version of AOP 202.]

Infant leukaemia (IFL) is a rare haematological disease (41 in 10⁶ newborns in the U.S., accounting for 10% of all childhood acute lymphoblastic leukaemias (ALL)) manifesting soon after birth (<1 year) and having a poor prognosis (Sanjuan-Pla *et al.* 2015). By comparison to the more frequent childhood leukaemia, IFLs show distinct features:

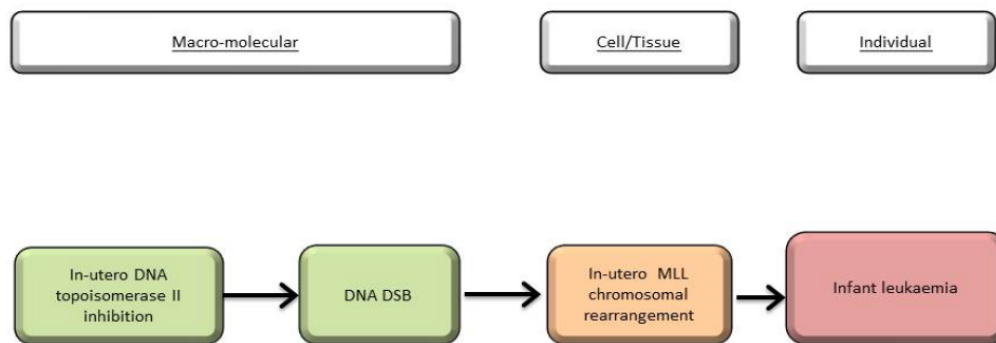
- An early neonatal onset linked to its plausible origin as a ‘intrauterine developmental disease’ (Greaves 2015; Sanjuan-Pla *et al.* 2015);
- Rearrangements of the mixed-lineage leukaemia (MLL) KMT2A gene on the q23 band of chromosome 11, as the hallmark genetic abnormality (Joannides and Grimwade 2010);
- MLL, however, is not the only translocation gene; for infant ALL, about 60-80% carry an MLL rearrangement (Sam *et al.* 2012; Jansen *et al.* 2007) and the percentage for infant acute myeloid leukaemia (AML) is about 40 %;
- The MLL rearrangement takes place at an early stage of development; the likely target cells (still unidentified) are the hematopoietic stem and progenitor cells (HSPC) in fetal liver and/or earlier (mesenchymal) stem cells in embryonic mesoderm (Bueno *et al.* 2009; Menendez *et al.* 2009);
- The infant MLL-rearranged ALL is associated with fewer somatic mutations (1.3 vs 6.5/case) than is the childhood disease (Andersson *et al.* 2015; Dobbins *et al.* 2013), pointing to the lack of a “second hit” and suggesting a “one big hit” origin.

In recognition of these distinct features a molecular Initiating Event (MIE), Key Events (KE) and an Adverse Outcome (AO) were identified. The MIE was identified as "*In-utero* exposure to DNA topoisomerase II poisons". Relationship to *in-utero* exposure was considered relevant to make a specific relationship with infant leukaemia for the AO; epidemiological studies suggested that *in-utero* exposure to topoisomerase II (topo II) may be involved in generation of the KE *in-utero* MLL chromosomal rearrangement.

Overall, based on the available evidence, IFL pathogenesis is thought to originate from a single, substantial hit to a target cell during early intrauterine development. The limited epidemiological studies do not allow any firm conclusion on a possible role for chemicals in IFL (Pombo-de-Oliveira *et al.* 2006; Ferreira *et al.* 2013); however, exposures to chemicals able to induce MLL rearrangements through topoII “poisoning”, (particularly etoposide and other topoII “poisons”, including some bioflavonoids), have been suggested as agents promoting the driver genetic oncogenic event. Experimental models for IFL have been developed, but a wholly satisfactory model reproducing the phenotype and latency is not yet available.

Nevertheless, the anticancer drug etoposide can be considered as a model chemical for a DNA topoII “poison”. Acute leukaemia is an adverse effect recorded in etoposide-treated patients, and these are observed with MLL rearrangements that are in many ways analogous to those in IFL leukaemia (Bueno *et al.* 2009; Joannides *et al.* 2010, 2011). Therefore, the proposed AOP is supported by convincing inferential evidence

by means of using etoposide as a model compound empirically supporting the linkage between the proposed MIE and the AO. In the meanwhile, this AOP identifies several knowledge gaps, the main ones being the identification of the initiating cell and the investigation of topoII poisons in a robust model; thus, the present AOP may be modified in the future on the basis of new evidence. The authors recognize that additional elements are limiting for the strength of this AOP, in particular that the empirical support is mainly based on one chemical stressor and that data for essentiality are also limited and difficult to generate; however, the biological plausibility for the proposed sequence of events for this AOP was considered strong.



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Sam TN, Kersey JH, Linabery AM, Johnson KJ, Heerema NA, Hilden JM, et al. MLL gene rearrangements in infant leukaemia vary with age at diagnosis and selected demographic factors: a Children's Oncology Group (COG) study. *Pediatr Blood cancer*. 2012; 58 (6): 836-839.

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2. Synthesis of main issues of the review

Major issues discussed in the review included updating and clarifying some of the literature supporting the Adverse Outcome Pathway, specificity of the Adverse Outcome (should it be specific to *in utero* exposure and IFL vs. other leukaemias), description of the Molecular Initiating Event, and weight of empirical evidence. These are described in detail under Section 3.

3. Summary record of the teleconference

3.1. TC agenda

AOP 202
Reviewers' and Authors' End of Review Teleconference
11:00 EST (New York Time), February 27, 2018
AGENDA

- Brief introduction of participants - Rita
- Time lines for completion of review report, AOP - Rita
- Discussion of issues, and /or disagreements between Authors and Reviewers [NB: agreement or consensus is desirable, but it is not required for the completion of the review report.]
 - Some items identified by Review Manager as not requiring discussion at the call: updating some literature; clarifying some discussion of the MIE and KEs; dealing with the vagaries of the wiki; typographical errors and minor edits.
 - Items identified for more discussion (see points 1 - 7 below) [To facilitate discussion, excerpts from reviewers' comments (black type) and authors' responses (red type) are provided below].
 - Some differing insights on regulatory applicability.
 - Copyright and quotation, both for text and figures.
 - Description of the MIE.
 - Weight of evidence scores for KER, in particular, some discussion of biological plausibility.
 - Is there value added in articulating a more generalizable AOP, with perhaps several AO including IFL?
- Next steps, action items.

Point 1

Original reviewer's comment: Of note, some of the text in the Wiki has been copied almost word-for-word from one of the sources. Rather than copying text, the authors need to give proper attribution to their sources. The authors have also shown a number of figures which are likely from copyrighted sources. Have they received permission for their use?

Authors' response: AOP development is based on current knowledge and there is nothing wrong to cut and paste text if this is making the clarity. Rewording is an unnecessary exercise as the relevant part is what is in the literature and not the interpretation of the developers. The citation is correct and the process in line with the AOP development guidance.

All the copyrights were addressed for the scientific opinion and there is no rules for the AOP development.

Reviewer Follow up: The issue of copying text from previously published material and copyright for using figures from other sources in an AOP is the same as in a regular scientific publication. AOPs are published in an OECD series (<http://dx.doi.org/10.1787/2415170X>) and proper attribution of the sources in critical. For the text, in this case, it may be sufficient to put the text in quotes so that the reader knows that it comes for the cited paper.

Review Manager: As copyright for figures was dealt with in EFSA Scientific Opinion (SO), could simply cite that.

Point 2

Original reviewer's comment: Substantial evidence indicates that translocations involving the MLL gene play a critical role in certain types of leukemia. However, these translocations may arise spontaneously, presumably either through topoII errors or through unidentified endogenous or exogenous inhibitors of topoisomerase II. There is little convincing evidence that I am aware of that xenobiotics induce MLL translocations in normal embryonic cells *in vivo*.

Authors' response: the comment is appreciated. The weakness of the epidemiological data is a strength for supporting this AOP from the regulatory perspective. We know that the epidemiological studies for pesticides are very weak. Relevant to this AOP is that a consistent human health effect observed through multiple metanalysis is leukaemia. We know (see also comment above) that this is a very general diagnostic criteria, which is basically impeding a proper hazard identification for many reasons. This triggered the idea of using *specific* AO, to include human health outcome in the process of hazard identification.

Reviewer follow up: The reviewer found the author response to be unclear, in particular, the statement underlined above.

Point 3

Original reviewer's comment: topoII poison is not a molecular initiating event (MIE). From my perspective, the MIE would be the inhibitor binding to the topoII enzyme and its interference with religation of the stabilized double stranded DNA break. A more disease-specific event would be the recombining of the DNA double strand breaks resulting in the critical translocation involving the MLL gene.

Authors' response: The authors understand this comment, but at least one author is quite resistant to change the name. It is difficult to find a correct compromise between an academic and regulatory position for the definition of the MIE (similarly to the AO) and the number and definition of the KEs. Based on the wealth of discussion we had in the past, one author would prefer to leave the definition of the MIE as it is with the main reason being that this would not change the intended regulatory application and that the 1st KE could be used to link this AOP to the network.

Reviewer follow up: According to the AOP users' Handbook the definition of a MIE is this: "A specialised type of key event that represents the initial point of chemical interaction on molecular level within the organism that results in a perturbation that starts the AOP".

Review Manager: Perhaps for MIE "topoII inhibitor binds to topoII enzyme" or "topoII inhibitor poisons (interferes with) topoII enzyme."

Point 4

Original reviewer's comment: The reviewer disagreed with the categorization of “strong” for the direct link between the MLL rearrangement and infant leukemia. The reviewer questioned whether a direct relationship was possible.

Authors' response: The authors understand the point made, but they tend to disagree with the sharp conclusion of the reviewer. The authors don't think it is necessary, at least based on actual knowledge, to prove in higher than given details, the direct relationship defined. The authors are fully buying into this AOP the “one big event” leading to IFL. This should not be seen as a simplification matter but as a differentiation from all the other AOPs, which are included in the development of the classical multiple hits theory.

Reviewer follow up: It is one thing to push for the “one big event” AOP, and another matter to show biological support for it. According to the Users' Handbook the definition of strong biological plausibility is: Extensive understanding of the KER based on extensive previous documentation and broad acceptance (e.g., mutation leading to tumours) -Established mechanistic basis. The authors have not convinced the reviewer that there is “extensive previous documentation” and “broad acceptance” that *in utero* exposure to topoisomerase II poisons is linked to infant leukemia.

Point 5

Original reviewer's comment: The authors are making this AOP specific to *in utero* exposure and to a specific chromosomal rearrangement, which makes it of limited regulatory applicability.

Authors' response: Knowledge of the details of the process is not necessary for regulatory decisions and detailed knowledge is not something required to support biological plausibility (not causality) in the AOP conceptual framework.

Reviewer follow up: True. Biological plausibility does not need empirical support, but this should be reflected in the level of confidence given to the relationship.

Point 6

Original reviewer's comment: To the author's knowledge, the mechanism by which topoisomerase II enzymes operate is the same in every cell type and so is the mechanism by which they create double strand breaks (DSB). Thus, the MIE (inhibition of topoisomerase II) and the first KE (formation of DSB) should be agnostic of the cell type. The authors can then make the next KE specific to fetal hematopoietic cells and to infant leukemia. Doing it in this way, would create a MIE and a KE that can be borrowed and used to build other AOPs, including one leading to secondary cancers in patients receiving Topoisomerase II inhibitors as part of their chemotherapy.

Authors' response: The reviewer seems very critical with this AOP but is proposing an AOP that will be even less substantiated, at least for the empirical point of view as we know for the big attempt we made to develop an AOP for a different 2 hits leukaemia (see our Scientific Opinion AOP 4).

