Organisation for Economic Co-operation and Development

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Adverse Outcome Pathway External Review Report

AOP 212: Histone deacetylase inhibition leading to testicular atrophy

Short name: Histone deacetylase inhibition leading to testicular atrophy

The title of the AOP was revised as a result of the review.
Original Title of the AOP: Histone deacetylase inhibition leading to testicular toxicity

This document has been prepared by the review manager of AOP 212 scientific review.
It compiles the views and comments of the reviewers and explains how the authors of the AOP plan to address these comments.
It provides the basis to EAGMST for determining if AOP 212 has been adequately revised by their authors following the review and if it can be released to the Working group of the National Coordinators of the Test Guidelines Programme and the Working Party on Hazard Assessment for endorsement.

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1. Introduction and background to specific AOP

1.1. Background

AOP 212 ("Histone deacetylase inhibition leading to testicular atrophy") passed internal review and was approved for release to external/scientific review at the OECD EAGMST annual meeting in June 2018. In response to requests to WNT (letters ENV/EHS/ND/2018.10 and ENV/EHS/ND/2019.01), the review manager was nominated in June 2019. Three external reviewers nominated by the review organiser completed their reviews between July and September 2019. These reviews were collated by the review manager and sent to the AOP developer on 8 October 2019. An end-of-review teleconference (TC) was held on 8 November 2019. The developer provided responses to the reviewers on 30 January and 10 February 2020.

This external review procedure is based on the "Draft Standard Operating Procedure for Adverse Outcome Pathway Scientific Review", Revised 20 November 2018.

Review organiser: Magda Sachana, OECD Secretariat

Review manager: Rex FitzGerald, Swiss Centre for Applied Human Toxicology (SCAHT), University of Basel

1.2. Introduction

AOP 212 ("Histone deacetylase inhibition leading to testicular atrophy ") includes the description and assessment of the critical elements of the pathway initiated by inhibition of histone deacetylase leading to testicular toxicity. Testicular toxicity is of interest for human health risk assessment especially in terms of reproductive and developmental toxicity. The mechanisms contributing to testicular toxicity have not been fully elucidated until now and this AOP describes one of the potential pathways involved.

The Molecular Initiating Event (MIE) of this AOP is the histone deacetylase (HDAC) inhibition followed by histone acetylation increase, disrupted cell cycle, apoptosis, and spermatocyte depletion as key events (KEs). As Adverse Outcome (AO) has been defined the testicular atrophy and the AOP describes an intracellular mechanisms of induction of the spermatocyte apoptosis.

A range of Histone deacetylase inhibitors (HDIs) that are approved as anti-cancer drugs have apoptotic effect in cancer cells and can initiate this pathway. HDIs include the short chain fatty acids, hydroxamic acids, benzamides and epoxides. The HDIs inhibit deacetylation of the histone, leading to the increase in histone acetylation. The apoptosis induced by disrupted cell cycle leads to spermatocyte depletion and testis atrophy.

2. Synthesis of main issues of the review

Main issues from the reviewers are summarised in Annex 2, which were sent to the developer on 8 October 2019. In the same Annex, the responses to the reviews provided by the developer on 5 November are included that indicate also the updates/changes in the AOP-Wiki.

The main issues identified in the reviews were:

Is KE1504 ("p21 (CDKN1A) expression, increase") necessary?

What is a more precise definition of AO testicular toxicity (KE1506)?

Are the EU-ToxRisk descriptions useful?

Is Quantitative Understanding of KERs 1709, 1715, 1716 and 1717 high or medium?

3. Summary record of the teleconference

8 November 2019

End-of-review teleconference (TC) was held on 8 November 2019. It was attended by all reviewers, the author, the review organiser and the review manager (Annex 1).

3.1. Main issues and responses during the call

Summary of revisions made and action list of revisions to be undertaken are shown in **bold**.

The main issues were addressed as follows:

Is KE1504 ("p21 (CDKN1A) expression, increase") necessary?

Outcome: No. **KE1504 will be delinked from AOP212**. It remains in the AOP-Wiki, since it may be useful to other AOP developer.

What is a more precise definition of AO testicular toxicity (KE1506)?

Outcome: Developer suggested and reviewers agreed that "**testicular atrophy**" is more appropriate, so **the title of KE1506 and associated KERs 1734 and 1717, and of the overall AOP212, need to be changed accordingly**.

