International Antimony Association

Scientific opinion, weight of evidence and read-across assessment, and further research options

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Human Health: Genotoxicity

Version 2

17 June 2019

Version management

Version	Date	Main change	Sections affected
1	31 July 2018	/	/
2	17 June 2019	New bio-elution data included, electrophilicity considered, ECHA's stepwise weight of evidence template followed, research strategy adjusted in consideration of REACH Evaluations Draft Decisions dated 18 April 2019	All

International Antimony Association

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International Antimony Association (VZW)
Avenue de Broqueville 12, 1150 Brussels, Belgium / Phone : +32 (0) 32 2 762 30 93 / Fax : +32 (2) 762 82 29
www.antimony.com

1. Introduction

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Genotoxicity studies have been performed for only a few of the 10 mono-constituent antimony (Sb) substances that are registered under REACH. Overall, around 15 studies have assessed the genotoxicity of Sb substances since 1991. Most of the information was generated using a small number of trivalent Sb substances, and the in vitro and in vivo studies do not yield comparable results. This dataset is used as the starting point to perform the ***weight of evidence-based genotoxicity assessment*** of Sb substances.

Annex XI of the REACH Regulation also opens the possibility of predicting properties of substances for which no data is available, on the basis of data available on other related substances, by applying read-across (so-called 'read-across approach') between one or more source and target substances. The ***read-across assessment*** is built upon the physico-chemical similarities and differences that can be observed among the Sb substances, and which are of relevance to the toxicological property to be assessed.

Along these two related assessments, a number of information gaps are identified and are the targets of a phased research program being conducted over the next several years, identified as further research options.

This "scientific opinion, weight of evidence and read-across assessment, and further research options" document therefore outlines the approach followed to assess and predict the genotoxicity properties of Sb substances under REACH. It implements the recommendations and principles laid down in relevant ECHA documents[1], in order to facilitate the examination of the read-across and weight of evidence justification by REACH authorities.

This document refers to evidence which is available in the REACH Registration dossier of the Sb substances and therefore avoids repetition of detailed description of evidence which is available in the dossier and/or the Chemical Safety Reports (CSRs). In addition to the technical annex provided at the end of this document, reading it in conjunction with the REACH dossiers and/or Chemical Safety Reports (CSRs) will bring a more complete picture to the reader.

i2a, on behalf of the registrants of its ten (10) Sb substances in scope, requests that the opinions and conclusions presented in this document, including the further research options that are outlined, are taken into account when preparing REACH Evaluation decisions. Future data generation may alter this scientific opinion and the REACH dossiers and CSRs. Accordingly, this document (and any updates of it) will be attached to the next REACH Dossier updates.

About i2a

The mission of the International Antimony Association is to inspire product stewardship along the antimony value chain. This mission is accomplished by generating and sharing information concerning the environmental and health safety and societal benefits of antimony and antimony compounds. Through a common evidence base, i2a promotes a harmonized risk management and continued safe use of antimony and antimony substances across the value chain and geographical borders.

For further information: www.antimony.com.

[1] ECHA Guidance on grouping of substances (2017), ECHA's Read-Across Assessment Framework (RAAF) (2017), and ECHA's Weight of evidence/uncertainty in hazard assessment background document and template (no date specified). Where necessary, the concepts and terms used in ECHA Documents are adapted to have greater compatibility with the unique properties of metals and metalloid substances.

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2. Assessment of genotoxicity applying a weight of evidence approach

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ECHA's recommendation for documenting a weight of evidence approach includes the following steps:

1) Problem formulation (cf. subsection 2.1 below)
2) Collection and documentation of all information (cf. subsection 2.2 below)
3) Assessment of quality of individual evidence (cf. subsection 2.2 below)
4) Integration and weighing of evidence (weight of evidence analysis), including the application of levels of confidence (cf. subsection 2.3 below)
5) Uncertainty analysis (cf. subsection 2.3 below)
6) Conclusion (cf. subsection 2.3 below)

2.1 Problem formulation

Establishing the genotoxic potential of Sb and its compounds is central to determinations of whether Sb substances are capable of inducing changes to DNA that may be transmitted to future generations via germ cells, or produce alterations to somatic cells that might initiate or otherwise progress the growth of neoplastic tissues.

The weight of evidence assessment therefore aims to respond to the following questions:

- Is the available evidence sufficient to assess the genotoxicity of Sb substances?
- What indication does the available evidence provide as to the potential genotoxicity of Sb substances?
- What research would increase the confidence/reduce the uncertainty of the genotoxicity assessment of Sb substances?

2.2 Genotoxicity information on Sb substances

This section covers steps 2) collection and documentation of all information, and 3) assessment of quality of individual evidence (reliability, relevance, adequacy) of ECHA's weight of evidence approach.

Literature addressing these issues was located by searching bibliographic data bases (ToxLine, PubMed). Initial literature searches focused on the period 1990 to May 2017. Supplemental searching to identify more recent publications were conducted in May of 2019, and focused upon May 2017 to May 2019. The capture of literature was broadened by reliance upon the primary search terms "antimon* followed by manual inspection of papers identified for relevance to Sb genotoxicity and other key toxicological properties. Approximately 200 papers concerned the use of antimonial compounds in the treatment of parasitic diseases. This clinical literature was included in the analysis since it potentially helped define toxic or toxicokinetic properties of antimonial compounds. Table 1 summarizes this literature search strategy.

Table 1: Toxicology literature search criteria for Sb substances.

Search dates covered	Number of papers identified	Search criteria	Substances included
1990 – May, 2017	~2500	"antimon*"	Antimony metal, Diantimony trioxide, Antimony sulfide, Antimony trichloride, Antimony tris (ethylene glycolate), Sodium hexahydroxoantimonate, Potassium hexahydroxoantimonate, Sodium antimonate, Antimony pentachloride, Diantimony pentoxide
May 2017 – May 2019	612	"antimon"	

Approximately 2500 publications were identified from 1990 to May 2017. Supplemental searching of more recent literature identified approximately 600 additional papers published from May 2017 to May 2019. Papers

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were sorted by date, the Sb substance evaluated, and characterization of the toxicological endpoints evaluated. Of the total 3100 candidate papers for evaluation, approximately one half merited more in-depth evaluation (although not all were used in CSR preparation).

Tables 2, 3 and 4 below provide an overview of the genotoxicity data that is available for the ten Sb substances registered under REACH, and the response observed after treatment with different Sb compounds. Studies in prokaryotic systems are presented first, followed by results in eukaryotic systems in vitro and in vivo. Within each subgroup, test results are ordered by relevance – tests for mutations are presented first, followed by results from "indicator assays".

Table 2: Overview of genotoxicity responses to Sb substances in prokaryotic systems.

Assay	Compound Tested	Concentration Tested	Response	Reference and Klimisch Score
Mutation Assays				
Ames Mutation	$SbCl_3$	625 – 5000 µg/plate	Negative	Kuroda, 1991 2: Reliable with restriction
Ames Mutation	Sb_2O_3	0.43 – 1.71 µg/plate	Negative	Kuroda, 1991 2: Reliable with restriction
Ames Mutation	Sb_2O_3	100 – 5000 µg/plate	Negative	Elliot, 1998 2: Reliable with restriction
Ames Mutation	Sb_2O_3	156 -5000 µg/plate	Equivocal positive	Asakura et al 2009 2:reliable with restrictions
Ames Mutation	$SbCl_5$	54 – 864 µg/plate	Negative	Kuroda, 1991 2: Reliable with restriction
Ames Mutation	Sb_2O_5	50 – 200 µg/plate	Negative	Kuroda, 1991 2: Reliable with restriction
Ames Mutation	$NaSb(OH)_6$	31.6 – 5000 µg/plate	Negative	Spruth, 2015 2: Reliable with restriction
Escherichia Coli	$NaSb(OH)_6$	31.6 – 5000 µg/plate	Negative	Spruth, 2015 2: Reliable with restriction
Indicator Assays				
B. subtillis rec	$SbCl_3$	6.3 – 23 µg/disk	Positive	Kuroda, 1991 2: Reliable with restriction
B. subtillis rec	$SbCl_3$	0.01 M	Positive	Kanematsu (1980) 2: Reliable with restriction
B. subtillis rec	Sb_2O_3	0.05 M	Positive	Kanematsu (1980) 2: Reliable with restriction
B. subtillis rec	Sb_2O_3	0.3 – 1.1 µg/disk	Positive	Kuroda, 1991 2: Reliable with restriction
B. subtillis rec	$SbCl_5$	65 - 260 µg/disk	Positive	Kuroda, 1991 2: Reliable with restriction
B. subtillis rec	$SbCl_5$		Positive	Kanematsu (1980) 2: Reliable with restriction
E. Coli SOS chromotest	$SbCl_3$	11 – 707 µM	Negative	Lantzsch, 1997 2: Reliable with restriction

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Indicator assays are assays that suggest genotoxic activity but do not themselves provide direct evidence of mutagenicity. For example, gene mutations or chromosome aberrations are indicative of a change in the primary sequence of DNA (i.e. are evidence for a mutagenic response). In a weight of evidence evaluation, mutagenic responses are generally accorded greater significance than responses in indicator assays (e.g. sister chromatid exchanges) that suggest impacts such as DNA damage may have occurred but do not determine if mutations have taken place in viable cells). Indicator assays can yield positive responses via mechanisms that do not produce mutagenic changes.

Sb 3+ compounds have been tested most frequently – tests of Sb 5+ compounds have been conducted less frequently and are in the shaded table rows. To further assist in data interpretation, positive assay responses indicative of genotoxicity are indicated in red. "Klimisch scores" indicative of study quality are presented in each table. Klimisch scores were derived in accordance with Klimisch et al. (1997) and compliance with international guidelines for genotoxicity testing. The recommendations of the International Workshop on Genotoxicity Testing (IWGT) or the Organization for Economic Cooperation and Development (OECD) often provided the most rigorous protocols defining acceptable testing methods and are cited as appropriate. In most instances, only studies with Klimisch scores of 1) Reliable without restriction and 2) Reliable with restrictions are included in this review. As regards in vitro studies, a number of low-quality studies have been published, and are summarized in the CSRs.

In vitro genotoxicity responses to Sb compounds are generally weak, inconsistent and often result from the use of high compound concentrations that sometimes exceed the solubility limits of sparingly soluble Sb compounds. The response profile that emerges from *in vitro* tests is mixed. Mutation assays in prokaryotic (bacterial) test systems are mostly negative, and indicative of no mutagenicity (Table 2). The main exception to this is a study (Asakura et al., 2009) that produced discordant negative and weak positive results in replicate experiments of Sb metal powder. The criteria applied for a positive response was a doubling of mutant colony frequency over that observed in solvent controls. This was not achieved in two experiments, but a third replicate experiment achieved this informal benchmark in a study in which the mutation frequency in solvent controls was unusually low and Sb treatment yielded mutation frequencies comparable to the negative controls in other studies run. Dose-dependent induction of mutants was not observed. This weak positive response is of questionable significance. Paradoxically, prokaryotic indicator assays (e.g. the B. subtillis rec test for DNA damage) generally provided positive responses. For a variety of technical reasons explained in the Technical Annex to this paper, results from prokaryotic systems can be unreliable when applied to metals in general and to Sb in particular and contribute little to a determination of genotoxic potential.

Table 3: Overview of genotoxic effects of Sb substances in cultured mammalian cells.

Assay	Compound Tested	Concentration	Test Result	Reference and Klimisch Score
Mutation Assays				
Mouse Lymphoma Cell Mutation	Sb_2O_3	6 – 50 µg/ml	Negative	Elliot 1998 2: Reliable with restriction.
Mouse Lymphoma Cell Mutation	$NaSb(OH)_6$	2.2 – 570 µg/ml	Negative	Stone, 2010 1: Reliable without restriction
Human Lymphocyte Chromosomal Aberrations	Sb_2O_3	10 – 100 µg/ml	Positive	Elliot 1998 2: Reliable with restriction.