Reviewer follow up: Consider if one is developing an AOP: Exposure to etoposide – directly linked to DSB -directly linked to MLL translocation – indirectly linked to secondary leukemia in cancer patients. In this case, the MIE and first two KEs can have much more empirical evidence to support their relationship. There is also empirical evidence that exposure to topoisomerase II inhibitors increases the incidence of MLL (which is currently lacking *in utero*). The strengths of these relationships would not be diminished by the fact that what happens once the MLL is formed may be more complicated and less established than what happens after the MLL is occurring *in utero*. A reviewer continues to be skeptical that the link between *in utero* MLL and infant leukemia can be described as strong.

Point 7

Original reviewer's comment: The main weakness is represented by the lack of direct evidence or extensive understanding of the events *in utero*; because of that, all the scoring calls “STRONG” described *in utero* should be evaluated as “MODERATE”.

Authors' response: This needs more discussion in the context of the AOP conceptual framework. The authors agree that the empirical support is moderate as based on indirect evidence, but the biological plausibility is strong and from a regulatory point of view is indicating that chemicals affecting this AOP are, at least, relevant risk factors for the AO.

3.2. Main issues and responses during the call

Notes vetted by authors and reviewers are below.

AOP 202
Reviewers' and Authors' End of Review Teleconference
11:00 EST (New York Time), February 27, 2018
Notes

All material from the dialog of 02/27/18 appears as Italics in these notes. Other typefaces indicate material from the agenda or other sources shared among the reviewers and authors.

- *Attending: Rita Schoeny, Andrea Terron, Olavi Pelkonen, David Eastmond, Francesca Marcon, Francesco Marchetti*
- Time lines for completion of review report, AOP 202.

In order for the OECD workplan deadlines to be met, the final Peer Review Report must be delivered to OECD on April 27, 2018. Review Manager will ensure that a draft is circulated to authors and reviewers no later than April 1, earlier if possible. Review Manager plans to circulate notes from today's meeting as well as the table of reviewer comments, author responses, and any subsequent dialog to enable the authors to finalize any responses to the review. Authors may choose to revise the AOP at any time.

- Discussion of issues, and /or disagreements between Authors and Reviewers [NB: agreement or consensus is desirable, but it is not required for the completion of the review report.]
 - Some items identified by Review Manager as not requiring discussion at the call: updating some literature; clarifying some discussion of the Molecular Initiating Event (MIE) and Key Events (KE)s; dealing with the vagaries of the wiki; typographical errors and minor edits.

All participants agreed with the above. Review Manager will send the marked-up copies of the AOP to Author 1. Reviewers will send papers to authors as requested. Author 1 will copy others on feedback. Reviewers are requested to note on AOP where papers should be added or cited.

- Items identified for more discussion.
 - Some differing insights on regulatory applicability.
 - Copyright and quotation, both for text and figures.
 - Description of the MIE.
 - Weight of evidence (WOE) scores for KER, in particular some, discussion of biological plausibility.
 - Is there value added in articulating a more generalizable AOP, with perhaps several AO including infant leukemia (IFL)?

Points 1 -7 below were discussed albeit not necessarily in the order shown.

- Next steps, action items.

Review Manager will send out notes from the 02/27/18 teleconference. She will also circulate the authors' responses and rebuttal and include subsequent dialog. [NB: As of 03/07/18, the first two items were completed.] Reviewers will send copies of papers as requested by the authors. Authors and reviewers may continue informal conversations on AOP 202. Authors will meet to discuss revisions as indicated under points 1-7. No later than April 1, Review Manager will send a Draft Final Review Report to authors and reviewers for their comment. Review Manager will at that time request a due date for these comments to be sent to her. Review Manager will deliver the Final Review Report to OECD no later than 04/27/18.

General Comment.

The authors were complimented on their thorough review of a complicated literature. The authors expressed their thanks for the review comments and suggestions.

Point 1

Original Reviewer's Comment: Of note, some of the text in the Wiki has been copied almost word-for-word from one of the sources. Rather than copying text, the authors need to give proper attribution to their sources. The authors have also shown a number of figures which are likely from copyrighted sources. Have they received permission for their use?

Discussion. Clarification from Authors: the EFSA Scientific Opinion (SO) that contains this AOP and related references and figures has been through the copyright process. It is not necessary to request copyright for OECD to use figures.

Action: AOP 202 will cite EFSA S. The authors will check with OECD to find out its policy.

Resolution. Authors will ensure that all quoted text is noted and cited.

Point 2

Original Reviewer's Comment: Substantial evidence indicates that translocations involving the MLL gene play a critical role in certain types of leukemia. However, these translocations may arise spontaneously, presumably either through topoII errors or through unidentified endogenous or exogenous inhibitors of topoisomerase II. There is little convincing evidence that I am aware of that xenobiotics induce MLL translocations in normal embryonic cells *in vivo*.

Discussion. Author 1 noted that since 2013, a group of the authors has been examining epidemiologic data from environmental exposures. EU pesticides regulation requires that epidemiologic data must be used in assessments when they are available. A number of weaknesses are recognized, which limit the use and applicability of epidemiological studies in the regulatory process of pesticides risk assessment. However, consistent observations though multiple meta-analyses included the observation of an association between pesticides exposure and Parkinson's disease and leukemia. For the latter, diagnostic criteria present difficulties, including a generic definition of the disease. The authors engaged in the AOP development as a way to identify KE at more molecular level in the hope that this would identify some early observable events likely to be associated with leukemia and used the AOP conceptual framework to support the mechanistic biological plausible link between the observation and the experimental evidence. Weak epidemiologic data were part of the impetus to engage in developing an AOP from a mechanistic perspective. A use of the AOP would be identification of experimental processes that may be used to identify additional agents contributing to the AO or to identify risk factors that can be considered in the overall risk assessment. The authors feel that the Biological Plausibility (BP) of the pathway can support the potential use of the AOP for the integration of data for human-based hazard identification in the process of risk assessment (for IFL in this case or other relevant AO).

A reviewer questioned how to bridge difference between IFL and adult leukemia (AL).

Response from authors: In experimental situations one observes a high level of risk from translocation in embryonic cells.

Author 2 noted that etoposide was used as an example for developing AOP 202. The authors found several papers for AL specific translocation, which can be observed in experimental situations in utero. The authors felt there is sufficient connection between the adult and infant observations – for etoposide -- for identifying the KE in the AOP. One use of the AOP can be to identify research experiments that would be used to support the pathway. The early observable KE could be used to identify relevant agents that may contribute to MIE. The authors think that the level of similarities between AL and IFL and the strong biological plausibility of this AOP are quite robust, and compensate for the fact that empirical support for the etoposide as is strong only for the AL. The authors are considering, however, a lower score for the overall empirical support; they note that evidence is indirect for etoposide and IFL. They continue to rank the overall biological plausibility for this AOP as strong.

A reviewer stated that there are differences at the sequence level between adult and infant translocations. It appears that the authors' written response to reviewer comments got the information reversed. The breakpoints in the infant leukemias are often more complex than those in therapy-related leukemias.

Author 2 responded that although there are differences in breakpoints, the authors feel there are enough similarities to support the AOP (see also the response above which is on the same subject). AL was a model for developing the AOP for IFL. Cellular environments probably explain differences in the breakpoints between in utero and adult.

Another reviewer noted that it is critical that the pathway be supported by empirical data. There are more observations and data for AL than for IFL.

Author 2 responded that there is less evidence, but there are good indications that the translocations associated with topoII poisoning are produced in utero. Animal experiments have been difficult, but there are models being developed, as well as in vitro work on embryonic stem cells, and they demonstrate that MLL rearrangements are occurring.

There was discussion about WOE of empirical support for KE. The authors consider that the biological plausibility for the pathway is strong. AOP 202 is perhaps a good example of moderate / indirect empirical evidence, but strong BP. Other AO could also be considered for this general pathway; perhaps childhood leukemia which presents a different situation from IFL.

The minimum or desirable degree of experimental support for AOP is still matter of discussion among those in the AOP area.

Resolution. There may remain some disagreement on this point. Some clarification of the discussion around BP will be useful. The authors may note that there is some continuing work by PM on testing some pesticides and etoposide in their model. This will be facilitated by the IFL AOP. The authors are however happy to review critically the overall WOE for the empirical support.

Point 3

Original Reviewer's Comment: topoII poison is not a molecular initiating event (MIE). From my perspective, the MIE would be the inhibitor binding to the topoII enzyme and its interference with religation of the stabilized double stranded DNA break [DSB]. A more disease-specific event would be the recombining of the DNA double strand breaks resulting in the critical translocation involving the MLL gene.

Discussion. Author 2 said that several names were proposed during development. One was "inhibition of topoII". There are different types of interaction / inhibition of the topoII process. Thus, the authors selected "poison" [as a verb].

A reviewer asked if poisoning occurs post binding? The authors said that this seems to be the case. Note that there are four different regions in topoII cycle. The authors wished in the AOP to distinguish the one that leads to the AO.

Another reviewer has been involved in developing an AOP for DNA alkylation leading to heritable germ cell mutation. The description of an AOP should consider that not all instances of the MIE will lead to the AO. KE are necessary events, but none alone is sufficient to result in the AO.

A description of the MIE could be "in utero topoII poison interacts with topoII enzyme". Stabilizing or de-stabilizing the topoII-DNA complex is part of the biological pathway.

Resolution: The AOP will describe MIE as an event rather than as an agent. Maybe "in utero topoII

poisoning.” The authors will check carefully in the description of MIE regarding the points made by the reviewers. The authors will make some change to ensure that the MIE is perceived as a process or event rather than as an agent. The authors are, however, keen to accept the proposal of the reviewer and consider “in-utero topoII poisoning” as the appropriate definition of the MIE.

Point 4

Original Reviewer’s Comment: The reviewer disagreed with the categorization of “strong” for the direct link between the MLL rearrangement and infant leukemia. The reviewer questioned whether a direct relationship was possible.

Discussion. This comment refers to the discussion of WOE for KER by contrast to the biological plausibility of the entire pathway.

Resolution. Authors will consider points in the above discussion of difference between experimental support / WOE for KERs, vs. biological plausibility of the entire AOP. They may modify some of the WOE for experimental evidence, but they continue to judge the biological plausibility of the pathway to be “strong”.

Point 5

Original Reviewer’s Comment: The authors are making this AOP specific to *in utero* exposure and to a specific chromosomal rearrangement, which makes it of limited regulatory applicability.

Discussion and resolution. Refer to the points made in earlier discussion. The authors will make some change to ensure that the MIE is perceived as a process or event rather than as an agent.

Point 6

Original Reviewer’s Comment: To the author’s knowledge, the mechanism by which topoII enzymes operate is the same in every cell type and so is the mechanism by which they create double strand breaks (DSB). Thus, the MIE (inhibition of topo I) and the first KE (formation of DSB) should be agnostic of the cell type. The authors can then make the next KE specific to fetal hematopoietic cells and to infant leukemia. Doing it in this way, would create a MIE and a KE that can be borrowed and used to build other AOPs, including one leading to secondary cancers in patients receiving Topo II inhibitors as part of their chemotherapy.