Are the EU-ToxRisk descriptions useful?

There was discussion as to whether the texts headed "Description from EU-ToxRisk deliverable" are too detailed and partly repetitive/redundant.

The developer considered them useful but not essential. Since they were written independently of the present AOP (by EU-ToxRisk collaborator Marvin Martens; see

history of changes in the AOP-Wiki), the **developer will need to confer with the author of this text** before making changes.

In any case, it was agreed that the **text "Description from EU-ToxRisk deliverable" should be deleted** from KE/KER/AOP texts, since it does not add useful information and there is no corresponding EU-ToxRisk reference.

Is Quantitative Understanding of KERs 1709, 1715, 1716 and 1717 high or medium?

Developer proposed to **change all to "Moderate"**. This was acceptable to all reviewers.

Other issues:

For KE1502 "Histone deacetylase inhibition", Sex Applicability refers only to males. Although there is good data for males, it was noted that this KE is in principle not gender-specific, so it was agreed to **change Sex Applicability to "unspecific"**.

For KE1506, the label for Process/Object/Action is "testicular atrophy/Testis/decreased". It was agreed to **change Action "decreased" to "N/A"**.

It was noted that **a new KER is needed for KE1502 - KE 1505**.

3.2. Action list with responses from author (after the end-of-review teleconference)

Responses to the reviewers' comments were sent by the developer to reviewers and review manager on 30 January 2020, and revised after feedback from the reviewers on 10 February 2020. These responses are summarized below:

Abstract

Revised in accordance with reviewer comments.

KE1502 Histone deacetylase inhibition (=MIE)

Description and measurement rewritten, life Stage Applicability revised to All life stages.

References corrected: Richon 2003, Villae-Garea and Esteller 2007.

Sentence "HDAC 1, 4, 6 are related to tumor size [Damaskos, 2016]" deleted (considered irrelevant to MIE by reviewer).

Sentences "MAA (2 or 5 mM) inhibited HDAC activity in dose-response manner in rat testis cytosolic and nuclear extracts" and "MAA (2 or 5 mM) inhibited HDAC activity in dose-response manner in rat testis cytosolic and nuclear extracts" deleted because reviewer considered them too specific for the overview of the MIE (but OK in KER).

Citation to Steffan 2001 "HDIs reduced lethality in Drosophila model" deleted because reviewer considered it misleading (paper showed HDAC inhibition led to reversal of inhibition of histone acetylase activity by abnormal huntingtin protein).

Description of Desjardins 2016 queried by reviewer, corrected to "HDAC inhibition restores the rate of resorption of subretinal blebs in hyper glycemia in brown Norway rat".

Description for Ansari (2016) queried by reviewer, changed from "HDIs were approved as drugs for multiple myeloma and T-cell lymphoma by FDA" to "Treatment of HDIs

inducing HDAC inhibition showed anti-tumor effects in human non-small cell lung cancer cells".

Citation to Miyanaga 2008 queried by reviewer (assumed effects of HDIs, not explicit HDAC inhibition), text changed correspondingly.

Jansen 2004 reference checked and updated.

Chung 2003 citation suggesting that HDAC inhibitors actively acetylate histones queried by reviewer, changed to "The treatment with HDAC inhibitors, phenylbutyrate (PB) (2 mM) and TSA (200 nM), inhibits HDAC in adjuvant arthritis synovial cells derived from rats, causing higher acetylated histone."

KE description modified as suggested by reviewer ("HDIs do not actively acetylate histones, although it is correct that HDI activity leads to hyperacetylation. It would be best to rephrase the sentence to "Histone deacetylase inhibitors (HDIs) inhibit HDAC and lead to hyperacetylation of histones, resulting in an open chromatin state in which DNA is more accessible by transcription factors."

In Description, sentence and reference referring to HDIs as cancer treatments (last sentence of first paragraph) deleted, as considered by reviewer to be not relevant.

Description from EU-ToxRisk deliverable rearranged in accordance with reviewer comment (redundant information, needs rephrasing and rearrangement).

Sentence "The measurement of HDAC inhibition monitors the decrease in histone acetylation" reworded to "The measurement of HDAC inhibition monitors changes in histone acetylation" in response to reviewer comment ("very confusing, since HDIs lead to increased acetylation").

