Commentary: NTP Draft Report 2-year Inhalation Carcinogenicity Studies Antimony Trioxide in Rate and Mice (NTP TR 590, 2016

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This commentary on the NTP Draft Report: 2-year Inhalation Carcinogenicity Studies Antimony Trioxide in Rats and Mice (NTP TR 590 draft, 2016) has been prepared at the request of the International Antimony Association located in Brussels, Belgium by CJB Risk Analysis, LLC. The International Antimony Association serves the common interests of antimony producers, traders, users and other stakeholders, concerning regulatory affairs and environmental, health and safety matters. CJB Risk Analysis, LLC is in turn a private consultancy based in Durham, North Carolina specializing in independent assessments of the toxicological impacts of metals upon humans and experimental animals. CJB Risk Analysis has examined the draft report for antimony trioxide, evaluating the technical rigor with which the studies were conducted, the adequacy of the histopathological examinations and supporting studies undertaken and the extent to which the overall conclusions of the studies are supported by the underlying data. A number of significant issues were identified during the course of this review and are outlined here in an effort to provide constructive input that would assist in revision of the draft report.

The inhalation studies of antimony trioxide were conducted in compliance with established guidelines for the conduct of cancer bioassays. Groups of 60 male and 60 female rats and mice were exposed to airborne antimony trioxide concentrations of 0, 3, 10 and 30 mg/m^3 for up to two years. Small supplemental groups of animals were included for separate evaluations of lung burden development. The protocol produced "some evidence" of carcinogenic activity of antimony trioxide for rat respiratory tissue, elevation of pulmonary neoplasms being observed that trended higher than those observed in controls but generally failed to achieve statistical significance. Genotoxic impacts of antimony trioxide were not observed in rats. Studies in mice were noted to provide "clear evidence" of carcinogenic activity with dose dependent elevations of neoplastic and preneoplastic lesions in respiratory tissues and dose dependent elevation of lymphoma incidence, especially in female mice. These observations are suggested to be linked to genotoxic effects reported from Comet assay studies of pulmonary tissue and micronucleus induction in circulating erythrocytes. Molecular analysis of oncogene mutations in control vs antimony trioxide exposed animals further observed a shift from *kras* alterations in spontaneously occurring lung lesions to a mix of *kras* and *egfr* lesions in tumors from antimony

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trioxide treated animals. This shifting fingerprint of oncogene alterations is cited as further evidence that neoplastic lesions in the respiratory tract were treatment induced.

The following comments concern the conduct of the bioassays and the ancillary mechanistic studies. General comments are presented first, usually addressing issues in the order in which they appear in the draft study report. These are followed by a series of specific recommendations for modification of the report and/or the analyses contained therein.

General Comments

Study Design and Execution:

Compliments are extended for execution of the basic study design. Procedures for aerosol generation, exposure control and monitoring were implemented with a high degree of precision that resulted in target exposure levels being accurately achieved on a regular basis.

The antimony trioxide aerosols produced were characterized by a somewhat finer particle size distribution (MMAD between 0.9 and 1.5 μm with a GSD between 1.8 and 2.1) than those likely employed by most other investigators. Groth et al (1986) reported a MMAD of 2.8 μm (no GSD given) at exposure concentrations of 45 mg/m^3. Newton et al (1993) similarly reported a MMAD of 3.0 (GSD 1.6) although the reliability of this PSD is uncertain since the material used for aerosol generation was a blend of antimony trioxide provided by eight different producers. Watt et al (1983) reported a PSD with a Feret's diameter of 0.4 μm and a GSD of 1.6 although the method of PSD distribution (SEM of dust samples of uncertain origin) may not provide reliable estimate of the actual characteristics of the experimental aerosols. If one assumes that a Feret's diameter of 0.4 μm is accurate, this would equate to aerosols with an MMAD of 5.2 μm and a GSD of 2.13 (Morrow and Oberdorster, 1993). The potential impact of PSD upon the toxicological findings relative to those in the existing published literature are not fully explored but likely are relevant to why the NTP studies observed greater toxicity to the lung than most other studies. The smaller PSD would be expected to result in higher rates of alveolar deposition from experimental aerosols. Greater alveolar deposition would result in higher toxicity, but one is then confronted with the relative deficit of rat lung neoplasms in the NTP study relative to some existing bioassays.

