



Ag and Ag compounds

Summary table - available reproductive toxicity data and EOGRTS added value for CLH process

Dear Sir, Dear Madam,

EPMF is convinced that the currently available reproductive toxicity studies are insufficient to conclude on the classification of ionic silver compounds. Only an **extended one-generation reproductive toxicity study (EOGRTS) on silver acetate can address the existing data gaps and allow a fully informed CLH decision** because:

- The study will provide one **consistent and comprehensive set of simultaneously generated data** according to a **fully conformant OECD 443 test design performed according to GLP**, instead of using information from a patchwork of studies (with variable reliability, performed with different substances, under varying test conditions and measuring different parameters) which prevents reliable comparison and could trigger misleading interpretations;
- **Simple ionic Ag compound as test substance** (silver acetate): the available studies have been performed on different silver substances, with some of them containing potential confounding toxicologically relevant moieties. The EOGRTS will be performed with silver acetate, a simple ionic Ag compound possessing comparatively high oral bioavailability, being of high read-across relevance to other ionic Ag forms;
- **Investigation of developmental immunotoxicity (DIT)**: the proposed study design includes Cohort 3 and will clarify the current 'suggestive' evidence of DIT caused by ionic Ag forms;
- **The test design will address existing data gaps** related to the reproductive toxicity potential of ionic Ag forms, e.g. **toxicokinetic parameter assessments** (covering adults and offspring), **mechanistically relevant parameters**, and investigation of **potential indirect effect confounders**.

The appended table provides perspectives on several animal studies considered to be key to the hazard assessment of ionic silver substances – such as silver nitrate – in respect of their reproductive toxicity potential. Coverage of fertility and development endpoints is included. For ease of cross-reference, all dose levels have been expressed as Ag equivalent values.



Data gaps and uncertainties in the currently available information for ionic silver are addressed in overview. Linked commentary is provided as to why progression of a pending EOGRTS on silver acetate¹ is expected to comprehensively improve the quality and reliability of the overall dataset, including that applicable to CLH needs.

a) Two-generation reproductive toxicity studies – Silver containing active substances (SCAS)			
Study	Main findings	Perspectives on applicability to ionic Ag	Relevance of proposed EOGRTS
<p>Silver Zinc Zeolite (SZZ) OECD 416 (rat; oral; dietary) Ref.: IIIA 6.8.2-04 [2002] 1.5/1.8, 9.8/11.3; and 20.3/22.9 mg Ag⁺ equiv./kg bw/d (F₀ ♂/♀)</p>	<p>Fetal mortality ↑ Stillbirth ↑ Pup bodyweight ↓ Thymus weight ↓ (F₁ and F₂ pups) Pup kidney and urinary tract abnormalities ↑ No statistically significant / dose-related effects on fertility parameters. CLH assignment (agreed by RAC): Repr. 2 H361d.</p>	<p>It can be argued that the developmental toxicity outcomes from these studies do have some congruence. However, if Ag⁺ is postulated as the reproductive toxicant, it is unclear why an obvious disparity in the degree of observed effect exists in the case of SSZHP even though the Ag equivalent intakes were higher than in the study on SZZ (and the predicted Ag bioavailability of SSZHP is greater²). There is also discordance with certain findings of a one-generation reproductive toxicity study on silver acetate (Table entry 'b' refers). In relating the outcomes for these SCAS to simple ionic Ag substances, a fundamental difficulty is the co-existence of other chemical moieties (e.g. zeolite, Zn), which may be responsible for independent or additive toxicities. Although certain effects, such as hydronephrosis, are more easily attributable to non-silver constituents, the overall reproductive effect position in respect of Ag⁺ is obscured. No Ag toxicokinetic (TK) investigations were linked to these studies. Key correlations which would be helpful in mechanistic interpretation such as serum Cu levels and ceruloplasmin status are also lacking; as were assessments as to whether Ag effects on animal biomes may have indirectly impacted on reproductive outcomes (see also Table entry 'e').</p>	<ul style="list-style-type: none"> • The test article is a simple ionic Ag compound (AgAc) with read-across relevance to other ionic Ag forms. The study will not be subject to potential confounding by other toxicologically relevant moieties, and it is expected to provide a definitive hazard assessment of fertility and developmental endpoints for ionic Ag. • In terms of conservative selection of the test substance, TK evaluations indicate that AgAc has comparatively high oral bioavailability (greater than silver nitrate). • Comprehensive TK parameter assessments will be integrated (covering adults and offspring). • Mechanistically relevant parameters will be addressed (refer to Table entry 'e').
<p>Silver Sodium Zirconium Hydrogen Phosphate (SSZHP) OECD 416 (rat; oral; dietary) Ref.: IIIA 6.8.2-03 [2002] 1.8/1.9, 9/9.9; and 37/40 mg Ag⁺ equiv./kg bw/d (F₀ ♂/♀)</p>	<p>Litter size ↓ (F₁/F₂ high-dose group) Litter wt ↓ (F₁/F₂ high-dose group) Thymic wt ↓ (slight; F₁/F₂ pups high-/mid-dose groups) Developmental effects were marginal. No statistically significant / dose-related effects on fertility parameters. CLH assignment (proposed by Keml): unclassified.</p>		

