Toxicology and Applied Pharmacology 403 (2020) 115156

Contents lists available at ScienceDirect

Toxicology and Applied Pharmacology

journal homepage: www.elsevier.com/locate/taap

Invited Review Article

Antimony and its compounds: Health impacts related to pulmonary toxicity, cancer, and genotoxicity

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ARTICLE INFO

Keywords:
Antimony
Review
Pulmonary Toxicity
Cancer
Genotoxicity
Mechanism

ABSTRACT

Although occupational exposure to antimony and its compounds can produce pulmonary toxicity, human carcinogenic impacts have not been observed. Inhalation studies with respirable antimony trioxide particles administered to rats and mice have, however, induced carcinogenic responses in the lungs and related tissue sites. Genotoxicity studies conducted to elucidate mechanism(s) for tumor induction have produced mixed results. Antimony compounds do not induce gene mutations in bacteria or cultured mammalian cells, but chromosome aberrations and micronuclei have been observed, usually at highly cytotoxic concentrations. Indirect mechanisms of genotoxicity have been proposed to mediate these responses. *In vivo* genotoxicity tests have generally yielded negative results although several positive studies of marginal quality have been reported. Genotoxic effects may be related to indirect modes of action such as the generation of excessive reactive oxygen species (ROS), altered gene expression or interference with DNA repair processes. Such indirect mechanisms may exhibit dose-response thresholds. For example, interaction of ROS with *in vivo* antioxidant systems could yield a threshold for genotoxicity (and cancer) only at concentrations above the capacity of antioxidant defense mechanisms to control and/or eliminate damage from ROS.

1. Introduction

Antimony is a silvery white metalloid of medium hardness often combined with other metals to form hardened alloys used in lead acid batteries, solder, sheet metal, pipes, metal bearings, castings and ammunition (ATSDR, 2019). A group V metalloid element, antimony commonly forms compounds with a valence state of (III) or (V) that have applications in products that include polyethylene terephthalate water bottles and fire retardants applied to fabrics. Occupational exposure to antimony occurs as can, to a much lesser extent, consumer exposures. Occupational aerosol exposure limits, such as those set by the American Conference of Industrial Governmental Hygienists (ACGIH, 1979) recommend that occupational inhalation exposures not exceed 0.5 mg/m^3. Exposures significantly in excess of this have been associated with pulmonary toxicity (pneumonitis), the health endpoint most modern exposure limits have been implemented to prevent (Cooper et al., 1968; McCallum, 2005; ACGIH, 1979). In recent years concerns for antimony exposure have shifted to encompass potential mutagenic and carcinogenic risks. This state-of-the-evidence review has been prepared to facilitate hazard classification and risk assessment procedures for antimony and antimony compounds for pulmonary toxicity, cancer and genotoxicity.

As reviewed by ATSDR, 2019 the uptake of antimony into the body can be dominated by the inhalation exposure route. Uptake from the gastrointestinal tract is low (< 1%) and self-limited by the emetic properties of antimony compounds (ATSDR, 2019). Uptake of antimony through the skin does not make a significant contribution to systemic exposure. Once taken up into the body, antimony and its compounds

Abbreviations: ARE, anti-oxidant response element; AQP, aquagylceroporin; ATSDR, Agency for Toxic Substances and Disease Registry; ACGIH, American Conference of Governmental Industrial Hygienists; APL, acute promyelocytic leukemia; 5caC, 5-carboxylcytosine; CHO, Chinese hamster ovary; ECHA, European Chemical Authority; EGFR, epidermal growth factor receptor; EU, European Union; 5-foC, 5-formylcytosine; GLP, Good laboratory practice; GSD, Geometric standard deviation; GSH, Glutathione; 5-hmC, 5-hydroxymethyl cytosine; HO, heme oxygenase; IARC, International Agency for Research on Cancer; IWGT, International Workshop on Genotoxicity Testing; 5mC, 5-methyl cytosine; MMAD, Mean mass aerodynamic diameter; NTP, National Toxicology Program; OECD, Oranization for Economic Cooperation and Development; PSA, prostate specific antigen; Sb, antimony; SbTO, diantimony trioxide; SCE, sister chromatid exchange; ROS, reactive oxygen species; TK, thymidine kinase

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https://doi.org/10.1016/j.taap.2020.115156
Received 31 March 2020; Received in revised form 4 July 2020; Accepted 20 July 2020
Available online 22 July 2020

Table 1
Summary of epidemiology studies workers of occupationally exposed to antimony trioxide.

Cohort	Results	Klimisch score and remarks	Reference
Prospective cancer mortality study of 1420 men employed at an antimony smelter which converted to antimony trioxide production in 1973. Selection criteria entailed 3 months of employment between 1961 and 1992.	Elevated lung cancer risk (37 observed *vs.* 23.9 expected). Lung cancer excess (32 cases) confined to workers with employment history prior to 1961.	2 (reliable with restrictions) Significant exposures to arsenic and lead were present until the early 1970s, precluding assumptions of causality between antimony exposure and lung cancer. No data on cigarette smoking rates.	Jones (1994)
Mortality study of 1014 men employed at an antimony smelter between 1937 and 1971. Worker population was largely Hispanic and an appropriate referent group was difficult to assemble. Occupational exposures included antimony ores, metal and diantimony trioxide.	Elevated incidence of lung cancer (SMR 1.39) when it was assumed that Hispanics have a lower incidence of smoking.	2 (reliable with restrictions) Pneumoconiosis also elevated (SMR 1.22) but ethnic specific rates were not available for comparison. Significant co-exposure to known lung carcinogens such as arsenic precludes attribution of health risk to antimony. No data available on cigarette smoking rates.	Schnorr et al., 1995
Mortality study of 1462 male workers employed at a tin smelter over an employment period of 1937–2001.	Elevated risk of lung cancer in the overall smelter population.	2 (reliable with restrictions) Co-exposures to lead, arsenic and cadmium were documented in air sampling 1972–1991. Recent personal exposure measurements for antimony trioxide not available for lung cancer cases– air samples from different process areas indicated low Sb levels (presumably SbTO) ranging from 0.01 to 0.11 mg/m^3. Median exposure prior to 1972 estimated at 0.63 mg/m^3. No control for smoking or other lifestyle confounders. Attribution of lung cancer to antimony Sb not possible.	Jones et al., 2007

can undergo a limited slow change in valence state from Sb(V) to Sb(III) *via* nonenzymatic mechanisms that likely entail a thiol-induced reduction (Ferreira et al., 2003). Sb(III) may also undergo conversion to methylated forms of Sb(V). Although methylation of Sb(III) is generally regarded as a detoxification pathway, the toxicity of methylated Sb has not been 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 because of gastrointestinal flora activity or within organs such as the liver (Wu et al., 2018). The extent of methylation in the rat appears to be low (*e.g.* 1–2%) with dimethyl forms of Sb exhibiting a high binding affinity for hemoglobin. Some level of methylation likely occurs in humans since Sb(III) is a good substrate for As(*III*)methyltransferase (Marapakala et al., 2012). However, the extent of Sb(III) methylation in humans has not been determined. Excretion of Sb(III) and Sb(V) compounds (administered by injection) from the body of rodents can exhibit multi-phasic kinetics with Sb(III) being eliminated preferentially *via* fecal excretion while urinary excretion is the preferred elimination route for Sb(V) (ATSDR, 2019). Fecal excretion of Sb(III) in humans has not been well characterized.

As reviewed by Gebreyohannes et al. (2018) antimonial compounds have been applied in the treatment of parasitic tropical diseases such as leishmaniasis. Sb(V) compounds such as meglumine antimoniate are administered *via* intravenous, intramuscular or intra-peritoneal injection to compensate for low gastrointestinal uptake and to avoid the emesis and gastrointestinal upset associated with oral intake. Daily injection of 20 mg Sb/kg bw for several weeks has been demonstrated to have clinical efficacy in the treatment of disease. The systemic levels effective in disease treatment are higher than those that can be achieved after oral or inhalation exposure but offer an opportunity to monitor potential systemic human health effects at the extreme high end of the exposure spectrum.

