Silver Tox Experts (TE) meeting

Minutes, Meeting 25 February 2020 (9:00-16:00 CET)

1 Welcome and Introduction

1.1 Reminder on Confidentiality and Competition Law

Participants were reminded on their obligation to comply with Confidentiality and Competition Law.

1.2 Tour de table and apologies

The list of participants is available in Annex 1.

1.3 Approval of the agenda

The agenda is available on slide 3 in Annex 2.

2 AgAc gut biome study: Autometallography (AMG) results and interpretation

2.1 AMG slides brain and testes

Cf. slides 4-10 in Annex 2 for background recap on AgAc gut biome study. Rats were dosed for 10 weeks with AgAc via dietary admin (0, 0.4, 4 and 40 mg AgAc/kg bw/d). The aim of the AM session of today's meeting is to view in person and discuss the results of the AMG analysis on brain and testis. Prof Lison discussed the slides with two pathologist ahead of the meeting. AMG was selected to check morphologically if Ag was present in the organs/parenchyma and the vascular system. Cf. slides prof. Lison for principle AMG: staining adds Ag° to spots where metals (mainly Ag in this case) are present - the technique amplifies and enlarges these spots. Note that also other molecules/organelles can be stained with the technique. AMG allows to qualitatively identify but quantification is not possible. **AP1**

Prof. Lison goes through the slides of the **brain AMG**:

- Slides were prepared for male and female brains. Tissue of one animal per 'cage' (whereby each cage contained 2 animals of similar sex) was taken. A slide was prepared of a frontal cut through the middle of the brain.
- In the control, only some incidental (non-relevant) spots and blood vessels are stained.
- For the HD (40 mg AgAc/kg bw/d) animals (consistent observations between male and female), there is clear black staining of the blood vessels & capillaries (basal membranes) as was expected, but also in the brain parenchyma tissue (dotted pattern in the cells). Lot of staining is observed in the choroid plexus. There is a homogenous distribution of the dotted pattern in the parenchyma tissue suggesting no preferred localisation. **This suggests presence of Ag deposits in the brain cells and suggests that Ag is able to pass the BBB** (Blood Brain Barrier) (it is noted that formally, we cannot prove that the staining is actually Ag and that cellular damage could also cause the AMG staining).
- As only one brain section has been investigated, it is unclear if this 'homogeneous' distribution is throughout the whole brain, or if there are other brain sections where no or even higher accumulation occurred (cerebellum might be a target according to literature, but this has not been sampled in the slide). Also, it is very difficult to identify cell types in the brain after AMG staining, and thus assign the Ag staining to a certain cell type.

- Electronmicroscopical investigations on the tissues is not possible since they are preserved in formaldehyde.
- Although neurotoxicological investigations have not been performed during the study, no 'abnormal' behaviour has been noted for any of the test animals up to 10 wks dosing.
- Neuropathologists have preliminary investigated the slides and they agree that there is Ag present in the neurons. Prof. Lison will organise another meeting with a human neuropathologist to better interpret the images. **AP2**
- In the MD animals (4 mg AgAc/kg/d), you mainly see staining in the parenchyma and much less in the blood vessels (compared to the HD animals) while there was a clear increase in blood Ag levels. This indicates that Ag passes the BBB to the parenchyma before the blood vessels are saturated, which is counter-intuitive (you would expect Ag to accumulate in the blood vessels first and only pass the BBB once the Ag concentration reaches a certain limit). A hypothesis to explain this observation is facilitated transport of Ag e.g. by Cu transporter (this might also explain passing of the BTB (Blood Testes Barrier)) or transferrin binding (although latter is less likely due to its metal(3+) binding specificity) and deposition in S/Se rich areas.
- The same trend is observed in different animals and there are no differences between males & females (although the Ag blood levels are slightly higher in the females than in the males).
- It is unlikely that Ag is transported and accumulated in the brain as ionic form. Ag precipitates immediately in biological media, and it is much more likely that it is transported & accumulated as a sulphide or selenide.
- It is not clear why some cells are stained and others are not. Possible hypotheses are this is related to cell activity or form of Ag transport.
- In literature, hippocampal damage has been described in different studies with Ag. What could be further done to demonstrate damage in the HD brains? It is suggested to further examine the adjacent slides without AMG staining, to see if there is any damage (compare control and HD blindly, and include MD if damage is observed at HD). Prof. Lison will further check with a neuropathologist, taking into account that we need the results ASAP, and latest by end of March. **AP2**
- Prof. Lison already looked microscopically at cells with AMG staining and did not observe any damage.