¹ REACH registration testing proposal - OECD 443 Extended One Generation Reproductive Toxicity Study on silver(1+) acetate [CAS number 563-63-3; EC number 209-254-9]. Registrant: Aurubis AG. Based on toxicokinetic and other considerations, silver acetate has been justified as the most appropriate read-across substance for silver(1+) ionic substances as a group.

² Outcomes from comparative bioelution modelling studies conducted by the European Silver Task Force estimated the oral bioavailability of SSZHP as greater than that of SZZ.



b) One-generation reproductive toxicity study (OGRTS) – Silver acetate (Sprando et al., 2017)

Study	Main findings	Perspectives on applicability to ionic Ag	Relevance of proposed EOGRTS
<p>Silver Acetate (AgAc) OGRTS per US FDA CFSAN (rat; oral; drinking water) Ref.: Sprando RL et al. (2017) Food Chem Toxicol. 106: 547-557.</p> <p>0.26, 2.6 or 26 mg Ag⁺ equiv./kg bw/d (F₀ ♂/♀)</p>	<p>Implantation number ↓ Pup mortality ↑ Pup bodyweight ↓ (delayed development) No effects on thymus (F₁) Apparent developmental LOAEL corresponded to mid-dose.</p> <p>Fertility: fertility index (nr. litters / ♀) ↓ (high-dose group only); historical control range and statistics unstated.</p>	<p>This study was not performed according to GLP and it conformed to a non-OECD test guideline. Evidence of developmental toxicity due to ionic Ag was presented in this study. However, the absence of historical control range information (and individual animal data) complicates its interpretation. Compared to an EOGRTS (OECD 443), important design disparities exist including, but not limited to, incomplete histopathology of primary and secondary reproductive tissues; and the omission of oestrus cycle and sperm parameter evaluations. In contrast to findings from the SCAS two-generation studies, thymic effects were absent³. In common with the SCAS studies, limitations exist in respect of the non-availability of TK and mechanistically relevant parameters.</p>	<ul style="list-style-type: none"> • The study will be a fully conformant OECD 443 design according to GLP. • Historical control range data and other enablers for a robust evaluation of reproductive outcomes will be assured. • See also above in respect of TK and mechanistic parameters.

c) Developmental immunotoxicity (DIT) study – Silver acetate (Babu et al., 2016)

Study	Main findings	Perspectives on applicability to ionic Ag	Relevance of proposed EOGRTS
<p>Silver Acetate (AgAc) Satellite DIT study linked to OGRTS (rat; oral; drinking water) Ref.: Babu US et al. (2016) Food Chem Toxicol. 98: 195-200.</p> <p>0.26, 2.6 or 26 mg Ag⁺ equiv./kg bw/d (F₀ ♂/♀)</p>	<p>Pup splenic CD8+ T cells ↓ Mitogen-induced splenic lymphocyte proliferation ↓ (pups) Only limited evidence of functional immune deficit due to omission of key T-cell dependent antibody response (TDAR) assay.</p> <p>Thymic functional immune parameter and innate immune response in offspring were unaffected.</p>	<p>This study was non-GLP and not equivalent to an OECD 443 DIT cohort design in terms of its reliability to discriminate effects on the developing immune system. Independent expert peer reviewer conclusions were that it does provide evidence which is 'suggestive' of DIT, whilst noting that dose-response patterns were not persuasive. The reviewer further commented that it is possible that the immune disturbances could have been the result of indirect stressor effects (such as might be due to Ag-induced dysbiosis). The current weight-of-evidence is that Ag is not significantly immunotoxic in adult animals, but it is acknowledged that the neonatal immune system can be more sensitive. Hence the industry position is that follow up of the report is justified, i.e. that it represents sufficient trigger level concern.</p>	<ul style="list-style-type: none"> • Inclusion of Cohort 3 (developmental immunotoxicity investigation) in the EOGRTS has been proposed. • Functional immune system assessment will incorporate a T-cell dependent antibody response (TDAR) assay. • The influence of non-specific indirect influences on neonatal immune development are part of the intended design.

