Silver Tox Experts (TE) Meeting

Draft minutes, Brussels, 15 March 2019 (13:00-16:15 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 4 in Annex 2. No remarks / additions; agenda approved.

1.4 Approval of the minutes of the last call (15 Feb) and status of action points

2 comments on the minutes were received from prof. Lison:

- The minutes mention that 2 months will be needed for estrogen analysis. However, prof. Lison will first need to check the literature and examine what should be measured before providing a proper time/cost estimate.
- Action point 2: checking the biological relevance of the observed changes in the gut microbiome is already covered by the contract with prof. Lison so no additional offer for this is needed.

The minutes of the call of 15 February 2019 were approved with abovementioned changes. A table with the status of the action points from this call is available on slide 5 in Annex 2. All action points have appropriately been addressed / are ongoing where possible, or will be discussed during the call.

2 Brief update on regulatory processes and timing

2.1 EOGRTS TP

TP process and timeline is available on slide 7 in Annex 2. Comments on the DD were submitted by 8 Feb 2019 and are now under consideration by ECHA, who may amend the DD accordingly. Afterwards, ECHA will notify the MSCA of the (amended) DD and they will have the chance to submit comments as well. The European Commission has informed us at the meeting of 13 March that the MSCAs have not been notified yet of our DD. Based on the decision-making process, we will not receive the final decision before June 2019 at the earliest. Our TP could be discussed at the June or October MSC meeting (if the TP is not handled via the written procedure). The TE stress that, if our TP goes to MSC, EPMF will need to be represented at the MSC meeting.

In any case, **EOGRTS test results would not be available before mid-2021 at the earliest**.

2.2 CLH silver nitrate

CLH process and timeline is available on slide 8 in Annex 2. Kemi has submitted the CLH proposal for silver nitrate in December 2018 and it is currently still in the accordance check phase. As soon as the dossier is compliant, the public consultation will start and 18 months later the RAC opinion has to be available. This means that the **RAC opinion on the silver nitrate CLH may be available before our EOGRTS test results are available**.

In the meantime, ESTF also reported that Kemi informed them that they will propose a Repr 1B classification for **elemental Ag** and that Kemi will submit the CLH dossier to ECHA soon.

The TE agree that further data to support read-across need to be generated soon in order to argue against classification of elemental Ag.

2.3 Outcome meeting with European Commission 13 March

Cf. slides 9-10 in Annex 2. The Commission suggested EPMF to submit comments during the CLH public consultation and Commission from their side could also inform RAC about the ongoing EOGRTS TP and ask RAC to take this in their opinion. RAC cannot delay its opinion but could include in their conclusions an opinion whether the classification may be changed or not by the EOGRTS outcome. This could be triggered by a specific question from the Commission but feasibility of this needs to be checked with ECHA and RAC. In order to do this, **the Commission needs a clear view from EPMF on the added value of the EOGRTS**. Therefore, EPMF will provide a table to the Commission soon listing the available evidence on Ag reprotox, what is currently missing and how the EOGRTS will address the data gaps.

(Post-meeting note: The requested table was drafted and sent to the Commission on 27 March 2019.)

3 AgAc gut biome study: results and interpretation

The original aim of the study and the previously discussed results are summarised on slides 12-15 in Annex 2. Additions / comments:

- The TE are reminded about the clear dose-dependent effect of AgAc treatment on **ferroxidase activities** (cf. slide 14 in Annex 2).
- While the **biome results** showed some statistically significant effects of AgAc treatment on rats (cf. slide 15 in Annex 2), it is recognised that the observed biome shifts are **not as remarkable as expected based on previous study results** (van den Brule et al. 2016, Williams et al. 2015). There are several possible explanations for this: different mode of administration (gavage in Williams et al. 2015), different species (mice in van den Brule et al. 2016) and different silver form (nanoAg in van den Brule et al. 2016) with different TK and different matrix influences. There is also the possibility of a very steep dose-response curve for biome effects. It is noted that the biological relevance of the observed biome changes in the EPMF study still needs to be determined but the TE note that the biome results of the study may be of limited use in our defence position around secondary effects.