Problematic deficiencies are noted in some of the ancillary studies (e.g. genotoxicity testing) designed to facilitate interpretation of bioassay results and will be discussed in greater detail.

Two-week range finding studies: short-term (two week) range finding studies were conducted at antimony trioxide exposure concentration of 0, 3.75, 7.5, 15. 30 and 60 mg/m^3. Based upon the results of these studies, combined with the results of studies with rats in the published literature, exposure concentrations of 3, 10 and 30 mg/m^3 were selected for use in the two year bioassays. No significant deficiencies were identified in the conduct of these short-term studies or the selection of exposures to be employed for the two-year bioassays. The subsequently conducted bioassays exposed groups of male and female Wistar Han rats and B6C3F1 mice (N=60 for each exposure group) to antimony trioxide for 105 weeks. Of these, 10 animals per sex, per species per exposure group were randomly selected for sacrifice at 12 months for interim evaluation and conduct of genetic toxicology studies. An additional 25 animals per sex and species were included for evaluation of antimony tissue burdens after approximately 2, 4, 9, 12 and 18 months of exposure (N=5 per time point).

Impacts of Two Year Antimony Trioxide Exposure Upon Rats

Two-year inhalation exposure of rats to antimony trioxide resulted in a variety of dose-dependent histopathological changes that mirrored those observed in the published literature. The primary histopathological findings indicate a time- and dose-dependent increase in inflammatory changes in apparent response to antimony trioxide exposure. These changes were accompanied by observations of increasing burdens of presumed antimony trioxide particles, the retention pattern of which appeared to mirror sites at which there was manifestation of acute inflammation, proteinosis and hyperplasia. Findings of neoplastic lesions indicated a relatively modest incidence of lung lesions. In male rats the incidence of alveolar/bronchiolar adenomas at 0, 3, 10 and 30 mg/m^3 of exposure were 3/50, 4/50, 6/50 and 8/50 and alveolar/bronchiolar carcinomas had an incidence of 0/50, 0/50, 2/50 and 0/50, respectively. No alveolar/bronchiolar carcinomas were observed at 105 weeks in female rats and the incidence of alveolar/bronchiolar adenomas was 0/50, 2/50, 6/50 and 5/50 respectively. One squamous cell carcinoma and two cystic keratinizing epitheliomas were observed in female rats (at 30 mg/m^3). A variety of other potentially treatment related neoplastic and non-neoplastic findings were made in the respiratory

tract and in other tissues (e.g. bone marrow) but the central focus of the analysis is placed upon the lung lesions. These studies provide only limited evidence for carcinogenic potential in rats.

Evaluation of lung tissue antimony trioxide burdens over time indicated that steady state concentrations of antimony trioxide were not achieved and that lung burdens progressively increased over the course of the study. The retention kinetics of antimony trioxide particles suggested that reduced pulmonary clearance might be associated with pulmonary overload. Calculations were thus made to determine whether volume and/or surface area based particulate loading met formal criteria for pulmonary overload. Overload criteria were not met at exposure concentrations of 3 mg/m^3, but 10 mg/m^3 exposure was associated with the gradual onset of volumetric overload by day 418 of exposure and surface area criteria for overload were nearly met by the end of the exposure period. Overload conditions were met for both volumetric and surface area criteria at 30 mg/m^3 after approximately 94 days of exposure.

Genotoxicity studies evaluated the incidence of micronuclei in circulating erythrocytes and DNA damage in lung tissue (assessed by the Comet assay) and observed little evidence of genotoxicity.