Discussion. There were several points discussed regarding developing a more generalized AOP, rather than concentrating on IFL as the only AO. One author has been thinking about this. Questions were raised as to ways of changing the MIE and KE1 to be more general, with focus on the in utero aspect around KE2. This needs careful consideration by the authors, as their initial impetus for developing the AOP was for pediatric leukemias in general. However, it may be of importance to stress that on the basis of the current evidence, the MLL rearrangement has to happen in utero. The authors said that they could not decide their path on this issue during the teleconference. Rather all authors will continue the discussion off line. Author 1 also noted that this point of not restricting the AOP to IFL was discussed during the OECD internal review of AOP 202. An alternative would be AOPs linking at the DSB, with various genotoxic MIE. There were questions about the specific sensitivity of the cells in utero. During the course of the discussion, the author expressed a preference to stay with the in utero specificity. Perhaps this AOP can link at other KE of more general pathways (e.g. DSB). Perhaps utility of an AOP specific to IFL could be discussed in sections on regulatory applicability or in introductory material. This topic will require some further discussion and thought among the authors. A reviewer noted that it would be useful to clarify what is meant by “one-hit” in the AOP (or in their response to the reviewers’ comments). Translocation is a multistep process. Perhaps this AOP describes a one-mutation process. The authors may consider that some agents will cause breaks in the MLL gene through other mechanisms, such as apoptosis. As a result, breaks in MLL might result from exposure to agents that are not topoII inhibitors. The authors clarified that “one-hit” in the context of this AOP means that the formation of MLL rearrangement (“one hit”) is sufficient to result in the AOP without the “second hit” (as in the conventional carcinogenesis model).

The point was considered again that the individual KE are necessary but not sufficient to produce the AO. The authors noted that this is described in the uncertainty section of the AOP; perhaps they can copy some of this discussion into description of the KEs.

Resolution: To continue discussion among the authors after the teleconference. They will share their choices as available with the Review Manager, who will include them in the Final Review Report, if feasible. [Note: as of finalization of the Final Review Report, the Review Manager had not received any further AOP text from the authors.]

Point 7

Original Reviewer's Comment: The main weakness is represented by the lack of direct evidence or extensive understanding of the events *in utero*; because of that, all the scoring calls "STRONG" described *in utero* should be evaluated as "MODERATE".

Discussion. This comment refers to the discussion of WOE for KER by contrast to the biological plausibility of the entire pathway.

Resolution. Authors will consider points in the above discussion of difference between experimental support / WOE for KERs, vs. biological plausibility of the entire AOP. They may modify some of the WOE for experimental evidence, but they continue to judge the biological plausibility of the pathway to be "strong".

3.3. Action list

- Review Manager will send out notes from the 02/27/18 teleconference. Review Manager will also circulate the authors' responses and rebuttal and include subsequent dialog. [NB: As of 03/07/18, the first two items were completed.]
- Reviewers will send copies of papers as requested by the authors.
- Authors and reviewers may continue informal conversations on AOP 202. Authors will meet to discuss revisions as indicated under points 1-7.
- No later than April 1, Review Manager will send a Draft Final Review Report to authors and reviewers for their comment. Review Manager will at that time request a due date for these comments to be sent to her.
- Review Manager will deliver the Final Review Report to OECD no later than 04/27/18.
- Authors are planning to revise and complete AOP 202.

4. Summary of planned revisions

- With the assistance of the reviewers the authors will update some of the literature.
- The authors will correct typographical errors and make minor edits as indicated on the reviewers' marked up copies of the AOP 202 snapshot.
- AOP 202 will cite EFSA Scientific Opinion document. The authors will check with OECD on the appropriate citation policy. Authors will ensure that all quoted text is noted and cited.
- There was substantial discussion on weight of empirical evidence for individual key event relationships, as well as on the biological plausibility of the pathway for IFL. The biological plausibility of the pathway rests to some extent on analogy among pathways for IFL, childhood leukaemia, and adult leukaemias, especially treatment-related (etoposide) adult leukaemia. The authors feel that the Biological Plausibility (BP) of the pathway can support the potential use of the AOP for the integration of human-based hazard identification (for IFL in this case or other relevant AO) in the process of risk assessment. The discussion is described under point 2 of the teleconference notes. AOP 202 is perhaps a good example of moderate / indirect empirical evidence, but strong BP. Other AO could also be considered for this general pathway; perhaps childhood leukemia, which presents a different situation from IFL. The minimum or desirable degree of experimental support for any AOP is still matter of discussion among those in the AOP area. The resolution is that there may remain some degree of disagreement among the reviewers and the authors. Some clarification of the discussion around BP was considered and will be undertaken by the authors. The authors may note that there is some continuing work by Author 3 on testing some pesticides and etoposide in their model. This will be facilitated by the completion of the IFL AOP. The authors are, however, happy to critically review the overall WOE for the degree of empirical support.
- There was discussion as to the wording of the MIE. The AOP will describe the MIE as an event rather than as an agent. A suggestion was "*in utero* topoII poisoning". The authors will check carefully in the description of MIE regarding the points made by the reviewers. The authors will make some change to ensure that the MIE is perceived as a process or event rather than as an agent. The authors are, however, keen to accept the proposal of the reviewers and consider "*in-utero* topoII poisoning" as appropriate definition of the MIE.
- There was discussion of the WOE for KER by contrast to the biological plausibility of the entire pathway. This is described under point 4 of the teleconference notes. The authors will consider points in the above discussion of difference between experimental support / WOE for KERs, vs. biological plausibility of the entire AOP. They may modify some of the WOE judgements for experimental evidence, but they continue to judge the biological plausibility of the pathway to be "strong".
- There were several points discussed regarding developing a more generalized AOP, rather than concentrating on IFL as the only AO, and some of the authors have been considering this option. Questions were raised as to ways of changing the MIE and KE1 to be more general, with focus on the *in utero* aspect around KE2. This needs careful consideration by the authors, as their initial impetus for developing the AOP was for pediatric leukaemias in general, and the immediate incentive to develop the AOP for infant leukaemia originated from the inability of epidemiological studies to discriminate between various types of pediatric leukaemias. Furthermore, it may be of importance to stress that on the basis of the current

evidence, the MLL rearrangement has to happen *in utero* for development of IFL. The authors said that they could not decide their path on this issue during the teleconference. Rather all authors will continue the discussion of the various options.

[In their review of the draft External Peer Review Report, the Authors subsequently made the following points: “1. For the MIE, the authors prefer to still have it as “*in-utero*” as this reflects the specificity of the AO. Having introduced an additional, more general, KE (i.e. KE1), would help in linking this AOP with others. 2. The authors are not supporting the idea to develop a generalized AOP. This AOP is specific for IFL and will help risk assessor to differentiate mechanism and chemical relevant for this AO. Other leukaemia will have other AOPs.”]

- There was discussion as to meaning of “one-hit” in the description of AOP 202. Following discussion, the authors think that although a single hit, as well as a two steps carcinogenesis paradigm, are of primary concern from the molecular point of view, they are not absolutely necessary from the toxicology pathways point of view. In the description and context of AOP development and regulatory use, this is very clear and the AOP summarizes the two different processes in a simple and transparent way. In this sense, although the authors agreed that MLL translocation might be not sufficient for development of IFL, it is sufficient from the biological, empirical, and regulatory point of view to support a direct KER. It is also recognized by the authors both in the literature and by key scientists operating in the field of IFL, that additional molecular events are necessary beyond the MLL translocation; however, this neoplastic /developmental disease is mechanistically different from other leukaemias wherein the “two hits” model would fit. The authors will consider moving or revising parts of the AOP discussion to enhance clarity of the concepts.
- There was continued discussion of WOE for KER by contrast to the biological plausibility of the entire pathway. Authors will consider points in the discussion of difference between experimental support and WOE for KERs, vs. biological plausibility of the entire AOP. The authors may modify some of the WOE for experimental evidence, but they continue to judge the biological plausibility of the pathway to be “strong”.

5. Further discussion

Discussion subsequent to the Reviewer / Author teleconference was conducted by email, and it is reflected in edits made to this Final External Peer Review Report.

6. Outcome of the external review

The reviewers encourage the authors to revise AOP 202 and to continue with the OECD process; that is, to send the revised AOP 202 for EAGMST approval as well as further WNT and WPHA endorsement. There was not total agreement either among reviewers or between reviewers and authors as to the recommendations for the specificity of the AOP. The reviewers supported consideration of perhaps several intersecting AOPs leading to various leukaemias, from exposure at different life stages. The authors have discussed this recommendation, but at this point they plan to limit their AOP to in utero exposure and infant leukaemia.

Annex 1: Table with reviewers' name

Reviewers:

David Eastmond, University of California, Riverside.

Francesca Marcon, Dept. Environment and Health, Istituto Superiore di Sanità.

Francesco Marchetti, Environmental Health Science Research Bureau, Health Canada

Naveed Honavar, Experimental Toxicology and Ecology, BASF SE

Annex 2: Individual reviewers' comments

Reviewer 1

Charge Questions: AOP 202: In utero DNA topoisomerase II inhibition leading to infant leukaemia.

My major concerns with the current AOP are explained in greater detail in the overall conclusion assessment of the AOP. However, they are also briefly mentioned in the answers to charge questions related to weigh of evidence and regulatory applicability.

- **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?**

I think the authors have done a reasonable job at incorporating the most relevant scientific literature on the subject. It should be highlighted that the supporting literature includes mostly studies that are not conducted in utero, as these latter are a few. The scientific content of the AOP accurately reflects the current scientific knowledge on the topic.

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

Although the MIE and first KE are clearly plausible, the data come principally from studies conducted in cell lines or after an adult exposure. That they happen in utero is inferred and, although this is acceptable, it should be reflected in the way the WOE is evaluated. It is my opinion that the authors are at times overrating the WOE scoring based on the available information.

For example, I disagree with calling “strong” the direct link between the MLL rearrangement and infant leukemia and I am even questioning that there can be a direct relationship. In their explanation of the relationship the authors state: “ It is believed that the fusion gene product block cell differentiation by inhibiting the normal transcriptional programs and recruiting repressor molecules such as histone deacetylase enzymes. Furthermore, the fusion gene product activates other key target genes, which ultimately lead to the propagation of the transformed cell lines without normal restrictions”. There are clearly more downstream events that have to take place in order to develop cancer. A direct relationship implies that there is no intermediate step between one event and the next one and, therefore, there is no possibility of preventing the downstream event from taking place once the more upstream event has occurred. Clearly, the MLL rearrangement by itself is not sufficient to lead to cancer, and it is not even essential, since it is not observed in all infant leukemia cases.

I find a contradiction in the abstract between the sentence “epidemiological studies suggested that topoisomerase II may be involved in generation of the KE, in utero MLL chromosomal rearrangement” and the sentence a few lines below “the limited epidemiological studies do not allow any firm conclusion on the possible role of chemicals in infant leukemia”. I accept that there is some epidemiological support for a role of topoII inhibitors on the induction of MLL rearrangements related to infant leukemia. But the data is not for etoposide, which is the topoII inhibitor that the authors heavily rely on for linking their MIE with the AO. Furthermore, there seem to be clear differences in the breakpoint organization of ET-induced MLL rearrangements in cancer patients and of those seen in infant leukemia. This makes it more critical that the link between etoposide exposure and MLL rearrangements be convincingly

demonstrated in utero. It is unquestionable that the stronger support for the involvement of etoposide in the formation of MLL-rearrangements comes from studies in adults and the authors cannot provide as strong direct data that the event is occurring in utero.

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

The authors are making this AOP specific to in utero exposure and to a specific chromosomal rearrangement which makes it of limited regulatory applicability. Furthermore, it reduces the strength of evidence that supports the AOP. The cell type where the rearrangement takes place is unknown and there is no experimental data to show that in utero exposure to topoII inhibitors increases the frequencies of the leukemia-inducing rearrangements. There is not even a clear understanding about how this is occurring. As the authors state: "...topoII inhibition has to occur in an especially vulnerable and correct hot spot in the MLL locus; however, details of this process and how it happens are not clear".