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Assay	Compound Tested	Concentration	Test Result	Reference and Klimisch Score
Chinese Hamster Lung Cells	Sb metal (10 □m average particle diameter)	Not specified	Positive?	Asakura et al (2009) 3: Not reliable Chromosome aberrations said to have been induced but there was low incidence of mitotic cells. No data on abberations observed are presented
MN CHO cells	$SbCl_3$	50 - 400 µM	Positive	Huang 1998 2: Reliable with restriction.
MN Human Fibroblasts	$SbCl_3$	50 - 400 µM	Positive	Huang 1998 2: Reliable with restriction,
MN Human bronchial epithelial	$SbCl_3$	50 - 400 µM	Positive	Huang1998 2: Reliable with restriction,
MN V79 cells	$SbCl_3$	0.1 – 50 µM	Positive	Gebel 1998 2: Reliable with restriction.
MN Human Lymphocytes	$SbCl_3$	0.1 – 25 µM	Positive	Schaumloffel 1998 2: Reliable with restriction.
MN Human Lymphocytes	$NaSb(OH)_6$	64 – 540 µg/ml	Negative	Whitwell, 2010 1; Reliable without restriction
MN Human Lymphocytes/FISH staining	$KSbO_3$	240 – 600 µM	Positive	Migliore, 1999 2: Reliable with restriction.
Indicator Assays				
SCE Human Lymphocyte	$SbCl_3$	0.1 – 10 µM	Weak Positive	Gebel, 1996 2: Reliable with restriction.
SCE Human Lymphocyte	Sb_2O_5	0.1 – 10 µM	Weak Positive	Gebel, 1996 2: Reliable with restriction.
Double strand DNA breaks in cultured human HeLa cells	$SbCl_3$	10 – 100 µM	Negative for DNA breaks Positive for inhibition of repair	Koch et al., 2017 2: Reliable with restriction
ToxTracker Assay				
GFP-based mouse embryonic stem (mES) reporter stem cell lines	Sb, Sb_2O_3, Sb_2S_3, $SbCl_3$, $Sb_2(C_2H_4O_2)_3$, $Na(Sb)(OH)_6$, $NaSbO_3$, $SbCl_5$, Sb_2O_5, $K(Sb)(OH)_6$	20 doses: 100 µg/ml and 19 consecutive 2-fold dilutions	• DNA damage: negative • Activation p53 response: negative • Induction of oxidative stress: positive for all test items except $Na(Sb)(OH)_6$ and $Sb2O_5$ • Unfolder protein response	Hendriks, 2017 2: Reliable with restriction (assay not yet validated)

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Assay	Compound Tested	Concentration	Test Result	Reference and Klimisch Score
			activation: positive for all test items except $SbCl_3$,$Na(Sb)(OH)_6$, $NaSbO_3$, and $Sb2O_5$	

A somewhat more consistent response profile emerges in tests using mammalian cells cultured *in vitro*. Two gene mutation tests have been done using a mouse lymphoma cell line (Table 3) and failed to detect mutagenic activity. A number of studies have evaluated the ability of Sb compounds to induce chromosome aberrations or micronucleus (MN) induction. Micronuclei are usually from fragmented chromosomes so tests for chromosome aberrations and micronucleus induction are expected to yield similar results. Positive assay results for the induction of micronuclei are the most consistent indication of genotoxic activity for Sb compounds reported in the scientific literature. However, as detailed section 2.3 and in the technical annex, recent studies have reported potential experimental artefacts in the staining procedures applied for the detection of micronuclei in cells treated with metals or metalloids (Wedel et al., 2013; Cohen et al., 2013), so the micronuclei evidence must be considered with the applicable caveat.

Mechanistic aspects of Sb genotoxicity have recently been evaluated in the "ToxTracker" assay, a novel test system that employs mammalian stem cell lines containing fluorescent reporters that respond to the expression of genes expected to be induced following the direct induction of DNA damage, oxidative stress and protein damage (Hendriks, 2017). None of the Sb compounds tested yielded responses indicative of direct DNA damage. However, all but two compounds (Sb pentoxide and sodium hexahydroxoantimonate) were found to be strong inducers of oxidative stress. All but three (sodium antimonate, Sb pentoxide and sodium hexahydroxoantimonate) exerted protein damage that could reflect interference with DNA repair functions. Although still in validation, the ToxTracker assay has generated a response profile for all Sb substances registered under REACH consistent with the induction of genotoxicity though indirect[2] mechanisms that entail oxidative stress or protein damage.

Oxidative stress reflects an imbalance between the systemic production of reactive oxygen species (ROS) and the ability of a biological system to readily detoxify the reactive intermediates or repair the resulting damage. Only genes that are up-regulated by oxidative stress were up-regulated by Sb compounds and could be of significance for multiple complex reasons. For example, ToxTracker observed induction of the SRXN1 reporter gene associated with the transcription factor Nrf2. Nrf2 is one of the critical regulators of endogenous antioxidant defense and promotes the transcription of a wide variety of antioxidant genes. Through binding to antioxidant response elements (AREs), Nrf2 is a transcription factor that activates antioxidant target genes, such as glutathione S-transferase and heme oxygenase (HO). Although cell signaling pathways triggered by the transcription factor Nrf2 prevents cancer initiation and progression in normal and premalignant tissues, in fully malignant cells, Nrf2 activity provides growth advantage by increasing cancer chemoresistance and enhancing tumor cell growth

Studies of the genotoxic effects of Sb compounds *in vivo* have focused upon the impacts of Sb trioxide. The results of testing in experimental animals are summarized in Table 4 and provide a mixed display of effects that lacks the consistency evident in the *in vitro* studies. Given the importance of *in vivo* findings for genotoxicity evaluations, low quality studies have been retained in the table. Several of the low-quality studies

[2] When genotoxicity is caused by other means than by direct interaction with DNA, e.g. induction of aneuploidy, oxidative stress, inhibition of DNA synthesis or cytotoxicity presents, the mode of action is assumed to be an indirect one. In short, indirect mechanisms of genotoxicity correspond to interactions of mutagens with non-DNA targets.

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have been extensively cited in the peer-reviewed literature and their inclusion in this section of the evaluation is intended as a demonstration of the value of considering study quality in weight of evidence evaluations.

Table 4: Genotoxic effects of Sb trioxide in vivo.

Assay	Compound	Dose	Result	Reference and Klimisch Score
Mutation Assays				
Chromosome Abb. Mouse Bone Marrow	Sb_2O_3	400 – 1000 mg/kg single oral dose	Negative	Gurnani, 1992 3, not reliable
Chromosome Abb. Mouse Bone Marrow	Sb_2O_3	400 – 1000 mg/kg 21-day oral dose	Equivocal Positive	Gurnani, 1992 3, not reliable.
Chromosome Abb. Rat Bone Marrow	Sb_2O_3	250 – 1000 mg/kg 21-day oral dose	Negative	Kirkland, 2007 1: Reliable without restriction
MN mouse bone marrow	Sb_2O_3	3200 and 5000 mg/kg oral gavage, one dose	Negative	Elliot 1998 2: Reliable with restrictions
MN mouse erythrocytes	Sb_2O_3	One-year daily inhalation exposure to 3, 10 and 30 mg/m^3	Equivocal Positive	NTP, 2017 2: Reliable with restrictions.
MN rat erythrocytes	Sb_2O_3	One-year daily inhalation exposure to 3, 10 and 30 mg/m^3	Negative	NTP, 2017 1: Reliable without restriction
MN rat bone marrow	Sb_2O_3	250 – 1000 mg/kg 21 day repeated oral dose	Negative	Kirkland, 2007 1: Reliable without restriction.
Genotoxicity Indicator Assay Data				
UDS rat liver	Sb_2O_3	5000 mg/kg oral gavage, single dose	Negative	Elliot 1998 2: Reliable with restrictions.
Comet Assay mouse lung	Sb_2O_3	One-year daily inhalation exposure to 3, 10 and 30 mg/m^3	Equivocal Positive	NTP, 2017 3: Not reliable.
Comet Assay rat lung	Sb_2O_3	One-year daily inhalation exposure to 3, 10 and 30 mg/m^3	Negative	NTP, 2017 3: Not reliable.
Human: SCEs and MNs in workers exposed to fire retardants	Sb_2O_3		SCE: Negative MN: Negative Fpg modified Comet: Positive	Cavallo et al., 2002 2 (reliable with restrictions)
Human: Induction of apurinic sites on DNA in workers exposed to Sb trioxide	Sb_2O_3	25 workers occupationally exposed to Sb trioxide. Urinary Sb output 10 -19 □g/L	Positive	Shanawany et al., 2017 2: Reliable with restrictions

Gurnani et al. (1992) reported that chromosome aberrations would result in the bone marrow cells of mice repeated oral exposures to Sb trioxide. Although single (acute) exposures to Sb trioxide did not produce effects, exposure of mice for up to 21 days produced chromosome changes that increased in frequency as a function of exposure duration and intensity. Interpretation of this positive study is problematic due to a number of technical deficiencies and deviations from Good Laboratory Practice (GLP) protocols. Kirkland et al., (2007) attempted to replicate the findings of Gurnani et al. (1992) in a study that strongly adhered to GLP guidelines and possesses technical rigor superior to other *in vivo* studies evaluating clastogenic effects of Sb compounds. No treatment related increases in chromosome aberrations or micronuclei were observed.

The National Toxicology Program of the United States recently conducted inhalation cancer bioassays upon rats and mice, exposing animals to 3, 10 and 30 mg/m^3 Sb trioxide for two years (NTP, 2017). After one year of inhalation exposure, a subgroup of rats and mice were evaluated for the presence of micronuclei in red blood cells. No impact was seen in rats whereas a slight increase in micronucleus induction was reported in mice. As detailed in the technical annex, this increase is most plausibly a reflection of hypoxia and not actually an indication of induced chromosome damage.

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DNA damage in lung cells was also evaluated via the Comet assay – no DNA damage was observed in rat lung cells whereas NTP (2017) indicated DNA damage could be detected in mouse lung cells. The detection of DNA damage in lung tissues exhibiting a toxic response (as occurred in the lung tissues of mice) is technically challenging (Speit et al., 2015) and NTP did not conduct any of the experimental controls needed to guard against false positive results in the Comet assay.
Thus, the NTP studies did not observe genotoxicity in rats, and produced at best, equivocal findings in mice. Lung tumors were subsequently observed in rats and mice, but the role of genotoxicity in the etiology of these tumors is unclear.

Other studies evaluating the genotoxic impacts of Sb in vivo followed protocols limited in scope. Elliot et al. (1998) examined the impacts of a single 5000 mg/kg oral gavage Sb trioxide dose upon micronucleus induction. No evidence was obtained for micronucleus induction. The same authors also examined the induction of unscheduled DNA synthesis in rat liver after a single dose of Sb trioxide administered by oral gavage at doses of 3200 and 5000 mg/kg. No treatment related impacts upon unscheduled DNA synthesis were observed.

A small number of studies have examined potential genotoxic impacts in workers occupationally exposed to Sb. Shanaway et al. (2017) observed an increase in apurinic sites in the DNA of workers occupationally exposed to Sb trioxide during Sb trioxide production. Apurinic sites increased in frequency with urinary Sb (10 – 19 µg/L) but only weakly correlated with length of employment. The significance of apurinic sites for mutagenesis is unclear since DNA repair would be expected to resolve apurinic lesions. The authors hypothesize that the presence of apurinic sites is either induced by Sb or the sites accumulate due to inhibition of DNA repair. Induction by reactive oxygen species is also possible, but evidence for this was not found. Whether any co-exposures that might have influenced results were present is not known – the use of Sb was noted to be taking place in a “primitive working environment”.