Descriptions of ChIP-chip and ChIP deleted ("Epigenetic modifications including the histone acetylation are measured using chromatin immunoprecipitation-microarray hybridization (ChIP-chip) [ENCODE Project Consortium, 2004, Ren, 2004]. ChIP detects physical interaction between transcription factors or cofactors and the chromosome [Johnson, 2007]"), in response to reviewer comment ("ChIP-chip and ChIP, in my opinion, can only be a very indirect measure for HDAC inhibition").

Description of HDAC activity assays added, as proposed by reviewer ("would advise to more focus on direct activity tests, as written in the EU-ToxRisk deliverable").

KE1503: Histone acetylation, increase

Description and measurement revised.

Measurement method (second bullet point of the EU-ToxRisk deliverable; Quantitative enzyme assays using acetylated peptides and purified HDAC enzyme) moved to KE1502, in accordance with reviewer suggestion ("should be moved to KE1502").

KE1504: p21 (CDKN1A) expression, increase

Delinked from AOP212, in accordance with end-of-review teleconference.

KE1505: cell cycle, disrupted

Description, measurement and Domain of Applicability revised.

KE description revised in accordance with reviewer comments ("KE description is too short. Maybe a general introduction to the cell cycle would be helpful in this respect"):

"The dysregulation of cell cycle leads to a decrease in the cell number. The cell cycle consists of G1, S, G2, M, and G0 phase. The cell cycle regulation is disrupted by the cell cycle arrest in certain cell cycle phase. The histone gene expression is regulated in cell cycle phases [Heintz, 1983]."

“The dysregulation of cell cycle leads to the decrease in cells” changed to “The disruption of the cell cycle leads to a decrease in cell numbers.” in accordance with reviewer suggestion.

Sentence “The phosphorylation of p21 …” deleted in accordance with reviewer comment ("too general and seems to better fit into the previous KE").

Following sentence also deleted in accordance with reviewer comment ("does not fit into a KE, because it is a direct effect of substances"

Description text moved to KER1715 in accordance with reviewer suggestion ("description would probably better fit into a KER"):

"The change in the amounts of cells in G1 phase and S phase of cell cycle was detected in mouse HDAC1 knock out fibroblast lines (Mus musculus) [Zupkovitz, 2010]."

Reviewer comment "For this KE description a general introduction is missing and most explanations probably better fit into KE1504. Since the measurements of both KEs are very different, it probably is good to leave them separately, but then both descriptions need a thorough revision." KE1504 has been delinked from this AOP212.

KE1262: Apoptosis

Description and measurement rewritten.

Specific information on HDIs deleted.

Domain of Applicability revised.

Description in Domain of Applicability moved to KER1716 with slight modification, in accordance with reviewer comments.

Three methods for detection of apoptosis (Parajuli 2014, Glaser 2003, Zupkovitz 2010) deleted, in accordance with reviewer comment ("[methods] are by no means apoptosis specific").

KE1515: spermatocyte depletion

Description, measurement and Domain of Applicability revised.

Bullet points in Domain of Applicability revised in accordance with reviewer comments.

New non-adjacent KER2010 added to link HDAC inhibition and spermatocyte depletion, and reference [Wade, 2008] cited there.

Sentence “The apoptosis of the cells leads to spermatocyte depletion” moved to KER1735, in accordance with reviewer suggestion.

Process of spermatogenesis described more in detail (ex de Kretser et al. 2016), in accordance with reviewer comments ("maybe concentrating on further molecular mechanisms controlling spermatogenesis").

Wade et al. (2008) methods for measuring apoptosis in spermatocytes moved to the method section for apoptosis, in accordance with reviewer suggestion.

KE1506: testicular toxicity (AO)

Name changed to “testicular atrophy, increased” from “testicular toxicity”, based on the discussion in end-of-review teleconference, and definition added.

Content revised in accordance with reviewer comments and suggestions:

Description in domain of applicability revised to focus on testicular atrophy;

Measurement or detection description revised to be more readable (bullet points);

Regulatory significance of the Adverse Outcome revised.

KE1709: Histone deacetylase inhibition leads to Histone acetylation, increase

Oryctolagus cuniculus and Brassica napus added in Taxonomic Applicability.

“SAHA and MS-275 hyperacetylates lysine of…” changed to “SAHA and MS-275 lead to the hyperacetylation of lysine…”.