2. Impacts of human occupational exposure upon the lung

The multiple uses of antimony have prompted examination of adverse effects that might be associated with occupational exposure. Long-term historical inhalation exposure to diantimony trioxide (SbTO) has been associated with changes in lung function (monitored *via* spirometry) or by X-ray evidence of changes in lung structure (pneumoconiosis) presumed to result from chronic inflammation and mild fibrosis (Cooper et al., 1968; Potkonjak and Pavlovich, 1983; McCallum, 2005). In general, the pneumoconiosis-related inflammation observed in workers tends to be limited, with only isolated cases of progressive pathological changes that may yield clinical changes in lung function (Cooper et al., 1968). The exposure levels to antimony compounds associated with human respiratory effects were not assessed in the preceding studies but, based upon the time period during which the studies were conducted (Vandenbroele et al., 2003), exposures likely exceeded the current ACGIH Occupational Exposure Limit of 0.5 mg/m^3 by a factor of 10 or more.

The pulmonary changes observed following high intensity occupational exposure bears qualitative similarity to histopathological changes produced by inhalation exposure of rats and mice exposed to respirable particulate SbTO (NTP, 2017). However, whereas Sb-induced pulmonary lesions in rodents are progressive and can produce significant decrements in pulmonary function, effects in humans tend to be benign fibrotic lesions that lack progressive development to the severe pulmonary toxicity evident in rodents (Cooper et al., 1968; Hoet, 2009). This could be an indication that humans are less sensitive to the inflammatory properties of SbTO, that the SbTO exposure levels and pulmonary deposition in rodent studies are significantly greater than experienced by humans in the workplace or that yet to-be-defined species-specific differences in pharmacokinetics and/or airway morphology enhance the development of lung function decrements in rodents.

Several epidemiology studies have evaluated the incidence of cancers in workers exposed to SbTO and are summarized in Table 1. Klimisch quality assessments, derived in accordance with Klimisch et al. (1997), have been made for each study in this and following study summary tables. High quality studies conducted in accordance with internationally accepted guidelines (under GLP if appropriate) are assigned a score of “1” indicating that the data are reliable without restriction. Studies with modest deficiencies in study design or data collection are assigned a Klimisch score of “2” (the data are reliable with restrictions) indicating that the basic findings of the study appear to have been generated by acceptable methods. Studies with significant flaws that compromise data acceptance or interpretation are assigned a score of “3” and the data are not judged to be reliable. The cohort mortality studies are all Klimisch score 2 and have detected small increases in lung cancer mortality in smelter environments. However, attribution of modest lung cancer increases to SbTO (or other antimony

Table 2
Overview of inhalation exposure impacts upon the lungs of experimental animals.

Method	Results	Klimisch score and remarks	Reference
Rat (Fischer 344) male and female exposed (65/sex/group) 6 h per day, days per week for 52 weeks to 0.0, 0.055, 0.511 or 4.50 mg/m^3 SbTO followed by 12 months of observation. The mean mass aerodynamic diameter (MMAD) of particles was 3.76 μm.	Interstitial inflammation, fibrosis and granulomas in treated animals and controls. No induced benign or malignant tumors observed. Evidence of particle overload being attained at highest dose tested. Lowest observable Adverse Effect Concentration (LOAEC) of 4.5 mg/m^3 based upon 80% inhibition of particle clearance. No Observable Adverse Effect Concentration (NOAEC) of 0.51 mg/m^3 based upon diminished clearance impacts.	2 (reliable with restrictions) Inhalation exposures for 52 weeks only – does not meet current criteria for the length of exposure in a cancer bioassay.	Newton et al. (1994)
Rat (Wistar) male and female exposed in groups of 90 /sex/substance to 45.0 mg/m^3 SbTO (MMAD 2.8 μm) or 36.0 mg/m^3 of Sb ore (MMAD 4.78 μm) for up to 52 weeks. (7 h/d and 5 d/wk) and then held for observation.	Lung tumors in 25% of female rats exposed to SbTO and 27% of female rats exposed to Sb ore. No lung tumors in exposed males or controls. Lung pathology showed interstitial fibrosis hyperplasia and metaplasia in response to treatment	2 (reliable with restrictions) Duration of inhalation exposures was 52 weeks, short the 2 yr currently recommenced. SbTO was impure with contamination by Pb and As.	Groth et al. (1986)
Groups of 20 female CDF rats exposed to 0, 1.9 and 5.0 mg/m^3 of SbTO 6 h/day, 5 days /week for one year followed by one year of observation. MMAD of 5.06 μm	Focal fibrosis at 3 months in high dose group that increased with exposure duration. Evident in the low dose group at 12 months. No malignant lung tumors in controls or low dose group but a scirrhous carcinoma of the lung found in 9 out of 18 high dose rats and squamous cell carcinomas in 2 out of 18 animals.	2 (reliable with restriction) Small size of exposure groups. Inhalation exposures of only 1 yr.	Watt (1983)
Rats (Wistar Han) male and female exposed in groups of 60 per sex per treatment concentration to SbTO (MMAD 0.9–1.5 μm) at concentrations of 0, 3, 10 and 30 mg/m^3 6 h per day, 5 days per week for up to two years.	Reduced body weight gain and survival at all treatment levels indicating MTD approached or exceeded. Dose-dependent increase in benign and malignant lung tumors and adrenal pheochromocytomas in both sexes. Significant lung inflammation and fibrosis accompanied by abnormal breathing and cyanosis indicative of hypoxia	1 (reliable without restriction)	NTP (2017)
Mice (B6C3F1) male and female exposed in groups of 60 per sex per treatment concentration to antimony trioxide (MMAD 0.9–1.5 μm) at concentrations of 0, 3, 10 and 30 mg/m^3 6 h per day, 5 days per week for up to two years.	Reduced body weight gain and survival at all treatment levels indicating MTD approached or exceeded. Dose-dependent increase in benign and malignant lung tumors in both sexes. Dose-dependent lymphoma increase (predominately B cell) especially in female mice. Benign and malignant skin neoplasms also observed. Significant lung inflammation, fibrosis and abnormal breathing.	1 (reliable without restriction)	NTP (2017)

compounds present) has not been possible due to significant levels of co-exposure to known lung carcinogens such as arsenic and cadmium. Indeed, Jones (1994) observed that lung cancer excess was only evident in workers employed during time frames when arsenical metal alloys were being produced or used. These studies also did not collect cigarette smoking data or determine levels of occupational exposure to SbTO experienced by individual workers, preventing assessment of whether there is a relationship between intensity of exposure to antimony and cancer. Epidemiology studies conducted to date do not provide evidence for carcinogenic effects associated with occupational exposure to SbTO.

3. Inhalation exposure studies of rodents

Animal studies (summarized in Table 2) have confirmed that substances such as SbTO can have toxic impacts upon the lung. Studies in Table 2 have been restricted to those with a duration of antimony exposure sufficient to permit development of neoplastic or preneoplastic lesions. Rodent inhalation exposures were usually conducted using respirable particle aerosols with a mean mass aerodynamic diameter (MMAD) < 5 μm that facilitates penetration into, and deposition within, the alveoli of the deep lung of rodents. Aerosols in industrial facilities typically possess a larger particle size distribution with an MMAD of 17 μm and a geometric standard deviation (GSD) of 2.7 (Hughson, 2005) that yields preferential deposition in the nose, throat and upper airways of the human respiratory tract. These larger particles, particularly when insoluble, are “cleared” to the gastrointestinal tract, will not directly impact pulmonary tissues and will be of systemic significance only to the extent permitted by limited ($< 1\%$) gastrointestinal uptake. Animal inhalation studies are thus designed to maximize the likelihood of damage to tissues of the deep lung. The proportion of inhalable aerosols of a respirable size range in occupational aerosols is variable but, at SbTO production facilities, has been estimated to be approximately 30% of the total inhalable aerosol (Vetter, 2018).

Initial experimental inhalation studies (Table 2) focussed upon the effects of SbTO (and in one case antimony ore) upon the rat and demonstrated that SbTO could impair particle clearance and cause lung toxicity and neoplasms. The significance of rat lung tumors induced *via* a pulmonary overload mechanism for humans could be questionable. These initial studies deviated from standard protocols for inhalation cancer bioassays in that only one year of SbTO exposure was performed (as opposed to the two years specified by most current cancer bioassay guidelines). The combined data from human epidemiology and experimental animal studies resulted in a Category 2 cancer classification within the European Union (ECHA, 2019). IARC had earlier deemed the human evidence to be inadequate and the rodent evidence to be adequate, resulting in a Category 2B (possible human carcinogen) classification (IARC, 1989).

