The International Antimony Association (i2a) is pleased to provide comments concerning the draft Report on Carcinogens Monograph on Antimony Trioxide.

The i2a is a commodity association based in Brussels, Belgium that represents the collective interests of Antimony producing and importing companies worldwide. The mission of i2a is to conduct studies, and to disseminate information concerning the safe use and benefits of Antimony and Antimony compounds. This entails generating data, giving access to data, sharing and providing information on the interpretation of scientific studies, and promoting awareness of worldwide environmental, health and safety regulations that may be relevant to Antimony compounds. It is from this perspective that i2a has reviewed the draft Monograph, and specifically developed a series of comments relative to the assessment.

These comments concern both the factual content of the draft Monograph and interpretations drawn from the recently finalized NTP Technical Report on the Toxicology and Carcinogenesis Studies of Antimony Trioxide in Wistar HAN[Crl:WI9Han)] Rats and B6C3Fi/N Mice (NTP 2017).

The i2a has identified a number of factual inaccuracies in the draft Report on Carcinogens Monograph and further questions technical interpretations provided of the recent NTP (2017) studies. We wish to express significant reservations regarding:

- The summary statistics provided of current patterns of antimony trioxide consumption, use and occupational exposure.
- The absence of data defining particle size distributions in the occupational setting and comparative analysis of particle size effects in studies with experimental animals.
- Potentially erroneous extraction of data from the primary scientific literature.
- Conclusions that non-pulmonary neoplastic lesions in the NTP (2017) inhalation bioassays are specific effects induced by antimony trioxide.
- The validity of the genotoxicity studies conducted during the course of the recent NTP antimony trioxide inhalation bioassays.

Specific comments concerning the draft Monograph follow:

1. Production and consumption (p. 13 – 15): i2a lacks specific information regarding patterns of antimony trioxide use in the United States. However, we are struck by the differences between the use profiles provided in the Monograph and the production and use data compiled by agencies such as the US Geological Survey for primary antimony production and use (USGS, 2017). We request that NTP consider the values reported by USGS (2017) and attempt to reconcile them with other cited sources so as to ensure an accurate portrayal of antimony trioxide production and use in the United States.

2. Occupational exposure (p. 15 – 19): The levels of occupational exposure provided in Table 2.3 are out of date, do not reflect current industry practice and create a misleading profile of the industry. Indeed, a number of the studies cited in the table are not, contrary to the table’s title, studies of US workers. In addition, in some instances the “other exposures” are mischaracterized
or inaccurately reported – for example Smith et al. (1995) believed that stibene exposure during the battery charging process elevated urinary Sb output. Use of personal protective equipment (respirators) would be expected in many of the work settings described, complicating both the assessment of true occupational exposure and the relationship with urinary excretion. The inclusion of data from the EU Risk Assessment of Antimony Trioxide in Table 2.4 is welcomed, but it is difficult to understand how the exposure values can be said to be similar to those in Table 2.3. All of the typical, and most of the worst case, exposure estimates in Table 2.4 are below the current ACGIH TLV. Citation of this more recent data in the subsequent exposure summary on page 26 is indicated as opposed to data that may have characterized industry practice 40 – 50 years ago. It should further be noted that the inhalation exposure estimates in Table 2.4 reflect respirator usage.

We request that the Monograph rely upon recent and relevant occupational exposure data for exposure estimation and any discussion of current occupational exposure levels.

Since antimony and antimony trioxide production largely occurs outside the borders of the United States, Table 2.3 could be eliminated and replaced by Table 2.4. Table 2.4 also introduces dermal exposure data but provides little reason for why dermal exposure data has been included and how it is to be interpreted. Moreover, many of the dermal exposure estimates are modelled values as opposed to measured data. In the absence of a rationale for why dermal exposure data is relevant we suggest that such exposure data be deleted from the Monograph. If retained, only measured data should be included.