The draft report's analysis of the rat bioassay findings is unusual in that little effort is made to align this study with the findings of several earlier (and admittedly somewhat deficient) studies suggesting carcinogenic potential of antimony trioxide for rats. Given that the particle size distribution of the experimental aerosols used in this study was likely somewhat smaller than those in most predecessor studies, greater alveolar deposition and subsequent carcinogenic impact might have been expected. Additional comparative analyses between the finding of this study and previously conducted rat studies would be beneficial.

Analysis of the rat data is also surprisingly dismissive of the potential role of pulmonary overload in modulating some of the responses observed. Given that previously published studies have in part been conducted with a view to evaluating the role of pulmonary overload in the production of neoplastic lesions in the respiratory tract in response to antimony trioxide exposure. More in depth discussion of this potential mechanism of action would have been expected. The potential role of progressive development of lung tissue burdens in modulating toxicological responses is seemingly dismissed because lower exposure concentrations did not meet fixed criteria for volumetric or surface area loading traditionally associated with overload.

This in turn implies that antimony trioxide possesses sufficient toxicity to impede lung clearance mechanisms prior to the onset of overload. As a result, the draft report seems to conclude that overload impacts are not germane to antimony trioxide toxicity do not merit further consideration. Strict adherence to arbitrary benchmarks to define overload results in little emphasis being placed upon mechanistic information that may dictate the exposure-response relationships that will modulate potential nonlinear dose response functions at low level exposures or the extrapolation of rodent dose response function to humans. For example, the present data demonstrate that antimony trioxide toxicity is amplified by inhibition of clearance mechanisms such that progressively larger lung burdens develop at high exposure levels. Indeed, although formal criteria for overload may not be met for a number of exposure groups. the cascade of histopathological changes observed in the rats of the NTP study mirror those associated with pulmonary overload impacts. The neoplastic lesions induced by antimony trioxide and the target tissues for antimony trioxide carcinogenesis (alveolar and bronchiolar cells) are further the target cells associated with overload related neoplastic lesions (MAK, 2014). By these measures, even though arbitrary volumetric or surface area trigger points for overload may not be met, antimony trioxide exposure is inducing the same cascade of histopathological changes that are associated with neoplasm development under conditions of pulmonary overload.

At lower levels of exposure, impairment of clearance mechanisms would be expected to attenuate and a lung burden equilibrium would be attained (as was evident in the parallel studies conducted in mice). The dose response for antimony trioxide impacts would be expected to exhibit significant non-linearity with disproportionately diminished toxicological impacts at exposure concentrations that do not impede clearance. Such mechanistic considerations should be brought forward in the document for more explicit and detailed discussion. Similarly, little guidance is offered on potential mechanisms of action that will also be important determinants of dose response. Are the negative findings of genotoxicity testing in the rat significant – or are they over-ridden by the purported suggestive findings of genotoxicity in the mouse.

Impacts of Antimony Trioxide Exposure Upon Mice

Inhalation exposure of mice to antimony trioxide produces a far more complex constellation of neoplastic and non-neoplastic effects with suggestions of significant impact at tissue sites distant

from the lungs. This commentary will predominantly focus upon the study outcomes that are the most significant from a toxicological and risk assessment perspective – the determination that there is clear evidence of carcinogenicity of antimony trioxide for both the lungs and the spleen and that these effects are likely are grounded in genotoxic properties of antimony trioxide.

Pulmonary neoplasms associated with antimony trioxide exposure were first detected at the 12 month interim sacrifice. Alveolar/bronchiolar adenomas were found in two male mice exposed to 10 mg/m^{3}antimony trioxide and one female exposed to 30 mg/m^3. Moreover, alveolar/bronchiolar carcinoma was observed in one of ten and two ten males exposure to 10 and 30 mg/m^3, respectively. No neoplastic lesions were observed in chamber controls at the interim evaluation.