Recent studies by the National Toxicology Program of the United States (NTP, 2017) rectified protocol deficiencies of earlier inhalation studies, conducting a two-year cancer bioassay of SbTO in both rats and mice. Exposure related lung tumors were evident in both sexes of both species, with stronger evidence of dose-dependent lung tumor formation in mice. In addition, adrenal gland tumors (pheochromocytomas) were evident in rats (both sexes) and lymphomas (mostly B cell) evident in female mice. The pulmonary damage induced by SbTO resulted in severe hypoxia that is the likely cause of pheochromocytoma induction in the rat (Greim et al., 2009) and not a specific response to SbTO. Interpretation of an increase in the incidence of lymphomas in female mice poses diagnostic challenges that were not addressed by NTP's histopathological analysis. Whereas lymphomas induced by chemicals are usually T cell in origin (Ward, 2005), those associated with SbTO exposure were predominantly B-cell or mixed B- and T-cell in origin and

many appeared to be reactive lesions responding to SbTO pulmonary toxicity. Mouse B-cell lymphomas are further difficult to interpret due to a high spontaneous incidence and a complex etiology that likely includes endogenous retrovirus activity (Ward, 2005). In NTP inhalation studies, the average historical control incidence of lymphomas in B6C3F1 female mice is 25.2% (range 14–36%). Thus, lymphoma incidence at 10 (40%) and 30 mg SbTO/m^3 (54%), but not 3 mg SbTO/m^3 (34%), was significantly elevated over historical controls. The complex and diverse mechanisms for B-cell lymphoma induction have prompted the development of histopathological diagnosis and classification strategies to distinguish between spontaneous and induced lesions (Ward, 2005). Unfortunately, none of these diagnostic criteria were applied in the NTP study. Based upon the limited data provided, the excess lymphomas associated with SbTO exposure appear to be similar to the naturally occurring lesions in the B6C3F1 mouse. The chronic inflammation and hypoxic conditions in the SbTO exposed mouse lung may have produced an adaptive response that promoted the development of what is already a high incidence spontaneous neoplasm. A low incidence of skin tumors was observed in male mice, but the frequency and histopathological diversity of observed skin lesions was such that they were most likely a secondary lesion and not of primary concern for cancer classification.

The observation of antimony induced lung tumors in both rats and mice puts a focus upon the genotoxic properties of antimony compounds, raising questions as to whether SbTO induced lung tumors were induced *via* direct genotoxic, indirect genotoxic or nongenotoxic mechanisms of action.

4. Genotoxicity test results

In vitro genotoxicity responses to antimony compounds are generally weak, inconsistent and often result from the use of highly cytotoxic compound concentrations that exceed the reported solubility limits of sparingly soluble antimony compounds. *In vitro* genotoxicity testing results are summarized in Tables 3–6. 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”. 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 (the basic definition for a mutagenic response). In a weight-of-evidence evaluation (ECHA, 2014), mutagenic responses are generally accorded greater significance than responses in indicator assays (*e.g.* Comet assay) 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 thus yield positive responses *via* mechanisms that do not entail mutagenic change.

Sb(III) compounds have been tested most frequently – tests of Sb(V) compounds are in the shaded table rows. To further assist in interpretation, positive assay responses indicative of genotoxicity are indicated in bold type. Klimisch scores indicative of study quality are presented to indicate the extent of study compliance with international guidelines for genotoxicity testing. The recommendations of the Organization for Economic Cooperation and Development (OECD) or the International Workshop on Genotoxicity Testing (IWGT) Procedures provided the most rigorous specification of acceptable testing protocols.

4.1. Genotoxicity results from prokaryotic test systems

Tests using prokaryotic systems provide negative responses for mutagenicity (*e.g.* in the Ames test with S. typhimuium) but positive responses in indicator assays (such as recombination in *B. subtills*) have been observed (Table 3). The main exception to negative Ames test data is a study (Asakura et al., 2009) that produced discordant negative and weak positive results in replicate experiments of Sb metal powder in Salmonella strain 1537. 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 the initial study, but a second and third replicate attained this informal benchmark. Dose dependent increases in strain 1537 revertants were not observed and the modest level of revertants attributed to Sb treatment was within the range of spontaneous revertants associated with strain 1537 in inter-laboratory comparisons (Levy et al., 2019). The application of current scoring recommendations (Levy et al., 2019) for interpreting strain 1537 positive responses (a three-fold increase in revertants) results in this being interpreted as a negative finding for mutagenic potential. Ames test mutagenicity data for Sb compounds are thus uniformly negative. A reverse mutation assay of pentavalent $NaSb(OH)_6$ in *E. coli* was similarly negative (Spruth, 2015).

Responses in indicator assays have been mixed. $SbCl_3$ was negative in the SOS chromotest in *E. coli* but positive responses were observed for several Sb compounds in the *B. subtilis* rec assay that assesses increases in post-replication DNA recombination events (Ayora et al., 2011) that are assumed to be the result of DNA damage induced by chemical treatment that halts the progression of DNA replication forks. Kuroda et al. (1991) suggested that this difference in response relative to the Ames test might be due to limitations upon the uptake of metalloids into Salmonella but not B. substilis. However, uptake of As(III) and Sb(III) into bacteria is facilitated by the ArsB protein, an aquaglyceroporin (AQP) channel analog (Meng et al., 2004) which is also responsible for As(III) and Sb(III) entry into mammalian cells (Garbinski et al., 2019). The Salmonella and *E. coli* ArsB proteins are 97% identical. (https://www.ncbi.nlm.nih.gov/protein/WP_094964486.1), an indication that the AQP gene is conserved and Sb uptake should be similar in different bacteria. Sb_2O_5 did not produce a response in Salmonella or *E. coli* but also seemed to lack toxicity as evidenced by lack of a zone of inhibition resulting from Sb_2O_5 treatment. The authors (Kuroda et al., 1991) attributed this to limited solubility of the pentoxide.

Every prokaryotic organism studied has metalloid efflux pumps that export As(III) and Sb(III). (Meng et al., 2004). While these efflux pumps can modulate toxicity, they do not eliminate it at high doses. Thus, the negative results in the Ames test cannot be explained by differential uptake or efflux since most compounds tested exhibited cytotoxicity. Rather, the negative Ames test findings are a probable indicator that Sb ions are not DNA reactive (MacGregor et al., 2015). In a weight of evidence evaluation, the multiple negative bacterial mutagenicity studies take precedence over the positive *B. subtilis* Rec assay results from a single lab.

4.2. Genotoxicity test results from cultured mammalian cells

Two studies have evaluated antimony compounds for forward mutation at the thymidine kinase (TK) locus of cultured L5178Y mouse lymphoma cells (Elliot et al., 1998; Stone, 2010; Table 4). SbTO, tested in the presence and absence of rat liver S9 supernatant for metabolic activation, failed to induce mutations after 4 h exposures. 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 (III) ions. Finally, the 4 h treatment time employed was shorter than the 24 h exposure duration recommended by some test guidelines (Moore et al., 2002). Similar negative results (Stone, 2010) were obtained in the testing of pentavalent $NaSb(OH)_6$ in the presence and absence of S9 using the microtiter fluctuation technique for the assay (Cole et al., 1983).

Although gene mutation assays are negative, clastogenic effects have been suggested. An early study by Paton and Allison (1972) observed possible chromosome damage by Sb compounds but is difficult to interpret due to the testing of a single dose and inadequate

Table 3
Genotoxicity responses to antimony compounds in prokaryotic systems (Kanematsu et al., 1980; Kubo et al., 2002; Lantzsch and Gebel, 1997).[a]