Cavallo et al. (2002) evaluated a number of different endpoints in 23 male workers exposed to Sb trioxide during the application of fire retardants and compared them to 23 healthy male controls. Workers were classified as having high (n=17) or low (n=6) Sb trioxide exposure but this was related to modest Sb in air levels averaging 12 and 5 µg/m^3, respectively. No increase in SCE or MN induction was associated with Sb trioxide exposure, but the levels of occupational exposure were quite low. Application of the “standard” Comet assay to worker lymphocytes yielded little by way of an assay response, but enzyme treatment (which cleaves DNA with oxidative damage) yielded a positive Comet assay result. The extent of oxidative damage was modest in absolute terms. If related to Sb exposure, the low levels of Sb in air would not be expected to yield a large response.

Table 5 below shows that although there is a good amount of information for trivalent substances, there is no in vivo genotoxicity data for pentavalent ones. In vivo mutagenicity testing was not required under REACH since no positive results were obtained from in vitro mutation assays (cf. Annex VII and VIII, Section 8.4 Column 2 of Regulation (EC) 1907/2006) and because the absence of mutagenicity in vivo, for Sb 3+ substances, which are generally considered to be more toxic than Sb 5+.
The ToxTracker assay findings confirm that Sb 3+ compounds will generally tend to exert oxidative stress and protein damage in a stronger manner than Sb 5+ compounds. Based on this, it can be assumed that if most in vivo studies with Sb 3+ compounds have not observed genotoxicity, it is very unlikely that the same studies conducted with similar Sb 5+ compounds would produce genotoxic effects.

Table 5 below presents an overview of the available genotoxicity studies per Sb substance (except the indicator assays and ToxTracker). While 8 out of 19 in vitro genotoxicity studies have reported positive responses for genotoxicity, 5 out of the 7 in vivo genotoxicity studies in rodents have failed to observe genotoxic effects. The positive or equivocal assay responses which have been observed are restricted to

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studies with significant technical deficiencies. A more detailed discussion on the available evidence is provided in the Technical Annex.

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Table 5: Overview of genotoxicity data (except indicator assays) available for Sb substances considered for grouping and read-across for genetic toxicity endpoints (x = Klimisch score 1 or 2, x = Klimisch score 3 or 4).

Name	CAS #	in vitro				in vivo	
		Ames, (OECD 471)	Chromosomal Aberrations (OECD 473)	Micronucleus (OECD 487)	Mouse Lymphoma Assay (OECD 490)	Chromosomal Aberrations (OECD 475)	Micronucleus (OECD 474)
Sb metal							
Sb –powder	7440-36-0	x	x				
Sb – massive	7440-36-0						
Trivalent Sb compounds							
Diantimony trioxide	1309-64-4	x, x	x		x	x, x, x	x, x, x, x
Antimony sulfide	1345-04-6						
Antimony tris(ethylene glycolate)	29736-75-2						
Antimony trichloride	10025-91-9	x		x, x, x, x, x			
Pentavalent Sb compounds							
Sodium hexahydroxoantimonate	33908-66-6	x, x	x	x	x		
Sodium antimonate	15432-85-6						
Antimony pentachloride	7647-18-9	x					
Antimony pentoxide	1314-60-9	x					
Potassium hexahydroxoantimonate	12208-13-8						

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2.3 Weight of evidence analysis of the genotoxicity of Sb substances

This section covers steps 3) integration and weighing of evidence (weight of evidence analysis), 4) application of levels of confidence, and 5) uncertainty analysis of the weight of evidence approach. A more detailed discussion on the available evidence is provided in the Technical Annex.

In order to assess the potential genotoxicity of Sb substances, it is important to understand the mechanism(s) by which Sb compounds produce positive response in some in vitro test systems, and the chemical species involved in such a response. The following hypothesis have been put forward based on the available genotoxicity information:

Most genotoxicity studies have been conducted using soluble Sb in the form of trivalent Sb trichloride and the assumption made that any activity observed in various test systems could be attributed to the release of the electrophilic Sb ion via hydrolysis to yield $Sb(OH)_3$. The behavior of trivalent Sb compounds in solution is likely to be complex and involve the sequential formation of Sb oxide chloride (SbOCl), Sb oxide hydroxide (SbO(OH) and ultimately the formation of Sb trioxide (Sb_2O_3) (Hashimoto et al., 2003). The pentavalent Sb pentachloride is similarly an electrophilic oxidizing agent which, as a function of pH, will also undergo a series of hydrolytic transformations to oxychlorides and oxide hydroxides that result in the formation of Sb_2O_5 (Zheng, Zhi and Chen, 2006). ***Although the chemical moiety that might be responsible for producing a possible genotoxic response is uncertain, hydrolysis products are the likely mediator for positive test responses.***

As oxyanions, ***Sb hydrolysis products would also be expected to undergo electrophilic interactions with cellular constituents such as thiol rich proteins*** (Verdugo et al., 2017). ***Such interactions provide the mechanistic basis for intracellular inclusion body formation.*** Documented to form after exposure to metals and metalloids, such inclusion bodies can be mistaken for micronuclei unless staining procedures are employed that are specific for the presence of DNA (Wedel et al, 2013; Cohen et al., 2013). Studies of micronucleus induction after treatment with Sb compounds have not routinely employed such high affinity staining procedures. As discussed in the Technical Annex, there is an element of uncertainty associated with the interpretation of existing micronucleus studies.

Direct covalent interaction of Sb 5+ with DNA has not been detected (Li et al., 2011) although ***binding of Sb 3+ to the ribose of guanosine appears to occur, leading to suggestions that interference with the function and/or fidelity of DNA polymerases may be impacted***. Consistent with this are impacts of Sb upon the repair of DNA strand breaks (Beyersmann and Hartwig, 2008; Koch et al., 2017) and excision repair (Grosskopf et al., 2010). Genotoxicity responses may thus be mediated by indirect impacts upon DNA repair. The relevance of these in vitro observations to in vivo exposure scenarios is uncertain, since the concentrations required to produce effects in vitro are generally significantly higher than plausible systemic levels of Sb in vivo. However, such concentrations may be within the range of feasibility for tissues exposure directly to Sb compounds (e.g. inhaled material in the lung).

De Boeck et al. (2003) suggest that the ***generation of oxygen radicals constitute another indirect pathway for inducing genotoxic responses***. Supportive evidence for this is derived from the calcium influx studies of Elliot et al. (1998). The cytotoxic effects of potassium antimony tartrate upon cardiomyocytes also appears to be associated with the generation of oxygen radicals (Tirmenstein *et al.* 1995). Finally, Jiang *et al.* (2016) have observed that apoptosis induced by Sb appears to be a response to the generation of active oxygen species.

Mechanistic aspects of Sb genotoxicity have recently been evaluated in the “ToxTracker” assay, a novel test system that employs mammalian stem cell lines containing fluorescent reporters that respond to the

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expression of genes expected to be induced following the direct induction of DNA damage, oxidative stress and protein damage (Hendriks, 2017). None of the Sb compounds tested yielded responses indicative of direct DNA damage. However, all but two compounds (Sb pentoxide and sodium hexahydroxoantimonate) were found to be strong inducers of oxidative stress. All but three (sodium antimonate, Sb pentoxide and sodium hexahydroxoantimonate) exerted protein damage that could reflect interference with DNA repair functions. Although still in validation, the ToxTracker assay has generated a response profile for all Sb substances registered under REACH consistent with the ***induction of genotoxicity though indirect mechanisms that entail oxidative stress or protein damage***.

If reactive oxygen species mediate most in vitro observations of genotoxicity, this could explain why most in vivo studies have not observed genotoxicity. Anti-oxidant system in an intact animal are robust and would mitigate against oxidative damage. ***Expression of genotoxicity would be absent in vivo or exhibit a threshold with genotoxicity only resulting when the protective capacity of anti-oxidant systems is exceeded*** (Kirkland et al., 2015). Consistent with this is the lack of correlation between urinary Sb and oxidative biomarkers in humans (Domingo-Relloso, 2019) although others have suggested oxidative damage detected by the Fpg modified Comet assay is induced by occupational exposure (Cavallo et al., 2002).

Not all evidence supports oxidative stress as a mechanism for Sb genotoxicity. Shaumloffel and Gebel (1998) did not observe attenuation of Sb induced Comet assay responses by the exogenous addition of superoxide dismutase or catalase, but it is not clear whether the positive Comet assay results reported were artifacts of cytotoxicity or apoptosis. The NTP inhalation cancer bioassays of Sb trioxide (NTP, 2017) observed ***activation of the EGFR oncogene*** in a number of mouse lung tumors and “fingerprinted” the DNA sequence changes presumed to be responsible for activation. The observed changes were not characteristic of DNA sequence changes associated with oxygen radicals. A high frequency of G to T transversions was not observed in activated oncogenes, the DNA sequence alteration that is most commonly associated with interaction of active oxygen species with DNA to form 8-hydroxyguanine and a subsequent G to T transversion (Tchou *et al.*, 1991; Hong et al., 2016). Sb 3+ binding to the ribose of guanosine also does not appear to impact the nature of mutations observed. Oncogene activation in mouse lung tumors may thus result from events unrelated to oxidative stress and/or may not be the critical event by which Sb trioxide induces mouse lung tumors.

In summary, the available data suggest that Sb hydrolysis products do not induce point mutations but that clastogenic events result from in vitro exposures. In vivo assessments of genotoxicity have generally produced negative or, at best, equivocal results. Several negative studies possess the highest technical rigor – those with equivocal findings have significant technical deficiencies. Thus, whereas in vitro studies suggest genotoxic properties, there is little evidence that this is expressed in vivo.

The overall weight of evidence analysis of the genotoxic potential of Sb and its compounds is summarized in Table 6 below.

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Table 6: Weight of evidence analysis of the genotoxic potential of Sb substances.

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Sb species	Type of evidence	Consistency and specificity	Likelihood and biological plausibility	Temporality	Conclusion	Confidence / Strength of evidence	Remaining uncertainty	Research options
Sb 3+	**in vitro**	No evidence of gene mutations. Micronucleus and clastogenicity positive.	Binding to DNA and evidence of indirect mechanisms support this	Lesions appear appropriately after treatment	Sb 3+ compounds are clastogens but do not induce point mutations in vitro	High - Strong evidence for clastogenicity in vitro but not point mutations	Potential staining artifacts in MN studies. Not validated in vivo (in vivo validation may be prevented by the fact that the concentrations that produce some indirect effects in vitro may not be achievable in vivo).	Verify possible MN staining artifact in vitro.
	in vivo	Most studies show consistent lack of genotoxicity	Anti-oxidant systems may prevent in vivo effects	Exposure is followed by radical oxygen species (ROS) induction and changes in other systems that may mediate an indirect response	Sb trioxide is not genotoxic in vivo	High – Systemic impacts have not been observed. Local effects cannot be precluded.	Only in vivo data for Sb trioxide. All in vivo data are not negative. Uncertain role of methylation.	In vivo research to clarify threshold (indirect) mechanism of action and lack of in vivo genotoxicity of Sb trioxide (include investigation of extent and significance of Sb 3+ methylation). In vitro research to show representativity of Sb trioxide for other Sb 3+ compounds.
	overall	Evidence of in vitro genotoxicity not reproduced in vivo	In vivo mechanisms 'control' Sb's threshold genotoxicity mechanisms	/	Sb 3+ compounds are not genotoxic.	Medium – to be demonstrated	Actual mechanism of observed genotoxicity unclear.	In vitro studies in respiratory cells, followed by in vivo validation research.
Sb 5+	**in vitro**	Little evidence for genotoxic activity. No binding to DNA.	Lack of interaction with DNA supports this	Not applicable	Sodium hexahydroxo-antimonate is not genotoxic in vitro	High – Strong evidence available	None	In vitro studies (in respiratory cells)
	in vivo	No data. Given that Sb 5+ is less potent than Sb 3+ as a toxin, lack of Sb 3+ in vivo genotoxicity should mean lack of Sb 5+ in vivo genotoxic activity too.	Sb 5+ can be converted to 3+	Not applicable	Sb 5+ are not genotoxic in vivo	Medium – Conclusion based on evidence on Sb 3+ compounds and expected conversion from Sb 5+ to Sb 3+.	No in vivo genotoxicity study has been conducted on any Sb 5+ compound. Conversion (and methylation) of Sb 5+ to Sb 3+ expected but not investigated fully.	Validate in vitro results with in vivo study, including investigation on the extent and significance of transformation (and methylation) from Sb 5+ to Sb 3+

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	overall	Data mainly available on Sb 3+ compounds	Sb 5+ can be converted to 3+, but is generally considered to be less toxic than Sb 3+	/	Sb 5+ compounds are not genotoxic.	Medium – to be demonstrated	Several evidence lacking	All the above

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The analysis summarized above is associated with different degrees of overall confidence/uncertainty which could be increased/decreased through further research options described below:

- **Verify possible Micronucleus staining artifacts:** The possible formation of inclusion bodies in vitro following treatment with Sb and its compounds should be evaluated for its potential impact upon micronucleus test data. The conduct of in vitro micronucleus tests with staining procedures with high specificity for DNA could provide a rapid means of determining whether staining artifacts have impacted the existing genotoxicity data base. This work is relevant for all Sb substances.