I guess one question that needs to be clarified is whether the authors see this AOP as a way to identify chemicals that have a role in infant leukemia or as a way to identify chemicals that acts as topo I inhibitors. If the former, which I suspect is what the authors envision, I remain very skeptical of the utility of this AOP for regulatory purposes because of its narrow, to say it in OECD lingo, applicability domain. If the latter, the AOP would have a much broader regulatory application but, it will require substantial changes that are described in more details below.

- **Conclusion:**
- **What are your overall conclusions of the assessment of this AOP?**

My main concern with this AOP is the authors' insistence in making it specific to an *in utero* exposure. I see that this issue was brought up during the internal review within EAGMST and that the authors have resisted making the change. It seems to me that by making the AOP specific to infant leukemia, they are creating an AOP for a MIE in a specific cell type and for a rearrangement in very specific region of the genome. As such, this AOP is an isolated AOP that does not contribute to expanding the network of KEs and is in my opinion of limited utility and almost outside of the spirit of the AOP program.

To my knowledge, the mechanism by which topoII enzymes operate is the same in every cell type and so is the mechanism by which they create DSBs. Thus, the MIE (inhibition of topoII) and the first KE (formation of DSB) should be agnostic of the cell type. The authors can then make the next KE specific to fetal hematopoietic cells and to infant leukemia. Doing it in this way, would create a MIE and a KE that can be borrowed and used to build other AOPs, including one leading to secondary cancers in patients receiving topoII inhibitors as part of their chemotherapy. I would venture to say that the authors should create this branching of the AOP within their current submission.

I appreciate that this would require significant work and rewriting, however, the evidence in support of their current AOP comes almost exclusively on the data in cancer patients. The authors have not convinced me that there is conclusive evidence that the MLL rearrangements found in infant leukemia arise through a mechanism that involves exclusively topoII enzymes. As the authors mention, the MLL gene is within a fragile site that is inherently susceptible to breakage and does not necessarily need topoII to create a DSB. Without the supporting evidence in cancer patients that rearrangements are seen in patients that have received topoII inhibitors, the authors

have little empirical data to support their AOP. Furthermore, making this AOP more general, and not limited to in utero exposure, would allow for the measurements of the MIE and KEs in any cell type, including *in vitro* cell lines, where dose response relationships, temporal concordance and consistency of the experimental evidence can be better investigated. These *in vitro* studies would also provide a quantitative understanding of the relationships between KEs, an aspect that is currently lacking in the present AOP. This would also expand the number of chemicals that have been shown to interfere with topo II function.

In summary, I believe that the present AOP require significant revisions before it can move on to the next in acceptance process at the OECD.

Other comments.

- 1) the abstract speaks of a MIE, a key event and the AOP. However, in the description of the AOP there are two key events: DSB and formation of the chromosomal rearrangement.
- 2) KEs should be presented in sequential order from the MIE to the AOP. Therefore, the description of the formation of double strand breaks (KE1) should be discussed before the formation of the MLL translocation (KE2).
- 3) There are various typographical mistakes throughout the document.

Reviewer 2

[Note: this reviewer also provided a marked copy of the AOP 202 snapshot; this is included as Annex 4.]

General comments

The current AOP combines a large amount of information related to topoisomerase II (topo II) inhibition and the development of infant leukemia. The authors have cited and extracted information from a large number of relevant review articles and primary research publications. However, the case that they have made is made up of a patchwork of evidence, some of which is very strong and some of which is very weak. For example, from my perspective, the evidence on infant leukemia is quite strong as is the evidence that etoposide, the selected model topoisomerase II poison, can cause leukemia in treated patients. However, the evidence that maternal exposure to etoposide can induce infant leukemia is very weak. And the evidence for other known topo II poisons (with the possible exception of dipyrone) is also quite weak. The mixing of evidence from the various diseases and mechanisms results in a pathway that is complicated and somewhat difficult to follow. The authors have also glossed over some key inconsistencies with their proposed pathway such as the fact that etoposide primarily induces acute myeloid leukemia (AML) in adults and children, but the proposed pathway is primarily focused on the genesis of acute lymphoblastic leukemia (ALL) in infants. AML and ALL are widely considered two different types of leukemia. I think that a stronger and more broadly applicable case would be made if the authors laid out the evidence for the association between etoposide (alone or combined with other topoII poisons) and therapy-related AML (t-AML) and ALL (t-ALL), and then made the case that topo II inhibition by an as-yet-unidentified topoII poison was responsible for infant leukemia (ALL and AML).

Charge Questions:

- Scientific quality: Does the AOP incorporate the appropriate scientific literature and does the scientific content of the AOP reflect current scientific knowledge on this specific topic?

Yes, for the most part, the AOP incorporates the appropriate scientific literature and the AOP reflects current scientific knowledge on the subject. However, some key facts are not presented and complications (as indicated above) seem to be glossed over. I also disagree with some of the details and interpretations of the study results. I believe that some additional background would be helpful. Some notable points are listed below. More detailed points can be found in the marked up pdf document.

- As an example (indicated above), the reader should know that there are several major and distinct types of leukemia – infant leukemia, child leukemia and adult leukemia (Wiemels, 2012). Adult and child leukemias can then also be subdivided into leukemias with no known cause (*de novo* leukemias) and those which are therapy-related (t-AML and t-ALL). The therapy-related leukemias are also classified by etiologic agent and characteristics. t-AML resulting from alkylating agents and ionizing radiation differ from those induced by topoII inhibitors. Leukemias induced by etoposide (one of the epipodophyllotoxin class of topoII poisons) differ from those induced by the anthracenedione or anthracycline classes of topoII poisons as well as the bisdioxopiperazine class of topoII inhibitors (catalytic inhibitors such as bimolane and ICRF 154). With this background the reader would have a better understanding of the context for the AOP and understand that the AOP is proposing a mechanism by which one class of topo II inhibitor would cause infant leukemia, an uncommon type of leukemia.
- Substantial evidence indicates that translocations involving the MLL gene play a critical role in certain types of leukemia. However, these translocations may arise spontaneously, presumably either through topo II errors or through unidentified endogenous or exogenous inhibitors of topoisomerase II. There is little convincing evidence that I am aware of that xenobiotics induce MLL translocations in normal embryonic cells *in vivo*. With the possible exception of dipyrone has been implicated in several studies, a topoII poison that can cause infant leukemia has not yet been identified. Elevated risks of infant leukemia were reported for the children of mothers who took dipyrone during pregnancy (see Pombo-de-Oliveira, 2016 and references therein). However, these studies appear to

have originated from the same group in Brazil and it is not clear to me whether these represent the same or different study populations.

- The distribution of breakpoints in the MLL gene seen in leukemias induced by topoII inhibitors shows a similarity to those seen in infant leukemias. However, they frequently differ when examined at the sequence level (Jung et al., 2010; Cimino et al., 1997). As described in Pendleton et al. (2014) “Rearrangements in 11q23 that are associated with infant leukemias often are more complex than those observed in t-AMLs, and breakpoints are distributed more heterogeneously in the 8.3-kb BCR.”
- TopoII poison is not a molecular initiating event. From my perspective, the molecular initiating event would be the inhibitor binding to the topoII enzyme and its interference with relegation of the stabilized double stranded DNA break. A more disease-specific event would be the recombining of the DNA double strand breaks resulting in the critical translocation involving the MLL gene.
- The AOP states in several places that the latency period for leukemias induced by topoII poisons is <2 years. Although this is stated in the reference cited, this is incorrect in a couple of ways. First, the number of interest is generally the median latency period. This is typically reported as 2-3 years but there is at least one prominent example where the median latency period was over 3 years. For example, the median latency for t-AML reported by Hijiya et al. (2007) was 3.4 years. The latency period also varies by translocation partner, with some being well over 3 years (Balgobind et al., 2010). The WHO (2008) describes the latency period for these types of therapy-related leukemias as being about 1-5 years.
- Etoposide clearly induces DNA breakage and translocations. However, the mechanism by which this occurs is not as well established as implied in the text. Various mechanisms have been proposed including those involving apoptosis, scaffold attachment regions, various nucleases in addition to topo II. In many cases, the breakpoints seen in MLL recombined leukemias induced by etoposide are not directly adjacent to a topoII recognition site (see for example, Wright and Vaughan, 2014).
- Of note, some of the text in the Wiki has been copied almost word-for-word from one of the sources. While the reference was provided at the end, this is not sufficient as the information was not in quotation marks. The one section where I noticed this is below.

AOP Wiki

The quinone is able to induce about 4 times more enzyme-mediated DNA cleavage than does the parent drug. Furthermore, the potency of etoposide quinone was about 2 times greater against topoisomerase II β than it is against topoisomerase II α , and it reacts about 2 to 4 times faster with the β isoform. The quinone metabolite induces a higher ratio of double- to single strand breaks than the parent chemical, and its activity is less dependent on ATP. Whereas etoposide acts as an interfacial topoisomerase II poison, etoposide quinone displayed all of the hallmarks of a covalent poison: the activity of the metabolite was abolished by reducing agents, and the compound inactivated topoisomerase II β when it was incubated with the enzyme prior to the addition of DNA (Smith et al. 2014).

Smith et al. (2014)

The quinone induced 4 times more enzyme-mediated DNA cleavage than did the parent drug. Furthermore, the potency of etoposide quinone was \sim 2 times greater against topoisomerase II β than it was against topoisomerase II α , and the drug reacted \sim 2–4 times faster with the β isoform. Etoposide quinone induced a higher ratio of double- to single-stranded breaks than etoposide, and its activity was less dependent on ATP. Whereas etoposide acts as an interfacial topoisomerase II poison, etoposide quinone displayed all of the hallmarks of a covalent poison: the activity of the metabolite was abolished by reducing agents, and the compound inactivated topoisomerase II β when it was incubated with the enzyme prior to the addition of DNA. These results are consistent with the hypothesis that etoposide quinone contributes to etoposide-related

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

I am not aware of any evidence in humans that maternal exposure to etoposide has resulted in infant or pediatric leukemia in humans. This includes case reports as well as epidemiological studies. As a result, I consider the evidence that etoposide is associated with infant leukemias to be weak. The evidence in adults and children is much stronger and IARC has classified etoposide as a Group 1 carcinogen. I agree that it has the potential to cause infant leukemia but there is no direct evidence to support the association in humans. My other comments on the strength of evidence can be found in the marked up pdf.

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

Infant leukemia is a rare type of leukemia so that increases induced by xenobiotics would be noticed if one was specifically looking for them. Otherwise, they would be difficult to detect. Since there is no convincing evidence showing that this type of leukemia has been caused by xenobiotics, the evidence will need to be strong to convince regulators and other stakeholders of a real association. In my opinion, the convincing evidence will need to come from appropriate cell and *in vivo* models as well as supporting evidence in humans. Some of bioflavonoids have shown topo II inhibitory activity in acellular systems but when tested in cells, the genotoxic effects that were observed occurred through an entirely different mechanism (c.f. Olaharski et al., 2005 and Gollapudi et al. 2014).

- Conclusion:
 - What are your overall conclusions of the assessment of this AOP?

The authors have made put together multiple lines of evidence and made a good case that maternal exposure to a topoII poison could cause infant leukemia. However, until sufficient evidence is generated to show that this can be caused by a xenobiotic, the pathway remains largely theoretical. As a result, I see the AOP as being closer to a promising hypothesis than a clearly established mechanism of action.

Reviewer 3

AOP 202 describes the potential association of events initiated by inhibition of the topoisomerase II (topoII) activity in an early life stage with infant leukemia.

The reviewers need to address the following points:

Scientific Quality:

The authors have done a thorough job in citing the most relevant literature regarding the etoposide related findings. However, the cited literature for other examples of topo II inhibitors do not completely cover the present literature. For example, Chlorpyrifos has been assessed by the EU in 2017 and a rapporteur assessment report (RAR) including several genotoxicity studies is available. The contradictory data in the RAR (the pesticide does not show any mutagenic activity) and the cited papers (mutagenicity observed) need to be addressed. Furthermore, although genistein has been used as a further example of a topoII inhibitor the cited literature do not directly link the observed effects to genistein intake.