3. The EU Risk Assessment Report further contained studies of the particle size distribution of occupational aerosols associated with antimony trioxide production. Given the subsequent focus of the Monograph upon carcinogenic impacts associated with the exposure of experimental animals to respirable antimony trioxide aerosols, it is difficult to understand why these data were not mentioned in a technical characterization of occupational exposure to antimony trioxide. Workplace monitoring (Hughson, 2005) indicated that particle agglomeration occurs in antimony trioxide occupational aerosols and that the respirable fraction of inhalable aerosols can be but a fraction of the total. We request that NTP considers actual workplace-relevant particle size distribution information for its assessment. A copy of the IOM study of particle-size distributions associated with antimony trioxide production (Hughson, 2005) is included with this submission. Although not published it was reviewed during the conduct of the EU Risk Assessment of Antimony Trioxide and (much as has been done for unpublished data cited by IARC) can be considered as having undergone peer review by the EU Technical Committee on New and Existing Substances.

4. The inclusion of a suggestion that low level exposure to antimony may be a potential public health concern (page 20) is entirely inappropriate in an exposure assessment review and should be removed. Concerns about noncancer endpoints, if they are to be introduced, should be reviewed within the broader evidence base of animal studies and further consider the findings of clinical studies using high doses of intravenous antimony compounds for the treatment of parasitic diseases. We request that NTP either reviews all evidence and reports of such effects in depth, or simply deletes any reference to noncancer adverse health effects in the exposure assessment section. Given the purpose of the RoC, deletion of these statements is probably appropriate.
5. See Wu and Chen (2017) Int. J. Env. Res. Publ. Health 14:689 for a recent study of relationships between occupational exposure antimony trioxide in air and antimony levels in blood and urine. These data will be relevant to the discussion on p. 30 and 33-34.

6. The discussion of occupational epidemiology studies (p. 43 – 58) appropriately concludes that there is inadequate evidence for carcinogenic impacts of occupational exposure to antimony. The confounders noted to be present in some of the studies could be expanded to include cadmium. Although the antimony exposure of individual workers was not established in any of the studies, general levels of potential antimony exposure were estimated and could be cited. The data cited for Jones et al. (2007) are cumulative exposure data – measured values for antimony in air are cited in the paper and would be more informative. The statement that an elevated relative risk of lung cancer mortality was observed in workers exposure to antimony is misleading - modest lung cancer elevations were observed in smelter workers but could not be attributed to antimony exposure. The present phrasing of such conclusions is misleading. Figure 4.1 provides SMR/RR estimates for antimony and lung cancer and states that a positive dose response relationship was observed (p. 52 – 53) based upon the studies of Jones et al. (2007). It is unclear how these conclusions were derived. Inspection of Jones et al. (2007) raises the possibility that RR and dose response functions observed for arsenic by Jones et al. in Table 4 of the paper have been mistakenly attributed to antimony and brought forward to the Monograph. We request that NTP fact-checks the information carried forward from the primary scientific literature on this.

7. The overview of animal cancer studies focuses upon the studies that are probably the most informative. A comparative review of the different study protocols should include the particle size of the antimony trioxide preparations used – all of the studies used experimentally generated respirable aerosols but we suggest that differences in the particle size distribution between the studies should be noted.

8. The Monograph appears to dismiss the possibility that rat lung neoplasms potentially result from pulmonary overload: Prior to the conduct of the NTP bioassays, the antimony trioxide inhalation studies cited in the Monograph had suggested that antimony trioxide induced pulmonary overload in the rat lung and that this was the likely cause of lung neoplasms. NTP (2017) studies conclude that overload does not occur at an antimony trioxide exposure of 3 mg/m3 and therefore that pulmonary overload is not required for the induction of neoplasms. The rationale for this conclusion is tenuous in that 3 mg/m3 is indeed associated with impaired clearance in the rat in the NTP studies – the departure from modeled clearance rates is just not sufficient to attain the lung burden levels that meet an arbitrary criterion for overload. Moreover, significant impairment of clearance has been reported at levels much lower than those used in the NTP studies (e.g. Newton et al., 1994). Finally, the incidence of lung neoplasms in both male and female rats is not statistically elevated over that in controls at by 3 mg/m3 antimony trioxide exposures. Dismissal of overload in the Monograph as a mechanism for the induction of lung neoplasms in the rat is premature and inappropriate. We request that the Monograph acknowledge these observations and indicate that the pulmonary overload which occurred at the 10 and 30 mg/m3 exposure levels is the most likely explanation for the appearance of rat lung tumors.