Non-neoplastic alterations were further observed at the interim evaluation (e.g. peribronchial and perivascular lymphocytic infiltrates, free and macrophage engulfed foreign bodies, fibrosis, hyperplasia and active inflammation) with lesion severity generally increasing as a function of exposure concentration.

By study week 105, significantly increased incidence of alveolar/bronchiolar adenomas and carcinomas were evident at an incidence above that observed in chamber controls. In male mice, animals with multiple alveolar/bronchiolar carcinomas were evident in all exposure groups but not in the chamber controls. Early mortality was significant in all groups (both exposure and chamber controls) making Poly-3 adjusted estimates of incidence perhaps the most appropriate and reliable indicator of effect. Adjusted rates of alveolar and bronchiolar adenomas at 0, 3, 10 and 30 mg/m^3 antimony trioxide were 21.5%, 32.9%, 21.8%, and 34.6% and alveolar and bronchiolar carcinomas observed with an adjusted incidence of 8.5%, 40.9%, 46.2% and 62.8%, respectively. Adenoma incidence exceeded the historical control ranges at 3 and 30 mg/m^3 while the incidence of carcinomas was significantly greater than chamber controls and exhibited (albeit weak) dependency upon the intensity of antimony trioxide aerosol exposure. Female mice similarly exhibited multiple alveolar/bronchiolar carcinomas in all exposure groups except the chamber controls. The adjusted rate of adenomas was 2.3%, 22.8%, 44.9% and 20.3% and alveolar/bronchiolar carcinomas 4%, 31.2%, 26.8% and 28.8% at exposure concentrations of 0, 3, 10 and 30 mg/m^3, respectively. The dose response for lesion induction in female mice did not exhibit dose dependency, but early mortality from pulmonary neoplasms and pulmonary toxicity

was significant – survival to week 105 was 72% in chamber controls and decreased to 62%, 52% and 30% with increasing exposure to antimony trioxide.

The dose dependency of effect is weaker than might have been expected, particularly given the antimony trioxide loading in pulmonary tissue over the course of the study, and may not be consistent with the dose response requirements for designation of clear evidence of carcinogenicity. However, the magnitude of the tumor induction response observed seems to be compatible with "clear evidence of carcinogenicity". A variety of secondary changes reflective of pulmonary toxicity were also observed and generally increased in severity as a function of exposure concentration. These secondary changes may have played a significant role in the development of neoplasms.

Time and Exposure Dependent Changes in Lung Burdens of Antimony Trioxide

Groups of five animals were sacrificed after approximately 2, 4, 9, 12 and 18 months of antimony trioxide exposure. Whereas rats exhibit continuous increases in lung weights and antimony trioxide lung burdens that increased as a function on inhalation exposure intensity, burden development in mice was more complex and the kinetics of deposition and clearance were difficult to model. This was in large part due to lung burdens at day 551 that were significantly higher than expected. In general, normalized lung burdens in mice at 10 and 30 mg/m^{3}exposure were higher than those at 3 mg/m^3. Indeed, lung burdens in female mice seemed to be attaining an equilibrium at 3 mg/m^3 (although model fit is admittedly poor) while burden at higher concentrations seems to continuously increase as a function of exposure time and intensity. Comparing the antimony trioxide lung burdens (normalized to g of lung tissue) for different exposure levels at day 551 in female mice yields burdens of 1,471, 4188 and 8398 ug antimony per gram of lung tissue at airborne concentrations of 3, 10 and 30 mg/m^3. Data for male mice are not provided, but are presumably similar. Lung burdens thus show roughly proportionate increases in antimony trioxide concentrations that reflect the duration and intensity of inhalation exposure. However, the "dose to target tissue" is not mirrored by proportionate increases in the incidence of neoplastic lesions.