Assay	Compound Tested	Concentration Tested	Response	Reference and Klimisch Score
Mutation Assays				
S. typhimurium Ames Mutation	$SbCl_3$	625 – 5000 µg/plate	Negative	Kuroda, 1991 2: Reliable with restriction
S. typhimurium Ames Mutation	Sb_2O_3	0.43 – 1.71 µg/plate	Negative	Kuroda, 1991 2: Reliable with restriction
S. typhimurium Ames Mutation	Sb_2O_3	100 – 5000 µg/plate	Negative	Elliot, 1998 2: Reliable with restriction
S. typhimurium Ames Mutation	Sb_2O_3	156 – 5000 µg/plate	Negative Authors describe a positive response in one bacterial strain that is negative by current scoring criteria.	Asakura et al., 2009 2: reliable with restrictions
S. typhimurium Ames Mutation	$SbCl_5$	54 – 864 µg/plate	Negative	Kuroda, 1991 2: Reliable with restriction
S. typhimurium Ames Mutation	Sb_2O_5	50 – 200 µg/plate	Negative	Kuroda, 1991 2: Reliable with restriction
S. typhimurium Ames Mutation	$NaSb(OH)_6$	31.6 – 5000 µg/plate	Negative	Spruth, 2015 1: Reliable without restriction
E. Coli WP2uvrA Reverse mutation	$NaSb(OH)_6$	31.6 – 5000 µg/plate	Negative	Spruth, 2015 1: Reliable without restriction
S. typhimurium Ames Mutation	Sb_2O_3	729 µg/disk	Negative	Kanematsu et al (1980) 3: not reliable Results indicated as negative but no data are provided
S. typhimurium Ames Mutation	$SbCl_3$	Not provided	Negative	Kubo et al., 2002 3: Not reliable due to lack of data presentation
Indicator Assays				
E. Coli SOS chromotest	$SbCl_3$	11 – 707 µM	Negative	Lantzsch and Gebel, 1997 2: Reliable with restriction
B. subtilis Rec assay	$SbCl_3$	6.3 – 23 µg/disk	**Positive**	Kuroda, 1991 2: Reliable with restriction
B. subtilis Rec assay	Sb_2O_3	0.3 – 1.1 µg/disk	**Positive**	Kuroda, 1991 2: Reliable with restriction
B. subtilis Rec assay	$SbCl_5$	65 - 260 µg/disk	**Positive**	Kuroda, 1991 2: Reliable with restriction
B. subtilis Rec assay	Sb_2O_5	60 µg/disk	Negative	Kuroda, 1991 2: Reliable with restriction

[a] Positive assay outcomes are in bold type. Shaded cells are assays of Sb(V) compounds, clear backgrounds are for Sb(III) compounds.

description of the chromosome changes observed. Elliot et al. (1998) examined the induction of chromosomal aberrations in cultured human lymphocytes. at nominal SbTO 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 (as assessed by mean mitotic index of cultured cells). Lymphocyte cultures from two human volunteers were studied, only one of which exhibited a weak positive

Table 4
Mutagenic and clastogenic effects of antimony compounds in cultured mammalian cells (Whitwell, 2010).[a]

Assay	Compound Tested	Exposure Time and Concentration	Test Result	Reference and Klimisch Score
Mouse Lymphoma Cell Mutation	Sb_2O_3	6 – 50 μg/ml 4 hr	Negative	Elliot et al 1998 2 Reliable with restriction
Mouse Lymphoma Cell Mutation	$NaSb(OH)_6$	2.2 – 570 μg/ml 4 hr	Negative	Stone, 2010 1: reliable without restriction
Human Lymphocyte Chromosomal Aberrations	Sb_2O_3	10 – 100 μg/ml 20 – 48 hr	**Positive**	Elliot et al 1998 2: Reliable with restriction
Hamster Lung Cell Line Chromosomal Aberrations	Sb metal (10 μm average particle diameter	6.25 – 200 μg/ml	Equivocal	Asakura et al (2009) 3: Not reliable Chromosome aberrations induced but responses are largely confined to treatments with excessive (>50%) cytotoxicity.
Human Leukocytes and Fibroblasts Chromosome aberrations	Antimony sodium tartrate	0.6 μg/ml 24 – 48 hr	**Weak Positive**	Paton and Allison, 1972 3: not reliable due to testing of a single dose and poor description of results
Hamster CHO ovary cells Micronucleus induction	$SbCl_3$	11 – 91 μg/ml 4 hr	Equivocal	Huang et al., 1998 3 Not reliable: Micronucleus induction is at excessive levels of cytotoxicity
Human Fibroblasts	$SbCl_3$	11 – 91 μg/ml 4 hr	Equivocal	Huang et al., 1998 3 Not reliable:
Micronucleus induction				Micronucleus induction is at excessive levels of cytotoxicity
Human Bronchial epithelial cells Micronucleus induction	$SbCl_3$	11 – 91 μg/ml 4 hr	Equivocal	Huang et al 1998 3 Not reliable: Micronucleus induction is at excessive levels of cytotoxicity

(*continued on next page*)

Table 4 (*continued*)

Hamster V79 lung cells Micronucleus induction	$SbCl_3$	0.2 – 11 μg/ml 24 hr	**Positive**	Gebel 1998 2: Reliable with restriction
Human Lymphocytes Micronucleus induction	$SbCl_3$	0.2 – 5.5 μg/ml 46 hr	**Positive**	Schaumloffel and Gebel 1998 2: Reliable with restriction
Human Lymphocytes Micronucleus induction	$NaSb(OH)_6$	64 – 540 μg/ml 24 hr	Negative	Whitwell, 2010 1; Reliable without restriction
Human Lymphocytes Micronucleus induction	$KSbO_3$	50 – 125 μg/ml 48 hr	**Positive**	Migliore, 1999 2: Reliable with restriction

[a] Positive assay outcomes are in bold type. Shaded cells are assays of Sb(V) compounds, clear backgrounds are for Sb(III) compounds.

response to SbTO in the absence of S9 mix. The addition of S9 mix increased the dose-dependent frequency of cells with aberrations, a curious finding since metabolism of SbTO would not be expected. Moreover, responses in the presence of S9 were strongest in cultures that had not been responsive to SbTO in the absence of S9. The nature of the aberrations was not explicitly described except to note that chromosome gaps had been excluded. Finally, Asakura et al. (2009) reported that Sb metal powder could induce chromosome aberrations, but the reported responses were not statistically evaluated and were predominantly observed at excessive (> 50%) levels of cytotoxicity. These studies provide at best limited evidence of clastogenic activity from Sb treatment.

Treatment with antimony compounds (usually water soluble $SbCl_3$) has been associated with micronucleus induction in different cell types. Huang et al. (1998) observed micronucleus induction in a series of studies using Chinese hamster ovary cells, human bronchial epithelial cells and human fibroblasts. Micronucleus induction was concentration dependent and associated with significant cytotoxicity. The authors also 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. Both mechanisms of action would be expected to exhibit non-linear dose-response functions although apoptosis would be associated with a loss of cell viability that would remove biological significance from any genotoxic response.

Dose-dependent increases in micronucleus induction were observed in V79 cells (Gebel et al., 1998) and cultured human lymphocytes (Schaumloffel and Gebel, 1998). Finally, Migliore et al. (1999) observed dose-dependent induction of micronuclei in cultured lymphocytes from two human volunteers following *in vitro* treatment with $KSbO_3$. Fluorescence *in situ* hybridization (FISH) indicated that micronuclei in antimony treated cells generally lacked centromeres, suggesting they resulted from clastogenic events as opposed to aneuploidy. The concentrations tested (50–125 μg/ml) are within the range expected for a moderately soluble compound although others have reported that the maximum solubility of the analogous $NaSbO_3$ in cell culture medium is 3 μg Sb/ml (Hendriks and Derr, 2018).

The absence of centromeres in antimony induced micronuclei, although consistent with chromosome breakage, raises technical concerns with respect to many of the early micronucleus studies conducted with antimony compounds. Relying primarily upon Giemsa staining for micronucleus detection, a staining method that lacks specificity for DNA (Nersesyan et al., 2006), potential false positive results have been suggested if protein inclusion bodies are induced. Metalloids such as arsenic have been reported to produce cytoplasmic inclusion bodies that can be mistaken for micronuclei if non-DNA specific stains (*e.g.* Giemsa) are used (Wedel et al., 2013; Cohen et al., 2013). Inclusion body formation is presumably due to electrophilic interaction with thiol groups on proteins and other macromolecules (Verdugo et al., 2017). Sb (III) has been shown to bind to sulfhydryl groups on proteins as well as on glutathione (Sun et al., 2016; Grosskopf et al., 2010; Koch et al., 2017).

In addition, analyses of historical genotoxicity test data from the 1990s and earlier have suggested that many older studies are not compliant with current guidelines for genotoxicity testing. Schisler et al. (2018) evaluated historical mutagenicity studies with the mouse lymphoma forward mutation assay conducted by the U.S. National Toxicology Program. Only 17% of 1900 studies of 342 chemicals met the strict study protocols and data interpretation guidelines specified by current OECD test guidelines (TG 490). Although not subjected to this detailed level of evaluation, much of the micronucleus and Comet assay data from this time frame were suggested to have inadequately controlled for cytotoxicity and apoptosis. For example, OECD TG 487 adopted in 2016 for the *in vitro* micronucleus test (OECD, 2016a) requires that the concentration of test substances in a micronucleus study include non-cytotoxic doses and that cytotoxicity from treatment does not exceed approximately 50%. The studies of Huang et al. (1998) evaluated the impacts of antimony trichloride upon micronucleus induction in CHO cells, human fibroblasts and human bronchial epithelial cells. Cytotoxicity, as measured by the sulforhodamine B dye assay, was evident at all concentrations tested and ranged from > 50% to 99% for most concentrations evaluated. It is not clear if significant micronucleus induction occurred within currently acceptable limits upon cytotoxicity. In contrast, Gebel et al. (1998) and Schaumloffel and Gebel (1998) observed approximate two-fold micronucleus induction in V79 and cultured human lymphocytes within acceptable cytotoxicity limits.