³ See also: Gao X et al. (2015) Toxicogenomic study in rat thymus of F1 generation offspring following maternal exposure to silver ion. Toxicology Reports 2: 341-350.



d) Prenatal developmental toxicity study – Silver acetate (US NTP)

Study	Main findings	Perspectives on applicability to ionic Ag	Relevance of proposed EOGRTS
Silver Acetate (AgAc) OECD 414 equivalent (rat; oral; gavage) Ref.: IIIA, 6.8.1-07 [2002]. 6.5, 19, 65 mg Ag ⁺ equiv./kg bw/d	No toxicologically relevant increases in incidences of fetal malformations or variations for treated groups. Equivocal effect on percent litters with late fetal deaths (high-dose group). NOAEL (developmental toxicity): 65 mg Ag ⁺ equiv./kg bw/d.	The investigation was an OECD guideline conform study, conducted to GLP within the US NTP regime. No teratogenic effects due to Ag ⁺ treatment were apparent. However, exposure to the test article was limited to gestation days 6-19 (covering organogenesis). Therefore, outcomes from this study do not cover the period of early embryonic development. The possibility exists that Ag ⁺ could affect embryos during this earlier developmental window (see also Table entry 'e').	<ul style="list-style-type: none"> • The EOGRTS study is a design capable of identifying early-stage embryofetal effects. • Correlation with mechanistically relevant parameters and potential indirect effect confounders will also be possible based on the design.

e) Studies pertaining to mechanisms – Ionic silver compounds

Study	Main findings	Perspectives on applicability to ionic Ag	Relevance of proposed EOGRTS
Silver Acetate (AgAc) 90-day repeat dose study (rat; oral; gavage) Ref.: Williams K et al. (2015) Nanotoxicology 9: 279-289. 65, 130, 260 mg Ag ⁺ equiv./kg bw/d	Evident general toxicity associated with Ag-induced gut microbiome disturbance (high-/mid-dose groups). Marked shifts in gut microbiome populations at the low dose level. NOAEL in relation to significant biome effects was not established.	A growing body of evidence in rodent models demonstrates that silver (Ag ⁺ as well as Ag nanoforms) can perturb the gut microbiome, affecting key symbiote populations, and hence has a potential to cause dysbiosis. Whether a causal linkage exists between a state of Ag-induced dysbiosis and indirect reproductive effects remains to be elucidated. Robust dose-response information in respect of microbiome effects at lower Ag exposure levels (relevant to reproductive toxicity study interpretation) does not yet exist.	<ul style="list-style-type: none"> • Microbiome investigations and linked reproductive function parameters (e.g. oestrogen hormones) will be examined as part of EOGRTS ancillary studies.



e) Studies pertaining to mechanisms – Ionic silver compounds (continued)

Study	Main findings	Perspectives on applicability to ionic Ag	Relevance of proposed EOGRTS
<p>Silver Chloride (AgCl)</p> <p>Non-guideline developmental toxicity study (rat; oral; dietary)</p> <p>Ref.: Shavlovski MM et al. (1995) BioMetals 8: 122-128.</p> <p>188 mg Ag⁺ equiv./kg bw/d</p>	<p>Treatment throughout gestation (GD 1-20) caused severe developmental alterations and fetal death (but not if exposure was restricted to GD 7-15).</p> <p>Ceruloplasmin co-administration mitigated the effect severity.</p> <p>The investigators ascribed the effect as likely due to disruption of the Cu-transporter ceruloplasmin, resulting in severe depression of maternal and/or embryofetal Cu levels (Cu being an essential micronutrient supporting reproduction).</p>	<p>This report is commonly cited as being one of few investigations which correlates the effect of ionic Ag on Cu/ceruloplasmin homeostasis as a mechanistic basis for embryotoxicity (in rodents). It should be noted that the study robustness is questionable for several reasons, e.g. the design included only a single dose level (at a high equivalent Ag⁺ dose known to be associated with indirect toxicity), whilst multiple maternal parameters and also certain key litter and fetal parameters applicable to a prenatal development study were omitted. Whilst it is plausible that ionic Ag could induce embryotoxicity via disturbance of Cu levels, properly correlated dose-response information is currently insufficient to support this conclusion. Other studies, e.g. in rodent models, related to the Cu axis cannot yield a complete picture, and do not provide insights on effect thresholds nor human hazard potential (given the adaptive reserve which operates during human pregnancy).</p>	<ul style="list-style-type: none"> • An extensive examination of the effects of Ag⁺ treatment on the Cu-axis will be undertaken as an integral part of the planned EOGRTS in a manner whereby any potential correlation with reproductive outcomes can be properly assessed. • Other parameters which are relevant to alternate mechanistic possibilities (direct and indirect effects) will also be embedded as ancillary investigations. For instance, regarding Se homeostasis, microbiome effects, and endocrine status.