- **Verify threshold/indirect mode of action for genotoxicity in respiratory cells:** A dedicated and carefully designed in vitro research would enable comparison and ranking of all Sb substances on the basis of their dose-response for key effects such as: cytotoxicity and viability impacts, intracellular reactive oxygen species (ROS) generation, ROS-induced damage, pro-inflammatory responses, Sb particle surface reactivity, oxidative potential, oxidative stress (intracellular glutathione and gene expression of HO-1), and genotoxicity in lung cells. This can be done in a phased approach, namely:
  - Phase 1: physical-chemical characterization of all Sb substances;
  - Phase 2a: assessment of effects in several cell phenotypes (cytotoxicity, oxidative potential, and inflammation); and
  - Phase 2b: assessment of genotoxicity in several cell phenotypes (oxidative stress, oxidative damage, and genotoxicity, including micronucleus and COMET assays).

  Testing for point mutation induction (establishing any mutation fingerprint) and micronucleus induction in vitro could be indicated.
  An extension of this work, incorporating complex in vitro tissue models and exposure methods akin to that of particle exposure via respiration, is a possible subsequent tier to further clarify the mode of action for genotoxicity in vitro.
  This work is relevant for all Sb substances.

- **Verify proposed mode of action in vivo:** The sequential genotoxicity studies are intended to clarify the mechanism/mode of action responsible for observed animal carcinogenicity. Factors such as the induction of ROS, pulmonary inflammation and inhibition of DNA repair have all been noted to potentially mediate genotoxic and/or carcinogenic responses. The in vitro genotoxicity research described above will thereby inform on the most optimal (combined) in vivo inhalation genotoxicity study(ies) to be performed to validate the genotoxicity weight of evidence assessment. Whenever in vivo studies are performed on Sb 3+ substances, they should also investigate the extent and significance of the methylation of Sb 3+.
  It is important to note that a number of advanced genotoxicity assays of potential relevance to Sb are currently under development, and would provide more precise and accurate results for a wider range of mechanistic events potentially relevant to Sb trioxide animal carcinogenesis. The development of these assays will be carefully monitored with a view to implementing studies that provide the basis for refining dose-response modelling and prediction of health effects in humans.
  This in vivo work would be specifically relevant for Sb 3+ substances, but also informative for the genotoxicity assessment of Sb 5+ substances.

- **Generate missing in vivo evidence for Sb 5+ compounds:** It is often assumed that Sb 3+ compounds are genotoxic and that Sb 5+ compounds are not. The testing literature for Sb 5+ compounds is limited, with only a small number of in vitro genotoxicity tests having been conducted. Pending resolution of the points above, additional in vivo testing of Sb 5+ compounds may be needed to support the assessment. Whenever in vivo studies are performed on Sb 5+ substances, they should foresee to also investigate the extent and significance of the conversion (and methylation) of Sb 5+.

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2.4 Conclusion of the weight of evidence assessment of the genotoxicity of Sb substances

This section covers step 6) conclusion of the weight of evidence approach.

Based on the available evidence and the assessment of it made above, questions formulated in section 2.1 above can be answered as follows:

- ***Is the available evidence sufficient to assess the genotoxicity of Sb substances?***
  Yes, although with a relatively medium level of confidence, due to remaining uncertainties pertaining to the difference in results between in vitro and in vivo assays, and the general lack of in vivo evidence on most Sb substances except Sb trioxide.

- ***What indication does the available evidence provide as to the potential genotoxicity of Sb substances?***
  The available evidence currently indicates that:
  - Sb 3+ compounds are clastogenic in vitro but do not induce point mutations; indirect mechanisms of action are probable.
  - Sb 5+ compounds are not mutagenic in vitro and do not bind to DNA. Genotoxic activity is unlikely unless there is conversion of Sb 5+ to Sb 3+.
  - Sb 3+ and 5+ substances are not genotoxic in vivo. Any genotoxicity observed (in vitro) would be the result of indirect, threshold, mechanisms, possibly mediated by changes in reactive oxygen species, valency and methylation.
  - The available data do **not** support a no classification for mutagenicity.

- ***What research would increase the confidence/reduce the uncertainty of the genotoxicity assessment of Sb substances?***
  The research necessary to increase the confidence and reduce the uncertainty of the genotoxicity assessment includes:
  - An in vitro micronucleus study to verifying the possible micronucleus staining artifacts caused by non-DNA-specific stains
  - An in vitro assay on respiratory cells to understand the mechanism of action yielding positive genotoxicity results in vitro but negative genotoxicity results in vivo, and to inform on subsequent in vivo research needs
  - One or more in vivo studies to validate the hypothesis developed on the basis of the in vitro evidence, and understand the extent and significance of the transformation (and methylation) and Sb compounds following absorption

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3. Assessment of genotoxicity applying a grouping/read-across approach

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ECHA's recommendation for documenting a read-across approach includes the following steps:

1) Properly identify and characterize the source and target substance(s)
2) Select of the most appropriate scenario to be used for the read-across assessment
3) Address each common assessment element (AE) of the selected scenario
4) Establish the read-across assessment based on the conclusions derived for all of the AEs

3.1 Identification and characterization of the source and target substance(s)

Metal and metalloid compounds are typically defined on the basis of the valence or oxidation state of the ion contained in the substance. In Table 7, the Sb substances and their corresponding CAS number are listed in order of valence state, namely 0, 3+ and 5+. The oxidation state (IUPAC Definition: the charge of the atom after ionic approximation of its heteronuclear bonds) will dictate the affinity and potential for interaction and chemical bonding of a given metal/metalloid substance with biological systems. As explained by Hashimoto et al. (2003), Zheng, Zhi and Chen (2006), and ATSDR (2017), Sb 3+ or 5+ will exhibit strong electrophilic characteristics, and be taken up as the (oxyan)ion after release from the parent compound due to hydrolysis of ionic bonds. ***Considering the specificity of interactions between a chemical and a cell, the differences in valence/ionic species may need to be considered for the purpose of read-across for genotoxicity evidence.***

Table 7 also provides information on the moiety (functional group) of each Sb substance that will normally influence the physico-chemical properties, and the bio-availability of the substance. The functional groups will dictate the ease with which Sb oxyanions are released from a substance and made available for systemic uptake. The primary impact of the moiety will be in determining the dissolution rate of compounds in the gastrointestinal tract (US EPA, 2007). The actual uptake of Sb ions/oxyanions from the gastrointestinal tract appears to be mediated by saturable carrier protein transport systems and will occur with low (<1 %) efficiency (ATSDR, 2017)[3].

The released moieties, many of which are essential nutrients or Essential Trace Elements (ETEs), may also be subject to independent gastrointestinal uptake (in many instances regulated by homeostatic control mechanisms). Given their essential nature, the anticipated genotoxicity impact of these moieties, compared to the possible genotoxicity impact of Sb, is expected to be negligible. Other moieties are normal metabolites (NM), often of carbohydrate metabolism, and are expected to be rapidly metabolized.

The chemical nature of the ligand moiety may exert its own toxicity in rare cases, but this is the exception and not the rule, and particularly not for the moieties reported in Table 7. The notable exception to this generalization will be moieties (e.g. chlorides) which, when administered in pure or concentrated doses, will have corrosive or irritant properties that serve to limit substance administration due to local effects that disrupt essential functions such as food ingestion or breathing. But again, this is not a reasonable and foreseeable situation under REACH. ***The difference in moieties can be omitted for the purpose of read-across for genotoxicity evidence.***

As regards the molecular weight and structure of each substance, the information in Table 7 shows that there are no specific trends or patterns among the molecular weight or structure that can inform the read-across approach. ***Considering the specificity of interactions between a chemical and a cell however, the difference in molecular weight may need to be considered for the purpose of estimating dosimetry in read-across comparisons of genotoxicity evidence.***

[3] This low uptake rate combines with the emetic properties of Sb compounds (ATSDR, 2017) to limit the systemic levels of Sb that can be achieved via oral exposure.

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Impurities in Sb substances are commonly arsenic and lead (in the relevant speciation)[4], but typically in concentration levels below 0,1% or their respective Specific Concentration Limits (SCL)[5]. This means that the assessment and read-across of the toxicity hazard and effect of the Sb substance will be driven by the Sb, and **not** by the impurities in the substances; and that the various *pure* Sb substances do not need to be distinguished on the basis of their impurities for the purpose of read-across. Table 7 confirms that ***the impurity profile is relatively comparable across the various Sb substances, and that there is no reason to discriminate between these on the basis of (im)purity for purposes of read-across for genotoxicity evidence.***

Finally, the table provides information on the physical form (powder, particle size) of each Sb substance. The physical form, and particle size, is relevant to the consideration of the exposure routes through which the various Sb substances may enter the body under realistic use conditions. Genotoxicity would require the systemic uptake of a relatively large amount of a given substance, able to interact directly or indirectly with the genetic material or genetic processes of the cells[6]. In this context, the oral exposure route offers the main physiological entry point for Sb substances into the human body; physical forms (and sizes) that can be ingested are those of relevance to genotoxicity (in theory, any of the Sb substances in Table 7). The ingestion route is also the route through which consumers will be more likely to be exposed to Sb substances[7]. ***The differences in physical form and particle sizes can be omitted for the purpose of read-across of genotoxicity evidence.***

The content of Table 7 shows that on the basis of identity or characterization, beyond valency and molecular weight, there are no major differences between the Sb substances subject to REACH that would challenge a grouping or read-across approach. In short, any Sb substance which does not contain a moiety with a more severe systemic toxicity than that of the moieties in Table 7, could in principle be part of the Sb substances read-across group for genotoxicity.

[4] Because of the geological affinity there is between the Sb, As and Pb in the predominant natural source of Sb (stibnite), As and Pb will typically be present as impurities in any Sb substance. Indeed, even following the transformation of stibnite into Sb "metal", and then into subsequent Sb compounds, these impurities will remain, albeit in controlled quantities. In Sb metal, the impurities will be present in metallic form whereas in e.g. Sb oxides or sulfides, they will be present in oxidic or sulfidic form, respectively.

[5] For carcinogens category 1A such as As oxides or acid the cut-off level is 0.1 %. For reprotoxicants category 1A such as Pb oxides the cut-off is 2.5 %, for Pb metal massive the SCL is 0.3 %, and for Pb metal powder the SCL is 0.03%.

[6] The fact that a substance is ingestible however, does not imply it can then be absorbed systemically and reach the relevant cells and have a genotoxic effect. Even if the substance is able to reach the relevant cells, it may express no genotoxic effect at all.