9. The Monograph further neglects to mention the impacts of antimony trioxide exposure upon the overall health status of rats and mice. Exposure of rats to 3, 10 or 30 mg/m3 antimony trioxide was associated with end of study body weight suppression of 7, 8 and 20% in male rats and 10,
20 and 28% in female rats, respectively. Corresponding body weight suppression in male mice was 8, 11 and 25% and 3, 8 and 21% in female mice. Much of the data generated by the NTP bioassays reflects effects near, or in excess of, the maximum tolerated dose for antimony trioxide. This conclusion is bolstered by the observations of labored breathing, hypoxia and premature mortality due to pulmonary inflammation in exposed animals. These observations do not negate the induction of pulmonary lesions but indicate that care must be exercised in the interpretation of other systemic effects that might be associated with inhalation exposure to antimony trioxide. We request that the significant impact of antimony trioxide exposure upon the overall health status of rats and mice should be explicitly noted and defined.

10. Adrenal gland neoplasms (pheochromocytomas) are cited by the Monograph (p. 65 – 66) as supporting the RoC criteria. There is a significant body of evidence that such adrenal lesions are to be expected under conditions of pulmonary inflammation and hypoxia. As reviewed by Greim et al. (2009), the association of this adrenal lesion with pulmonary impairment is sufficiently robust that, within the context of the EU REACH process, pheochromocytomas secondary to pulmonary impairment are not considered as relevant for cancer classification or risk assessment. The adrenal lesions are most properly regarded as a response to pulmonary damage induced by antimony trioxide and not a direct substance-specific effect of antimony trioxide. Indeed, they can be interpreted as confirmation that MTDs have been exceeded in the rat. The Monograph notes the probable association of pheochromocytomas with hypoxia but still judges them to be significant lesions in support of the RoC. We request NTP to consider the probable association between pheochromocytomas and hypoxia, and revise its judgement on the (limited) significance of the adrenal lesions in the context of cancer classification or risk assessment.

11. The incidence of lymphomas is noted to be increased in female mice (p. 66 – 67) and judged to be clear evidence of carcinogenicity. This conclusion is reached without acknowledgement of the special diagnostic challenges that are posed by the high spontaneous incidence and complex etiology of lymphomas in mice, with higher prevalence of spontaneous lesions being present in female mice (Ward, 2005). As the NTP (2017) inhalation study 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/m³, but not 3 mg/m³ 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 most often early onset T-cell lymphomas while spontaneous lesions are generally 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 were applied in the NTP study. The lymphomas associated with antimony trioxide are predominantly B-cell in nature with some T-cell characteristics. On this basis, they appear to be 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 responses in the lung and spleen that promoted the development of what is already a high incidence spontaneous neoplasm in the female mouse. As such they would not provide clear evidence of carcinogenicity.

In the absence of proper diagnostic criteria, we request NTP to limit the interpretation and use of the lymphoma induction in the cancer assessment of the RoC.
12. A low incidence of skin lesions was observed in mice exposed to antimony trioxide. Benign histiocytomas occurred with low frequency in male mice. Although antimony trioxide is not generally considered to be a dermal irritant, skin irritation and dermatitis have been reported in workers exposed to high levels of antimony trioxide (EU, 2008). Given the high levels of whole body exposures, the appearance of histiocytomas is mostly likely an immunological as opposed to neoplastic response. The Monograph further notes a low incidence of fibrosarcomas in male mice and pools the incidence of both lesions to yield a dose response suggested to support listing in the RoC. Histiocytomas are not, so far as i2a is aware, precursor lesions to fibrosarcomas and there appears to be no legitimate scientific rationale to support data pooling. The presentation of pooled data for the male mouse skin lesions in table 5.8 should be deleted. The observation of two squamous cell carcinomas in antimony trioxide treated female mice is unusual but difficult to interpret in the absence of preneoplastic precursor lesions. Moreover, no other study has suggested skin as a target organ for antimony trioxide carcinogenesis. Skin lesions do not support listing in the RoC and the Monograph should be modified accordingly.