Weight of Evidence

The inhibition of topoII activity may (amongst others) lead to a transformation in the MLL gene. The transformed MLL gene (only in conjugation with certain other genes e.g. AF4, ENL, AF9 etc.) is apparently related to leukemia. Thus, the mutation of the MLL per se via topo II is not sufficient to induce leukemia (as demonstrated by the MLL-K.O. mice). Therefore, although the focus of this AOP has been set on the topoII induced DSB in the MLL gene, it is also important that certain other genes (e.g. AF4) are also mutated in order for conjugation to take place. Thus, in my opinion this is not simply an associative event but also a key event.

MLL-AF-4 knock in transgenic mice develop leukemia with a latency period. This is not reflecting the course observed in infant leukemia. This could either mean that the topoII inhibition in an early life stage (leading to the generation of the relevant fusion proteins) is not solely associated with infant leukemia but also with other types of leukemia. On the other hand, it could mean that the KE and KER are not reflected in the assessed rodent models. If this is the case, it remains uncertain whether animal models can be used for assessment of the AOP in regulatory studies.

Regulatory acceptability

The AOP is definitely a relevant issue for regulatory purposes. However, under the indicated circumstances it is difficult to envisage how this parameter could be integrated in toxicological studies. The authors have suggested addressing the issue under OECD 443. However, they have not defined which endpoint needs to be looked at for this AOP. For example, should induction of leukemia in the F1 or F2 generation be used as an endpoint or should F1 or possible F2 generations be assessed for MLL transformations? Nevertheless, as described above it remains uncertain whether rodent studies are appropriate models for this AOP.

The use of *in vitro* cell systems may be an alternative (e.g. by using liver hematopoietic stem cells). However, the *in vivo* relevance of the effects has not been clearly demonstrated by agents other than etoposide.

Thus, it would be necessary for authors to make a more explicit recommendation on how to incorporate this AOP in toxicological assessments for regulatory use.

Conclusion:

The described AOP has fundamental potential for use for regulatory purposes. However, it is presently very much focused on effects observed from etoposide studies. The other examples used do not contribute as solidly to define the AOP.

The authors should be more explicit on which parameters may be used within toxicological assessments to assess the relevance of this AOP for regulatory purposes.

Reviewer 4

Charge questions: AOP 202: In utero DNA topoisomerase II inhibition leading to infant leukemia

Scientific quality:	Answer
Does the AOP incorporate the appropriate scientific literature?	<p>The authors made a big effort to include the appropriate scientific literature; however, in some sections of the AOP, more references would be valuable.</p> <p>In particular:</p> <p>pag.2, Background: “the hallmark of the AOP is the formation of MLL gene rearrangements.....acute leukemia by global (epi)genetic dysregulation” (refs? i.e. Gill Super et al., Cancer Genet 2015, 208(5), 230)</p> <p>pag.2, Etoposide: recent ref on topoII inhibitors and poisons: Delgado et al, 2018, Biochem J., 475:373</p> <p>pag 3, etoposide quinone: refs on the different mechanism of action of etoposide and quinone metabolite;</p> <p>pag 5: Udrouiu et al. 2015 is a review: please cite the original studies</p> <p>pag. 6, how is measured topoII activity: there is a recent paper that could be included, NAR, 2017, 45(13): 7855</p> <p>In addition, inhibition of topo2 induces block of the replication fork: methods could be reported measuring this effect.</p> <p>In this section, methods measuring the DNA damage response are reported; since they are a measure of DNA damage and there is a section on DSB, the authors could evaluate whether present these tests only in the DSB paragraph.</p> <p>pag. 12, how are measured DSBs: the analyses of chromosomal aberrations and micronuclei should be mentioned.</p> <p>pag. 17, possible facilitating mutated genes: there is a recent paper that could be included, Int J Cancer 2017, 140, 864</p>
Does the scientific content of the AOP reflect current scientific knowledge on this specific topic?	<p>Yes; however, the question on how topoII cleavage and specific translocations are related, in my opinion, deserves to be more specifically described, possibly taking into account the paper by Yu et al., 2017 Genome Res, 27, 1238.</p>
Weight of evidence:	Answer
Are the weight-of-evidence judgment/scoring calls provided by AOP developers for KE, KERs and	The main weakness is represented by the lack of direct evidence or extensive understanding of the

the overall AOP justified?	events <i>in utero</i> ; because of that, all the scoring calls “STRONG” described <i>in utero</i> should be evaluated as “MODERATE”.
Regulatory applicability:	Answer
Considering the strength of evidence and current gaps/weaknesses, what would be the regulatory applicability of this AOP, in your opinion?	Taking into consideration uncertainties and inconsistencies summarized pag. 28-29, this AOP, in my opinion, cannot be used directly for regulatory applications because other factors in addition to MLLr or topoII inhibition seem to be needed for the development of infant leukemia. However, I agree with the authors that this AOP can be useful in the MOA framework for specific chemicals, and could serve in guiding testing strategies.
Conclusion:	Answer
What are your overall conclusions of the assessment of this AOP?	The overall assessment of the AOP is well done.

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

Comments by the reviews were collated by the review manager into tabular form. This was distributed to the authors for their response. The reviewers supplied some additional comments in advance of the teleconference. All dialogue that occurred in advance of the teleconference is included on the table below.

COLLATED REVIEW OF AOP 202: *IN UTERO* DNA TOPOISOMERASE II INHIBITION LEADING TO INFANT LEUKEMIA

February 5, 2018

Reviewers:

David Eastmond, University of California, Riverside.

Francesca Marcon, Dept. Environment and Health, Istituto Superiore di Sanità.

Francesco Marchetti, Environmental Health Science Research Bureau, Health Canada

Naveed Honavar, Experimental Toxicology and Ecology, BASF SE

In this version of the review, the reviewers are referred to by number; the numbers do not correspond to the order above. Page numbers refer to the AOP snapshot.

General Comments		
	<p><i>R2:</i> The current AOP combines a large amount of information related to Topoisomerase II (topoII) inhibition and the development of infant leukemia. The authors have cited and extracted information from a large number of relevant review articles and primary research publications. However, the case that they have made is made up of a patchwork of evidence, some of which is very strong and some of which is very weak. For example, from my perspective, the evidence on translocations consistent with topoisomerase II inhibition and infant leukemia is quite strong as is the evidence that etoposide, the selected model topoisomerase II poison, can cause leukemia in treated patients. However, the evidence that maternal exposure to etoposide can induce infant leukemia is very weak. And the evidence for other known topoII poisons (with the possible exception of dipyrone) is also quite weak. The mixing of evidence from the various diseases and mechanisms results in a pathway that is complicated and somewhat difficult to follow. The authors have also glossed over some key inconsistencies</p>	<p>IFL is difficult to study in ‘real-life’ conditions and that was the reason to try to search an analogous condition in adults, i.e. treatment related leukemia.</p> <p>Author 2 questions whether there are substantial mechanistic among topoII poisons. There are mechanistic similarities and analogies.</p>

	<p>with their proposed pathway such as the fact that etoposide primarily induces acute myeloid leukemia (AML) in adults and children, but the proposed pathway is primarily focused on the genesis of acute lymphoblastic leukemia (ALL) in infants. AML and ALL are widely considered two different types of leukemia. I think that a stronger and more broadly applicable case would be made if the authors laid out the evidence for the association between etoposide (alone or combined with other topoII poisons) and therapy-related AML (t-AML) and ALL (t-ALL), and then made the case that topoII inhibition by an as-yet-unidentified topoII poison was responsible for infant leukemia (ALL and AML).</p>	<p>Secondary acute leukaemia carrying MLL-r is an adverse effect observed in patients treated with etoposide and a few other anticancer agents. Characteristics of the disease are in many ways analogous to those in infant leukaemia (Joannides and Grimwade 2010; Joannides et al. 2011) (Table 1). This so-called therapy-associated acute leukaemia (t-AL) in adults is characterised by its short latency, <2 years between the treatment of the primary malignancy with epipodophyllotoxins and the clinical diagnosis of the secondary disease, and by the poor prognosis (Relling et al. 1998; Cowell and Austin 2012; Ezoe 2012; Pendleton et al. 2014). It is recognised that the MLL-r fusion genes are caused by etoposide, other epipodophyllotoxins or anthracyclines, because MLL-r has not been detected in bone marrow samples banked before the initiation of the treatment for the first malignancy (Cowell and Austin 2012; Pendleton et al. 2014). Overall, the evidence supporting the causal relationship between etoposide-induced topoII inhibition and further formation of cleavage complexes leading to MLL-r is strong and could be regarded as 'beyond reasonable doubt'. Also, the breakpoints in MLL or partner genes fall within a few base pairs of a drug-induced enzyme-mediated DNA cleavage site (Cowell and Austin 2012; Pendleton et al. 2014; Gole and Wiesmüller 2015). All the above disease characteristics, MLL-rearrangement, short latency and poor prognosis strongly suggest that infant leukaemia and treatment-related leukaemia are sufficiently similar to allow for inferences to be made regarding tentative aetiological factors, molecular events and disease progression and manifestation. Thus, etoposide can be considered as a</p>
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	<p>Of note, some of the text in the Wiki has been copied almost word-for-word from one of the sources. While the reference was provided at the end, this is not sufficient as the information was not in quotation marks. Copying of other sections may also have occurred elsewhere in the Wiki. The one section where I noticed this is below.</p> <p>AOP Wiki: “The quinone is able to induce about 4 times more enzyme-mediated DNA cleavage than does the parent drug.</p>	<p>model chemical for DNA topoII inhibition and MLL-rearrangement, and it was used here as a tool compound to empirically support the linkage between MIE and AO in the AOP.</p> <p>Author 3 noted that there is solid evidence of that. Therapy-related acute leukemia usually comes in AML format due to myeloablation consequences of chemotherapy regimens and impairment of hematopoietic progenitors that cope with myeloablation and bone marrow reconstitution. extensive work by C.A. Felix lab in cooperation with worldwide leaders in AML</p> <p>The reason for us using mouse and human-based models addressing the etoposide-mediated damage is due really on old work from t-AML patients treated with etoposide. furthermore, much evidence has shown that topoII drugs disrupt genomic hot spots such as MLL locus which render genotypes such as MLL-PTD (partial tandem duplication) and MLL-fusions. the stochastic nature of this is further confirmed by the more than 120 partners fusing with MLL.</p> <p>Importantly, topoII exposure is quite specific for hematopoietic immature cells (stem and progenitors). The mechanisms may be both epigenetic and structural (Yu X. <i>Genome. Res.</i> 2017). Beyond etoposide and other common topoII inhibitors we have now evidence (data not shown, unpublished, Rodriguez V <i>et al.</i>) that both permethrin and chlorpyrifos include specific genomic breaks at MLL locus in human embryonic and fetal stem cells (Rodriguez V <i>et al.</i>) unpublished).</p> <p>Without linking topoII poison exposure and MLL breaks no AOP could be made.</p>
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	<p>Furthermore, the potency of etoposide quinone was about 2 times greater against topoisomerase IIβ than it is against topoisomerase IIα, and it reacts about 2 to 4 time faster with the β isoform. The quinone metabolite induces a higher ratio of double - to single strand breaks than the parent chemical, and its activity is less dependent on ATP. Whereas etoposide acts as an interfacial topoisomerase II poison, etoposide quinone displayed all of the hallmarks of a covalent poison: the activity of the metabolite was abolished by reducing agents, and the compound inactivated topoisomerase IIβ when it was incubated with the enzyme prior to the addition of DNA (Smith <i>et al.</i> 2014).”</p> <p>Smith <i>et al.</i> (2014) “The quinone induced ~4 times more enzyme-mediated DNA cleavage than did the parent drug. Furthermore, the potency of etoposide quinone was ~2 times greater against topoisomerase IIβ than it was against topoisomerase IIα, and the drug reacted ~2–4 times faster with the β isoform. Etoposide quinone induced a higher ratio of double- to single-stranded breaks than etoposide, and its activity was less dependent on ATP. Whereas etoposide acts as an interfacial topoisomerase II poison, etoposide quinone displayed all of the hallmarks of a covalent poison: the activity of the metabolite was abolished by reducing agents, and the compound inactivated topoisomerase IIβ when it was incubated with the enzyme prior to the addition of DNA.”</p> <p>Rather than copying text, the authors need to give proper attribution to their sources. The authors have also shown a number of figures which are likely from copyrighted sources. Have they received permission for their use?</p> <p>R3: The overall assessment of the AOP is well done.</p>	<p>AOP development is based on current knowledge and there is nothing wrong to cut and paste text if this is making the clarity. Rewording is an unnecessary exercise as the relevant part is what is in the literature and not the interpretation of the developers. The citation is correct and the process in line with the AOP development guidance.</p> <p>All the copyrights were addressed for the EFSA scientific opinion (SO), and there are no rules for the AOP development.</p> <p>1)Noted, the text in the abstract need to be revised to include DSB in the abstract</p>
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	<p><i>R4:</i> My major concerns with the current AOP are explained in greater detail in the overall conclusion assessment of the AOP. However, they are also briefly mentioned in the answers to charge questions related to weigh of evidence and regulatory applicability.</p> <p>Other comments.</p> <p>1) The abstract speaks of a molecular initiating event (MIE), a key event (KE) and the adverse outcome (AO). However, in the description of the AOP there are two key events: double strand breaks (DSB) and formation of the chromosomal rearrangement.</p> <p>2) KEs should be presented in sequential order from the MIE to the AO. Therefore, the description of the formation of double strand breaks (KE1) should be discussed before the formation of the MLL translocation (KE2).</p> <p>3) There are various typographical mistakes throughout the document.</p>	<p>2) This is a technical problem in the wiki dealing with KE added in a later time</p> <p>3) Noted</p>
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Charge questions: AOP 202: *In utero* DNA topoisomerase II inhibition leading to infant leukemia