[7] Lung carcinogenicity would be mediated by inhalation exposure, as discussed in the scientific opinion on lung toxicity and carcinogenicity.

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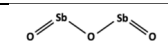
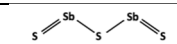
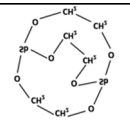
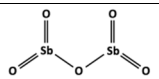
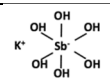
Table 7: Identity, characterization and structural (dis)similarity of Sb substances considered for grouping and read-across.

Name	CAS #	Form and typical particle size	Molecular weight (g/mol)	Chemical formula	Structure	Moiety	Purity (% w/w)	Impurities
"Metallic" Sb								
Sb – powder	7440-36-0	Powder (< 1 mm)	121.76	Sb	Sb	--	>89.45 - <100	As: <2.5 Pb: <9
Sb – massive	7440-36-0	Massive (> 1 mm)[3]	121.76	Sb	Sb	--		
Trivalent Sb substances								
Diantimony trioxide	1309-64-4	Powder 0.2-0.44 µm	291.5	Sb_2O_3		--	>97 - <100	As_2O_3: <0.1 PbO: <2.5
Antimony sulfide	1345-04-6	Powder D_{50}: 32.7 µm	339.7	Sb_2S_3		SO_4^{2-}		
Antimony tris(ethylene glycolate)	29736-75-2	Crystal D_{50}: 1600 µm	495.7	$Sb_2(C_2H_4O_2)_3$		$(C_2H_4O_2)^{2-}$	>99	n.s. [2]
Antimony trichloride	10025-91-9	Crystal D_{50}: 897 µm	190.7	$SbCl_3$		Cl^-	>99	n.s. [2]
Pentavalent Sb substances								
Sodium hexahydroxoantimonate	33908-66-6	Powder MMAD: 26.2 µm[1]	246.8	$Na(Sb)(OH)_6$		Na^+	>94.8 – <99.75	PbO: <2.5
Sodium antimonate	15432-85-6	Powder/Crystals: 1-180 µm	192.7	$NaSbO_3$		Na^+	>95 – <99.9	n.s. [2]
Antimony pentachloride	7647-18-9	Liquid	299,02	$SbCl_5$		Cl^-	>98	$SbCl_3$: <1 As: <0.1 Pb: <0.1
Antimony pentoxide	1314-60-9	Powder/colloidal suspension D_{50}: 24.4 µm	323.5	Sb_2O_5		--	>87 - <99.9	As_2O_3: <0.1 PbO: <0.25
Potassium hexahydroxoantimonate	12208-13-8	Crystal	262.9	$K(Sb)(OH)_6$		K^+	> 94 - < 97	n.s. [2]

[1] Mass Median Aerodynamic Diameter; [2] Non-specified impurities for which the individual composition does not exceed 0.1% and/or which are not classified; [3] Cf. Guidance on the Application of the CLP Criteria Version 5.0 – July 2017, page 600, Section IV.5.5 Particle size and surface area.

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3.2 Selection of the most appropriate scenario to be used for the read-across assessment

As genotoxicity information is available from more than one source substance, and used for more than one target substance, the read-across approach applied to fill in genotoxicity data gaps for Sb 3+ and 5+ substances is a ***category*** (as opposed to analogue) or group one.

The read-across hypothesis is that different Sb 3+ or 5+ substances, respectively, will ***give rise to/release (the same) common compound*** to which an organism will be exposed. On this basis the substances can actually be grouped into two subgroups which are each releasing a valence-specific (3+ or 5+) soluble metal (oxyan)ion. The release of the specific Sb ion can be considered a common transformation product within each subgroup (no matter how the transformation occurs).

As concluded in section 2.4 above, the genotoxicity data available on the Sb substances reveal a general trend of no (in vivo) genotoxicity. Mechanistic information indicates that the in vitro genotoxicity observed in some cases are most likely the result of indirect mechanisms, which differ between Sb 3+ and Sb 5+ substances. Whereas Sb 3+ substances appear to induce oxidative stress or interfere with DNA repair processes, Sb 5+ substances are of lower potency and refractile to binding to DNA. The trends observed, while not strikingly different, provide sufficient toxicological and toxicokinetic differences (Filella and Williams, 2010; Wu et al., 2018; ATSDR, 2017) to indicate a qualitative similarity of effects inside each subgroup.

As a result, the read-across approach applied to the genotoxicity assessment of Sb substances corresponds to Scenario 5:
"*This scenario covers the category approach for which the read-across hypothesis is based on (bio)transformation to common compound(s). For the REACH information requirement under consideration, the property investigated in studies conducted with different source substances is used to predict the property that would be observed in a study with the target substance if it were to be conducted. Similar properties are observed for the different source substances; this may include absence of effects for every member of the category. No relevant differences in predicted properties are observed for several source substances.*"

3.3 Common assessment elements for the selected scenario

This section will cover the following assessment elements of scenario 5: 1) Formation of common (identical) compound(s); 2) Biological target(s) for the common compound(s); 3) Exposure of the biological target(s) to the common compound(s); and 4) Impact of parent compounds.

Assessment element 5) Formation and impact of non-common compounds was addressed in section 3.1, in the discussion about the moieties and functional groups of the Sb substances in scope.

The systemic toxicity of most metal(loid)s and their compounds occurs upon: i) the release of soluble metal ions; and ii) their uptake by the body and/or interaction at their target organ sites. It is the bioavailability of the released metal at the site of action (for local effects) or uptake (for systemic effects) in the organism that can be the most important determining factor modulating toxicity.

Information on bioavailability can be predicted using in vitro models, such as bioelution, that simulate processes governing uptake rates in vivo. Bio-elution measures the bio-accessibility of a substance in simulated biological fluids. A bio-elution test will therefore measure the amount of released metal "available for absorption" under physiological conditions.

There are artificial fluids for every relevant route of exposure to be assessed. For the oral exposure route, which is relevant for the systemic uptake of Sb substances, fluids exist which simulate the stomach and the

intestinal conditions. A key difference between these two fluids is the pH they mimic: pH of 1.5 for gastric, and pH of 7.4 for intestinal. Although the uptake of metals is known to occur in the intestine, at neutral pH, the highest release of metal can be expected to take place in the stomach, at acidic pH. Because of this, the gastric fluid is often selected for bio-accessibility testing, as it represents 'worst-case conditions' for release following ingestion.

A relationship can be assumed between in vitro bio-accessibility in an artificial biological fluid, and relative in vivo bioavailability. Bio-accessibility methods are generally considered to overestimate absolute bioavailability and toxicity of inorganic compounds since bio-elution tests do not assess absorption after release. Based on this overestimation, it can be safely assumed that a (worst-case) relationship can be defined between in vitro bio-accessibility in the artificial fluid and relative in vivo bioavailability of the ions that may additionally be absorbed systemically following release.

Table 8 provides information on the release and behavior of Sb species in a number of physiologically relevant media.

Table 8: Solubility and bio-accessibility data of Sb substances considered for grouping and read-across.

Name	CAS #	Solubility in water(1)	Bio-accessibility in artificial gastric fluid (2)	Extraction in culture medium
Sb – metal powder	7440-36-0	18.2 µg/ml	29.6 µg Sb/ml	60 µg Sb/ml
Sb – massive metal	7440-36-0			
Diantimony trioxide	1309-64-4	0.370 µg/ml	8.15 µg Sb/ml	0,8 µg Sb/ml
Antimony sulfide	1345-04-6	43.5 µg/ml	14.6 µg Sb/ml	5.6 µg Sb/ml
Antimony tris(ethylene glycolate)	29736-75-2	0.4 µg/ml	15.3 µg Sb/ml	32 µg Sb/ml
Antimony trichloride	10025-91-9	Technically not feasible	15.9 µg Sb/ml	30 µg Sb/ml
Sodium hexahydroxoantimonate	33908-66-6	594 µg/ml	260 µg Sb/ml	30 µg Sb/ml
Sodium antimonate	15432-85-6	247 µg/ml	325 µg Sb/ml	2.5 µg Sb/ml
Antimony pentachloride	7647-18-9	Decomposes in water	580 µg Sb/ml	29 µg Sb/ml*
Antimony pentoxide	1314-60-9	453 µg/ml	1.16 µg Sb/ml	4.7 µg Sb/ml
Potassium hexahydroxoantimonate	12208-13-8	17,100 µg/ml	184 µg Sb/ml	42 µg Sb/ml

(1) Extracted from the Chemical Safety Reports (2019)
(2) 2 hours Bio-elution Study on 10 Sb substances at a 0.2 and 2 g/L loading in a simulated gastric fluid (Brouwers, 2019)
(3) ToxTracker test reports on Sb substances – Hendriks (2017 and 2018)
* Comparison between water solubility and extraction results under examination

Water solubility data is important as most in vitro test systems designed to assess potential mutagenic hazard are conducted at neutral pH. Figure 1 indicates where, on the basis of water solubility only (second table), and the definition of approximate solubilities described by the US Pharmacopeia and National Formulary[8] (first table), the cut-off or subgroup delimitation between these two subgroups could be positioned. The substances which decompose in water have not been included in this provisional subgrouping.

Sb glycolate, Sb trisulfide, Sb metal powder[9], and Sb trioxide can be considered to be insoluble, and likely to have a limited systemic[10] absorption and toxicity. As regards Sodium antimonate, Sb pentoxide, Sodium hexahydroxoantimonate, and Potassium hexahydroxoantimonate they are either only very slightly soluble or sparingly soluble. Their potential to be taken up and reaching systemic levels high enough to produce toxicity is low.

[8] Available from: https://www.uspnf.com/sites/default/files/usp_pdf/EN/USPNF/usp-nf-notices/usp38_nf33_gn.pdf and repository.uobabylon.edu.iq/2010_2011/4_10975_328.doc. ECHA does not provide any quantitative reference to water solubility thresholds. The only Q&A addressing water solubility ('What are the criteria for deciding if a substance is highly insoluble in water or poorly water soluble?', ID: 0836, dated November 2016) relates to determining aquatic toxicity testing requirements.
[9] This further suggests that the reported effects of Sb metal powder, which was administered orally to rabbits, is most probably associated to maternal toxicity, and not to a systemic uptake and direct developmental toxicity.
[10] Local effects are addressed in the lung toxicity and carcinogenicity scientific opinion.

Table I. Description and Relative Solubility of US Pharmacopeia and National Formulary Articles

Description form (Solubility definition)	*Parts of solvent required for one part of solute*	*Solubility range (mg/mL)*	*Solubility assigned (mg/mL)*
Very soluble (VS)	< 1	> 1000	1000
Freely soluble (FS)	from 1 to 10	100 – 1000	100
Soluble	from 10 to 30	33 – 100	33
Sparingly soluble (SPS)	from 30 to 100	10 – 33	10
Slightly soluble (SS)	from 100 to 1000	1 – 10	1
Very slightly soluble (VSS)	from 1000 to 10000	0.1 – 1	0.1
Practically insoluble (PI)	> 10000	< 0.1	0.01

Table II. Solubility of Sb substances according to US Pharmacopeia and National Formulary Articles' definition

Sb Substance	Water solubility from Table 3 (µg/ml)	Recalculated water solubility (mg/ml)	Solubility definition according to US Pharmacopeia
Sb glycolate	0.0004-0.0012	0.0000004-0.0000012	Practically insoluble
Sb trisulfide	0.944	0.000944	Practically insoluble
Sb metal powder	18.2	0.0182	Practically insoluble
Sb trioxide	19.7-28.7	0.0197-0.0287	Practically insoluble
Sb triacetate	'Moderately soluble'	Not applicable	Very slightly soluble?
Sodium antimonate	247	0.247	Very slightly soluble
Sb pentoxide	453	0.453	Very slightly soluble
Sodium hexahydroxoantimonate	594	0.594	Very slightly soluble
Potassium hexahydroxoantimonate	20,000	20	Sparingly soluble
Sb potassium tartrate	83,000	83	Soluble
Sb pentavalent drugs	'Very soluble'	Not applicable	Freely soluble?