Description in Response-response relationships revised according to reviewer suggestion ("more passive").

"EU-ToxRisk Deliverables" text rewritten, in accordance with reviewer suggestion ("make more readable" and "direct references to HDIs should not be included in the biological plausibility").

Text based on Choudhary (2009) revised according to reviewer suggestion.

KER1710: Histone acetylation, increase, leads to p21 (CDKN1A) expression, increase

Delinked from AOP212, in accordance with the end-of-review teleconference.

KER1711: p21 (CDKN1A) expression, increase, leads to cell cycle, disrupted

Delinked from AOP212, in accordance with the end-of-review teleconference.

KER1712: cell cycle, disrupted, leads to Apoptosis

KER revised in accordance with reviewer comments ("as it is now, it is not understandable").

Description revised in accordance with reviewer comments ("[text] very difficult to understand").

Third bullet in empirical evidence revised in accordance with reviewer comments ("could probably be improved by rewording the statements").

Uncertainties and Inconsistencies revised in accordance with reviewer comments ("do not understand").

Response-response relationship revised.

KER1735: Apoptosis leads to spermatocyte depletion

Publication by Brinworth et al (1995) cited and emphasized in biological plausibility and KER description; original references and description in biological plausibility moved to empirical evidence, in accordance with reviewer comments.

Empirical Evidence revised (to define Sucla2).

KER1734 spermatocyte depletion leads to testicular toxicity

Rewritten in response to reviewer comments (testicular toxicity such as testicular atrophy, which is the new AO).

KER1714: Histone deacetylase inhibition leads to p21 (CDKN1A) expression, increase

Delinked from AOP212, in accordance with end-of-review teleconference.

KER1715: Histone deacetylase inhibition leads to cell cycle, disrupted

Species conservation, biological plausibility, empirical evidence/support bullet point (including Richon et al 2000 citation) and Uncertainties and Inconsistencies text revised according to reviewer suggestions.

KER1716: Histone deacetylase inhibition leads to Apoptosis

Species Conservation, Biological Plausibility, Empirical Evidence and Uncertainties and Inconsistencies text revised according to reviewer suggestions. Weight of evidence and quantitative understanding set as moderate.

KER1717: Histone deacetylase inhibition leads to testicular toxicity

Title changed to Histone deacetylase inhibition leads to Testicular atrophy.

Added new KER2010 entitled Histone deacetylase inhibition leads to Spermatocyte depletion. References Yerby et al and Kose-Ozlece et al added to new KER2010. References Oishi (1994), Oishi (2001), El-Awady et al (2015), Yamazoe et al (2015), Dayan et al (2014), and Oishi (2004) deleted from KER1717.

Uncertainties and Inconsistencies revised.

Weight of evidence and quantitative understanding changed to moderate (reviewer said "For this KER I suggest changing weight of evidence to moderate and quantitative understanding to low.").

Overall

Description "…, of which MAA especially focused on have the testicular toxicity such as testis atrophy in vivo" deleted, in accordance with reviewer suggestion ("restricts the AOP too much onto one substance").

Quantitative Understanding revised to Moderate, according to discussion in end-of-review teleconference

Considerations for Potential Applications of the AOP revised.

References now in consistent format and in alphabetical order.

4. Outcome of the external review

Developer has implemented all agreed changes and provided responses to the reviewers (30 January and 10 February 2020); the AOP-Wiki has been correspondingly updated (10 February 2020).

AOP 212 is now (11 February 2020) considered acceptable by the reviewers.

Annex 1: List of Reviewers, Author and Review manager

Expert name	Affiliation	Representing country
Josephine Kugler	German Federal Institute for Risk Assessment (BfR)	Germany
Iva Sovadinova	Research Centre for Toxic Compounds in the Environment Faculty of Science Masaryk University Brno	Czech Republic
Mike Wade	Health Canada	Canada

Author	Affiliation
Shihori Tanabe	Division of Risk Assessment Centre for Biological Safety and Research National Institute of Health Sciences, Japan

Review Manager	Affiliation
Rex FitzGerald	Swiss Centre for Applied Human Toxicology (SCAHT), University of Basel

