Silver Work Group

Expert meeting Reprotoxicity

Draft minutes, Brussels, 23 November 2017 (09:00-16:00 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 in Annex 1. No remarks / additions; agenda approved.

The aim of the meeting is to discuss the strategy for the EOGRTS TP (defence, adjustments) and for possible enabling work and to have sufficient arguments to avoid a worst-case scenario of a Repr Cat.1B classification for silver.

2 Silver reproductive toxicity current status

Cf. Annex 2 slides 7-14 presented by M. Raffray and slides 15-22 presented by M. Holsapple:

- Over the last 2 years, the overall balance of evidence on silver reprotox shifted adversely, and the fear is that ionic silver is on a trajectory for **Repr classification, which could be as severe as Repr 1B**. Participants were reminded about the RAC discussions on the silver zinc zeolite (SZZ) CLH, which focused mainly on silver (and largely disregarded the zinc and zeolite moiety). Here, we managed to argue classification down from Repr Cat.1B to Cat.2.
- **Developmental tox** is considered the main battleground (pre- and post-natal effects), while the evidence for fertility effects is not very strong (in contradiction to Kemi's assessment of the Sprando et al. study fertility effect outcome). Only the OGRTS showed fertility effects (Sprando et al.) but not the 2-gen studies with silver containing active substances (SCAS), while the doses in the studies overlap.
- The key developmental studies for silver are summarised on slides 9-13 in Annex 2. The absence of effects in the silver sodium zirconium hydrogen phosphate (SSZHP) study could be used to our advantage: if effects are all silver-related, then why is there a difference in effects between the different SCAS? Furthermore, we should underline that the effects in studies with SCAS can be confounded by non-silver moieties.
- There is still uncertainty on the **mode of action (MoA)** and the possibility for **indirect effects causing secondary impacts on reproductive parameters** (indirect effects in isolation would not justify classification). In the absence of a confirmed MoA, RAC will probably fall back on the effects of Ag on Cu homeostasis and its relevance to human reproduction. Building a main defence argument around the latter point seems problematic (see for example, Alebic-Juretic and Frkovic 2005).

- There is a disparity between the SCAS studies and Sprando/Babu studies regarding **thymic effects**. The SZZ study showed thymic effects at mid and high dose, while Sprando et al. study did not (which is further argumentation to perform an EOGRTS).
- There are considered to be 3 key parameters in RAC decision making for silver reprotox: pup mortality & implantation loss, growth retardation, and thymic effects.
- Babu et al. study:
    - Although there are some weaknesses in the study approach (which could be addressed in the EOGRTS), this study provides suggestive evidence for the possibility that silver exposure can cause **developmental immunotoxicity** (**DIT**). There was consensus from meeting participants that this indicative finding is a sufficient trigger for inclusion of the DIT cohort in our EOGRTS.
    - In isolation, the study would not be sufficient for classification but in combination with other recent studies, a Repr Cat.1B classification for ionic Ag is now a bigger risk.
    - M. Holsapple confirms the adversity / dose-response of the splenic lymphocyte immunophenotyping in the study is not convincing.

      AP1: M. Holsapple to write an expert paragraph on the adversity / dose-response of the Babu et al. study's immunophenotyping effect with depression in cell populations.
- In our original TP in 2015, we argued **against the developmental neurotoxicity (DNT)** cohort but understood our position was marginal (for instance some TK studies would probably be considered by regulators as cause for concern given that long half-life silver depots in the CNS have been described in certain reports).
- If we are allowed to perform an EOGRTS, we are now looking at the **most complex EOGRTS design**, including all cohorts. It is noted that the number of labs who can perform such a complex test is limited.

Given the risk of a Repr classification, PMC needs to prepare the defence and update of the EOGRTS TP asap. The ideal outcome would be no Repr classification but we **need to defend against Repr Cat.1B.** Ideal final positions could include (and are not necessarily exclusive) robust evidence that:

- Ag reprotox is a secondary effect (preferred outcome), and/or
- MoA is of no / limited relevance to humans (preferred outcome), and/or
- Ag reprotox is confined to exposure levels significantly above those relevant to human risk assessment (less preferred, as this would still mean that soluble Ag is classified as Repr Cat.1B/2 but risk assessment could mitigate).