13. The mechanisms of action underlying the induction of lung tumors in rats and mice are evaluated by the Monograph with a strong focus upon genotoxicity. The mixed genotoxicity testing profile of antimony trioxide in vitro is noted, but the review of in vivo genotoxicity is unbalanced and does not adequately factor summaries of study quality into its conclusions. Kirkland et al. (2007) strongly adhered to GLP guidelines for genotoxicity testing and this study possesses the greatest technical rigor compared to the rest of the in vivo studies evaluating the clastogenic potential of antimony compounds. It is not clear which studies are the determinants for a designation of “P” for in vivo micronucleus induction in table 6.1. Indeed, assessments of study quality are generally lacking in the derivation the genotoxicity summary for all endpoints in vitro and in vivo. We further note that the addition of studies of antimony trichloride adds little to the overall summary and is probably inappropriate in an evaluation of antimony trioxide. We request that the Monograph clarify which genotoxicity studies are judged to be of highest quality how they relate to the derivation of the summary provided in Table 6 -1. Heavy reliance appears to be placed upon studies by NTP (2017) which, as noted below, we find to be of marginal quality. Finally, the nature of “P” findings for in vivo DNA damage in prokaryotes should be explained.

14. The Monograph places emphasis upon genotoxicity studies conducted during the NTP inhalation bioassays (NTP, 2017). 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 were reported for cells within mouse lung tissue. Although the Monograph attributes significance to the positive Comet assay results, the protocols employed for conduct of the Comet assay do not meet minimal quality standards for conduct of the Comet assay as established by the International Working Group on Genetic Toxicology Testing. 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 et 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. The NTP (2017) study controlled for none of these sources of artefactual false positives. The deficiencies of the studies should be noted and any inferences removed that they are suggestive of in vivo genotoxicity from antimony trioxide exposure. We suggest that the Monograph should not consider the Comet assay results as providing evidence of genotoxicity – a conclusion that would be in line with the findings of the Peer Review Panel that provided an earlier evaluation of this work.
15. Flow cytometric procedures were also applied to study the possible induction of micronuclei in the erythrocytes and white blood cells from rats and mice in the NTP (2017) bioassays. 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. For 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 perturb erythropoiesis. Indeed, the study report notes 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, in the absence of data or citation of peer-reviewed articles, that NTP studies 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. Undocumented and unpublished observations are thus given greater weight than publications in the peer-reviewed literature. We request that the Monograph should not consider the NTP genotoxicity studies in isolation and without consideration for the results of other studies performed at equivalent or higher quality.

16. The critical evaluation of the NTP (2017) genotoxicity studies described above was shared by the Peer Review Panel that evaluated the initial draft of the NTP study report. The Panel judged there was little evidence for genotoxicity provided by the Comet assay or micronucleus test results. The comments of the Peer Review Panel were noted in the NTP (2017) study report but were not incorporated into the final NTP report conclusions or noted in the Monograph. We request that Monograph’s genotoxicity assessment considers the limitations identified by i2a and at the Peer Review Panel in the overall assessment of genotoxicity.