Scientific quality		
<p><i>Does the AOP incorporate the appropriate scientific literature?</i></p>	<p><i>R1:</i> The authors have done a thorough job in citing the most relevant literature regarding the etoposide related findings. However, the cited literature for other examples of topoII inhibitors do not completely cover the present literature. For example, Chlorpyrifos has been assessed by the EU in 2017, and a rapporteur assessment report (RAR) including several genotoxicity studies is available. The contradictory data in the RAR (the pesticide does not show any mutagenic activity) and the cited papers (mutagenicity observed) need to be addressed. Furthermore, although</p>	<p>Literature not available at the time of AOP will be checked for revision Chlorpyrifos is currently under renewal and more precisely under comments evaluation, meaning that the comments are still not publicly available as well as the final conclusion. The genotoxicity issue for Chlorpyrifos is far from being resolved considering both the quality of the studies included in the RAR as well as the results. In addition, some comments are also likely related to the SO published by EFSA that is including the proposed AOP in the Appendix. Being the regulatory process undergoing, the</p>

	<p>genistein has been used as a further example of a Topo II inhibitor, the cited literature does not directly link the observed effects to genistein intake.</p> <p>R2: Yes, for the most part, the AOP incorporates the appropriate scientific literature, and the AOP reflects current scientific knowledge on the subject. However, some key facts are not presented, and complications (as indicated in General Remarks above) seem to be glossed over. I also disagree with some of the details and interpretations of the study results. I believe that some additional background would be helpful. Some notable points are listed below. More detailed points can be found in the marked-up pdf document.</p> <ul style="list-style-type: none"> • As an example (indicated above), the reader should know that there are several major and distinct types of leukemia – infant leukemia, child leukemia and adult leukemia (Wiemels, 2012). Adult and child leukemias can then also be subdivided into leukemias with no known cause (<i>de novo</i> leukemias) and those which are therapy-related (t-AML and t-ALL). The therapy-related leukemias are also classified by etiologic agent and characteristics. t-AML resulting from alkylating agents and ionizing radiation differ from those induced by topoII inhibitors. Leukemias induced by 	<p>quotation of the RAR was not considered appropriate by the authors of this AOP and only what is the open literature was considered</p> <p>The comment on ginestein is not clear. Ginestein was quoted as topoII poisons and as such of potential interest for further work for exploring the AOP. The quotation of potential additional stressors is valuable in the context of the AOP framework as additional research of works are frequently warranted. In addition, this will give the opportunity to the reader of the AOP on the reasons why the empirical support has limitation in terms of number of available stressors. AOP are chemically agnostic, meaning that the pathway is more linked to the hazard identification/characterization steps if used in a risk assessment process; so the comment on ginestein intake (exposure) is not understood.</p> <p>The suggested point will be addressed in the background of the AOP which is actually describing only the IFL. Authors will include the papers from many labs including Felix CA Lab.</p> <p>The comment is appreciated. The weakness of the epidemiological data is</p>
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	<p>etoposide (one of the epipodophyllotoxin class of topoII poisons) differ from those induced by the anthracenedione or anthracycline classes of topoII poisons as well as the bisdioxopiperazine class of t inhibitors (catalytic inhibitors such as bimolane and ICRF 154). With this background the reader would have a better understanding of the context for the AOP and understand that the AOP is proposing a mechanism by which one class of topoII inhibitor would cause infant leukemia, an uncommon type of leukemia.</p> <ul style="list-style-type: none"> • Substantial evidence indicates that translocations involving the MLL gene play a critical role in certain types of leukemia. However, these translocations may arise spontaneously, presumably either through topoII errors or through unidentified endogenous or exogenous inhibitors of topoisomerase II. There is little convincing evidence that I am aware of that xenobiotics induce MLL translocations in normal embryonic cells <i>in vivo</i>. With the possible exception of dipyrone, which has been implicated in several studies, a topoII poison that can cause infant leukemia has not yet been identified. Elevated risks of infant leukemia were reported for the children of mothers who took dipyrone during pregnancy (see Pombo-de-Oliveira, 2016 and references therein). However, these studies appear to have originated from the same group in Brazil, and it is not clear to me whether these represent the same or different study populations. • The distribution of breakpoints in the MLL gene seen in leukemias induced by topoII inhibitors shows a similarity to 	<p>strength for supporting this AOP from the regulatory perspective. We know that the epidemiological studies for pesticides are very weak. Relevant to this AOP is that a consistent human health effect observed through multiple metanalysis is leukaemia. The authors know (see also comment above) that this is a very general diagnostic criteria which is basically impeding a proper hazard identification for many reasons. This triggered the idea of using <i>specific</i> AO, to include human health outcome in the process of hazard identification.</p> <p>Dipyrone requires further experimental studies. There is epidemiological but not experimental supporting data since it was simply not tested. However, data from the UK Epidemiological group on childhood leukemia confirms Pombo-de-Oliveira's data and suggest that transplacental exposure to Dipyrone is associated with higher risk of MLL and ALL. It should be noted that non-transplacental exposure (i.e. direct exposure, intramuscular, respiration etc.) to this compound has not been tested in non-infants.</p> <p>There is good information from the MLL recombinome consortium (Meyer Cet al. Leukemia 2009, 2013, 2017). MLL breaks occur in a 8.3 BCR, but differences exist with age, geographical distribution and disease. Breakpoints in t-AML are more complex and heterogeneously distributed because leukemia-inducing hits are not transplacental and therefore are received in a more heterogeneous fashion (waves of overexposure in a systemic manner). Besides, the adjuvant contributions of other chemotherapy drugs are unknown. Finally, the genetic and epigenetic contribution in t-AML is very variable since one cannot account for common and recurrent predisposing SNPs or genetic variants.</p>
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	<p>those seen in infants leukemias. However, they frequently differ when examined at the sequence level (Jung <i>et al.</i>, 2010; Cimino <i>et al.</i>, 1997). As described in Pendleton <i>et al.</i> (2014) “Rearrangements in 11q23 that are associated with infant leukemias often are more complex than those observed in t-AMLs, and breakpoints are distributed more heterogeneously in the 8.3-kb BCR.”</p> <ul style="list-style-type: none"> • topoII poison is not a molecular initiating event (MIE). From my perspective, the MIE would be the inhibitor binding to the topo II enzyme and its interference with religation of the stabilized double stranded DNA break. A more disease-specific event would be the recombining of the DNA double strand breaks resulting in the critical translocation involving the MLL gene. 	<p>It has to be stressed that the goal (perhaps the main goal) of the draft AOP was to supply a plausible pathway for the etiology of IFL, with all the available scientific knowledge. The authors understand this comment but are quite resistant to change the name of the MIE. We discussed a lot, not only among the authors of this AOP, but also among the different scientists belonging to the WG who are authors of multiple AOPs in advanced stage of development. The issue is that the AOP is based on a scientific ground, though it is intended for regulatory application and use. Academically, there is no doubt that a more specific definition (as proposed) has an appropriate reasoning. However, we were already accused of being too specific (OECD internal review) and going in details will possibly deviate from the AOP concept. It is difficult to find a correct compromise between an academic and regulatory position for the definition of the MIE (similarly to the AO) and the number and definition of the KEs. Based on the wealth of discussion we had in the past, the authors would prefer to leave the definition of the MIE as it is with the main reason being that this would not change the intended regulatory application and that the 1st KE could be used to link this AOP to the network.</p> <p>The suggested literature will be checked and added as appropriate.</p>
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	<p><i>R3:</i></p> <p>The authors made a big effort to include the appropriate scientific literature; however, in some sections of the AOP, more references would be valuable.</p> <p>In particular:</p> <p>Page 2, Background: “the hallmark of the AOP is the formation of MLL gene rearrangements.....acute leukemia by global (epi)genetic dysregulation” (refs? i.e. Gill Super <i>et al.</i>, Cancer Genet 2015, 208(5), 230)</p> <p>Page 2, Etoposide: recent ref on Topo II inhibitors and poisons: Delgado <i>et al.</i>, 2018, Biochem J., 475:373</p> <p>page 3, etoposide quinone: refs on the different mechanism of action of etoposide and quinone metabolite;</p> <p>page 5, Udroui <i>et al.</i> 2015 is a review: please cite the original studies</p> <p>page 6, how topoII activity is measured: there is a recent paper that could be included, NAR, 2017, 45(13): 7855</p> <p>In addition, inhibition of topoII induces block of the replication fork: methods could be reported measuring this effect.</p> <p>In this section, methods measuring the DNA damage response are reported; since they are a measure of DNA damage and there is a section on double strand breaks (DSB), the authors could evaluate whether to present these tests only in the DSB paragraph.</p> <p>page 12, how DSBs are measured: the analyses of chromosomal aberrations and micronuclei should be mentioned.</p> <p>page 17, possible facilitating mutated genes: there is a recent paper that could be included, Int J Cancer 2017, 140, 864.</p> <p><i>R4:</i></p> <p>I think the authors have done a reasonable job at incorporating the most relevant scientific literature on the</p>	
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	<p>subject. It should be highlighted that the supporting literature includes mostly studies that are not conducted <i>in utero</i>, as these latter are few.</p>	
<p>Does the scientific content of the AOP reflect current scientific knowledge on this specific topic?</p>	<p>R2: The AOP states in several places that the latency period for leukemias induced by Topo II poisons is <2 years. Although this is stated in the reference cited, this is incorrect in a couple of ways. First, the number of interest is generally the <u>median</u> latency period. This is typically reported as 2-3 years, but there is at least one prominent example where the median latency period was over 3 years. For example, the median latency for t-AML reported by Hijiya <i>et al.</i> (2007) was 3.4 years. The latency period also varies by translocation partner, with some being well over 3 years (Balgobind <i>et al.</i>, 2010). The WHO (2008) describes the latency period for these types of therapy-related leukemias as being about 1-5 years.</p> <p>Etoposide clearly induces DNA breakage and translocations. However, the mechanism by which this occurs is not as well established as implied in the text. Various mechanisms have been proposed including those involving apoptosis, scaffold attachment regions, and various nucleases in addition to topo II (Gole and Weismuller, 2015). In many cases, the breakpoints seen in MLL recombined leukemias induced by etoposide are not directly adjacent to a topoII recognition site (see for example, Wright and Vaughan, 2014).</p>	<p>The latency period is the median-mean of latency. The 2 year threshold is used to refer to infant leukemia.</p> <p>The authors agree we need to describe this point in the KE description, in the description of the stressor and in the uncertainties part of the overall assessment of the AOP. The authors agree that this is still an open field actively working on topoII mechanisms. CA Felix just reported that TOP2A cleavage is also a broad DNA damage mechanism in oncogenic translocations such as MLL. Furthermore, please note that a topoII inhibitor inhibits the enzyme to facilitate <i>bona fide</i> DNA repair; therefore, the consequence is that many molecular pathways involved in DNA damage repair become no longer functional.</p> <p>The fact that the exact mechanism is still unresolved does not invalidate the AOP.</p> <p>See answer to the comment above.</p>