It was collective opinion that this will require some **enabling work** before the EOGRTS, which would preferably be started **before we receive the draft decision / before we have the informal discussion with ECHA**, in order to be prepared for the discussion (cf. timeline slide 24 in Annex 2). The enabling work would include *in vivo* work but unless it is the study for the actual REACH endpoint, you are allowed to perform other studies if properly justified (e.g. TK study with additional parameters).

It is noted that **effects on the gut microbiome** at silver treatment levels corresponding to the latest reprotoxicity studies have not been assessed, and if present could offer a basis to **argue for important indirect effects, including silver developmental immunotoxicity.**

EOGRTS dose-setting:

- Original TP intentionally included dose level known to impact on GI tract microbiome (placeholder levels). Enabling work was intended/signalled in the TP (lower dose DRF; biome studies; TK etc.).

- It is acknowledged that Sprando / other recent data requires a re-think of dose-setting:
  - From OECD TG 443: ideal is to base dosing on **pre-determined TK** before a main study and also justification of dose-levels vs **human exposure**. It was acknowledged by meeting participants that the latter consideration is unlikely to be a focus in ECHA decisions.
  - In addition, the sector going-in position is to avoid a dose level adversely affecting biome.
  - It is essential **to defend any suggestion to base doses of an EOGRTS on standard considerations of systemic Ag toxicity / MTD etc.!**
- M. Holsapple's opinion re argyria & dose-setting is duly noted (cf. his previous expert opinion on the Babu et al. study). Some complications exist from a PMC perspective:
  - Argyria has always been argued as not toxicologically significant.
  - Industry contention is that it is a visible sign of a protective sequestration mechanism, which is actually detectable at low Ag exposures (e.g. by careful histopathology; ultrastructural studies).
- WHO have estimated human silver intake in adults as approximately 8 µg/day total. Some other publications mention differing estimates of 20-80 µg/day total, so that circa 100 µg/day could be viewed as a published worst case (apart from therapeutic situations). The oral NOAEL for argyria in humans for a total lifetime intake is 10 g of silver, which is in the order of the WHO intake estimate of 8 µg/day (based on 70 years exposure; 70 kg adult).
  AP2: M. Holsapple to send WHO reference that mentions NOAEL of 5 µg/kg bw/day, as mentioned on the slides.

  (Post-meeting note: Action completed.
  - *The reference to the WHO NOAEL of 5 µg/kg bw/day is found on p 4-5 and p 76 of the 2014 SCENIHR opinion on nanosilver: "Current human risk assessments are mainly based on the development of argyria. In workers the threshold limit value for metallic silver is 0.1 mg/m³ and 0.01 mg/m³ for silver salts. For the general population the WHO has set a NOAEL related to the sum of all exposure routes of 5 µg/kg bw/d." The calculation of this NOAEL is explained on p 76 and is based on the total lifetime oral intake of about 10 g of silver.*
  - *The basis for the 10 g / lifetime NOAEL is described in the 2014 doc http://www.who.int/water_sanitation_health/dwq/chemicals/Silver_water_disinfection_toxicity_2014V2.pdf: "There is currently no WHO health-based guideline value for silver in the drinking-water guidelines (WHO, 2011). Silver was last reviewed by the WHO for the drinking-water guidelines in 1993, when it was concluded that on the basis of epidemiological and pharmacokinetic knowledge at the time a total lifetime oral intake of about 10g of silver could be considered the human NOAEL. As it was felt that the contribution of drinking-water to this NOAEL would normally be negligible it was not deemed necessary to establish a health-based guideline value. However, it was suggested that where silver salts are used for drinking-water treatment that a concentration of 0.1mg/l could be tolerated without risk to health (a concentration that would give a total dose over a 70 year period of half of the NOAEL outlined above). The 0.1 mg/l level is thus a health advisory rather than a guideline value, a distinction that is rarely appreciated by researchers (e.g. Pelkonen et al., 2003) who often refer to 0.1 mg/l as a guideline or allowable amount")*
- Reference is made to the argumentation of the dose-setting for molybdenum compounds, as this may be relevant for the Ag case (cf. also agenda point 4).
  AP3: PMC Secretariat to check with IMOA (S. Carey) argumentation of the dose-setting for Mo.