Table 5
Effects of antimony compounds upon indicator assay endpoints in cultured mammalian cells (Gebel, 1998; Gebel et al., 1997).[a]

Assay	Compound Tested	Concentration Tested	Results	Reference and Klimisch Score
Human Lymphocytes SCE induction	$SbCl_3$	0.02 – 2.3 μg/ml 24 hr	**Weak Positive**	Gebel, 1996 2: Reliable with restriction.
Human Lymphocytes SCE induction	Sb_2O_3	0.3 – 1.5 μg/ml 24 hr	Equivocal	Gebel et al., 1997 3: Not reliable Insufficient experimental detail provided for evaluation
Mouse Embryonic stem cell lines ToxTracker GFP Assay	Sb metal powder, 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, $K(Sb)(OH)_6$ $Sb_(C_2H_3O_2)_3$ $K_2Sb_2(C_4H_2O_6)_2$	20 doses: 100 μg/ml and 19 consecutive 2-fold dilutions up to 50% cytotoxicity or maximum solubility	• DNA damage: negative • Activation of p53 response: negative except for a positive response for $Sb(C_2H_3O_2)_3$ • Induction of oxidative stress: p**ositive for all test compounds** except for $Na(Sb)(OH)_6$ and $Sb2O_5$ • Unfolder protein response activation: **positive for all test items**	Hendriks and Derr , 2018 2: Reliable with restriction (assay not yet validated)

(*continued on next page*)

Table 5 (*continued*)

			except $SbCl_3$, $Na(Sb)(OH)_6$	
Human HepG2 liver blastoma and LS-174T colon adenocarcinoma cells Phosphorylation of histones H2AX.	$SbCl_3$	2.3 – 115 μg/ml for 24 hr	**Positive** Dose-dependent increased histone phosphorylation evident at noncytotoxic (HepG2) or low toxicity (LS174T) concentrations	Kopp et al., 2018 2: reliable with restriction (assay not yet validated)
Human HeLa cervical carcinoma cells Double strand DNA breaks	$SbCl_3$	2.3 – 23 μg/ml 24 hr	Negative for DNA strand breaks. **Positive for inhibition of DNA repair of radiation damage**	Koch et al., 2017 2: Reliable with restriction
Human lymphocytes Comet Assay	Glucantime	1.06 – 4.25 mg Sb (V)/ml 3 and 24 hr	Negative	Lima et al., 2009 3: not reliable Inadequate measure of cytotoxicity
Hamster V79 lung cells Comet Assay	$SbCl_3$	0.3 – 3.0 μg/ml 24 hr	Equivocal	Gebel et al, 1998 3: not reliable due to inadequate measure of cytotoxicity
Human Lymphocytes Comet Assay	$SbCl_3$	0.2 – 5.5 μg/ml 2.5 hr	**Positive**	Schaumloffel and Gebel, 1998 3: not reliable due to inadequate measure of treatment cytotoxicity

[a] Positive assay outcomes are in bold type. Shaded cells are assays of Sb(V) compounds, clear backgrounds are for Sb(III) compounds.

These studies reinforce cytogenetic studies suggesting that antimony compounds may exert weak clastogenic activity *in vitro*.

The study of antimony compounds in indicator assays has yielded some positive results (Table 5). Sister chromatid exchange (SCE) induction and DNA damage assay (*e.g.* Comet assay) results have been generated most frequently but the quality of most studies is low. Both assay types 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 (Lima et al., 2010; Gebel et al., 1998; Schaumloffel and Gebel, 1998). Such findings do not make significant contributions to a weight of evidence evaluation of genotoxicity.

Data from other types of indicator assays are also available. Increased histone phosphorylation has been observed in human tumor cell lines following 24 h treatment with $SbCl_3$ (Kopp et al., 2018) with initial increases evident at concentrations that produce no or low levels of cytotoxicity (as determined by increases in cell counts during treatment). Increased histone phosphorylation has been associated with DNA adducts, DNA abasic sites, DNA strand breaks and DNA replication or transcription blocking lesions. However, increases in histone phosphorylation have not been validated as an assay for genotoxicity.

Sb compounds have also been evaluated in the new "ToxTracker" assay that evaluates chemically induced changes in gene expression diagnostic of different mechanisms for genotoxicity (Hendriks and Derr, 2018). The findings of the ToxTracker assay will be discussed further from the perspective of mechanisms of action – the assay suggests direct damage to DNA does not occur after Sb treatment but that indirect mechanisms of action may mediate any genotoxic responses.

4.3. *In vivo* genotoxicity test results

Gurnani et al. (1992) evaluated single and repeated doses of SbTO upon chromosome aberrations in mouse bone marrow. Oral gavage of 400–1000 mg SbTO/kg in a single dose, followed by analysis of chromosome aberrations, did not produce an increase in aberration frequency. In a repeated dosing protocol, mice were exposed to 400, 667 and 1000 mg/kg SbTO by oral gavage for up to 21 days and animals sacrificed 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). 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 present in Gurnani et al. (1992) are significant and indicate a need for confirmation from other studies. A later publication by Gurnani et al. (1993) would at first seem to provide confirmation but, as noted by Kirkland et al. (2007), is duplicate publication of data originally published in 1992. Gurnani et al. (1993) has thus been excluded from Table 6 since it is not a new study.

Kirkland et al. (2007) replicated the protocols of Gurnani et al. (1992) in a study of male and female rats administered 250, 500 and 1000 mg/kg SbTO 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., 1992 study). Treatment with SbTO 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 antimony into blood and the presence of antimony in bone marrow. Animals were 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 to other *in vivo* studies evaluating clastogenic effects of antimony compounds. The most significant study limitation was a failure of SbTO treatment to produce toxicity in bone marrow.

Other studies evaluating the genotoxic impacts of antimony *in vivo* followed protocols more limited in scope. Elliot et al. (1998) examined the impacts of a single 5000 mg/kg oral gavage SbTO dose upon micronucleus induction. No evidence was obtained for micronucleus induction but the use of only a single treatment and one concentration 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 SbTO 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 recently conducted inhalation cancer bioassays upon rats and mice, exposing animals to 3, 10 and 30 mg/m^3 Sb_2O_3 (SbTO) for two years (NTP, 2017). The NTP conducted studies evaluating the genotoxic effects of exposure to SbTO after one year of inhalation exposure. Flow cytometric procedures were applied to enumerate induction of micronuclei in erythrocytes and white blood cells from rats and mice. Increased micronucleus frequencies 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 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}antimony trioxide. While the response observed was statistically significant, the biological significance of the small 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 can cause modest increases in micronucleus incidence - generally accompanied by the appearance of immature reticulocytes in the blood (Tweats et al., 2007; Molloy et al., 2014). The pulmonary toxicity of SbTO produced severe hypoxia and that perturbed erythropoiesis as evidenced by increased prevalence of immature reticulocytes in the blood of mice and erythroid hyperplasia (NTP, 2017). Although NTP (2017) interpreted the induction of micronuclei in mice as evidence of genotoxicity, the small magnitude of the response and evidence of altered red blood cell production indicates that designation of this as a positive response is not appropriate.

Lung tissues from rats and mice exposed to SbTO for 12 months were analyzed for DNA damage by the Comet assay. No DNA damage was observed in rats but positive assay responses were reported for cells within mouse lung tissue. Although NTP did not attribute great significance to the positive Comet assay results, the protocols employed for conduct of the Comet assay do not meet current minimal quality standards (OECD, 2016b; Speit et al., 2015). Application of the Comet assay to intact tissues should carefully control for natural processes 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 NTP 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 potentially valid observation since uncontrolled sources of experimental artifact, such as excessive cytotoxicity or apoptosis, would not create a false negative assay response.