Annex 2 Main issues from the reviewers and responses

MIE	KER	KE1	KER	KER*	KE2	KER	KER*	KE3	KER	KER*	KE4	KER	KE5	KER	KER*	AO
1502	1709	1503	1710	1714	1504	1711	1715	1505	1712	1716	1262	1735	1515	1734	1717	1506
Histone deacetylase inhibition	**1502-1503**	**Histone acetylation, increase**	**1503-1504**	**1502-1504**	**p21 (CDKN1A) expression, increase**	**1504-1505**	**1502-1505**	**Cell cycle, disrupted**	**1505-1262**	**1502-1262**	**Apoptosis**	**1262 - 1515**	**Spermatocyte depletion**	**1515-1506**	**1502-1506**	**Testicular toxicity**
Review1																
OK	OK	OK			Weak (not clear that p21 is necessary event, suggest remove this KE)	Weak (data suggest that p21 is not the sole mediator of disrupted cell cycle)		Reasonable			Agree		Agree			Agree
Response																
				Deleted	Removed											
Review2																

Too much detail. Add EU-ToxRisk deliverable text.	Add taxa from bullet points (Oryctolagus cuniculus, Brassica napu). Possibly rewrite text in collaboration with EU-ToxRisk. HDI selectivity example too detailed and not relevant	OK, but methods redundant cf. MIE 1502. Suggest use EU-ToxRIsk text. [Overall] Empirical support section for MIE to KE1 is too long and too detailed	Too detailed. Rewrite short description to stress correlation of increased histone acetylation with increased p21 expression. Add "p21 expression is regulated by several pathways. "Quantitative understanding" belongs in KER 1714	Biological plausibility too detailed and does not state the "normal" status. Cite source paper s not Falkenberg and Johnston (2014) review. Declare non-histone HDAC targets as uncertainty	Some literature for KE 1505 seems to better fit in KE 1504	Taxonomic applicability, add Xenopus laevis. [Overall] needs major revision	Taxonomic applicability, add Mus musculus. Second part of text too detailed. Inconsistencies section, Don't understand 1st sentence; no inconsistency.	Description is too short. Most explanations probably better fit into KE 1504.	Very difficult to understand; need less and clearer details. Move description details to biological plauisibility or empirical evidence. Change weight of evidence and quantitative understanding to moderate.	Henderson et al (2016) text too detailed. Add other studies e.g. Brinkworth et al (1995). Parajuli et al (2014) not inconsistent.	Bullet points (spermatocyte depletion) before description belong in KER 1735	Add Brinworth et al 1995.	Use methods from Wade et al 2008. Bullet points before the KE description belong in KER 1735 (apoptosis to spermatocyte depletion)	Define what is meant by testicular atrophy. [Overview] should be independent of MIE	Many refs not appropriate; some OK for KER 1734. Add valproate testicular tox. Change weight of evidence to moderate and quantitative understanding to low.	Define what is meant by testicular atrophy. Methods described in too much detail. Measuring spermatogenic cells via flow cytometry belongs in KE 1515.
Response																
The EU-ToxRis	Oryctolagus cunicul	Empirical Eviden					Mus musculus has	The Key Event	Details in Descript	Simplified.	Moved to KER 1735.	Have been	Methods have	Defined.	The valproic acid-	Defined.

k Deliverable description has been added.	us and Brassia napus has been added in taxonomic applicability. Methods have been updated with EU-ToxRisk.	ce for MIE to KE1 (KER1706) has been updated with EU-ToxRisk Deliverable.					been added in Taxonomic applicability. The 1st sentence in the Empirical Evidence describing "In HDAC1-/- fibroblast lines, increase in the amount of cells in G1 phase and decrease in the amount of cells in S phase were observed, which indicates the importance of HDAC inhibition in cell cycle	Description has been updated with the sentences "The dysregulation of cell cycle leads to the decreases in cell number. The cell cycle consists of G1, S, G2, M, and G0 phase. The cell cyle regulation is disrupted by the cell cycle arrest in certain cell cycle phase."	ion has been moved to Evidence Supporting the KER Description in unvertainties and inconsistencies as "Methoxyacetic acid (MAA), a HDAC inhibitor, induced cell cycle arrest, apoptosis, leading to suppression of human prostate cancer cell growth [Parajuli, 2014]. It is not fully elucidated	Added.		added.	been added. Added to KER1735.	Updated to be independent of MIE.	induced testicular toxici with new reference Kallen B (2004) has been added Changed.	Moved.