- It is noted that **further data are urgently needed on which Ag levels cause gut microbiome effects**. There is an important difference between rodents and humans in the sense that the rodent microbiome is not fully developed yet when born, this happens mostly during weaning (the gut microbiome helps develop the immune system in neonates). It is suggested to contact an **expert on the gut microbiome** - options:
  - Dominique Lison (Université Catholique de Louvain) - cf. Van den Brule et al. 2016
  - Vincent Young (Michigan State University) - M. Holsapple can introduce us
  - Corrie Whisner (Arizona State University)
  - Rodney Dietert (Cornell University) - probably not best option

  AP4: PMC Secretariat to contact expert on the gut microbiome.

For the **DIT cohort** in our updated EOGRTS TP, the design should include the **T-Dependent Antibody Response (TDAR = "gold standard") assay, Natural killer (NK) cell activity** and **immunophenotyping.** M. Holsapple agreed that predictivity and sensitivity based on these parameters is optimal.

The following table from a previous review by M. Raffray provides an overview of DIT triggers relevant to EOGRTS and the current evidence for silver:

Key criterion	Evidence Summation Conclusion	Remarks
SAR or chemical class linked to immunotoxicity	**TENTATIVE**	Only if Ag viewed as aligned to other immunotoxic / 'heavy' metals. Some regulators lean toward this speculative viewpoint.
Evidence of mechanistically-relevant endocrine disruption	**NO**	
Altered weight or pathology of lymphoid organs	**EQUIVOCAL**	Some thymic effects, principally organ weight depression, were observed in 2-gen studies on SCAS (including an effect in offspring). However, this was not replicated in Babu et al.
Altered cellularity of bone marrow, spleen, thymus, or lymph nodes	**YES**[1]	Babu et al. is an important study (in respect of spleen cell effects) which does align with previous less reliable evidence.
Altered differential white cell counts	**NO**	Fragmentary evidence only in humans and animal models.
Functional immunotoxicity in adults	**NO**	No robust evidence from conventional RDT and 2-gen studies on SCAS. Conflicting reports exist for Ag NP (e.g. i.v. route) of questionable relevance to Ag^+.
Functional immunotoxicity in neonates	**EQUIVOCAL**	Babu et al. included only limited evaluation of functional immune system status. E.g., a TDAR assay was not included.

This results in 1 definite and 2 equivocal conclusions, which is considered enough to trigger the DIT cohort.

3 Process / timeline EOGRTS Testing Proposal (TP)

The public consultation on our EOGRTS TP on silver acetate ran from 27 Sep to 13 Nov 2017. Now a scientific and legal evaluation is ongoing and a draft decision will be prepared and sent to the lead registrant

[1] In terms of guidance applicable to EOGRTS, this is sufficient to represent an external trigger for inclusion of the DIT cohort extension to the study.

afterwards. Current assumption is that we will receive the draft decision in January 2018. A commenting period of 30 days will then start. During this period we will have the opportunity for an **informal call with ECHA**, which is considered the best opportunity to alert ECHA about the fact that our TP is outdated / that we need to do some enabling studies before the actual EOGRTS. It is suggested to **update the registration dossier / TP after receiving the draft decision**.

In addition to the ongoing process for our EOGRTS TP, Sweden has recently submitted **CLH proposals for 3 SCAS**. For silver zeolite (SZ) and silver copper zeolite (SCZ), a Repr Cat. 2 classification has been proposed (no substance-specific data available but conservative approach suggested using same classification as SZZ). For SSZHP, no Repr classification is proposed (2-gen study with SSZHP available that 'did not result in effects meeting criteria for classification'). The 60-day public commenting period is expected to start soon. Even though these SCAS are not in the PMC portfolio but in the scope of the European Silver Task Force (ESTF), PMC is planning to submit comments on the Repr classification of SZ and SCZ, given the possible effect of the CLH of these substances on the silver classification.

