Health Sciences

Review of In Vivo Genotoxicity Studies Used in the Proposed CLH Mutagenicity Classification of Divanadium Pentoxide

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Limitations

The purpose of this analysis is limited to an evaluation of the basis upon which divanadium pentoxide (V_2O_5) has been proposed for harmonized classification and labelling in the European Union under Regulation (EC) No. 1272/2008 (CLP Regulation), Annex VI, Part 2. The assessment presented herein is based on review of the arguments set forth to classify V_2O_5 for germ cell mutagenicity (classification category Muta 1B; Suspected of causing genetic defects) (ANSES, 2019), the data upon which this classification proposal is based, other information available in the peer-reviewed scientific literature, and the author's scientific expertise in the area of genotoxicity/mutagenicity data. The findings presented herein are made to a reasonable degree of scientific certainty. Exponent reserves the right to supplement this report and to expand or modify the conclusions and findings based on the review of additional materials as they become available through additional work or the review of additional work performed by others. The scope of services performed during this investigation may not adequately address the needs of other users of this report, and any re-use of this report or its findings, conclusions or recommendations as presented herein are at the sole risk of the user.

Executive Summary

In a proposal for harmonized classification and labelling (CLH Report) for divanadium pentoxide (V_2O_5), ANSES on behalf of French Member State Competent Authority (MSCA) concluded that germ cell mutagenicity classification category Muta 1B (Suspected of causing genetic defects) was warranted (ANSES, 2019). Herein, the available *in vivo* studies upon which the classification is proposed were reviewed. As a result of this analysis, the following conclusions were drawn.

- In the most stringent and relevant studies in which the genotoxicity of V_2O_5 was investigated using high, tumorigenic concentrations, no evidence for either mutagenicity or chromosomal damage or aneugenicity was shown
- A number of other studies that claimed positive findings for *in vivo* genotoxicity suffer from methodological issues.

Given the preponderance of weight available from those studies that either followed a validated protocol or were conducted according to the state-of-the-art methodology, it is the opinion of this reviewer that V_2O_5 is not likely to be an *in vivo* genotoxicant.

It is thus concluded that the Muta 1B classification is neither warranted nor justified for V_2O_5.

Introduction

In a proposal for harmonized classification and labelling (CLH Report) for divanadium pentoxide (V_2O_5), ANSES on behalf of French Member State Competent Authority (MSCA) concluded that germ cell mutagenicity classification category Muta 1B (Suspected of causing genetic defects) was warranted (ANSES, 2019). This conclusion was driven, for the most part, by positive findings in a number of *in vivo* studies, some of which were conducted using validated protocols and others with irrelevant routes of administration and/or use of non-validated test systems. The purpose of this document is to critically evaluate the available *in vivo* studies to determine whether the proposed Muta1B classification is indeed justified.

On the following pages, the *in vivo* studies identified in the CLH document were re-examined in order to arrive at a weight of evidence conclusion regarding the *in vivo* genotoxicity of V_2O_5 in somatic and germ cells of experimental systems. For the purposes of completing this analysis, I relied primarily on information available from the CLH report (ANSES, 2019), the in vivo mutagenicity/genotoxicity studies cited therein, the peer-reviewed scientific literature, and my scientific expertise (~40 years) in the area of genotoxicity/mutagenicity.

Review of *In Vivo* Studies

Manjanatha et al. (2015) – cII Mutation Analysis in Transgenic Big Blue Mice

The CLH report on V_2O_5 accurately states that this substance is not mutagenic in a transgenic rodent somatic cell gene mutation assay by inhalation (Manjanatha et al., 2015). This study, which examined the induction of mutations at the cII locus of the transgenic Big Blue mouse, meets or exceeds the OECD 488 guideline requirements. This gene mutation study was erroneously rated in the CLH report as not reliable (RL=3), without further explanation of how this rating was derived. In the expert judgment of this reviewer, Manjanatha et al. (2015) is a high-quality study for which the methods and results have been clearly described in a peer-reviewed publication. As such, the study should be considered reliable without restriction (RL=1).

This study is unique in that it examined the mutagenicity of V_2O_5 in a tissue (lung) that is considered the target for the induction of tumours based on results of the NTP inhalation carcinogenicity bioassay (NTP, 2002): male mouse lung tumours. In the mutagenicity and carcinogenicity studies, the same strain of the mouse was used, viz., $B_6C_3F_1$ mouse. The transgenic Big Blue mice were exposed, nose-only, to particulate aerosols containing 0, 0.1, or 1 mg/m^3 for 4 or 8 weeks (6 h/day, 5 days/week). The 1 mg/m^3 exposure level represented the concentration at which lung tumours were increased in the NTP bioassay. The primary purpose of this investigation was to examine whether V_2O_5 is mutagenic *in vivo*, and if so, whether the V_2O_5-induced mutagenicity is likely an initial key event in the aetiology of lung tumorigenicity by this substance. One of the key impetuses for the study was the reported small (2-fold) increase in oxidative DNA damage (8-oxodGuo) in the lung tissue of mice exposed to carcinogenic levels of V_2O_5 (Schuler et al., 2011). The Big Blue mouse study examined whether the small increase in the oxidative DNA damage translated into mutagenicity in a test system capable of detecting such events.