3.1 Additional results

Cf. Annex 3 and slides 16-23 in Annex 2. Additions / comments:

- The **dosing expression** on the different graphs is inconsistent and confusing: the same dosing expression should be used in all graphs of the study report, mentioning both the calculated AgAc and Ag dose in mg/kg bw/d. Furthermore, the standard deviation should be mentioned in the graphs. **AP1**
- Slide 16: **Ag levels in blood** of rats are consistent with earlier results. Comparison of the blood Ag content to other oral TK studies shows that oral bioavailability is higher via gavage than dietary administration. Saturation kinetics were not achieved since exposure levels were too low.
- Slide 17: **Ag levels in reproductive organs**:
  - Females: Ag levels in uterus + ovaries (it was not possible to reliably separate the uterus from the ovaries during dissection) of the high-dose group are comparable with earlier results.

- Males: there is a dose dependent increase in Ag levels in testis of the mid- and high-dose groups. It is noted that the concentrations are significantly higher than the van der Zande et al. 2012 study (silver nitrate study in rat, via gavage), which already reported high Ag levels in testis. In our current study, the absolute values / blood Ag ratio are an order of magnitude higher than in van der Zande et al. 2012 (cf. Annex 4). This inconsistency will be checked with prof. Lison (**AP1**). Other reports on testicular levels / distribution are limited / conflicting and it is uncertain if Ag depots are outside of the blood testis barrier (BTB) and/or have penetrated the BTB. If the currently reported Ag levels in testis in the biome study are accurate, it is suggested to perform ultrastructural localisation of Ag in the testis (**AP2**). In addition to reporting high Ag levels in testis, van der Zande et al. also showed slow Ag clearance from testis.

- Slide 18-20: **Cu / Cp oxidase levels in serum**:
  - Serum Cu levels were not depressed in the low- and mid-dose groups but were depressed in the high-dose groups of both sexes to ± 40-50% of serum Cu levels in the control groups (slightly higher depression for males). In the high-dose female group (28 mg AgAc/kg bw/d), the serum ceruloplasmin (Cp) oxidase activity was depressed to 12% of the control.
  - The inferences of this moderate Cu deficiency regarding reprotox are not clear cut (cf. slide 20) but the link between Cu depletion and reproductive performance is already known. A further complication is that not only Cu levels are affected by AgAc exposure but also Cp and Se such that potential additive impacts on reproduction become a consideration . From other work, it is known that the structure of Cp can be distorted by Ag so it can no longer transport Cu.
  - The Sprando et al. study did not assess Cu markers, but dev tox was reported at 40 mg AgAc/kg bw/d and the study had a claimed dev tox LOAEL of 4 mg AgAc/kg bw/d.
- Slide 18 and slides 21-23: **Se levels in serum**:
  - Serum Se levels were not depressed in the low- and mid-dose groups but were depressed in the high-dose groups of both sexes to ± 50-70% of control group means values, dependent on timepoint of measurement (slightly higher depression for males).
  - Se has a major role in healthy reproduction (cf. slide 21).
  - There is a previous report of Ag treatment effect on Se: Yoshida et al. 1983. In this study, the achieved dose was higher than the high-dose in our biome study (confirmed by TK) and the Se reduction in plasma was also higher (but no reprotox parameters were assessed).
  - As an independent effect, the degree of Se depression in the biome study is probably not sufficient to seriously affect reproductive capacity in males/females (most sensitive parameter of Se in reproduction is the potential for adverse effect on pup growth). However, in the case of Ag we need to be mindful of the potential for combined effects factoring in also the Cu/Cp axis.
  - Se is now confirmed as a parameter in respect of the EOGRTS design (main study or enabling study) so it is suggested to measure Se in blood and key tissues (especially repr tract) and assess the activity of one or more Se-enzymes as these more closely relate to actual biological effects.
  - Se is central in thyroid hormone processing (via selenoprotein deiodinases). If effects on Se levels are observed, it is predictable that regulators would raise questions about ED via thyroid hormone axes. Sinc the EOGRTS already includes thyroid hormone assessments, this is a further reason to perform the EOGRTS.