ESTF has been alerted to the Babu et al. paper and PMC's assessment of its impact / the possibility of a Repr Cat.1B classification but it seems they are not planning any actions to avoid this. It is suggested to contact ESTF again and highlight the seriousness of the current situation.

AP5: PMC Secretariat to approach ESTF again on possible Repr classification.

4 RAC mindset on reprotox classifications

The implications of a Repr Cat.1B classification are that silver would be restricted for consumer use and would fall under Authorisation. Silver could be banned in the EU from certain electronics, brazing alloys, silverware, medical devices, etc.. Furthermore, biocidal use would be adversely impacted, to the point of being disallowed.

PMC will consider sending letters to the authorities if we are faced with a **Repr Cat.1B** classification for silver: cf. proposal Annex 2 slide 25. The aim is to have sufficient arguments available to highlight to the authorities what the DU consequences are of a Repr classification based on insufficient data (cf. also letters sent to several MS for the SZZ CLH proposal).

A number of case studies on how RAC has previously assessed Repr classification were presented (cf. Annex 2 slides 28-40):

- At the RAC meeting where the **SZZ** CLH was discussed, the decision was very finely balanced between Repr Cat.1B and Cat.2. All discussions focused on silver, not zinc or zeolite.
- For the organic compound (cf. slides 31-40, presented by O. Lemke), the RAC opinion was published Sep 2017. There were indications for maternal stress but these were difficult to spot. This case shows that in your study design, **careful scrutiny is needed for potential indirect effects at all dose levels** and you need to ensure that there is a **good linkage between dams and pups**.
- **Co** case:
  - Different from the Ag case in the sense that it looks into dissimilar reproductive effects but the case is a good illustration of **issues with dose-setting and maternal toxicity**. The case has not been discussed at RAC yet.
  - 5 soluble Co compounds have Repr classification mainly based on fertility (28 Co compounds in total in consortium).
  - Because of gaps in the risk assessment for developmental tox, a prenatal developmental toxicity (PNDT) study with $CoCl_2$ was initiated. Industry consider that the reprotox / adverse testicular

effects were a result of haemodynamic adverse effects leading to tissue hypoxia (i.e. reprotox is a secondary effect of systemic Co toxicity) but RAC/CA seems to disagree.
    - Discussion on dose-setting for 2nd species DNT and definition of maternal toxicity (adversity) is currently ongoing. There might be developmental tox effects beyond hematopoietic effects. There is not so much TK info on Co.
    - An EOGRTS is proposed for the low bioavailable group. Classification is already accepted for the high soluble group.
    - This case shows the importance of **building a sound argumentation around what is the leading effect**.
- **Mo** case:
    - PNDT results were rejected by ECHA because dosing too low. ECHA asked for 2nd species PNDT with different dose setting.
    - The doses used were relevant to human exposure, but this is not a good argument: it mixes up hazard and exposure. The EOGRTS guideline does mention you can take into account human exposure levels.

M. Holsapple's assessment of impact on situation for silver: guidance is still lacking on how positive findings in DIT would impact overall reproductive toxicity classification criteria (which is a different situation to trigger application to EOGRTS cohorts).

5 Stress-testing examples

Cf. agenda point 10. No further suggestions for 'stress-test' questions or defence points were proposed at this point.

6 Defence of EOGRTS TP

Cf. Annex 2 slides 47-48:

- In addition to the fact that the existing studies show a number of shortcomings that could be addressed in our EOGRTS (e.g. inclusion of TDAR as definitive endpoint for immunotox), it is noted again that the **possible effect of silver on the neonate gut microbiome is a plausible MoA** for a unique developmental immunotoxicant. Testing this hypothesis would require at least a OGRTS (i.e. cannot be tested in adults).
- The rationale to include the DIT cohort would be diminished if 1) biodistribution of silver to the **spleen** of the F1 generation was not seen, and 2) if exposure to silver did not affect the **gut microbiome** in the F1 generation.
- The interpretation of the results from the DIT cohort would be compromised if unreasonably high doses of silver were used, so **we need to be clear about the rationale for the dose selection**.