MIE	KER	KE1	KER	KER*	KE2	KER	KER*	KE3	KER	KER*	KE4	KER	KE5	KER	KER*	AO
1502	1709	1503	1710	1714	1504	1711	1715	1505	1712	1716	1262	1735	1515	1734	1717	1506
Histone deacetylase inhibition	**1502-1503**	**Histone acetylation, increase**	**1503-1504**	**1502-1504**	**p21 (CDKN1A) expression, increase**	**1504-1505**	**1502-1505**	**Cell cycle, disrupted**	**1505-1262**	**1502-1262**	**Apoptosis**	**1262 - 1515**	**Spermatocyte depletion**	**1515-1506**	**1502-1506**	**Testicular toxicity**
							regulation [Zupkovitz, 2010]." has been deleted.		whether MAA-induced apoptosis is involved in p53/p63/p73 pathway [Parajuli, 2014]" has been deleted. Changed.							

MIE	KER	KE1	KER	KER*	KE2	KER	KER*	KE3	KER	KER*	KE4	KER	KE5	KER	KER*	AO
1502	1709	1503	1710	1714	1504	1711	1715	1505	1712	1716	1262	1735	1515	1734	1717	1506
Histone deacetylase inhibition	**1502-1503**	**Histone acetylation, increase**	**1503-1504**	**1502-1504**	**p21 (CDKN1A) expression, increase**	**1504-1505**	**1502-1505**	**Cell cycle, disrupted**	**1505-1262**	**1502-1262**	**Apoptosis**	**1262 - 1515**	**Spermatocyte depletion**	**1515-1506**	**1502-1506**	**Testicular toxicity**
Review3																
Need concise clear description for non-experts. Add assays. Why is life-stage only adult	Taxonomic applicability defined quite narrowly. Quantitative Understanding "high" not justified.	Description not clear as single isolated measurable event. Add assays.	Evidence how general? Taxonomic applicability defined quite narrowly	Biological plausibility text not clear	Add additional p21 arrest cell cycle mechanism [shouldn't this be in KER1711?]. Simplify assays. Taxonomic applicability not specified	Diabetes evidence relevant? Taxonomic applicability defined quite narrowly	Quantitative Understanding "high" not justified.	Description should be general; p21 info too specific. Simplify assay description. Generalize Domain of Applicability. Taxonomic applicability not specified	Add dose-concordance.	Empirical Evidence unclear. Quantitative Understanding "high" not justified.	Add general description. Assays not specific.	Define Sucla2	Omit CDKI specific info. More general assay description. Generalize Domain of Applicability. Taxonomic applicability not specified		Add uncertainty ref(s). Quantitative Understanding "high" not justified.	AO testicular atrophy? Add assay methods. Taxonomic applicability not specified
Response																

The Western blots and fluorimetric and colorimetric kits have been added as the description from EU-ToxRisk deliverable. Life stage has been changed into All life stages.	The taxonomic applicability has been updated with addition of Oryctolagus cuniculus and Brassia napus. Quantitative Understanding has been changed into "moderate".	Description from EU-ToxRisk Deliverable has been added.			This will be modified without KE1504.		Quantitative Understanding for KER1715 has been changed into "Moderate."	Description has been updated to be general with new reference by Heintz et al. Taxonomic applicability has been specified as Homo sapiens and Mus musculus.	The description as "Cell proliferation which was determined at daily intervals agter a 24-hr pulse of [3H]thymidine changed as the amount of DNA in the cultures changed. Cell death which was measured by lactic dehydrogenase (LDH) activity in the medium changed in parallel with the changes in cell proliferation [Lynch,	Evidence and Quantitative Understanding has been changed into Moderate.	The general description with new reference [Susan 2007] has been added. TUNEL assay with new reference Kressel M and Groscurth P (1994) has been added.	Sucla2 has been defined as a β subunit of succinyl coenzyme A synthase.	Omitted. Generalized. Rattus norvegicus has been added.		Changed.	Have been added. Rattus norvegicus has been added.

MIE	KER	KE1	KER	KER*	KE2	KER	KER*	KE3	KER	KER*	KE4	KER	KE5	KER	KER*	AO
1502	1709	1503	1710	1714	1504	1711	1715	1505	1712	1716	1262	1735	1515	1734	1717	1506
Histone deacetylase inhibition	1502-1503	Histone acetylation, increase	1503-1504	1502-1504	p21 (CDKN1A) expression, increase	1504-1505	1502-1505	Cell cycle, disrupted	1505-1262	1502-1262	Apoptosis	1262 - 1515	Spermatocyte depletion	1515-1506	1502-1506	Testicular toxicity
									1986]. The decrease in total DNA was measured, the increase in cell death was observed [Lynch, 1986]" has been added in the quantitative understanding.							