As with the rodent data, the impacts of antimony trioxide in mice are suggestive of pulmonary overload. Higher concentrations of antimony trioxide seem to inhibit the clearance of particles and the spectrum of inflammatory changes observed are qualitatively similar to those seen in

rats. The sites of impact, and cell targets for toxicity and neoplasia are further similar to those impacted in the rat. This is further reflected in the data from the two week studies that evaluated the impacts of antimony trioxide concentration upon clearance rates with more rapid clearance being suggested at the lowest airborne exposure concentrations. While too limited to support definitive conclusions, the combined data suggest that antimony trioxide, if present at sufficient concentrations, will inhibit clearance. This would be expected to introduce nonlinearity into the development of lung burdens and the dose response relationships for different toxicological endpoints with significant enhancement of effects at elevated levels of exposure.

Lymphoma Incidence in Mice

Whereas significant alterations in the incidence of malignant lymphomas were not observed in rats or male mice, malignant lymphoma incidence seemed to exhibit exposure dependent increases in antimony trioxide exposed female mice. Lymphoma incidence (Poly-3 adjusted for intercurrent mortality) increased from 15.5% in chamber controls to 38.1, 47.5 and 60.7% at antimony trioxide concentrations of 3, 10 and 30 mg/m^3, respectively. This seeming dose dependent increase in lymphoma incidence is interpreted as "clear evidence" of carcinogenic activity.

This conclusion is reached without acknowledgement of the special diagnostic challenges that are posed by the high spontaneous incidence of lymphomas in mice, with higher prevalence of spontaneous lesions being present in female mice (Ward, 2005). As the NTP draft accurately notes, the average historical control incidence of lymphomas in B6C3F1 female mice is 25.2% (range 14 – 36%). Thus, incidence at 10 and 30 mg/m^3, but not 3 mg/m^3 is significantly elevated over historical controls. However, interpretation of any increased incidence of lymphomas in a mouse cancer bioassay must be evaluated carefully. Chemically induced lymphomas are generally early onset T-cell lymphomas while spontaneous lesions are generally late arising B-cell lymphomas (Ward, 2005). Because of the difficulty of interpreting increases in lymphoma induction in mice, diagnosis and classification schemes have been developed to help distinguish induced from spontaneous lesions. None of these diagnostic criteria appear to have been applied in this study. The lymphomas associated with antimony trioxide are described in the report as being predominantly B-cell in nature with some T-cell characteristics. On this basis, they appear to be quite similar to the naturally occurring lesions in the B6C3F1 mouse and it can be plausibly

postulated that the chronic inflammation and hypoxic conditions in the antimony trioxide exposed lung produced adaptive response in the spleen that promote the development of what is already a high incidence spontaneous neoplasm in the female mouse.

Studies of Genotoxicity

The draft report describes the conduct of studies to evaluate the genotoxic effects of exposure to antimony trioxide. Lung tissue from a rats and mice exposed to antimony 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 draft document 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 in this study do not meet minimal quality standards for conduct of the Comet assay. 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 (Henderson et al., 2008; Fairbairn etl al., 1996), apoptosis (Choucroun et al., 2001; Fairbairn et al., 1996), oxidative stress and terminal differentiation must all be carefully assessed for their impact upon assay outcomes. Even then, discrimination between effects that might be directly induced by antimony trioxide and those that would be associated with indirect genotoxicity mediated by processes associated with pulmonary overload would not be possible. The study described in the draft report controlled for none of these sources of artifactual false positives. Information can also be obtained from detailed evaluation of the shape of Comet tails and the distribution of DNA within the tail (Lee et al., 2003). Little meaningful information is provided on any of these accepted response parameters. The deficiencies of the studies should be noted and any inferences removed that they are suggestive of in vivo genotoxicity from antimony trioxide exposure.