- It is concluded that **the biome study has definitely moved forward our knowledge on AgAc effects on Cp, Cu and Se, and these parameters need to be taken into account for the EOGRTS test design and for further TK work**. There appears to be an effect on Cu + Cp + Se, and the main question is: at what point does Ag^+ trigger a composite deficiency state which is significant enough to impact on reproduction?
- The EOGRTS will be performed with AgAc but what about elemental Ag? Further comparative TK data will be generated for key Ag substances (including elemental Ag). For elemental Ag (bulk forms), it was speculated as unlikely that the critical Ag^+ level would be reached where Cu, Se and Cp are seriously affected.

3.2 Planned follow-up actions and additional investigations

Cf. slides 24-25 in Annex 2. Additions / comments:

- The TE agree to have the **uterus and ovaries histopath** performed by prof. Marbaix. **AP1**
- There is currently no need for further investigations on the uterus / ovaries (like e.g. ultrastructural localisation) so the full samples can be used for histopath.
- **Ultrastructural localisation of Ag in the brain**: since we are unsure if the autometallography technique will be sensitive enough for localisation, the received offers are considered too costly. However, the offer from Antwerp University includes quantification. The TE agree to start with visualisation and there is no need for quantification of Ag in the brain at this moment; making the analysis qualitative should reduce costs. As tissue set for localisation, the TE agree on brain, testis (if high Ag levels confirmed) and gut (if stored; highest levels of Ag predicted) or kidney (existing ultrastructural papers on Ag will permit cross-checking of technique outcomes) as positive control; 4 animals per sex. Liver could be done as well, but is considered non-essential. **AP2**
- We are still awaiting an estimation of costs / time for the short-chain fatty acid (**SCFA)** microbial metabolite markers **and estrogen analysis**.

3.3 Relevance to Ag+ defence and EOGRTS

Cf. slides 26-29 in Annex 2. Additions / comments:

- Classification of ionic Ag is likely (at least Repr Cat. 2). As stated previously, we need to investigate the **differentiation between elemental Ag and ionic Ag but also between elemental bulk Ag (micron plus size) and nanoAg**, in order to have evidence-based argumentation against a classification of elemental Ag. In this respect, a **comparative TK study** is essential as a first step. Key substances to be included are: elemental Ag (≥ micro), elemental nanoAg, AgAc and $AgNO_3$ (+ possibly AgCl and Ag_2O).
- Is there a precedent for a substance having a different classification for the bulk and the nanoform? Reference is made to Ni, where a separate TP for micron sized Ni was submitted because of issues with results for nanoNi (https://echa.europa.eu/information-on-chemicals/testing-proposals/current/-/substance-rev/22801/term).
- The biome study has confirmed the importance of the Cu/Cp axis. The TE agree that the **mechanistics of the Cu axis and relevance to human hazard assessment** needs to be further explored with regard to Repr Cat. 2 clasification.
- Have we considered what human data there are? Epidemiology data on reprotox and Ag do not exist. In addition, RAC (and some other regulators) tend to favour animal reprotox evidence over human epidemiology. **AP3**

(Post-meeting note: Below excerpt is taken from an industry (ECETOC) panel paper and gives more formal insight into the challenge of building a case based on human data, especially if based on retrospective studies:

> *'However, convincing human evidence of reproductive toxicity for a specific chemical is rarely available because it is often impossible to identify a population suitable for study that is exposed only to the chemical of interest. Human data may provide limited evidence of reproductive toxicity that indicates a need for further studies of the chemical; the test method selected should be based on the potential effect suspected.*
> *When evidence of a reproductive hazard has been derived from animal studies it is unlikely that the absence of evidence of this hazard in an exposed human population will negate the concerns raised by the animal model. This is because there will usually be methodological and statistical limitations to the human data. For example, statistical power calculations indicate that a prospective study with well-defined exposure during the first trimester with 300 pregnancies could identify only those developmental toxins that caused at least a 10-fold increase in the overall frequency of malformations; a study with around 1000 pregnancies would have power to identify only those developmental toxins that caused at least a 2-fold increase (EMEA/CHMP Guideline, 2006). Extensive, high quality and preferable prospective, data are necessary to support a conclusion that there is no risk from exposure to the chemical.')*