	<p><i>R3:</i> Yes; however, the question on how topo II cleavage and specific translocations are related, in my opinion, deserves to be more specifically described, possibly taking into account the paper by Yu <i>et al.</i>, 2017 Genome Res, 27, 1238.</p> <p><i>R4:</i> The scientific content of the AOP accurately reflects the current scientific knowledge on the topic.</p>	
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Weight of evidence:		
<p><i>Are the weight-of-evidence judgment/scoring calls provided by AOP developers for KE, KERs and the overall AOP justified?</i></p>	<p><i>R1:</i> The inhibition of topoII activity may (amongst others) lead to a transformation in the MLL gene. The transformed MLL gene (only in conjugation with certain other genes e.g. AF4, ENL, AF9 etc.) is apparently related to leukemia. Thus, the mutation of the MLL per se via topoII is not sufficient to induce leukemia (as demonstrated by the MLL-K.O. mice). Therefore, although the focus of this AOP has been set on the topoII induced double strand breaks (DSB) in the MLL gene, it is also important that certain other genes (e.g. AF4) are also mutated in order for conjugation to take place. Thus, in my opinion this is not simply an associative event but also a key event (KE).</p>	<p>The comment is appreciated. We extensively discussed this point during the development of this AOP. The first point where it is necessary to have an agreement is to have a feedback from the reviewer if the description of the production of the fusion gene is sufficiently described under the KE2 description and if the molecular and cellular processes behind the KER 3 is correctly reported in the overall assessment of the AOP, in the list of uncertainties and in the specific list of uncertainties in the KER3. The authors will check for completeness and appropriateness as this is an important molecular process for the development of the disease. The second point, where Author liskeener to leave the AOP sequence as it is, regards the appropriateness of including the mutation of additional genes as an additional KE. We included DNA DSB as additional KE. This is measurable (in a regulatory setting) and leads to the second KE through accumulation of DSBs. Thus, the MLL gene can be rearranged with a number of other genes that have been simultaneously cleaved, resulting in the formation of fusion genes, which represent the second key event. Molecular and cellular processes of</p>

	<p>MLL-AF-4 knock-in transgenic mice develop leukemia with a latency period. This is not reflecting the course observed in infant leukemia. This could mean that the topoII inhibition in an early life stage (leading to the generation of the relevant fusion proteins) is not solely associated with infant leukemia but also with other types of leukemia. On the other hand, it could mean that the KE and KER are not reflected in the assessed rodent models. If this is the case, it remains uncertain whether animal models can be used for assessment of the AOP in regulatory studies.</p>	<p>the second key event relationship (KER1) have been clarified at least on a general level in the text of the AOP. The product of the fusion gene encompasses many preserved and acquired functions of the fusion partners associated with differentiation block of HSPCs and expansion of clones expressing the fusion product (KER3). Originally an additional key event, differentiation block and clonal expansion, was envisaged, but ultimately (at least for the time being), we decided that the information is in effect contained in the fusion protein and, finally, the process) from the rearranged fusion gene leads to the manifest leukaemia. 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. Though the MLL rearrangement remains a KE of regulatory relevance, essential and measurable in a regulatory setting, the associated mutation events are more complex to frame in the linear process of the AOP, likely essential but still not completely scientifically understood and much more complex to measure in a regulatory setting. In conclusion, the authors support the scientific argumentation but don’t see the “regulatory benefit” of including, at this time, this additional KE. This is not precluding that in the future, when the aspects mentioned before, if clarified and relevant for the AOP network, this KE will be included in the AOP.</p> <p>It is obvious that MLL breaks <i>per se</i> are not oncogenic. Oncogenesis requires the generation of an aberrant fusion protein, in which n-terminal MLL fuses to c-terminal f creating a</p>
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	<p><i>R2:</i> I am not aware of any evidence in humans that maternal exposure to etoposide has resulted in infant or pediatric leukemia in humans. This includes case reports as well as epidemiological studies. As a result, I consider the evidence that etoposide is associated with infant leukemias to be weak. The evidence in adults and children is much stronger, and IARC has classified etoposide as a Group 1 carcinogen. I agree that it has the potential to cause infant leukemia, but there is no direct evidence to support the association in humans. My other comments on the strength of evidence can be found in the marked-up pdf (attached).</p> <p><i>R3:</i> The main weakness is represented by the lack of direct evidence or extensive understanding of the events <i>in utero</i>; because of that, all the scoring calls “STRONG” described <i>in utero</i> should be evaluated as “MODERATE”.</p> <p><i>R4:</i> Although the molecular initiating event (MIE) and first key event (KE) are clearly plausible, the data come principally from studies conducted in cell lines or after an adult exposure. That they happen <i>in utero</i> is inferred and, although this is acceptable, it should be reflected in the way the WOE is evaluated. It is my opinion that the authors are at times overrating the WOE scoring based on the available information.</p> <p>For example, I disagree with calling</p>	<p>fusion that displaces h4k4 activity towards the recruitment of dot11 and consequent H3K79 histone markers on MLL target genes. However, MLL fusions require a MLL break within the 8.3 kb BCR to create an in frame oncogenic fusion. This AOP reflects how topoII inhibitors impact MLL genomic loci to promote unspecific and random MLL breaks. many (99% perhaps) of the MLL breaks eventually are managed by the cell DNA machinery and are successfully repaired. However, a minor percentage (based on epidemiological data) escape DNA repairs and give rise to cells carrying the fusion genes.</p> <p>The frequency of cord blood carrying a fusion gene is 100x higher than the frequency of leukemia indicating that many healthy kids carry a non-leukemic tel-AML1 or MLL fusion. Whether a fusion-carrying individual will develop or not the leukemia depends in the cell-of-origin whether the fusion has occurred and the presence of secondary oncogenic hits that fully transforms the original founder clone.</p> <p>Lack of relevant animal models have fully been admitted in the AOP, but it does not mean that in the future animal models recapitulating IFL would not be possible to generate. Indeed, the intention was to describe the limitation of the animal models for the exploration of this specific hazard. The authors add more text in the overall assessment of the AOP and in the uncertainties pointing out that not only the experimental model specifically designed for the IFL have limitation, but that the standard animal testing, pivotal for regulatory authorization is basically not exploring this hazard. In any case, as formulated by the reviewer, it is worth</p>
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	<p>“strong” the direct link between the MLL rearrangement and infant leukemia, and I am even questioning that there can be a direct relationship. In their explanation of the relationship the authors state: “It is believed that the fusion gene product block cell differentiation by inhibiting the normal transcriptional programs and recruiting repressor molecules such as histone deacetylase enzymes. Furthermore, the fusion gene product activates other key target genes, which ultimately lead to the propagation of the transformed cell lines without normal restrictions”. There are clearly more downstream events that have to take place in order to develop cancer. A direct relationship implies that there is no intermediate step between one event and the next one and, therefore, there is no possibility of preventing the downstream event from taking place once the more upstream event has occurred. Clearly, the MLL rearrangement by itself is not sufficient to lead to cancer, and it is not even essential, since it is not observed in all infant leukemia cases.</p> <p>I find a contradiction in the abstract between the sentence “epidemiological studies suggested that topoisomerase II may be involved in generation of the KE, <i>in utero</i> MLL chromosomal rearrangement” and the sentence a few lines below “the limited epidemiological studies do not allow any firm conclusion on the possible role of chemicals in infant leukemia”. I accept that there is some epidemiological support for a role of topoII inhibitors on the induction of MLL rearrangements related to infant leukemia. But the data are not for etoposide, which is the topoII inhibitor that the authors heavily rely on for linking their MIE with the adverse outcome (AO). Furthermore, there seem to be clear differences in the breakpoint organization of ET-induced MLL</p>	<p>to consider the point as additional one in the uncertainties chapter</p> <p>This needs more discussion in the context of the AOP conceptual framework. The authors agree that the empirical support is moderate as based on indirect evidence, but the biological plausibility is strong and from a regulatory point of view is indicating that chemicals affecting this AOP are, at least, relevant risk factors for the AO.</p> <p>See reply above. We can re-evaluate the score though to me the biological plausibility should remain strong and this needs to be discussed</p> <p>The authors understand the point made here, but they tend to disagree with the sharp conclusion of the reviewer. The authors don't think it is necessary, at least based on actual knowledge, to prove in higher than given details, the direct relationship. The authors are fully buying in this AOP into the “one big event” leading to IFL. This should not be seen as a simplification matter but as a differentiation from all the others AOPs which are including in the development the classical multiple hits theory. One author also disagrees with the final sentence, as the MLL is essential for the majority of the cases and for the EFSA PPR Panel this was seen as an extremely important, likely poorly investigated Key Event. The author does do agree to a tailored revisit of the WOE but would be quite reluctant to give up to the strong score to the biological plausibility, at least</p>
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	<p>rearrangements in cancer patients and of those seen in infant leukemia. This makes it more critical that the link between etoposide exposure and MLL rearrangements be convincingly demonstrated <i>in utero</i>. It is unquestionable that the stronger support for the involvement of etoposide in the formation of MLL-rearrangements comes from studies in adults and the authors cannot provide as strong direct data that the event is occurring <i>in utero</i>.</p>	<p>on this argumentation.</p> <p>Only 80% of infant b-ALL display MLL fusions. 20% of infant b-ALL are normal karyotype. MLL fusion are were known to be leukemogenic (Barabe F and Dick <i>Science</i> 2007 among others....). However, distinct MLL fusions display differential latency and a requirement of further oncogenic hits depending on the cell of origin. Infants with MLL leukemia display a silent mutational landscape (Andersson A. <i>Nat Genetics</i> 2015 and Agraz-Doblas A <i>Nat. Comm.</i> 2018) indicating that no further genetic instability is required for leukemogenesis (Greaves M <i>Cancer Cell.</i> 2015. “When one mutation takes it all”). However, normal karyotype MLL, although also genetically stable, are thought to display a distinct epigenetic landscape account for MLL-independent oncogenesis.</p> <p>The authors are happy to check the quoted sentence in the abstract about the epidemiological studies.</p> <p>One author disagrees on the final part of the comment unless it is not exclusively referring to the empirical support, but rather to biological plausibility. Also, although indirect, embryonic stem cells and their hematopoietic derivatives are much more sensitive than cord blood-derived CD34+ cells to etoposide-induced MLL-r. In addition, undifferentiated human embryonic stem cells (hESCs) were concurrently predisposed to acute cell death (Bueno <i>et al.</i> 2009).</p> <p>Indeed, the evidence for MLL rearrangement <i>in utero</i> is quite strong based on IFL clinical data cord blood analysis.</p> <p>Published and unpublished (for permethrin and Chorpyrifos) data have</p>
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		<p>shown that topoII inhibitors induce MLL rearrangements in human prenatal cells (embryonic and fetal) at much higher frequency than in adult stem cells (Moneypenney, <i>Carcinogenesis</i> 2006, Bueno C, <i>Carcinogenesis</i> 2009, Blanco JG <i>FASEB J</i> 2004, Libura <i>Eur. J.Haematol.</i> 2005, van Waalwijk van Doorn-Khosrovani, S.B. <i>Carcinogenesis</i> 2007 etc.)</p> <p>Besides, this study has identified MLL rearrangements in Guthrie cards and neonatal blood spots (Ford, A.M. <i>et al.</i> 1993. <i>Nature</i>, Gale, K.B. <i>et al.</i> 1997 <i>PNAS</i>, Ross, J.A. <i>et al.</i> 1996 <i>Cancer Causes Control</i>) unequivocally demonstrating a prenatal (<i>in utero</i>) origin of MLL fusions in infant ALL.</p>
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Regulatory applicability		
<p>Considering the strength of evidence and current gaps/weaknesses, what would be the regulatory applicability of this AOP, in your opinion?</p>	<p><i>RI:</i> The AOP is definitely a relevant issue for regulatory purposes. However, under the indicated circumstances it is difficult to envisage how this parameter could be integrated in toxicological studies. The authors have suggested addressing the issue under OECD 443. However, they have not</p>	<p>This AOP presents a framework for potentially critical processes to be measurable when designing developmental toxicology studies. The intention of mentioning the regulatory studies is to show where in the regulatory process the endpoint potentially linked with IFL are explored</p>