A limited number of publications have also evaluated genotoxicity in humans exposed to Sb compounds. Cavallo et al. (2002) evaluated SCE, micronucleus induction and Comet assay responses in 23 workers exposed to low levels of SbTO during the fireproofing of fabrics. There was no induction of SCE or micronuclei, but levels of SbTO exposure were low (0.12 μg/m^3). A modified version of the Comet Assay that includes a glycosylase that recognizes and removes oxidized bases such as 8-hydroxydeoxyguanosine from DNA suggested low levels of oxidative damage to DNA that appeared to be SbTO dose-related but could not be related to more established genotoxicity endpoints. Given the low levels of SbTO exposure, lack of response for validated genotoxicity endpoints and the novel version of the Comet Assay applied, the significance of the suggested oxidative DNA damage is difficult to ascertain. A case study (Hantson et al., 1996) has evaluated chromosome changes in a single patient before and after being administered 42.5 grams of pentavalent of meglumine antimoniate intravenously over the course of 15 days. No changes were observed in structural chromosome aberrations or SCE induction but a modest increase in cells with possible micronuclei were observed. The authors concluded that treatment did not pose a mutagenic or carcinogenic risk to humans, but such conclusions are difficult to accept based upon observations of a single individual. However, suggestions of oxidative DNA damage have been obtained in studies of mice administered meglumine by intraperitoneal

Table 6
Mutagenic and genotoxic effects of antimony trioxide in vivo (Shanawany et al., 2017).[a]

Assay	Compound	Dose	Result	Reference and Klimisch Score
Mutagenicity Test Results				
Mouse Bone Marrow Chromosome Abb.	Sb_2O_3	400 – 1000 mg/kg single oral dose	Negative	Gurnani, 1992 3: Not reliable Multiple deviations from GLP protocols
Mouse Bone Marrow Chromosome aberrations.	Sb_2O_3	400 – 1000 mg/kg 21 day oral dose	Equivocal	Gurnani, 1992 3: Not reliable: Multiple deviations from GLP protocols
. Rat Bone Marrow Chromosome aberrations	Sb_2O_3	250 – 1000 mg/kg 21 day oral dose	Negative	Kirkland, 2007 1: Reliable without restriction
One patient treated for leishmaniasis Chromosome aberrations	Meglumine antimoniate	42.5 g i.v. over 15 days	Negative	Hantson et al., 1996 3: Not reliable Results collected from a single patient.
Mouse bone marrow Micronucleus induction	Sb_2O_3	3200 and 5000 mg/kg oral gavage, one dose	Negative	Elliot et al, 1998 2: Reliable with restrictions
Mouse erythrocytes Micronucleus induction	Sb_2O_3	One-year daily inhalation exposure to 3, 10 and 30 mg/m^3	**Weak Positive**	NTP, 2017 2: Reliable with restrictions
Rat erythrocytes Micronucleus induction	Sb_2O_3	One-year daily inhalation exposure to 3, 10 and 30 mg/m^3	Negative	NTP, 2017 1: Reliable without restriction
Rat bone marrow Micronucleus induction	Sb_2O_3	250 – 1000 mg/kg 21 day repeated oral dose	Negative	Kirkland, 2007 1: Reliable without restriction

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Table 6 (*continued*)

Lymphocytes from 23 male workers Micronucleus induction	Sb_2O_3	Low inhalation exposure of 0.12 μg/m^3	Negative	Cavallo et al., 2002 2: reliable with restriction but airborne exposure very low. Co-exposures not determined.
Genotoxicity Indicator Assay Data				
Rat liver Unscheduled DNA synthesis	Sb_2O_3	5000 mg/kg oral gavage, single dose	Negative	Elliot et al., 1998 2: Reliable with restrictions
Mouse lung Comet Assay	Sb_2O_3	One year daily inhalation exposure to 3, 10 and 30 mg/m^3	Equivocal	NTP, 2017 3: Not reliable No control over cytotoxicity or apoptosis
Rat lung Comet Assay	Sb_2O_3	One year daily inhalation exposure to 3, 10 and 30 mg/m^3	Negative	NTP, 2017 3: Not reliable No control over cytotoxicity or apoptosis
Lymphocytes from 23 male workers SCE and Comet Assay	Sb_2O_3	Low inhalation exposure of 0.12 μg/m^3	Negative for SCE induction. **Positive for Comet Assay**	Cavallo et al., 2002 2: Reliable with restriction Airborne exposure very low. Co-exposures not determined.
25 workers exposed to SbTO Induction of apurinic sites on DNA.	Sb_2O_3	25 workers occupationally exposed to Sb trioxide. Urinary Sb output 10 -19 μg/L	**Positive**	Shanawany et al., 2017 2: Reliable with restrictions

[a] Positive assay outcomes are in bold type. Shaded cells are assays of Sb(V) compounds, clear backgrounds are for Sb(III) compounds.

injection (Lima et al., 2010; de Jesus et al., 2017; Moreira et al., 2017 and De Jesus et al. (2018). Administered doses in these studies are often high (up to 810 mg/kg) and elicit responses in the modified enzyme digestion version of the Comet Assay (inclusive of the addition of formamidopyrimidine-DNA glycosylase and endonuclease III for removal of oxidized DNA bases) that suggests the induction of oxidative DNA damage which can be reduced or blocked by the administration of antioxidants such as ascorbic acid (De Jesus et al., 2018). The presumed oxidative damage in these studies is difficult to interpret given the high doses administered *via* injection and the testing of only one dose. Micronucleus induction was also examined in these studies and low-level responses lacking dose dependency were observed. Parallel studies conducted *in vitro* did not exhibit these effects (Lima et al., 2010), supporting the suggestion that *in vivo* impacts are mediated by the conversion of Sb (V) to Sb (III). The results of such studies are difficult to extrapolate to *in vivo* studies conducted *via* physiological routes of administration, since the systemic antimony levels achieved (although not reported in these studies) were likely far higher than those that could be achieved *via* inhalation or oral exposure in occupational or consumer exposure scenarios. Such animal studies are primarily relevant to clinical applications of pentavalent antimonials in the treatment of leishmaniasis – a disease which in and of itself appears to induce oxidative DNA damage (Moreira et al., 2017; Xiong et al., 2017).

The available data suggest that antimony hydrolysis products do not induce point mutations but that weak clastogenic effects might 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 weak genotoxic properties, there is little evidence that this is expressed *in vivo*.

5. Possible mechanism(s) of antimony genotoxicity

The studies described above indicate that Sb and its compounds may possess weak genotoxic potential. Most studies have been conducted using soluble antimony such as trivalent antimony trichloride and the assumption made that any genotoxic activity observed could be attributed to the release of antimony ions *via* hydrolysis to yield oxyanions that vary in chemistry and valence state as a function of pH and the presence of other ions. The hydroxide oxyanions that may be formed from Sb metal have been best characterized in environmental samples (Nakamaru and Altansuvd, 2014) as opposed to cell culture medium. The dissolution of antimony trichloride in cell culture medium is likely to be complex (Hashimoto et al., 2003) and involve the sequential formation of antimony oxide chloride (SbOCl), antimony oxide

hydroxide (SbO(OH) and ultimately the formation of SbTO. The chemical moiety responsible for producing a genotoxic response is thus uncertain.

Most pentavalent antimony compounds are insoluble and difficult to test for genotoxicity. Sb(V) genotoxicity testing has thus focused upon compounds anticipated to release antimony ions in aqueous environments such as antimony pentachloride. Antimony pentachloride undergoes rapid hydrolysis in water to release hydrochloric acid and, as a function of pH, will undergo a series of hydrolytic transformations to oxide hydroxides that result in the formation of Sb_2O_5. Once again, hydrolysis products are the likely mediator for any positive test responses. The highly acidic and corrosive nature of $SbCl_5$ has placed limitations upon its use in animals.

Multiple studies have attempted to define the mechanisms of action for Sb-induced genotoxicity. These analyses have frequently been conducted on a comparative basis with arsenic compounds since both are Group V metalloids. The tissues and cell types studied are frequently targets for arsenic carcinogenesis but need not be target tissues for antimony genotoxicity or carcinogenesis. Impacts upon ROS generation, DNA repair and gene expression have been evaluated with the greatest frequency. These mechanisms are not mutually exclusive.