General remarks

Reviewer 1:

the two most critical issues to improve this AOP would be to better understand how HDAC inhibition links to spermatocyte apoptosis. The current pathway – through p21 – is poorly supported by available evidence. Perhaps, for now, this KE could be removed from the AOP.

The second big data gap is to understand why vorinostat, one of the most potent HDAC inhibitors, does not cause testis toxicity.

Answer to Reviewer 1:

Thank you very much for your valuable comments. The KE2 (KE1504) entitled "p21 (CDKN1A) expression, increase" may be removed from the current AOP 212.

The reason why vorinostat does not cause testis toxicity is a very important and interesting issue and need to be investigated.

Reviewer 2:

description and biological plausibility parts need to be revised.

- involvement of p21 in apoptosis seems to be rather indirect

- p21 can have very different effects from cellular proliferation or cell cycle arrest

- are all spermatocytes lost through apoptosis? Possible other mechanisms could be growth arrest / cell cycle arrest with resulting less spermatocytes or other forms of cell death like necrosis…

- KER 1712 is not easy to understand and needs to be rewritten

- KE testicular toxicity is unclear in regard to what actually is meant by toxicity

- Some of the texts are nicely written, while others are mere bullet points in text form. Often facts / details are not understandable, because an explaining sentence is completely missing.

Answer to Reviewer 2:

The description of p21 will be revised as suggested, and KE2 (KE1504) entitled "p21 (CDKN1A) expression, increase" will be deleted as suggested by Reviewer 1.

KER 1712 will be revised to be easier to read.

KE testicular toxicity will be revised to include the more detailed description about how it is related to the toxicity.

Explaining sentence was added to be more understandable.

Reviewer 3:

The EU-ToxRisk deliverables are provided. I'm not sure why. These deliverables do not bring nothing more for AOP/KE/KER descriptions than the authors already mentioned

Taxonomic applicability is not specified for KE1504, 1505, 1515, 1506

The weight of evidence judgement/scoring for KEs, KERs and the overall AOP are mostly adequately described, justified and follow the User's Handbook guidance. In my opinion, the authors did not provide such information for HIGH evidence for the Quantitative Understanding of the KER1709, 1715, 1716 and 1717. The authors did not define how much change in the upstream KE, and/or for how long, is needed to elicit a detectable and defined change in the downstream KE. I'd rather describe the level as MODERATE

AOP focuses on drugs/therapeutics inhibiting HDACs; suggest keep less specific.

Answer to Reviewer 3:

EU-ToxRisk deliverables can be deleted, although I think that the content of the KER has been modified by different person other than authors. I have checked the history of KERs and noticed that someone other than authors is kind enough for providing the EU-ToxRisk deliverables. We may discuss with that person.

Taxonomic applicability for KE1504, 1505, 1515, and 1506 will be specified as possible.

The weight of evidence for the Quantitative Understanding of the KER1709, 1715, 1716 and 1717 will be changed into MODERATE.

I agree with the idea to keep less specific. Is there any specific descriptions to be less specific? Please specify the descriptions.

Main issues for discussion during the "End of review" teleconference (TC):

Is KE2 (1504; p21 (CDKN1A) expression, increase) necessary?

A. KE2 will be deleted.

What is a more precise definition of AO testicular toxicity (1506)?

A. I think that it would be testicular atrophy, but any more precise definition would be welcomed.

Are the EU-ToxRisk descriptions useful?

A. I am not sure whether these descriptions can be included or not. It can be useful, meanwhile the AOP can consist without the description. Either way is fine at least personally. We may need to discuss about this addition of EU-ToxRisk by Marvin Martens according to the history of changes in the AOP-Wiki, all together further.

Is Quantitative Understanding of KERs 1709, 1715, 1716 and 1717 high or medium?

A. These will be changed into Moderate.

Any remaining conflicts between reviewers, or are all other comments OK?

A. All comments are OK, thank you very much for your time and efforts for reviewing the manuscript. I don't have any conflicts between reviewers.