3.4 TK segment: mode of administration influence on Ag TK

Cf. slides 30-31 in Annex 2. Reprotox literature data on Ag often involve a different mode of administration. There is no study for Ag which directly compares the effect of different oral modes (gavage / drinking water / diet) but there is a comparative study for Mn (Mn is in terms of TK profile very similar to Ag): Foster et al. 2015; http://europepmc.org/articles/PMC4490190. this study shows higher bioavailability for gavage administration versus diet / drinking water (and some evidence that toxicity also corresponds with this precedence order). If we had chosen gavage for the biome study, we might have seen higher toxicity and a different picture of the biome results.

It is noted that for an EOGRTS, gavage administration has a significant cost impact because of handling. There is a preference for dietary administration since this was also the administration mode for the biome study (there may be some sequestration in food but we do see dose dependent Ag levels in blood). **There is TE consensus around dietary mode of admin for the AgAc EOGRTS.**

4 Preparation EOGRTS

Cf. slides 33-35 in Annex 2. Dose setting for the EOGRTS: available dose level versus effect information is summarised on slide 34:

- The TE consider the argument on human exposure of secondary relevance to dose-setting.
- Currently available effect data are considered insufficient for definitive dose setting for the EOGRTS and **further DRF work is needed**.
- Given dietary admin for EOGRTS, enabling DRF work (and reprotox specific TK if performed) has to be configured accordingly. A disadvantage of dietary admin is that you cannot get the same precision than with gavage so we will need to be careful in selecting a lab.
- In the Sprando et al. drinking water study, no systemic toxicity was observed at the high-dose of 40 mg/kg bw/d, so we would need to dose higher than that.

- A total loss of Cu and Cp activity was observed at a AgCl level corresponding to 60 mg AgAc/kg bw/d, but since we currently cannot consider this an 'adverse effect', we need to dose higher. It is suggested to get an expert opinion on the Cu/Cp effect.
- In the Williams et al. study, 'some toxicity' was observed at 100 mg AgAc/kg bw/d via gavage. We would have to dose higher in a dietary EOGRTS or at least explore this via the DRF.
- Saturation kinetics would be well above 100 mg AgAc/kg bw/d.
- Based on the Williams et al. study (using gavage), it is expected that clear biome effects will be observed at > 100 mg AgAc/kg bw/d. Whether this is replicated using dietary administration is to be determined. Biome parameters should be measured during the DRF.
- **Suggested high-dose for DRF: 125 mg AgAc/kg bw/d.** If half-log spacing of levels is then employed, there would be a risk of non-assured NOAEL for devtox, if outcomes mirror those of Sprando. Therefore, 4 dose-levels may be needed in the EOGRTS. **Total of 4 doses suggested to ensure proper identification NOAEL/LOAEL: 125 - 40 - 13 – 4 mg AgAc/kg bw/d.**
- Proposed study design:
    - **Dietary OECD 422 study with AgAc as DRF**, measuring the usual parameters + Cu, Se, Cp parameters (given biome study has confirmed importance of Ag^+ effect on Cu, Se, Cp) + TK parameters.
    - **Concurrent dietary TK studies for elemental Ag (≥ micro), elemental nanoAg and $AgNO_3$.** Repeated dosing is suggested for the TK studies, in order to reach steady-state.
    - Dietary admin for both DRF and TK would allow direct comparison between results.

    *(Post-meeting note: After the meeting, M Raffray suggested an **alternative study design** for the Ag substance comparative TK work, whereby gavage is main mode for TK with linkage to dietary DRF via integrated TK in that study. This because there are some disadvantages related to running the TK work via the dietary mode (e.g. not possible to derive absolute bioavailability, higher inter-individual TK variability). A more conventional TK design gavage-i.v. couplet (test substance(s) dosed by gavage, repeat dose; with i.v. only needed as a single dose on d1 in satellite animal groups) could give us better information: possibility to derive absolute bioavailability, plus some other technical advantages when considering our aim of establishing definitive comparative TK between Ag substances for read-across, and maybe also a dataset that could be used to calibrate a PBTK model. A further practical advantage is study setup time – if gavage is used this will avoid startup work on dietary analysis, stability, palatability etc. for the substances other than AgAc.)*
- It is suggested to reduce costs by going for a non-GLP study, as this is not a formal toxicity study we would use for regulatory compliance, and reporting would be accelerated.