Figure 1: Provisional subgrouping of Sb substances based on water solubility, for the purpose of genotoxicity read-across assessment.

Three subgroups can provisionally be identified based on water solubility only:

1. Sb substances which are considered to be water soluble (water solubility > 33 mg/ml), and likely to be absorbed and reach high systemic levels following oral exposure;
2. Sb substances which are considered to be slightly or sparingly soluble in water (water solubility between 0.1 and 33 mg/ml), and less likely to be absorbed and reach high systemic levels following oral exposure; and
3. Sb substances which are considered to be insoluble in water (water solubility < 0.1 mg/ml), and unlikely to be absorbed and reach high systemic levels following oral exposure.

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Whereas water solubility may thus be of primary importance in the assessment of in vitro genotoxicity, dissolution in gastric fluid may give a more realistic assessment of potential systemic uptake that could permit in vivo genotoxicity to be expressed.

Bio-elution assays in artificial gastric fluid (0.07 N HCl) have been (repeated or) launched on all ten (10) Sb substances in scope in 2018, with results reported in 2019. The assays were conducted according to the Standard Operating Procedure for Bio-elution Testing of Metals, Inorganic Metal Compounds (Eurometaux, October 31, 2018) currently in the process for validation by ECVAM; as well as in artificial gastric fluid supplemented with physiological constituents, naturally present in the human gastric juice, such as: urea, glucuronic acid, glucose, glucosamine, mucin, bovine serum albumin and pepsin.

The results of these assays provide various observations:

- All substances release a minimum amount of Sb ions (Sb3+ for the trivalent substances and Sb5+ for the pentavalent substances), except Sb pentoxide which releases levels near the limits of analytical detection in such conditions.
- The trivalent substances seem to be less bio-accessible than the pentavalent substances, and could be foreseen as a sub-group for further systemic toxicity assessment.
- The pentavalent substances, excluding Sb pentoxide, present a significantly higher bio-accessibility, and could constitute a second sub-group in terms of read-across for systemic toxicity endpoints.
- The presence of physiological gastric proteins did not cause any significant difference in the dissolution of the Sb compounds except for the Sb tris(ethylene glycolate) and Sb trichloride, for which the capacity to release Sb3+ ions was three times higher than in a medium without proteins. This last observation could be explained by the fact that the molecular structure and moieties of these two Sb substances could increase their chemical affinity for proteins, resulting in a further redox reaction, resulting in a higher release of Sb ions.

When comparing the water solubility and bio-elution test results, it appears that, except for Sb pentoxide, the synthetic gastric fluid provokes a higher release of Sb ions to the medium than water. Whereas Sb 3+ substances will show the same relative insolubility in both media, Sb 5+ substances behave differently in water and the artificial physiological fluid. The solubilization or release of Sb ions seems to be more medium dependent for Sb 5+ substances than for Sb 3+ ones, probably because of the chemical affinity between their moieties and the pH and composition of the medium (even without proteins).

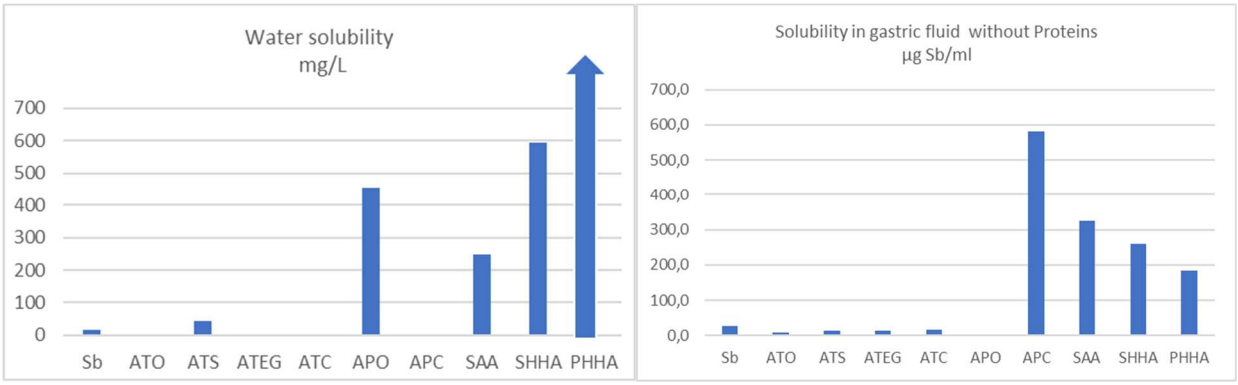

Figure 2: Water solubility and release of Sb in synthetic gastric fluids for 10 Sb substances

There is little data detailing speciation or changes in valency following uptake of Sb into mammalian systems (ATSDR, 2017). The information in Table 9 below may however provide an indication of general trends.

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Table 9: Summary of available ADME information on Sb substances.

Absorption	Distribution	Metabolization	Excretion
The valence state of Sb compounds has been suggested to impact uptake from the gastrointestinal tract but differences reported are generally small (ATSDR, 2017). As a generalization, uptake efficiency is very low (less than 1%) and likely nonlinear, with saturation of uptake processes as administered doses increase.	Valency (and changes in valency) of systemic Sb in mammalian systems, has not been well characterized but appears to influence Sb distribution within the body and subsequent excretion. Systemic Sb 5+, after ingestion or injection, tends to partition to blood plasma and the spleen whereas Sb 3+ is preferentially found with the erythrocyte and the liver (Edel et al., 1983; Poon et al., 1998; Coelho et al., 2014). Sb ions cross the placenta, but there is no comparative data that documents the impact of valency state upon placental transfer. The half-life of systemic Sb appears to be in the order of 10 days.	Conversion of 23% of Sb 5+ to Sb 3+ has been documented in studies of substances administered via intramuscular injection to humans (Vasquez et al., 2006). This conversion appears to be thiol mediated (Ferreira et al., 2003) and not enzymatically controlled. Whereas metabolism of Sb 3+ was not thought to occur, recent studies have documented di and tri-methyl Sb 5+ metabolite formation from Sb 3+ (He et al., 2019). Initially documented in plants, bacteria and fungi, in crops, methylation varies as a function of cultivar and section of the plant that is sampled (Ji et al., 2018). Human populations residing in Sb contaminated areas excrete methylated Sb in their urine, a probable reflection of ingestion of methylated Sb (Li et al., 2018) whereas inorganic Sb in urine is associated with occupational exposure. Some level of Sb methylation is suspected to occur in the liver, with subsequent accumulation of methylated Sb within the erythrocyte (Wu et al., 2018). The mechanism for this metabolism has yet to be determined but appears to result in the conversion of Sb 3+ to di- and tri-methyl Sb 5+ compounds. This metabolism is thought to be a detoxification mechanism that facilitates excretion and reduces the systemic burden of more toxic Sb 3+ moieties.	The impact of valency, and changes in valency, of systemic Sb once taken up into mammalian systems has not been well characterized but appears to influence Sb distribution within the body and subsequent excretion. Valency does affect excretion routes with Sb 5+ exhibiting urinary excretion while fecal metabolism predominates for Sb 3+.

The information above shows that Sb 3+ and Sb 5+:

- Have a comparable limited gastrointestinal absorption of around 1%;
- Will follow different distribution paths after absorption, with Sb 3+ found mainly in erythrocyte and the liver, while Sb 5+ found in blood plasma and the spleen;
- Will convert (from Sb 5+ to Sb 3+), especially where they are soluble (insoluble forms will remain unchanged); and be subject to methylation that converts Sb 3+ to di- and tri-methyl Sb 5+;
- Will follow different excretion paths, with Sb 3+ being excreted via feces and Sb 5+ via urine.

Two valency states of Sb ions can be released by Sb substances: Sb 3+ and Sb 5+ forms. Although sharing a number of properties, differences are evident in toxicokinetics, potential metabolism and, possibly also, toxicity of the two valency states. The significance of these differences will vary as a function of possible valency state interconversion such that exposure to Sb 5+ may result in the formation of Sb 3+, and Sb 3+ may undergo conversion to methylated forms of Sb 5+. Although methylation of Sb is generally regarded as a detoxification pathway, the toxicity of methylated Sb has not been well characterized. The extent of

methylation in plants is highly variable and contributes to methylated Sb in the diet (Ji et al., 2018). In vivo methylation in mammals may occur as a result of gastrointestinal flora activity or within organs such as the liver (Wu et al., 2018). The extent of methylation appears to be low (e.g. 1 – 2%) but requires further study.

For metals under homeostatic control, uptake is regulated and bioavailability can be of secondary importance. For others, such as Sb, uptake is not necessary a linear function of dose since sites of uptake (carrier systems) can saturate. The actual uptake of Sb ions/oxyanions from the gastrointestinal tract appears to be mediated by saturable carrier protein transport systems and will occur with low (<1 %) efficiency (ATSDR, 2017). Actual systemic exposure will hence be limited by this very low absorption for any Sb substance.

As regards the species that is released to become available for exposure, as explained in section 3.1 above, Sb 3+ compounds will normally hydrolyze to release an electrophilic Sb ion $Sb(OH)_3$. The behavior of Sb 3+ compounds in solution is likely to be complex and involve the sequential formation of Sb oxide chloride (SbOCl), Sb oxide hydroxide (SbO(OH)) and ultimately the formation of Sb trioxide (Sb_2O_3) (Hashimoto et al., 2003). The pentavalent Sb pentachloride is similarly an electrophilic oxidizing agent which, as a function of pH, will also undergo a series of hydrolytic transformations to oxychlorides and oxide hydroxides that result in the formation of Sb_2O_5 (Zheng, Zhi and Chen, 2006). Although the precise nature of the chemical moiety released in both cases remains unknown, the bio-accessibility data indicates that Sb 3+ substances will likely release Sb 3+ ions, whereas Sb 5+ substances (except Sb pentoxide which releases at levels near the limits of analytical detection) will release Sb 5+ ions.

Considering bioavailability as a conservative approach in the assessment of Sb uptake and hazard, and using bio-accessibility results as a worst-case prediction of bioavailability potential, ***for the purpose of read-across, the following subgroups can be established on the basis of bio-accessibility data:***

- ***Subgroup 3+: Show limited release of Sb 3+ in bio-elution tests***
- ***Subgroup 5+ except Sb pentoxide: Show higher release of Sb 5+ in bio-elution tests***
- ***Subgroup Sb pentoxide: Releases levels of Sb near the limits of analytical detection***

3.4 Consistency of the genotoxicity information for Sb substances

The assessment of the genotoxicity dataset on Sb substances is made in Section 2.3 above and concludes the following:

- Sb 3+ in vitro genotoxicity: Sb 3+ compounds are clastogens but do not induce point mutations in vitro
- Sb 3+ in vivo genotoxicity: Sb trioxide is not genotoxic in vivo
- Sb 3+ overall genotoxicity: Sb 3+ compounds are not genotoxic
- Sb 5+ in vitro genotoxicity: Sodium hexahydroxoantimonate is not genotoxic in vitro, there is no binding with DNA
- Sb 5+ in vivo genotoxicity: Sb 3+ evidence used to infer that Sb 5+ are not genotoxic in vivo
- Sb 5+ overall genotoxicity: Sb 5+ compounds are not genotoxic.

There is some indication of consistency that neither Sb 3+ compounds, nor Sb 5+ compounds (including Sb pentoxide) are genotoxic. The consistency will be confirmed upon assessment and consideration of the evidence which will be completed with the research options described in Section 2.3.