7 Adjustments to EOGRTS TP

Cf. Annex 2 slides 50-52, and discussion under previous agenda points:

- The **DIT cohort design** in our updated EOGRTS TP should include the **TDAR assay** (and **Natural killer (NK) cell activity** and **immunophenotyping (IPT)**?)**.**

  (Post-meeting note: While there is no question that including all three of these parameters may increase the predictive value of the EOGRTS from an immunotox perspective, the only parameter identified in the OECD TG 443 EOGRTS study design is the TDAR, as noted in the OECD document,

and in Moore et al. (2016). M. Holsapple believes that we should revisit the inclusion of all three of these parameters, to ensure that we have a solid rationale for including two additional immunotox parameters in an already complex study design. During the development of the EOGRTS DIT cohort, the group did consider endpoints in addition to the TDAR, but chose not to formalize that concept because of the difficulty in integrating the NKC assay and IPT – e.g., they really shouldn't be included in the animals who are immunized with a TD antigen.)

- The **dose-setting** needs to be revised (see also above). We do not need to confine ourselves to specific numbers in the TP but need to mention further enabling work (**TK** with additional parameters) is needed to define dosing, and need to ask sufficient time to conduct this enabling work.
- **Biodistribution of silver to immunocompetent organs of the F1 generation** (primarily spleen) needs to be checked.
- Possible **MoA** needs to be addressed, by determining if exposure to silver affects the **gut microbiome in the F1 generation:**
  - For speed, enabling work should first focus on the gut microbiome effects in mothers, not in the F1 neonates.
  - Effects on the gut microbiome are mostly reversible, but any resultant effects on the immune system are often not.
  - Learning cases? There must be other biocides that have produced indirect reprotox effects when tested.

    (Post-meeting note: From M. Holsapple's perspective, studies to determine if a biocide - like silver - could be a developmental immunotoxicant, have not been conducted.)
  - We will need to **educate RAC** on the importance of the gut microbiome (cf. Annex 2 slide 60, Williams et al. 2014).
  - Is this a valid MoA for humans? Van den Brule could give argumentation of why silver is a unique developmental immunotoxicant to the rodent, and this could also be relevant to human hazard assessment.

    (Post-meeting note: Another expert that could help us in the discussion on the human relevance of this MoA is Jeroen Raes (VIB / Leuven University), an expert on the human gut microbiome.)
  - To what extent is an effect on the gut microbiome an adverse effect?
  - Reference is made to the ETAP discussions on ionic vs nano, where it was concluded that the main difference in effects is for microorganisms. Toxicity of the nanoform to microorganisms may be amplified through phagocytosis / nanosilver uptake and resulting persistent internal release of ionic silver. This may be an explanation for why the nanoform could be more biocidal/more impactful on the biome. This would also bear on any dose-selection for nanosilver.

    (Post-meeting note: A further reason why nanosilver could be more active is that ionic silver will suffer precipitation in high chloride environments such as the stomach. Also, the pronounced effect of nano vs ionic for microorganisms seems related to possible 'local hotspots' for nano exposure (places where particles are concentrated, e.g. by agglomeration or interaction with organic matter), resulting in very high local exposure, whereas exposure to ions is more homogeneous.)
  - Taking into account the Van den Brule paper, there is a chance that ECHA will ask for the nanoform because of concern.

AP4: PMC Secretariat to get opinion from gut microbiome expert on the Van den Brule et al. paper and whether or not results can be extrapolated to the rat.

AP6: S. Verberckmoes to include human relevance gut microbiome MoA in stress-test.

AP7: O. Lemke to check what parameter to use to measure adverse effect.

8 Mode of action (MoA) insights

Cf. Annex 2 slides 54-62.

For the Ag-Cu axis effect, there are some analogues with Zn and they seem to be successful in avoiding a Repr classification. Zn is known to interfere with Cu update from the gut, thus resulting in depressed circulating Cu levels and a potential for indirect effects due to this MoA.