Prof. Lison goes through the slides of the **testes AMG**:

- Slides were prepared for a single animal per cage, and tissue samples of both testes were included. Depending on the positioning of the testes during slide preparation, sections were taken longitudinal or transversal.
- Observations are similar to brain tissue. In control treatments, besides some isolated / non-relevant spots / areas, all is clear. The basement membrane of the seminiferous tubules is stained as well (known phenomenon to pathologist). For the MD and HD animals, there are again clear observations of stained dots in the tissue / tubules (mainly the Sertoli cells) and a strong staining all basement membranes. Gonadal cells are occasionally stained as well (logic as they have limited time to potentially accumulate Ag internally).
- Primarily the Sertoli cells are stained, which is hypothesised to be related to the presence of Cu transporters to these cell types.
- To further localise and identify (speciation) Ag in the cells, immunohistochemistry, EDX, XFT… can be considered, but **no analysis with either of these techniques has been agreed upon**.

The brain and testes slides will be further examined and discussed with a neuropathologist. **AP2**

Reduction of **ferroxidase activity** (and consequent potential indication of anaemia) in AgAc biome study was striking to prof. Lison (in MD and HD, at 4 weeks and 10 weeks). Unfortunately, no haematology has been performed on the test animals (like haemoglobin (Hb) or red blood cells (RBC) levels). Prof Lison suggests to look at Hb or RBC levels in future studies (included in EOGRTS, not in TK study). EPMF to look at ESTF study ($AgNO_3$, oral gavage, 4 weeks) and Boudreau study to check if they observed anything in the haematology parameters. **AP3**

From a first check during the meeting, there seems to be no notable effect on the haematology parameters in the ESTF study (despite increase in blood Ag levels). M Raffray doesn't remember clear effects on the haemoglobin in other Ag studies. It needs to considered as well that the Lison study applied dietary administration whereas the ESTF and Boudreau studies used gavage.

It is suggested to do a desktop study on the link between Ag and Cu transport (to check the hypothesis that Ag 'hijacks' the Cu transport system). The available evidence looks conflicting but there is some evidence that the Cu transporter can transport Ag. **AP4**

The results of the AgAc gut biome study can be considered for publishing in at least two papers:

1. Gut biome results (key message: AgAc less potent than nanoAg)
2. Systemic effects (incl. blood parameters) and AMG results.

The participants agree that especially the latter results should not be published yet. The EPMF will inform Prof Lison shortly if results on the gut biome can be published. For the second publication, prof Lison agrees to wait with publishing until the EPMF approves the submission. The EPMF secretariat will also provide some useful references on potential neurotox of Ag to Prof. Lison. **AP5-6**

2.2 Changes Ag workplan triggered by AMG results

A short recap of the meeting with prof. Lison is given for those dialling in. It is noted that prof. Lison's observations are not new; Danish groups have already demonstrated Ag presence in the brain in the 70s & 80s but the pattern in the neurons was not clear. **AP7**

There are different levels where potentially further scientific evidence / reassurance can be obtained:

1) Ultrastructural / localisational level: chemical identification of deposits (technique to be defined).
2) Pathological level: what does the presence of the observed lesions mean?
3) Functional level: is there an effect on the behaviour of the exposed animals? This aspect will be investigated in the EOGRTS.

Questions / comments by the Ag TE:

- It is difficult to make any statements about the impact of the distance between the neurons and the blood vessels on the staining, as it is difficult to see small vessels on the slides and we only have a 2D representation of a 3D structure.
- **Putting all evidence together, the TE agree that the AMG results suggest the likely occurrence of (dose-related) Ag deposits in the brain after Ag exposure and it is concerning that Ag seems to pass the BBB**.

Agreed follow-up actions:

- **Desktop study on Ag and Cu transport. AP4**
- **Neuropathologist to read slides blind (first HD and control to see if they can differentiate, with special attention to the hippocampal region) and see if they can find any damage. If so, the MD should be included as well AP2**

The AgAc gut biome study is limited in terms of further info we can get out. It is suggested to use the **EOGRTS DRF** (or 'prelim OECD443' as referred to in the slides / by Covance) to do some further investigations. More neuropath could be performed on the pups, or TEM / EDX on pup brains to investigate where / in which form Ag deposits are. This will give more information before running the main EOGRTS.