Sensitive flow cytometric procedures were also applied to study the possible induction of micronuclei in the erythrocytes and white blood cells from rats and mice. Micronuclei were not observed in cells from rats or mouse white blood cells but a low level of micronucleus induction was observed in mouse erythrocytes. The incidence of micronuclei increased in both male and female mice in a dose-dependent fashion but the overall magnitude of response was small. By way of example, normochromatic erythrocytes exhibited an average of 1.04 micronuclei per 1000 cells, increasing to a maximum of 1.38 per 1000 in female mice exposed to 30

mg/m^{3}antimony trioxide. This level of response is statistically significant by virtue of 1,000,000 cells having been scored, but would not have been detectable prior to the application of flow cytometry to screen large numbers of cells. While the response observed may be statistically significant, the biological significance of such small responses is uncertain.

Interpretation of the micronucleus studies is further complicated by the observations of other laboratories that conditions that accelerate or perturb erythropoiesis produce small increases in erythrocytes micronuclei (Tweats et al., 2007; Molloy et al, 2012). Thus, induction of anemia by blood loss or dietary restriction has been associated with modest increases in micronucleus induction and is generally accompanied by the appearance of immature reticulocytes in the blood. The impacts of antimony trioxide produce conditions such as hypoxia and bone marrow hyperplasia that would be expected to perturb erythropoiesis. Indeed, the draft study report mentions an increased prevalence of immature reticulocytes in the blood of mice and acknowledges that erythropoiesis is likely affected in the exposed mice. However, the assertion is made by the study researchers, in the absence of data or citation of peer-reviewed articles, that they have never observed increased micronucleus production as a consequence of accelerated erythropoiesis and that the increase in micronucleus production must be a result of antimony trioxide genotoxicity. Such assertions on a significant mechanistic issue are difficult to accept when undocumented and unpublished observations are given greater weight than publications in the peer-reviewed literature.

As summarized the recent EU Risk Assessment Report (2008) for antimony trioxide the genotoxic potential of antimony trioxide has also been intensively examined *in vivo* and test results have been almost universally negative. For example, Kirkland et al (2007), exposed rats to high oral doses of antimony trioxide at levels close to the maximum tolerated dose for 14 – 21 days. Uptake of the antimony trioxide was monitored and toxicokinetic studies confirmed that high levels of antimony trioxide were achieved in the bone marrow of the test animals. Even under these extreme exposure conditions, complete with confirmation that high exposures were being achieved in the bone marrow of the test animals, no impact of treatment was observed upon the incidence of chromosome aberration or micronucleus production. In any weight of evidence evaluation, such meticulously conducted studies would take precedence over the marginally positive and poorly documented data presented in the NTP draft report.

Oncogene Alterations

The draft report includes an evaluation of oncogene alterations observed in the lung neoplasms of rats and mice, comparing the alterations observed in chamber controls to those in antimony trioxide treated animals. Comments here will focus upon the mouse tumors that were observed with far higher frequency and thus provide more robust indicators of the "molecular pathology" of spontaneous and induced neoplasms. Spontaneous neoplasms were found to contain altered Kras genes with mutations generally mapping to documented hot spots. Altered Kras oncogenes were also observed in 43% of the tumors observed in antimony trioxide treated animals. The report annex describes the oncogene alterations observed in some detail and postulates, not unreasonably, that the Kras altered genes observed in the tumors of antimony trioxide treated animals were spontaneous lesions present in the mouse lung and permitted to undergo clonal expansion by the impacts of antimony trioxide.

In addition to Kras alterations, 46% of lung tumors in antimony trioxide treated mice were observed to contain altered Egfr oncogenes. The high prevalence of tumors with Egfr alterations in exposed animals is interpreted as evidence of an oncogene "fingerprint" which, when combined with suggestions of genotoxicity, could be interpreted as antimony trioxide inducing a neoplastic process via a mechanism distinct from that responsible for tumors in chamber controls. The report goes on to note that mutations in Kras and EGFR are commonly observed in human non-small cell lung cancer and occur in a mutually exclusive manner. Both genes are also major components of the MAPK signaling pathway.