Recently generated data from the ToxTracker assay has suggested compound-specific differences in the ability to induce oxidative stress and/or protein damage. It remains to be seen if the in vitro genotoxic properties of these compounds exhibit differences concordant with their ToxTracker response profiles. Effects that correlate with physical chemical properties such as solubility in aqueous cell culture medium would validate read-across strategies for in vitro genotoxicity.

3.5 Read-across analysis of the genotoxicity of Sb substances

Table 10 below summarizes the evidence available for each one of the common and specific assessment elements to be considered to justify the read-across approach applied to predict the genotoxicity of each subgroup of Sb substances.

Table 10: Assessment elements and read-across justification evidence.

Assessment Element/Details	Supporting evidence
Characterization of source and target substances	
Identity and characterization of all substances in category	**Subgroup Sb 3+:** Source substances: Any Sb 3+ substance releasing Sb 3+ ion and having moieties or impurities which do not have a more toxic systemic toxicity profile than Sb 3+. Target substances: Other Sb 3+ substances releasing Sb 3+ ion and having moieties or impurities which do not have a more systemic toxicity profile than Sb 3+. **Subgroup Sb 5+:** Source substances: Any Sb 5+ substance (except Sb pentoxide) releasing Sb 5+ ion and having moieties or impurities which do not have a more toxic systemic toxicity profile than Sb 5+. Target substances: Other Sb 5+ substances (except Sb pentoxide) releasing Sb 5+ ion and having moieties or impurities which do not have a more systemic toxicity profile than Sb 5+. **Subgroup Sb pentoxide:** Source/Target substance: Sb pentoxide as described in Table 7. Cf. Table 7 for more detailed identification and characterization information.
Structural similarity and dissimilarity within the category (category description)	
The structural similarities and differences identified for all category members	**Subgroup Sb 3+:** All substances in the category have in common that they have one or more Sb 3+ atoms bond through ionic or covalent bonding with moieties, many of which are essential nutrients or Essential Trace Elements (ETEs), with none or negligible genotoxicity, or normal metabolites (NM), which are expected to be rapidly metabolized. **Subgroup Sb 5+:** All substances in the category have in common that they have one or more Sb 5+ atoms bond through ionic or covalent bonding with moieties, many of which are essential nutrients or Essential Trace Elements (ETEs), with none or negligible genotoxicity, or normal metabolites (NM), which are expected to be rapidly metabolized. **Subgroup Sb pentoxide:** The only substance in the category is Sb pentoxide. Cf. Table 7 for more detailed identification and characterization information.
Structural differences that are allowed within the category are specified	**Subgroup Sb 3+:** Differences in molecular weight, moieties and release rates are allowed as long as there is evidence that the final speciation of the released (oxyan)ions, i.e. Sb 3+, remains comparable. **Subgroup Sb 5+:** Differences in molecular weight, moieties and release rates are allowed as long as there is evidence that the final speciation of the released (oxyan)ions, i.e. Sb 5+, remains comparable. **Subgroup Sb pentoxide:** The only substance in the category is Sb pentoxide. Cf. Table 7 for more detailed identification and characterization information.
Link of structural similarities and structural differences with the proposed regular patterns (presence of hypothesis) - It is explained why and how the category members should behave in a predictable manner	
Formation of common (identical) and non-common compounds	**Subgroup Sb 3+:** All substances in the category have in common that they release a common Sb (oxyan)ion 3+ form in vivo. This Sb 3+ form can be considered as a common transformation product.

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Assessment Element/Details	Supporting evidence
	Systemic exposure will be to this common transformation product, no matter the original form of the substance originally present and/or administered. Low level metabolism to methylated Sb 5+ forms may occur, but is believed to be a detoxification pathway. **Subgroup Sb 5+:** All substances in the category have in common that they release a common Sb (oxyan)ion 5+ (which may partially convert into a Sb 3+ form in vivo). The Sb 5+ form can be considered as a common transformation product. Systemic exposure will be to this common transformation product, no matter the original form of the substance originally present and/or administered. Some conversion to Sb 3+ may occur. **Subgroup Sb pentoxide:** The only substance in the category is Sb pentoxide. Cf. Section 3.3 for more information on transformation products.
Degradation, bioaccumulation and impact of non-common compounds	**Subgroup Sb 3+, Sb 5+ or Sb pentoxide:** The moieties of the various Sb substances of the group will be absorbed for essential functions in the body or metabolized as any other normal metabolite. Cf. Section 3.2 for more information.
Impact of impurities on the prediction	
The identified impurities have an impact on the prediction	**Subgroup Sb 3+, Sb 5+ or Sb pentoxide:** All Sb substances will typically have some levels of As and/or Pb as impurities, because of the geological affinity/common primary origin of these three elements in nature. These impurities are not expected to have an impact on the (predicted) effect as long as they are present in concentrations below the classification threshold. Cf. Table 7 for more detailed information on impurities.
Consistency of properties in the data matrix	
A data matrix with experimental data for source and target substances is needed to support the read-across	**Subgroup Sb 3+:** The genotoxicity dataset available for Sb 3+ substances contains only one endpoint for which comparable studies are available on more than one Sb 3+ substance: Ames Test. Negative assay responses were observed, but this may be due to inefficient uptake of Sb and active efflux pumps that limit the intracellular concentrations of Sb. Bacterial test systems are generally recognized as inappropriate for the study of metalloids. Overall there is test data for two Sb 3+ substances (Sb trioxide and Sb trichloride), and the ToxTracker assay provides a comparative overview for all other Sb 3+ substances. When comparing available data: - Most in vitro studies in mammalian cells with Sb 3+ substances yield positive results for clastogenic events but not point mutations; - All high-quality in vivo studies with Sb trioxide yield negative genotoxicity results; and - ToxTracker shows a comparable induction of oxidative stress and interference with DNA repair processes for all Sb 3+ substances. **Subgroup Sb 5+:** The genotoxicity dataset available for Sb 5+ substances contains only one endpoint for which comparable studies are available on more than one Sb 5+ substance: Ames Test. Beyond Ames, there is mutation test data (i.e. excluding indicator assays) for one Sb 5+ substance, but the ToxTracker assay provides a comparative overview for all other Sb 5+ substances. When comparing available data: - Most in vitro studies with Sb 5+ substances yield negative genotoxicity results (cf. interpretation!); - There are no in vivo genotoxicity results for Sb 5+ substances (but Sb 5+ is generally assumed to be less toxic than Sb 3+, so genotoxicity of Sb 5+ can only be lower, if any); - ToxTracker shows a comparable absence of induction of oxidative stress and interference with DNA repair processes for all Sb 5+ substances. **Subgroup Sb pentoxide:** There is no genotoxicity data for Sb pentoxide. More information in Sections 2.2 and 2.3 of this document.

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Assessment Element/Details	Supporting evidence
Reliability and adequacy of the source data	
The source study(ies) needs to be reliable and adequate as requested for any other key study	Only adequate and reliable data has been used to support the read-across justification. More information in Section 2.2 of this document.

3.6 Conclusion of the read-across assessment of the genotoxicity of Sb substances

Table 11 provides, for each Sb substance, the result of the hazard assessment and classification constructed on the basis of the read-across approach.

Table 11: Classification resulting from read-across approach for Sb substances.

Name	CAS #	Mutagenicity classification	Further research options
Subgroup Sb 3+			• In vitro micronucleus study to verify the possible micronucleus staining artifacts caused by non-DNA-specific stains • In vitro assay on respiratory cells to understand the mechanism of action yielding positive genotoxicity results in vitro but negative genotoxicity results in vivo, and to inform on subsequent in vivo research needs • One or more in vivo studies to validate the hypothesis developed on the basis of the in vitro evidence, and understand the extent and significance of the transformation (and methylation) and Sb compounds following absorption
Sb –powder	7440-36-0	Not classified	
Sb – massive	7440-36-0	Not classified	
Diantimony trioxide	1309-64-4	Not classified	
Antimony sulfide	1345-04-6	Not classified	
Antimony tris (ethylene glycolate)	29736-75-2	Not classified	
Antimony trichloride	10025-91-9	Not classified	
Subgroup Sb 5+ (except Sb pentoxide)			
Sodium hexahydroxoantimonate	33908-66-6	Not classified	
Sodium antimonate	15432-85-6	Not classified	
Antimony pentachloride	7647-18-9	Not classified	
Potassium hexahydroxoantimonate	12208-13-8	Not classified	
Subgroup Sb pentoxide			
Antimony pentoxide	1314-60-9	Not classified	

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4. Technical Annex

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Prokaryotic Test Systems

Tests using prokaryotic systems generally provide negative responses for mutagenicity, but interpretation of this negative finding must be qualified by recognition that uptake of ions for metalloids such as Sb by prokaryotic organisms is generally considered to be limited. Negative findings in the Ames test must be qualified by recognition that uptake of ions for metals such as Sb by prokaryotic organisms is seldom measured and is generally considered to be quite limited (Kuroda et al., 1991, Zangi et al., 2012). Genetic resistance to antibiotics, often carried by DNA plasmids transmissible from one bacterial strain to another, can impart properties of a "metalloid pump" that actively reduces intracellular concentration of Sb ions (Xu et al., 1998). The presence of such a gene in a bacterial test strain would predispose to (potentially false) negative test results. Five structural proteins that can function as efflux transporters were initially identified and the metalloid binding domains of the transporter proteins established (Ruan et al., 2008). A sixth family of novel efflux transporters that will be activated by, and reduce intracellular concentrations of, Sb, has recently been identified (Shi et al., 2018). The multiplicity and prevalence of metal-resistance genes in bacteria is such that negative Ames tests in bacteria have limited significance unless it is demonstrated that Sb is taken up and retained by the test organisms.

Although gene mutations were not observed in bacterial mutation test, positive response were observed for Sb compounds in the *B. subtilis* rec assay for DNA damage (Table 2). This "indicator assay" assesses increases in recombination events that are most likely the result of DNA damage induced by chemical treatment. Sb_2O_5 did not produce a response but also seemed to lack toxicity as evidenced by lack of a zone of inhibition resulting from Sb_2O_5 treatment. The authors attributed this to limited solubility of the pentoxide but data to substantiate this are not presented. Independent of the reasons, the rec assay results for Sb_2O_5 do not appear to have resulted in significant exposure to Sb ions. The authors further hypothesized that the difference in response in the two bacterial test systems might have been produced by differences in compound uptake or toxicity in the two bacterial strains. False negatives would result in the Ames test if inadequate Sb uptake occurred, whereas false positives can occur in the rec assay if cytotoxicity results in lysosomal nuclease release. In the absence of information that discriminates between these alternate hypotheses, response inconsistency between the bacterial test systems, and between compounds in the rec assay, make it difficult to derive definitive conclusions regarding mutagenicity or genotoxicity from studies using bacteria.

In Vitro Tests with Mammalian Cells

Two studies have evaluated Sb compounds for forward mutation at the thymidine kinase (TK) locus of cultured L5178Y mouse lymphoma cells (Elliot *et al*., 1998; Stone 2010; Table 2). Sb trioxide, tested in the presence and absence of S9 for metabolic activation, failed to induce mutation after 4 h exposure. Tested concentrations were nominal (i.e. not measured in the cell culture medium) and may have exceeded the aqueous solubility of the test compound. Little cytotoxicity was observed, further suggesting limited release of Sb 3+ ions. Finally, the 4 h treatment time employed was shorter than the 24 h exposure duration currently recommended by international guidelines (Moore *et al.,* 2002). Thus, while Sb trioxide was not mutagenic, positive responses might have been induced by longer duration of chemical exposure or the study of more soluble Sb compounds that would yield higher Sb concentrations. Similarly, negative results were obtained in the testing of Sodium hexahydroxoantimonat ($NaSb(OH)_6$) in the presence and absence of S9 using the microtiter fluctuation technique for the assay.