	<p>defined which endpoint needs to be looked at for this AOP. For example, should induction of leukemia in the F1 or F2 generation be used as an endpoint or should F1 or possible F2 generations be assessed for MLL transformations? Nevertheless, as described above it remains uncertain whether rodent studies are appropriate models for this AOP.</p> <p>The use of <i>in vitro</i> cell systems may be an alternative (e.g. by using liver hematopoietic stem cells). However, the <i>in vivo</i> relevance of the effects has not been clearly demonstrated by agents other than etoposide.</p> <p>Thus, it would be necessary for authors to make a more explicit recommendation on how to incorporate this AOP in toxicological assessments for regulatory use.</p> <p>R2: Infant leukemia is a rare type of leukemia; it would be expected that increases induced by xenobiotics would be noticed if one were specifically looking for them. Otherwise, such increases would be difficult to detect. Since there is no convincing evidence showing that this type of leukemia has been caused by xenobiotics, the evidence will need to be strong in order to convince regulators and other stakeholders of a real association. In my opinion, the convincing evidence will need to come from appropriate cell and <i>in vivo</i> models as well as supporting evidence</p>	<p>and make clear the wealth of limitations. If this was not clear the authors will revise the write- Our intention is also describing the limitation of the current regulatory genotoxicity battery. This is not intended to challenge the overall genotoxicity paradigm, but rather to underline the fact that a different sensitivity can exist among the cell systems. This is one of the reasons for describing the MIE associated with the <i>in utero</i> window of exposure. Chlorpyrifos is an additional chemical for which this AOP is attracting a lot of interest. The authors will check about pyridone.</p> <p>A goal of this AOP is to better contextualize epidemiological studies in the backbone of risk assessment. If this is not clear it needs to be addressed because this was the main target of the work done and this AOP was included in a PPR Panel scientific opinion. The authors are now (in EFSA) proposing this approach with the hope of having a guidance for the interpretation of epidemiological studies ready in a relatively short timeframe that will include the use of the AOP to substantiate the mechanistic biological plausibility for epidemiological studies, foster the causality link on a mechanistic base and identify risk factors.</p> <p>The observation is relevant but does not invalidate the regulatory use of this AOP for the identification of risk factors or for an inclusion of the AO in the process of hazard identification for environmental chemicals. As mentioned above, if the biological plausibility for the KERs is strong the empirical support should be not considered as more relevant; this would be not in line with the AOP conceptual framework. In</p>
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	<p>in humans. Some of the bioflavonoids have shown Topo II inhibitory activity in acellular systems, but when tested in cells, the genotoxic effects that were observed occurred through an entirely different mechanism (c.f. Olaharski <i>et al.</i>, 2005 and Gollapudi <i>et al.</i> 2014).</p> <p><i>R3:</i> Taking into consideration uncertainties and inconsistencies summarized page 28-29, this AOP, in my opinion, cannot be used directly for regulatory applications because other factors in addition to MLLr or Topo II inhibition seem to be needed for the development of infant leukemia. However, I agree with the authors that this AOP can be useful in the MOA framework for specific chemicals, and could serve in guiding testing strategies.</p> <p><i>R4:</i> The authors are making this AOP specific to <i>in utero</i> exposure and to a specific chromosomal rearrangement, which makes it of limited regulatory applicability. Furthermore, it reduces the strength of evidence that supports the AOP. The cell type where the rearrangement takes place is unknown, and there is no experimental data to show that <i>in utero</i> exposure to Topo II inhibitors increases the frequencies of the leukemia-inducing rearrangements. There is not even a clear understanding about how this is occurring. As the authors state: "...topoII inhibition has to occur in an especially vulnerable and correct hot spot in the MLL locus; however, details of this process and how it happens are not clear".</p>	<p>addition, the regulators would appreciate the possible lack of sensitivity for the standard genotoxicity cell systems when dealing with such hazard and the poor reliability of the standard rodent carcinogenesis for capturing complex human diseases that could have an environmental component.</p> <p>For the reasons mentioned above the authors disagree with this comment. The biological plausibility (BP) linked to the cell type is very high, confirmed by the correspondence between the blood cord examination and the IFL. The <i>in utero</i> exposure is not explored in the regulatory process and such a strong non explored BP is considered by EFSA a strong weakness with a need or regulatory actions (see also the paper on Chlorpyrifos). Knowledge of the details of the process is not necessary for regulatory decisions and detailed knowledge is not something required to support BP (not causality) in the AOP conceptual framework. IFL has all the possibility of being associated with environmental toxicants and the metanalysis conducted with pesticides are indicating leukaemia as a consistent human health outcome associated with pesticides exposure. Considering the limited possibility of comparing regulatory toxicological studies with a so broad definition of the disease, the authors see a strong case of this AOP for including or excluding this MIE (and associated risk factors) in the risk assessment process of potential environmental toxicants like pesticides</p> <p>A link between etoposide/topoII inhibition and adult b-ALL cannot be</p>
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	<p>I guess one question that needs to be clarified is whether the authors see this AOP as a way to identify chemicals that have a role in infant leukemia or as a way to identify chemicals that act as topoII inhibitors. If the former, which I suspect is what the authors envision, I remain very skeptical of the utility of this AOP for regulatory purposes because of its narrow, to say it in OECD lingo, applicability domain. If the latter, the AOP would have a much broader regulatory application, but it will require substantial changes that are described in more details below.</p>	<p>ruled out. There are not enough epidemiological data to define the etiology of adult acute leukemia. Exposure to such compounds is likely to be oncogenic; however, the lack of access to the natural history of the disease impede us from having well-established unique oncogenic hits to be evaluated. The natural history is likely composed of a bunch of cooperating overlapping or sequential oncogenic hits so that weighting the impact topo II inhibitors on MLL fusions in an environment free of masking/confounding cooperation hits is very challenging, there is no way to track the impact of a compound from the initial hit to disease onset (many years).</p>
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Conclusion		
<p><i>What are your overall conclusions of the assessment of this AOP?</i></p>	<p>R2: The authors have put together multiple lines of evidence and made a good case that maternal exposure to a topoII poison could cause infant leukemia. However, until sufficient evidence is generated to show that this can be caused by a xenobiotic, the pathway remains largely theoretical. As a result, I see the AOP as being closer to a promising hypothesis than a clearly established mechanism of action.</p> <p>R4: My main concern with this AOP is the authors' insistence in making it specific to an <i>in utero</i> exposure. I see that this issue was brought up during the internal review within EAGMST and that the authors have resisted making the change. It seems to me that by making the AOP specific to infant leukemia, they are creating an AOP for a molecular initiating event (MIE) in a specific cell type and for a rearrangement in very specific region of the genome. As such, this AOP is an isolated AOP that does not contribute to expanding the network of key events (KEs) and is, in my</p>	<p>The authors support finalization of this AOP based on the strong BP</p> <p>The proposal of eliminating the <i>in utero</i> localization of the MIE was proposed by one out three of the OECD internal reviewers and was not supported, at least unanimously at the EAGMST meeting. In particular, the representative at the meeting of the US EPA was not supportive at all of the internal reviewer proposal. The authors understand that it is always a complex task to find a balance between the specificity of the MIE, KEs and MOA (as requested in the AOP guidance). The R4 seems very critical with this AOP but is proposing an AOP that will be even less substantiated, at least for the empirical point of view as we know for the attempt we made to develop an AOP for a different 2 hits leukaemia (see our EFSA Scientific Opinion, AOP</p>

	<p>opinion, of limited utility and almost outside of the spirit of the AOP program.</p> <p>To my knowledge, the mechanism by which topoII enzymes operate is the same in every cell type and so is the mechanism by which they create double strand breaks (DSB). Thus, the MIE (inhibition of topo II) and the first KE (formation of DSB) should be agnostic of the cell type. The authors can then make the next KE specific to fetal hematopoietic cells and to infant leukemia. Doing it in this way, would create a MIE and a KE that can be borrowed and used to build other AOPs, including one leading to secondary cancers in patients receiving topoII inhibitors as part of their chemotherapy. I would venture to say that the authors should create this branching of the AOP within their current submission.</p> <p>I appreciate that this would require significant work and rewriting; however, the evidence in support of their current AOP comes almost exclusively from the data in cancer patients. The authors have not convinced me that there is conclusive evidence that the MLL rearrangements found in infant leukemia arise through a mechanism that involves exclusively topoII enzymes. As the authors mention, the MLL gene is within a fragile site that is inherently susceptible to breakage and does not necessarily need topoII to create a DSB. Without the supporting evidence in cancer patients that rearrangements are seen in patients that have received topoII inhibitors, the authors have little empirical data to support their AOP. Furthermore, making this AOP more general, and not limited to <i>in utero</i> exposure, would allow for the measurements of the MIE and KEs in any cell type, including <i>in vitro</i> cell lines, where dose response relationships, temporal concordance and consistency of the experimental evidence can be better investigated. These <i>in vitro</i> studies would also provide a quantitative understanding</p>	<p>4). One could link AOP 202 by linking the KE1 (DNA DSB) which is common to many genotoxicity events, out of the <i>in utero</i> issue) and from this develop additional AOs more or less specific than this one.</p>
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	<p>of the relationships between KEs, an aspect that is currently lacking in the present AOP. This would also expand the number of chemicals that have been shown to interfere with topoII function.</p> <p>In summary, I believe that the present AOP requires significant revisions before it can move on to the next step in the acceptance process at the OECD.</p>	
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Annex 4: Reviewer 2 Marked copy of AOP 202 snapshot

Please see attached pdf file:  AOP topoisomerase
snapshot Eastmond r