The TE would like 1 week to go through the AMG slides (incl. zoomed in slides) and the DRF study design to suggest modifications to the study design. **AP7-8**

3 Ag TK testing

3.1 Status update

Cf. slides 14-18 in Annex 2. Questions / comments:

- The **dosing** of AgAc in the TK study has been based on previous discussions for the EOGRTS DRF dose setting and we might re-consider the dosing of the EOGRTS DRF (see below). There is however no need to change the TK dosing regime. If the studies proceed as expected, both the results of the TK study and the results of the palatability study will inform on the dose setting for the EOGRTS DRF.
- **AgNP and AgMP characterisation program** (cf. slide 17 in Annex 2): the AgNP behaviour is ok in both vehicles but the AgMP behaviour in the gavage vehicle (1% MC) is problematic with only 20-50% total Ag recovery. This is most likely due to sedimentation. Follow-up investigations for the AgMP: use more vigorous vortex mixing by magnetic stirring combined with use of lower viscosity MC or lower MC concentration (of same viscosity).
- For **read-across** purposes, it is suggested to also perform Ag localisation in the brain / testes for the different Ag substances if this doesn't delay the TK program and if this doesn't impact on the Ag analysis in the brain / testes (i.e. if enough tissue remains for Ag distribution analysis). It is suggested to ask prof. Lison for the required tissue processing protocol for Ag localisation and if he is willing to perform the Ag localisation. Proposal would be to send half of brain and 1 testis to prof. Lison and use the other half for Ag analysis (needs to be verified with Covance/Arcinova). Female reproductive organs don't need to be included for Ag localisation work. The TE agree to investigate feasibility of this suggestion. **AP14-15**

3.2 SZZ TK testing

Cf. slides 19-23 in Annex 2. Comments / questions:

- It will not be possible to add SZZ as test substance to the current TK study as this would delay the study. It will have to be a follow-up study (also including at least one AgAc test dose as reference).
- Without TK data for SZZ, we cannot defend read-across from the SZZ carcinogenicity study to other Ag substances and an additional carcinogenicity study will most likely be requested with an ionic Ag substance. However, even with TK data for SZZ, it will be difficult to defend read-across (because of anticipated lower bioavailability of SZZ compared to ionic Ag / AgNP).

The TE agree and recommend that EPMF does not perform TK testing on SZZ.

4 Ag EOGRTs

4.1 Status update

Cf. slides 25-36 in Annex 2.

Dose setting:

- In line with ECHA requirements, the high dose animals need to show some signs of systemic effects (e.g. decrease in body weight), and dose setting should be supported and justified by a well-designed dose-range finding study.
- Cf. slides 25-28 in Annex 2. In the Williams 2014 study (gavage, 13 weeks), severe gastroenteritis was observed at 200 mg AgAc/kg bw/d so we want to stay below that for the DRF study.
- It is suggested to go for 120 or 160 for the DRF study to be a factor 3/4 of the Sprando HD (40 mg AgAc/kg bw/d). A dose of 100 mg AgAc/kg/d is reported to be well tolerated.
- If we go up to 160, we might encounter palatability issues, which would make the dose unusable in the DRF study. In that case, we might be challenged by ECHA as to why we didn't administer by gavage.
- If we assume the reprotox MoA of Ag is Cu driven, we need to select the LD so that it doesn't interfere with Cu.
- Cf. slide 36: the diet to be used by Covance is a breeder diet, with Se and Cu content higher than the Sprando study. It is suggested to **confirm the Se and Cu content of different diet lots in a pre-study.** **AP9**
- Cf. slide 30: dosing: a dose needs to be added between 4 and 40 (12 or 16 mg AgAc/kg bw/d).
- The dose setting will also depend on the results of the Ag TK study.

Sampling and analysis:

- Pup neuropath: it is suggested to look at brains of PND21 pups (brain development largely completed at that point) but also look later if possible. Covance was asked if they routinely see any difference between PND21 and PND70-80 for neuropath but we are still waiting for a reply.
- It is suggested to reconsider / **improve sampling and analyses in the DRF study** to maximise information we get out of this study, e.g. we might not need GD17 measurements but we might want to focus on Ag/Cu/Se measurements in tissues. Additional (tissue) samples can be taken at PND21. **It was agreed to create a wishlist with analysis vs different sampling occasions during the study, and organise a meeting with Covance (possibly at Arcinova site) to discuss EPMF wishlist.** **AP9-11**
- If you want to look at pup homogenates (e.g. measure Ag/Cu/Se content), PND4 is recommended to PND21.
- Arcinova is responsible for the analytical work but they are slow. **It is suggested to consider other CROs for the analytics if we do not get a clear update / timelines from Arcinova shortly.** **AP12**
- The palatability study could be brought forward in time without diet formulation analysis being performed, but then the study would not be fully GLP. This would give us more time to discuss the EOGRTS DRF setup before initiation. **AP13**
- Microbiome analysis: samples can be taken and stored for potential later analysis. It needs to be decided if samples should be taken from F0 and/or F1 (PDN21?) animals.