AP8: PMC Secretariat to check with IZA (N. Lombaert) how they have argumented the Zn case.

9 Enabling work

Cf. Annex 2 slides 64-78:

- As discussed before, enabling work will need to include investigation towards silver effects on the **gut microbiome** (MOA_4 + MOA_5; could be done together). **Before we engage in any enabling work, it is important to involve a gut microbiome expert.**
- A short-term study on the rat gut microbiome in parent animals encompassing lower Ag treatment levels could be a good quick way to show the regulators the complexity of the issues.
- If high level exposure of the mother animals shows a devastating effect on the microbiota, the exposure levels of the neonates will need to be much lower.
- **TK** data will also need to be generated (MOA_2).
- We will also need to defend **argyria** at some point but this is not a priority for now (MOA_7).
- M. Holsapple's report mentions: 'DIT protocol could be extended to include juvenile animals and young adults; an important consideration would be to continue exposure until a DIT assessment is done'. How does it impact on the design of the EOGRTS?

  AP9: M. Holsapple to look at EOGRTS guidance (OECD TG 443) and see what adjustment is needed to extend DIT protocol to include juvenile animals and young adults.

  (Post-meeting note: Action completed. M. Holsapple believes that the protocol is designed to address possible effects on "juvenile animals and young adults", and the need "to continue exposure until a DIT assessment is done". In Section 51 of OECD TG 443, it is noted that F1 animals are immunized on PND 56.)

  AP10: M. Holsapple to reflect on DNT expert.

10 Re-visit stress-test / Confirm technical defence plan

Cf. Annex 2 slides 77-78.

It is concluded that we will need to initiate a study on silver effects on the gut microbiome after consultation with expert (looking at maternal + F1 generation) = extended TK / biome study.

AP11: S. Verberckmoes to contact D. Lison re the van den Brule et al. paper/expert consultancy possibilities.

(Post-meeting note: Action completed.

- *Lison's interpretation of the low effective doses of nanosilver on the microbiome is that it is the only form that gives a stable and steady release of silver ions in the intestine that kills/changes the biome. Ionic silver will precipitate as AgCl and will be presented as a much less bioavailable form to the gut. Therefore, he expects that a much higher dose of ionic silver is required to elicit comparable effects.*
- *PMC Secretariat to follow up on consultancy possibilities and experimental work.)*

Annexes

1. Agenda & list of participants
2. Slides presented at the meeting

Actions

Table 1. Actions agreed at the 23 November 2017 Ag expert meeting Reprotox in Brussels

	What?	Who?	When?
1.	Write an expert paragraph on the adversity / dose-response of the Babu et al. study's immunophenotyping effect with depression in cell populations	M. Holsapple	Dec. 2017
2.	Send WHO reference that mentions NOAEL of 5 µg/kg bw/day	M. Holsapple	Done
3.	Check with IMOA (S. Carey) argumentation of the dose-setting for Mo	PMC Sec	Dec. 2017
4.	Contact expert on the gut microbiome (D. Lison?) for experimental design of enabling studies / get opinion on the Van den Brule et al. paper and whether or not results can be extrapolated to the rat	PMC Sec	ASAP
5.	Approach ESTF again on possible Repr classification	PMC Sec	Dec. 2017
6.	Include human relevance gut microbiome MoA in stress-test	S. Verberckmoes	Dec. 2017
7.	Check what parameter to use to measure adverse effect	O. Lemke	Dec. 2017
8.	Check with IZA (N. Lombaert) how they have argumented the Zn case	PMC Sec	Dec. 2017
9.	Look at EOGRTS guidance (OECD TG 443) and see what adjustment is needed to extend DIT protocol to include juvenile animals and young adults	M. Holsapple	Done
10.	Reflect on DNT expert and send suggestions to PMC Secretariat	M. Holsapple	Dec. 2017
11.	Contact D. Lison re the van den Brule et al. paper/expert consultancy possibilities	S. Verberckmoes	Done