The draft report interprets the high prevalence of Egfr altered oncogenes as evidence of antimony trioxide inducing tumors via a probable genotoxic mechanism different from that which produces spontaneous neoplasms. While there may be a correlation between antimony trioxide exposure and lung tumors with Egfr alterations, the assumptions of causality made are potentially premature. In humans, lung cancer tumors are increased in subjects with disease syndromes (e.g. chronic obstructive pulmonary disease) that impair lung function and lead to hypoxic conditions. Signaling pathways involving EGFR appear to play a role in the growth of such tumors under hypoxic condition (Karoor et al., 2012). Egfr alterations are further linked to the ability of cancer cells to survive in hypoxic microenvironments (Murakami et al., 2014). The prevalence of Egfr alterations in antimony trioxide treated animals may thus be a result of selection for

tumors capable of undergoing rapid clonal expansion under the hypoxic conditions associated with the pulmonary toxicity produced by antimony trioxide. Taken further, just as the draft report suggests that antimony trioxide tumors with Kras alterations represent spontaneous lesions permitted to undergo clonal expansion, tumors with Egfr alterations may result from cells with spontaneous Egfr lesions selected to clonally expand by antimony trioxide induced hypoxia. If true, this proposed mechanisms of action would result in an interesting prediction for the observed limited dose response for tumor incidence. This is to say, tumors are arising from a finite number of spontaneously altered cells and are stimulated to grow under the conditions of toxicity produced by high levels of antimony trioxide exposure. In such a scenario, there would be poor dose dependence for tumor formation once threshold toxicity levels were attained. Indeed, the predicted dose response functions would be very similar to those observed for the induction of lung tumors in mice exposed to antimony trioxide.

Priority Recommendations for Report Revision

This commentary has noted a number of issues that merit consideration in a revision of the draft report on antimony trioxide. Some of the more critical issues raised are quickly summarized below:

1. Several bioassays have investigated the carcinogenic effects of antimony trioxide in rats. The present document does not adequately review and analyze this existing data base.

2. The authors judge that pulmonary overload is not a factor in the development of lung tumors following antimony trioxide treatment. However, others have concluded differently and this should be acknowledged. The present work further demonstrates that antimony trioxide effects are in large part modulated by strong inhibition of clearance mechanisms at higher exposure levels that likely attenuates at lower exposure levels to permit equilibrium states to be established for lung burden. This implies nonlinearity will be observed for neoplastic and non-neoplastic endpoints.

3. The role of genotoxicity in the induction of lung tumors remains to be determined and the limitations of the genotoxicity studies should be specifically acknowledged. The Comet assay data has little scientific value and alternate interpretations of the micronucleus data are

indicated by the published literature. The studies in the present document are largely uninformative with respect to genotoxicity.

4. Mechanistic conclusions should be deferred concerning the "fingerprint" of Egfr alterations that appear in antimony trioxide exposed animals but not in the lung tumors of chamber controls. Egfr alterations may facilitate clonal expansion of neoplastic cells in the hypoxic environment of the antimony trioxide exposed lung – their prevalence may be the result of selection for the clonal expansion of existing spontaneous lesions and not the induction of mutations in oncogenes.

5. The significance of the lymphomas that have been observed in mice must be reevaluated within the context of the higher historical incidence of lymphoma in the mouse strain used and the physiological stress imposed by the pulmonary damage induced by antimony trioxide. In the absence of more sophisticated procedures recommended for the classification and diagnosis of mouse lymphomas, lymphoma incidence does not provide clear evidence of carcinogenicity. Indeed, based on the limited data provided, the lesions observed appear to be the related to the spontaneous lymphomas that are common to many mouse strains.

6. From a mechanistic perspective, if lung cancer formation from antimony trioxide exposure in mice is due to clonal expansion of existing spontaneous oncogene altered cells under the hypoxic conditions induced in the mouse lung, can it truly be said that there is clear evidence of carcinogenicity for the lung?

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