17. The Monograph notes that activated oncogenes can be detected in a number of the lung tumors observed in antimony trioxide treated rats and mice (NTP, 2017) and suggests that the presence of activated oncogenes is evidence that antimony trioxide had mutagenic activity. The presence of activated oncogenes in tumors can be the result of a myriad of direct and indirect processes, a number of which are subsequently discussed in the Monograph. Mouse lung tumors were observed with far higher frequency and thus permit more robust analysis of the “molecular pathology” responsible for activated oncogenes in spontaneous and induced neoplasms. Spontaneous lung tumors were found to contain altered Kras genes with the activating mutations generally mapping to established “hot spots” (i.e. G to A transitions in codon 12). Altered Kras oncogenes were detected in 43% of the tumors observed in antimony trioxide treated animals. NTP (2017) notes that tumors in antimony trioxide treated animals possessed base sequence changes in hot spots similar to those observed in spontaneous tumors and suggests that the Kras
altered genes observed in the tumors of antimony trioxide treated animals were the result of spontaneous lesions permitted to undergo clonal expansion by the pulmonary toxicity of antimony trioxide. This suggestion is consistent with the observation that spontaneous activated oncogenes are now known to be present in the normal tissues of animals used in cancer bioassays (Parsons et al., 2009), exhibiting both tissue and animal strain specificity with respect to the prevalence of different activated oncogenes. **We request NTP to revise its conclusion that the prevalence of activated oncogenes alterations indicates that antimony trioxide is mutagenic.**

18. 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 in the Monograph as evidence of mutagenic oncogene alterations induced by antimony trioxide. However, the origin of Egfr alterations is potentially more complex than is described. 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 (Karooor 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. Activated Egfr may thus be spontaneous in origin or produced by a variety of indirect processes (e.g. ROS generation, error prone DNA repair) with an increased prevalence in tumors that is more indicative of the conditions that permitted clonal expansion of neoplastic lesions. The mere observation of an activated oncogene in a tumor in and of itself confers little information that permits determination of the mechanism(s) that may have produced it. **We request NTP to revise its conclusion that the prevalence of Egfr oncogenes alterations indicates that antimony trioxide is mutagenic.**

19. The integrated summary of mechanistic information (p. 95) appropriately notes multiple indirect mechanism that might contribute to lung tumor induction by antimony trioxide but lacks consistency on the issue of mutagenicity stating that “it is generally not mutagenic” and then that it “causes mutation in Egfr in lung tumors”. Clarity is needed on this issue. **We request NTP to clarify its genotoxicity assessment and eventually distinguish, where possible, between direct genotoxic effects, and indirect ones.**

20. Multiple aspects of the Preliminary Listing Recommendation are problematic:

- The summary of cancer induction includes lesions at tissue sites (adrenal, lymphoma and skin) that are likely side effects of pulmonary toxicity or the irritant properties of antimony trioxide. These lesions are not relevant to an evaluation of the carcinogenic properties of antimony trioxide or justification for RoC listing.
- Contrary to the assertions of the Monograph, there is no statistically significant increase in rat lung tumors at antimony trioxide concentrations (3 mg/m³) that do not produce overload. Rat lung tumor incidence at higher exposure levels is low, lacking in dose response and most likely the result of pulmonary overload. As such, the rat pulmonary lesions are not reflective of human risk and not supportive of RoC listing.
- NTP suggests that many of the lung tumors in mice originate from cells with spontaneous Kras oncogene activation that are permitted to undergo clonal expansion in response to the pulmonary toxicity induced by antimony trioxide.
• Mouse lung tumors with Egfr lesions most probably reflect selection for, and clonal expansion of, cells with enhanced proliferative capacity under hypoxic conditions. It is not possible to ascertain whether Egfr alterations are spontaneous or induced.
• Given the above, data from experimental animal studies do not yield compelling evidence of cancer risk at exposure levels, or via mechanisms, that are likely to be relevant to present occupational or consumer exposure scenarios. The animal cancer data thus do not provide evidence of a compelling need for RoC listing.
• Plausible mechanisms are noted for genotoxicity, particularly via indirect mechanisms, but there is little quality data that suggests these mechanisms produce genotoxic impacts in vivo.
• Epidemiological studies have failed to demonstrate elevated cancer risk that can be attributed to antimony trioxide exposure.

i2a respectfully requests that the designation of “reasonably anticipated to be a human carcinogen” be reviewed in light of the above, and a determination made of whether such a designation is indeed justified and/or would have any real-world relevance under current standards regulating human exposure.
References (restricted to articles not already cited in the Monograph)