Timelines: cf. slide 35 in Annex 2.

4.2 Adding additional low dose

Cf. slide 37 in Annex 2. **The TE agree to the EPMF Secretariat's proposal to perform the EOGRTS as foreseen and decide on dose levels based on the results of the EOGRTS DRF study**, irrespective of the external request to include an additional low dose of 0.2 mg/kg bw/d**.**

5 AOB

No AOB.

Annexes

1. List of participants
2. Slides presented at the meeting

Actions

Table 1. Actions agreed at the 25 February Ag Tox Experts meeting

	What?	Who?	When?
Follow-up AgAc gut biome study – AMG results			
1.	Distribute Danscher & Stoltenberg 2006 publication to TE	EPMF Sec	w/e 28 Feb
2.	Discuss adjacent brain and testes slides in further detail with a neuropathologist (blind read – start with control and HD, include MD if differences observed at HD slides)	Prof. Lison	asap, but <end March
3.	Check ESTF study and Boudreau study for info on blood parameters	EPMF Sec	ongoing
4.	Inform EPMF Sec by when a desktop study could be performed on Ag and Cu transport	M Raffray	By mid-March
5.	Inform prof. Lison: (1) if AgAc gut biome results can be published (shortly) and (2) when the systemic results/AMG can be published	EPMF Sec	(1) By mid-March (2) As deemed appropriate by EPMF
6.	Share PfA neurotox defence doc with Prof. Lison	EPMF Sec	Done
7.	Share AMG slides AgAc gut biome study (incl. high magnifications) and neurotox references / PfA neurotox defence doc with Ag TE + send doodle to discuss follow up actions in EOGRTS DRF	EPMF Sec	ongoing
8.	Go through the AMG slides (incl. zoomed in slides) and the DRF study design to suggest modifications to the study design.	Ag TE	By mid-March
Ag EOGRTS			
9.	Make wishlist / matrix of samplings / analyses we want to include in the EOGRTS DRF study + analytically confirm Se & Cu content in breeder diet	L Aveyard	By end March
10.	Discuss and agree on wishlist of samplings / analyses to include in the EOGRTS DRF study	Ag TE	Early April
11.	Discuss and agree on wishlist of samplings / analyses to include in the EOGRTS DRF study	L Aveyard and EPMF Sec with Covance	At F2F meeting with Covance (timing tbc)
12.	Request clear update / timelines from Arcinova for the analytics and decide on that basis if we want to continue working with Arcinova + France to call Covance management (Mark Terry)	EPMF Sec	asap
13.	Keep pushing Covance to start palatability study ASAP	L Aveyard	asap
Ag TK testing			
14.	Check if prof. Lison can do Ag localisation in brain / testes from the Ag TK study & check way of processing the tissues	EPMF Sec	ongoing

15.	Check with Covance if they can sample and process half of the brain and 1 testis in the TK study for further external analysis without impacting on timeline / Ag distribution analysis	M Raffray	asap

Annex 1: Participants

Katrien ARIJS, consultant for EPMF (ARCHE, Belgium)

Lindsay AVEYARD, Consultant, GPC Consulting Ltd (United Kingdom) - reprotox study monitor

Arno BUTHE, Heraeus (Germany)

Marie-Laure LEDRICH, Consultant for Traxys (Luxembourg)

Dominique LISON, UCL (Belgium) - prof. responsible for AgAc gut biome study - only for agenda point 2.1

Jelle MERTENS, EPMF (Belgium)

Mark RAFFRAY, Consultant, Raffray Biosciences Ltd (United Kingdom) - TK study monitor

Steven VERBERCKMOES, Umicore (Belgium)

Via conference call; as from agenda point 2.2 onwards:

Eliot DEAG, Johnson Matthey (United Kingdom)

Olga LEMKE, BASF (Germany)

Nissanka RAJAPAKSE, Johnson Matthey (United Kingdom)