  (Post-meeting note: If any of the studies will end up being submitted, e.g. in the respective dossiers, then a rationale for GLP is strengthened. The availability of GLP compliant TK (OECD 417) will also be useful in defence of any advocacy we need. Assuming the decision is GLP at a CRO, there likely won't be much difference in the time to the point of a preliminary report and the outcome information we need.)
- **TK study duration**: suggested 14 days. Although definitive data is not available for each of the Ag substances being considered, a case can be made that steady-state kinetics is predicted by 14 days (section in the TK data mining report relates).

(Post-meeting note: Suggestion to run the TK to 28 days as this would remove all doubt (from the mind of any doubting third party) that steady-state has been achieved. The option exists to truncate duration at 14 days if steady-state is reached rather than go to 28 days.)

- **The TE ask for 1 week to reach consensus on the proposed study design. AP4**

5 Ag read-across / TK program design

Cf. slides 37-42 in Annex 2 and agenda point 4. Given the limited time available, the agenda point on the PBTK model is deffered to the next call / meeting.

6 CLH proposal silver nitrate

Cf. slide 44-46 in Annex 2. Comments/additions:

- **Skin Corr 1A versus 1B**: TE to give business impact assessment in the short term. **AP5**
- **Repr 1B**: It is suggested to check the mentioning of Cp effects in the CLH proposal and refer to our EOGRTS in our comments.

Annexes

1. List of participants
2. Slides presented at the meeting
3. Results AgAc gut biome study - Ag, Cu, Se analysis
4. Supplemental data from van der Zande et al. 2012 (https://doi.org/10.1021/nn302649p)
5. Ag TK report 'Assessment of silver toxicokinetic parameters: desktop review and critique of key published data' (M. Raffray, 21 Jan 2019)
6. Feedback G. Truisi PBTK model
7. Draft CLH report silver nitrate *(note: not for circulation outside TE group)*

Actions

Table 1. Actions agreed at the 15 March 2019 Ag Tox Experts meeting

	What?	Who?	When?
AgAc gut biome study			
1.	Ask prof. Lison: • to use same dosing expression in all graphs of the study report (calculated AgAc and Ag dose in mg/kg bw/d) + mention deviation in graphs; • to check inconsistency between reported Ag levels in testis in current study and study van der Zande et al. 2012; • to inform prof. Marbaix that we want to go ahead with uterus & ovaries histopath; • if gut is stored for possible ultrastructural localisation; • what the possible reasons are for the discrepancy between the achieved dosing and the nominal dosing (concentrations ca 25% below nominal); is this an issue simply with the formulator or something substance specific which we need to better establish?	EPMF Sec	Mar 2019
2.	Follow-up ultrastructural localisation of Ag:	EPMF Sec	Mar 2019

	• request updated offer with qualitative analysis only, in brain and gut (if stored) or kidney for 4 animals per sex; • request localisation in testis as well if currently reported Ag levels in testis in the biome study are accurate.		
3.	Check if there are human data on Ag and reproductive toxicity that can be used.	Ag Tox Experts	Mar-Apr 2019
Ag read-across / TK / preparation EOGRTS			
4.	Provide feedback to EPMF Sec about proposed study design for DRF + TK work.	Ag Tox Experts	By 22 Mar 2019
CLH proposal silver nitrate			
5.	Provide business impact assessment to EPMF Sec on Skin Corr 1A classification versus 1B.	Ag Tox Experts	By 22 Mar 2019

Annex 1: Participants

Katrien ARIJS, consultant for EPMF (ARCHE, Belgium)

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Marie-Laure LEDRICH, consultant for Traxys (Luxembourg) – *via conference call*

Jelle MERTENS, EPMF (Belgium)

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Nissanka RAJAPAKSE, Johnson Matthey (United Kingdom) – *via conference call*

Steven VERBERCKMOES, Umicore (Belgium)

Apologies

Olga LEMKE, BASF (Germany)