5.1. Covalent interactions with DNA

Covalent interactions between Sb compounds and DNA do not seem to occur. No interaction or binding of Sb(V) (administered as $KSb(OH)_6$) with herring sperm DNA was detected by Li et al. (2011) using short column capillary electrophoresis coupled with ICP-MS. An apparent binding of Sb(III) (administered as $K(SbO)C_4H_4O_6$) to DNA was reported as evidenced by the appearance of a new DNA peak associated with Sb(III) after 12 h of incubation under physiological conditions. However, interpretation of this observation is uncertain. The product specification sheet for the DNA used (Sigma-Aldrich Lot 3159) indicates that it is a degraded DNA oligomer (< 50 base pairs). Prolonged incubation under physiological conditions may have yielded an additional DNA peak as a function of DNA reannealing and/or resulted from interaction of $K(SbO)C_4H_4O_6$) with sites on degraded oligomers that would not be accessible on intact native DNA. It is further uncertain whether the association of Sb(III), administered in the form of antimony potassium tartrate, with a new DNA peak was due to binding by Sb(III) or the product of binding resulting from the chelating properties of the tartrate moiety.

Li et al. interpret their work as evidence of covalent binding to DNA, likely mediated by the 2′ and 3′ hydroxyl groups of ribose, a surprising interpretation in that DNA contains deoxyribose (not ribose) and therefore lacks a 2′ hydroxyl group. The 3′ hydroxyl of deoxyribose is further bound to the phosphate backbone of DNA. There thus are no 2′ and 3′ hydroxyl groups available in intact DNA to mediate the proposed interaction. While the study suggests comigration of antimony potassium tartrate with degraded DNA oligomers, there is insufficient data to support a covalent mechanism for this interaction. The negative profile of Sb compounds in the Ames test is further indicative of a probable lack of direct interaction with DNA (MacGregor et al., 2015).

5.2. Induction of reactive oxygen species

De Boeck et al. (2003) suggest that the generation of oxygen radicals constitutes an indirect pathway for inducing genotoxic responses by antimony. If reactive oxygen species (ROS) mediate *in vitro* observations of genotoxicity, this could explain why most *in vivo* studies have not observed genotoxicity. Antioxidant systems 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 antioxidant systems is exceeded (Kirkland et al., 2015).

Indirect mechanisms involving ROS have been the most intensively studied pathways for antimony genotoxicity with multiple lines of evidence indicating that ROS generation is key. Oxidant stress reflects an imbalance between the systemic production of ROS and the ability of a biological system to readily detoxify the reactive intermediates or repair the resulting damage. In the Toxys analysis of gene expression changes in the newly developed ToxTracker Assay, only genes that are responsive to oxidative stress were up-regulated by antimony compounds at non-toxic concentrations (Hendriks and Derr, 2018). Nrf2 is a critical regulator of endogenous antioxidant defense and promotes the transcription of a wide variety of antioxidant genes. Through binding to antioxidant response elements (AREs), Nrf2 activates antioxidant target genes such as glutathione S-transferase and heme oxygenase (HO). Although cell signaling pathways triggered by the transcription factor Nrf2 prevent 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 (Kansenen et al., 2013). The ToxTracker assay was also positive for oxidant activation of Blvrb, which is activated without Nrf2 involvement. However, activation of genes responding to DNA damage was not observed (Hendriks and Derr, 2018). Immortalized human keratinocytes also show oxidant-induced gene expression after exposure to trivalent (but not pentavalent) antimony (Patterson, 2003).

SbTO also caused dose-dependent cytotoxicity and apoptosis in human embryonic kidney 293 (HEK293) cells that was closely correlated with excessive ROS (Jiang et al., 2016). Nrf2 expression and nuclear translocation were significantly induced by SbTO treatment, and Nrf2 knockdown increased SbTO-induced cell apoptosis, showing that it has a protective effect. They also reported that SbTO increased Gadd45b expression (*via* Nrf2), resulting in phosphorylation and activation of MAP kinases. Gadd45b has been implicated as an anti-apoptosis factor. Together, this study demonstrated that the Nrf2-Gadd45b signaling axis exhibited a protective role in Sb-induced cell apoptosis.

$SbCl_3$ was also shown to induce ROS-dependent autophagy and loss of cell viability in human bronchial epithelial cells (BEAS-2b). The NAD-dependent deacetylase sirtuin1 (SIRT1) plays a crucial role in oxidative stress, mitochondria function and apoptosis *via* deacetylation of substrates. In an investigation of SIRT1 under Sb exposure, it was found that decreased gene expression and accelerated decay of the protein led to lowered SIRT1 expression *via* ROS, which in turn triggered apoptosis (Zhao, 2018).

Antimony compounds also exert toxicity and growth inhibition of cells from acute promyelocytic leukemia (APL), a disease which is sometimes treated with arsenic trioxide. SbTO also induced growth inhibition, apoptosis and terminal differentiation in these cells. SbTO-induced ROS correlated with increased apoptosis markers *via* activation of JNK and its downstream target AP-1, an effect enhanced by GSH depletion (which would increase ROS). Arsenic-resistant APL cells with increased glutathione levels (which would decrease ROS) exhibit increased cross-resistance to SbTO (Mann et al., 2006). Although these results appear opposite to effects on the human keratinocytes, it is not unusual for a toxicant to have opposite effects at sub-toxic doses and toxic doses (*e.g.* hormesis). For example, in the case of oxidant stress, the term “oxidative eustress” has been proposed for low-level oxidative stress used for redox signaling and regulation, and the term “oxidative distress” proposed for pathological ROS levels that can damage biomolecules and lead to cytotoxicity and apoptosis (Sies, 2018, Fig. 1).

Multiple lines of evidence indicate that treatment with Sb compounds can be associated with the enhanced production of ROS and it is plausible to assume that ROS may have mediated genotoxic responses seen *in vitro* at high concentrations. Attenuation of genotoxicity *in vivo* would be expected due to the presence of antioxidant defense systems that would not be present in cultured mammalian cells or bacteria

5.3. Changes in gene expression

Next generation sequencing has shown that when keratinocytes are

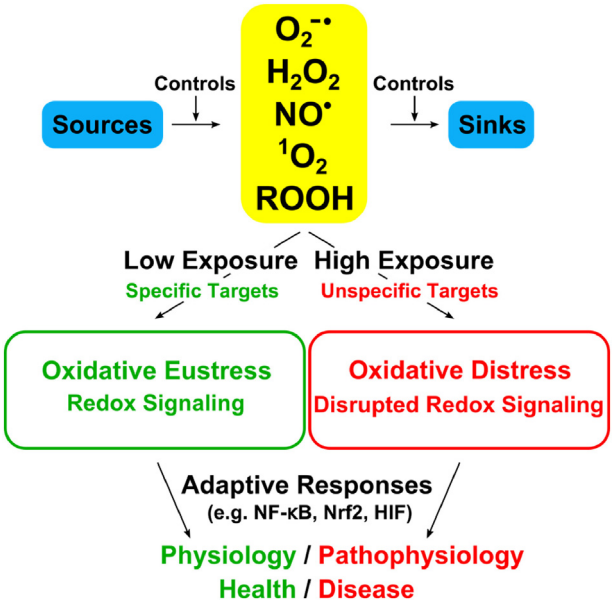

Fig. 1. Distinction between normal and pathological levels of ROS induction. Reprinted from Curr. Opin. Toxicol 7, Sies, H., On the history of oxidative stress: Concept and some aspects of current development, pp. 122–126, 2018, with permission from Elsevier.

treated with 3 μM sodium arsenite or 6 μM antimony tartrate, the transcriptional responses are similar, with more genes suppressed than stimulated Phillips, 2016). Parallel changes were seen for genes in the Nrf2 pathway (up-regulated), genes for some metabolic enzymes (up-regulated), and genes for differentiation (down-regulated) (Phillips, 2016). In some cases, the authors made determinations of protein levels that confirmed these results. In addition, among other newly discovered mRNAs down-regulated by arsenite and antimonite are those encoding LRIG1, a negative regulator of epidermal growth factor receptor (EGFR) signaling, and ryanodine receptor 1, a positive regulator of calcium signaling that promotes cell differentiation. Down-regulation of a negative regulator of EGFR would tend to promote growth factor activity, resulting in increased cell proliferation. Increased cell proliferation is one of the hallmarks of cancer (Hanahan and Weinberg, 2011) and could facilitate the development of neoplastic lesions in the absence of genotoxic impacts. Impacts upon keratinocytes are particularly relevant to arsenic which is known to cause hyperkeratosis and skin cancers in humans (Cohen et al., 2013). Skin has not been identified as a target for Sb-induced genotoxicity or carcinogenesis.