Elliot *et al*. (1998) also examined the induction of chromosomal aberrations in cultured human lymphocytes at nominal Sb trioxide concentrations that ranged from 10 to 100 µg/ml. Setting aside concerns over possible exceedance of solubility limits, a dose-dependent increase in chromosome aberrations was observed in the absence of cytotoxicity. The nature of the aberrations was not explicitly described except to note that chromosome gaps had been excluded. Asakura et al. (2009) also reported that Sb metal powder induced chromosome aberrations. However, no data on toxicity, doses used or aberrations observed was provided to permit evaluation of this claim.

Given the chromosome aberrations, it is not surprising that studies have reported that treatment with Sb compounds (usually $SbCl_3$) is associated with micronucleus (MN) induction in a variety of different cell types. Huang *et al*. (1998) observed MN induction in a series of studies using Chinese hamster ovary cells, human bronchial epithelial cells and human fibroblasts. MN induction was concentration-dependent and, at higher concentrations, associated with significant cytotoxicity. The authors further observed an influx of calcium into cells after $SbCl_3$ treatment followed by time-delayed apoptosis and DNA fragmentation. Calcium influx was noted to potentially be an indication of oxidative stress and to provide a mechanistic pathway for DNA damage via indirect pathways. Induction of apoptosis was similarly noted to provide an additional pathway for DNA damage to occur independent of direct Sb ion interaction with DNA. Both mechanisms of actions would be expected to exhibit non-linear dose-response functions (i.e. thresholds).

Similar dose-dependent increases in MN induction were observed in V79 cells (Gebel et al., 1998) and cultured human lymphocytes (Schaumloffel and Gebel, 1998). Finally, Migliore et al. (1999) observed strong dose dependent induction of micronuclei in cultured lymphocytes from two human volunteers following in vitro treatment with $KSbO_3$ (potassium antimonate). Fluorescence in situ hybridization was used to examine micronuclei for the presence of centromeres – micronuclei in Sb treated cells generally lacked centromeres suggesting the occurrence of clastogenic events as opposed to aneuploidy. The concentrations tested (240 – 600 □M) are within the range expected for a moderately soluble compound but higher than others have reported as being possible in cell culture medium.

The absence of centromeres in Sb induced MN, although consistent with chromosome breakage, also raises technical concerns with respect to the majority of the micronucleus studies conducted of Sb compounds. Studies conducted to date have primarily relied upon Giemsa staining for micronucleus detection, a staining method that lacks specificity for DNA (Nersesyan et al., 2006). Metalloids such as arsenic have recently been reported (Wedel et al, 2013; Cohen et al., 2013), presumably due to electrophilic interaction with thiol groups on proteins and other macromolecules, to produce cytoplasmic inclusions bodies that can be mistaken for micronuclei if non-DNA specific stains (e.g. Giemsa) are used. There thus remains the possibility that inclusion body formation by Sb may have produced staining artifacts misinterpreted as micronuclei. Further research would be required to determine if this potential source of experimental artifact is applicable to Sb.

The study of Sb compounds in indicator assays yields positive results (Table 3). Sister chromatid exchange induction and Comet assay results have been generated most frequently but the quality of most studies is low. Both assays require careful monitoring of, and control for, cytotoxicity, terminal differentiation and/or apoptosis to permit meaningful interpretation of results. Most studies have failed to implement proper controls for these sources of experimental artifact and have been excluded from consideration here. Moreover, given the preponderance of positive micronucleus data, indicator assay data adds little to a weight of evidence evaluation. Indicator assay data considered but excluded from evaluation here are summarized in the CSRs.

In vivo test results

Gurnani et al. (1992) evaluated the effects of single and repeated doses of Sb trioxide chromosome aberrations in mouse bone marrow. Oral gavage of 400 -1000 mg/kg in a single dose, followed by analysis of chromosome aberrations after dosing did not detect an increase in aberration frequency. In a repeated dosing protocol, mice were exposed to 400, 667 and1000 mg/kg Sb trioxide by oral gavage for up to 21 days and animals sacrifice at 7, 14 and 21 days for evaluation of chromosome aberrations. Day 21 evaluations were restricted to the 400 and 667 mg/kg dosing group since lethality occurred on day 20 in the 1000 mg/kg treatment group. The authors reported a variety of chromosome alterations including chromatid gaps and breaks, polyploid cells and “centric fusions” that increased as a function of dose through day 7 and 14 and then declined at day 21. Presentation of the data is less than straightforward and statistical evaluations were conducted after pooling of data for aberration types that should have been evaluated independently (e.g. chromatid breaks and polyploid cells should have been evaluated separately). Kirkland et al. (2007) have noted a number of deviations from GLP protocols in the conduct of the study of Gurnani et al. (1992), questioned the purity of the test substance used and noted irregularities in the nature of the chromosomal changes observed (i.e. breaks

and centric fusions should have been associated with chromosome fragments but were not). The study deficiencies are significant and indicate a need for validation from other studies. A later publication by Gurnani et al. (1993) would at first seem to provide confirmation of Gurnani et al. (1992) but, as also noted by Kirkland et al. (2007), is merely republication of the data originally published in 1992. Gurnani et al. (1993) has thus been excluded from Table 4 since it is not a new study.

Kirkland et al. (2007) mirrored the protocols of Gurnani et al. (1992) in a study of male and female rats administered 250, 500 and 1000 mg/kg Sb trioxide by oral gavage for 21 days. Six male and six female rats were included in each treatment group and the protocol included a positive control treatment group (lacking in the Gurnani et al., 2002 study). Treatment with Sb trioxide produced few signs of clinical toxicity other than a modest reduction in weight gain in the highest dosing group. Additional toxicokinetic studies confirmed both the uptake of Sb into the blood and the presence of Sb in bone marrow. Animals were then evaluated for the induction of both bone marrow chromosome aberrations and micronuclei in polychromatic erythrocytes on day 22. No treatment-related increases in chromosome aberrations or micronuclei were observed. This study strongly adhered to GLP guidelines and possesses technical rigor superior other *in vivo* studies evaluating clastogenic effects of Sb compounds.

Other studies evaluating the genotoxic impacts of Sb *in vivo* followed protocols limited in scope. Elliot et al. (1998) examined the impacts of a single 5000 mg/kg oral gavage Sb trioxide dose upon micronucleus induction. No evidence was obtained for micronucleus induction but the use of only a single treatment and one dose limits the significance of this negative finding. The same authors also examined the induction of unscheduled DNA synthesis in rat liver after a single dose of Sb trioxide administered by oral gavage at doses of 3200 and 5000 mg/kg. No treatment-related impacts upon unscheduled DNA synthesis were observed.

The National Toxicology Program of the United States (US NTP) recently conducted inhalation cancer bioassays upon rats and mice, exposing animals to 3, 10 and 30 mg/m^3 Sb trioxide for two years (NTP, 2017). The NTP also conducted studies to evaluate the genotoxic effects of exposure to Sb trioxide after one year of inhalation exposure. Sensitive flow cytometric procedures were also applied to enumerate induction of micronuclei in the erythrocytes and white blood cells from rats and mice. Increased micronuclei were not observed in cells from rats but a low level of micronucleus induction was observed in mouse erythrocytes. The incidence of micronuclei increased in both male and female mice generally increased in a dose-dependent fashion but the response magnitude was small. For example, normochromatic erythrocytes exhibited an average of 1.04 micronuclei per 1000 cells in controls, increasing to a maximum of 1.38 per 1000 cells in female mice exposed to 30 mg/m^3 of Sb trioxide. This level of response is statistically significant by virtue of 1,000,000 cells having been scored but would not have been detectable or significant without the application of flow cytometry to screen large numbers of cells. While the response observed may be statistically significant, the biological significance of the response is unclear.

Other laboratories have observed that conditions which accelerate or perturb erythropoiesis produce small increases in erythrocyte micronuclei. Thus, induction of anemia by blood loss or dietary iron restriction causes modest increases in micronucleus incidence - generally accompanied by the appearance of immature reticulocytes in the blood (Tweats et al., 2007; Molloy et al, 2012). The pulmonary toxicity of Sb trioxide produced hypoxia and bone marrow hyperplasia that perturbed erythropoiesis as evidenced by increased prevalence of immature reticulocytes in the blood of mice. Although NTP (2017) interprets the induction of micronuclei in mice as evidence of genotoxicity, the small magnitude of the response and evidence of disturbed red blood cell production indicates that designation of this as a positive response is not inappropriate. Indeed, as acknowledged by NTP (2017) an independent Peer Review Panel had evaluated the genotoxicity study results and indicated that evidence of genotoxicity was lacking in the NTP studies.

Lung tissues from a separate cohort of rats and mice exposed to Sb trioxide for 12 months were analyzed for DNA damage by the Comet assay. No DNA damage was observed in exposed rats while positive assay responses are reported for cells within mouse lung tissue. Although the NTP report does not attribute great significance to the positive Comet assay results, it must be noted that the protocols employed for conduct of

the Comet assay do not meet current minimal quality standards (Speit et al., 2015). Application of the Comet assay to intact tissues must carefully control for natural process that can produce DNA fragmentation and false positive assay outcomes. Cytotoxicity, apoptosis and terminal differentiation must all be carefully assessed for their impact upon assay outcomes. The study controlled for none of these sources of artifact, casting doubt upon the significance of the modest positive response observed in mice. Lack of genotoxicity in rats remains a significant observation since the uncontrolled sources of experimental artifact would create false positive assay response and would not mask genotoxicity to create a false negative response.

Potential Mechanisms of Action

The mechanism(s) by which Sb compounds exert genotoxic effects *in vitro* remain(s) to be determined. There is no evidence that Sb ions undergo covalent interaction with DNA but the nature of interactions is influenced by valence state (Li et el., 2011). No interaction or binding of Sb 5+ with DNA was detected by Li et al. (2011). An apparent binding of Sb 3+ to DNA was observed, with initial binding of Sb 3+ to the ribose group of guanosine. This binding action is generally slow to take place and may have a covalent basis. In binding to ribose, Sb 3+ does not alter the base pair specificity of guanine and primarily poses an impediment to the efficacy of DNSA polymerase function during replication or DNA repair. Genotoxicity is thus believed to involve indirect mechanisms. De Boeck et al. (2003) suggest that the generation of oxygen radicals constitute an indirect pathway for inducing genotoxic responses.

Not all evidence supports oxidative stress as a mechanism for Sb genotoxicity. Shaumloffel and Gebel (1998) did not observe attenuation of Sb induced Comet assay responses by the exogenous addition of superoxide dismutase or catalase, but it is not clear whether the positive Comet assay results reported were artifacts of cytotoxicity or apoptosis. The NTP inhalation cancer bioassays of Sb trioxide (NTP, 2017) observed activation of the EGFR oncogene in a number of mouse lung tumors and “fingerprinted” the DNA sequence changes presumed to be responsible for activation. The observed changes were not characteristic of DNA sequence changes associated with oxygen radicals. A high frequency of G to T transversions was not observed in activated oncogenes, the DNA sequence alteration that is most commonly associated with interaction of active oxygen species with DNA to form 8-hydroxyguanine and a subsequent G to T transversion (Tchou *et al.*, 1991; Hong et al., 2016). Oncogene activation in mouse lung tumors may thus result from events unrelated to oxidative stress and/or may not be the critical event by which Sb trioxide induces mouse lung tumors.

5. References

International Antimony Association (VZW)
Avenue de Broqueville 12, 1150 Brussels, Belgium / Phone : +32 (0) 32 2 762 30 93 / Fax : +32 (2) 762 82 29
www.antimony.com

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International Antimony Association (VZW)
Avenue de Broqueville 12, 1150 Brussels, Belgium / Phone : +32 (0) 32 2 762 30 93 / Fax : +32 (2) 762 82 29
www.antimony.com

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International Antimony Association (VZW)
Avenue de Broqueville 12, 1150 Brussels, Belgium / Phone : +32 (0) 32 2 762 30 93 / Fax : +32 (2) 762 82 29
www.antimony.com