Studies on micro-RNAs have shown similar keratinocyte responses to arsenic and antimony compounds that could lead to increased cell proliferation (Phillips, 2016). The authors conclude that arsenite and antimonite have parallel effects on major signaling pathways that result in suppressing differentiation (by down-regulating pro-differentiation pathways such as NOTCH1 and BMP6) and preserving proliferative potential (by prolonging EGFR signaling). This forced cell proliferation could result in ROS-induced DNA damage yielding mutagenic changes if cell division is stimulated prior to the repair of DNA damage that might otherwise be successfully repaired without error. Thus, like As (III) (Rossman and Klein, 2011), Sb(III) could act as a comutagen, by both inhibiting DNA repair and stimulating proliferation in DNA damaged cells. However, there is little empirical data suggestive of comutagenic properties for Sb(III).

Following up on their observation that patients with prostate cancer have higher serum antimony concentrations than controls, and that high serum antimony levels correlated with poorer outcome in cancer patients, Zhang (2018a) found that low-dose antimony promoted growth of prostate cancer cells *in vitro*. Low-dose antimony treatment was found to enhance c-Myc protein stability and promote prostate cancer cell proliferation. C-Myc is a proto-oncogene whose alterations exist in a significant fraction (> 30%) of human cancers (Meyer and Penn, 2008). Exposure of these cells to antimony also triggered the phosphorylation of androgen receptor (AR), which transcriptionally regulates the expression of androgen-related targets, including prostate-specific antigen (PSA), a clinically used biomarker for screen and diagnosis of prostate cancer, and NKX3.1, which plays a role in antioxidant regulation. The authors suggest that antimony could promote tumor growth by mimicking androgen activity in androgen-dependent prostate cancer cells (Zhang, 2018b). However, occupational epidemiology and animal bioassays have evaluated the carcinogenic potential of SbTO and no carcinogenic impacts upon the prostate have been reported.

Finally, changes in gene expression can be regulated by DNA methylation that does not alter the primary sequence of DNA. While methylation of cytosine in DNA to 5-methyl cytosine (5-mC) is the most important epigenetic modification in DNA, 5-mC in DNA can be converted to 5-hydroxymethyl cytosine (5-hmC), 5-formylcytosine (5-foC), and 5-carboxylcytosine (5-caC). Like 5-mC, these newly identified cytosine modifications are considered to play a role in the regulation of gene expression. One recent study has reported an association between urinary metal concentrations and the amount of DNA methylation and hydroxy-methylation in DNA extracted from blood. The level of global 5-hmC and to a lesser extent 5-mc, was positively associated with antimony exposure (Tellez-Plaza, 2014). In contrast, levels of 5-hmC, 5-foC, and 5-caC are significantly decreased in both the DNA and RNA of cultured mouse embryonic stem cells exposed to $SbCl_3$ (Xiong et al., 2017). The latter study is difficult to interpret due to use of only a single treatment concentration and a failure to monitor cytotoxicity.

5.4. Impaired DNA repair

Sb(III) has been shown to interfere with DNA repair processes and this may facilitate genotoxic responses by other agents. Both $SbCl_3$ and antimony potassium tartrate inhibit the repair of radiation induced double-strand DNA breaks in cultured CHO cells (Takahashi et al., 2002; Koch et al., 2017). Inhibition of nucleotide excision repair (Grosskopf et al., 2010) will inhibit repair of lesions induced by UV irradiation or chemical treatment with activated polycyclic aromatic hydrocarbons in A549 human lung carcinoma cells. The inhibition of DNA repair was dose-related and a small effect was seen at doses as low as 10 μg/ml (Koch et al., 2017). This is still a high concentration for cultured cells, so the relevance of this to *in vivo* scenarios is uncertain.

5.5. Interactions with cellular proteins

Antimony hydrolysis products would also be expected to undergo electrophilic interactions with cellular constituents such as thiol rich proteins (Verdugo et al., 2017; Grosskopf et al., 2010). Such interactions provide a mechanistic basis for intracellular inclusion body formation and for oxidant stress *via* reactions with cellular glutathione. Interaction with cellular proteins likely mediates aspects of cytotoxicity and may mediate effects upon DNA repair and replication. Specific target proteins for most Sb protein interactions remain to be determined.

6. Summary and conclusions

Elevated inhalation exposure to SbTO in the workplace is associated with mild pneumonitis, a health impact that has been the basis for modern occupational exposure limits of 0.5 mg/m^3 for inhalable SbTO particulate matter. Adherence to this occupational exposure limit appears to have limited, if not eliminated, pneumonitis resulting from SbTO exposure. Inhalation exposure of rats and mice to respirable SbTO (3, 10 and 30 mg/m^3) produces significant pulmonary toxicity and is associated with the appearance of tumors in the lung and several

Table 7
Possible mechanisms of genotoxicity.

Potential mechanism	Mechanism plausibility
Covalent interaction with DNA	Unlikely due to lack of reliable data to support covalent interaction.
Induction of ROS	Probable. ROS induction consistently observed and could either induce DNA damage or stimulate cell proliferation to convert promutagenic lesions to mutations.
Altered Gene Expression	Possible. Altered DNA expression observed for genes controlling cell proliferation and differentiation. Could facilitate conversion of promutagenic lesions.
Inhibition of DNA repair	Possible. Altered by high *in vitro* Sb concentrations that may not be relevant *in vivo*, inhibition of repair could result in persistence or error-prone repair of promutagenic lesions.
Interaction with cellular proteins	Possible. Protein binding occurs but specific cellular targets have not been identified.

related tissue sites. The severity of lesions induced in rodents is greater than that observed in most humans. The seemingly higher sensitivity of rodents to SbTO toxicity could represent a species-specific difference in susceptibility and/or a reflection of the higher deposition rate of respirable SbTO in the deep lung of rodents. Lung tumors attributable to antimony exposure have not been observed in human epidemiology studies, but existing studies are limited in their control of critical confounders, lack information on known co-exposures to human carcinogens and have very limited documentation of SbTO exposure levels. The observation of tumors in experimental animals but not in human epidemiology studies is consistent with the classification of SbTO as a Category 2 or possible human carcinogen.

Genotoxicity testing of SbTO and other antimony compounds have produced mixed results. Assays for mutagenicity in bacteria are negative, but assays for DNA recombination (*B. subtilis* rec assay) are positive. Negative bacterial mutagenicity studies suggest lack of direct covalent interaction between Sb and DNA and are assigned precedence over *B. subtilis* findings.

Gene mutation assays in mammalian cells *in vitro* are limited in number but are negative. In contrast, cytogenetic impacts (chromosome aberrations and micronucleus induction) are sometimes observed, but these studies often utilize treatment concentrations that produce grossly excessive cytotoxicity. The limited available evidence indicates that Sb (III) compounds are at best weakly clastogenic *in vitro*. Less data is available for Sb(V) compounds and both positive and negative micronucleus test results have been reported.

Expressions of genotoxicity *in vivo* are inconsistent. Positive study results exist for micronucleus induction, chromosome aberrations and the Comet Assay, but study quality is suspect for many of these responses. Negative study results have characterized the higher quality studies and have suggested that genotoxic impacts observed *in vitro* entail mechanisms that are not expressed *in vivo*.

The most probable mechanisms for genotoxicity (summarized in Table 7) do not appear to entail mutagenic changes produced by direct interaction of Sb with DNA. Rather, indirect mechanisms appear to be at play, most likely involving the generation of ROS, that could exhibit thresholds as a function of the antioxidant defenses present in tissues exposed to antimony. Alternatively, or in addition, effects involving DNA replication and repair, DNA methylation changes and/or interaction with growth factors or regulators of DNA transcription, may serve to induce or amplify genotoxic responses. Response thresholds would be probable for these alternate mechanisms of action as well. Within the context of the Global Harmonized System for classification, the available evidence does not indicate that antimony would fulfil the criteria for triggering classification as a direct-acting mutagen. Instead, indirect genotoxic or nongenotoxic mechanisms are most likely responsible for adverse effects (*e.g.* cancer in animals). In terms of risk assessment for pulmonary toxicity, and cancer, the dose-response functions for the most probable and other possible mechanisms of action suggests an effect threshold can be established for all endpoints with a relatively high level of certainty.

Declaration of Competing Interest

There are no conflicts of interest to declare.

Acknowledgements

The preparation of this paper was supported by an unrestricted research award from the International Antimony Association, Brussels, Belgium that granted the authors full editorial control over the contents of this review. We thank Dr. Barry P. Rosen, Department of Cellular Biology and Pharmacology, Florida International University, Herbert Wertheim College of Medicine, Miami, FL 33199, for his helpful discussions on bacterial proteins involved in antimony uptake and efflux.

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