In the study by Manjanatha et al. (2015), the exposure of lung tissues to V_2O_5 was well-controlled and confirmed by measuring the vanadium burden in the tissue at the time of necropsy;

concurrently-exposed wild-type mice were used for this analysis by inductively-coupled plasma mass spectroscopy (ICP/MS). The results showed a dose-proportional increase in the concentration of vanadium in the lungs at both the 4-week and 8-week necropsy time points. Consistent with this finding is the significant increase in lung weights of mice exposed to 1 mg/m^3 V_2O_5 at both necropsy time points. Despite this demonstrated exposure, there was neither an increase in the frequency of mutations, nor a change in the mutational spectra in this tumour target tissue. These results clearly support the conclusion that V_2O_5 is not a somatic cell mutagen. nor is mutagenicity an initial key event in the inhalation tumorigenicity associated with V_2O_5. Based on these results, it can be concluded that the reported small increase in the oxidative DNA damage reported by Schuler et al. (2011) was simply an "indicator" effect, with no consequential adverse impact on the integrity of the mouse lung cell genome.

Banda et al. (2015) – K*ras* Mutations in Mouse Lungs Exposed to V_2O_5

Lung tissues from the study by Manjanatha et al. (2015) were also interrogated for the induction of mutations at codon 12 of endogenous K*ras* gene. In this study, two different K*ras* mutations were quantified, i.e., GGT to GAT and GGT to GTT, using the sensitive technique allele-specific competitive blocker PCR (ACB-PCR). These two mutant codons accounted for nearly 65% of the K*ras* mutations observed among the lung tumours induced in mice by V_2O_5 inhalation exposure (NTP, 2002). Accordingly, the possibility existed that induction of these mutations in the endogenous K*ras* gene might represent the early key event(s) in the development of lung tumours in V_2O_5-exposed mice. As part of this investigation, three hypotheses were examined: a) a significant increase in GGT to GTT mutations would be consistent with V_2O_5 causing oxidative DNA damage, b) a concomitant increase in both mutations (i.e., GGT to GAT and GGT to GTT) is consistent with amplification of pre-existing K*ras* mutations, and c)the increase in K*ras* mutations is not an early key event, but instead, a late event in the aetiology of V_2O_5-induced lung tumorigenesis. Results of these analyses indicated that exposure of mice for up to 8 weeks did not significantly increase either of the K*ras* mutant fractions. This finding provides strong support to the third hypothesis that an increase in K*ras* mutations is not an early event in the induction of lung tumours in V_2O_5-exposed mice.

Thus, results from the Banda et al. (2015) study are considered to be of high quality and inform once again that V_2O_5 is not a somatic cell mutagen and that mutagenicity is not likely an early key event in the tumorigenic mode of action for this compound. It should be further noted that the CLH report makes an erroneous statement in Table 16 that positive controls were not used in this study. A closer examination of the publication by Banda et al. (2015) indicates that duplicate mutation frequency standards were, in fact, analysed by ACB-PCR in parallel with the mouse lung DNA samples.

Schuler et al. (2011) – Oxidative DNA Damage and DNA Strand Breaks in Mouse Lungs

As part of a comprehensive study to gain insight into the possible mode of action responsible for the lung tumours observed in V_2O_5-exposed mice, Schuler et al. (2011) investigated DNA oxidative stress by measuring nine specific DNA-oxo-adducts in the lungs of mice inhalation exposed, nose-only, to V_2O_5 for up to 16 days at concentrations identified in the NTP bioassay (NTP, 2002) as tumorigenic (1 and 4 mg/m^3). Using an alkaline comet assay, they also examined DNA strand breaks in lung tissue cell suspensions and in bronchioalveolar lavage (BAL) cells. To confirm exposure of the portal of entry tissue as well as systemic exposure, the authors measured vanadium concentrations in the lungs and blood, respectively. In addition, cell proliferation analysis of the lung tissue using Ki-67 and PCNA immunohistochemical staining indicated concentration- and time-dependent increases in mitotic activity, further confirming adequate delivery of the test compound to the target tissue.

Of the nine specific DNA-oxo-adducts analysed, only two (8-oxodGuo and dCyd341) were found at sufficient levels for quantitation with the technique used. Of these, only the levels of 8-oxodGuo were slightly, but significantly, higher (approximately 2-fold) at the tumorigenic exposure levels. As discussed in connection with the study reported by Manjanatha et al. (2015), the biological significance of a small increase in this oxidative adduct is questionable since there was no increase in mutagenicity in the lung tissue of mice exposed to the tumorigenic doses of V_2O_5.

Furthermore, in the study by Schuler et al. (2011), there was also no increase in DNA strand breakage in either the lung cells or BAL cells. The absence of DNA strand breaks supports the

conclusions that V_2O_5 had no genotoxic effect in the tumour target tissue and that genotoxicity is not likely to be an initial key event in the aetiology of the mouse lung tumours. In light of the recent analysis by the International Workshops on Genotoxicity Testing (IWGT) on the most appropriate *in vivo* test to conduct as a follow-up to positive findings in the *in vitro* genetic toxicology assays (Kirkland et al., 2019), the negative findings for the induction of DNA strand breaks in V_2O_5-exposed mouse lungs are very significant. The IGWT examined the performance of two *in vivo* assays for which OECD test guidance are available and that can be conducted in any tissue of interest (i.e., transgenic rodent mutation assays and the comet assay). The group concluded that both assays are equally sensitive to detect *in vivo* genotoxicants, although intuitively, the comet assay is expected to be responsive to a wider variety of mechanisms, including clastogenicity. With V_2O_5, the concordance of negative results in the transgenic and comet assays in a tissue receiving maximum exposure is remarkable and reinforces the conclusion that this substance is not an *in vivo* genotoxicant.

Black et al. (2015) – Gene Expression Profiling of Mouse Lungs Exposed to V_2O_5.

Black et al. (2015) is one of the most significant investigations to shed light on the potential *in vivo* genotoxicity of V_2O_5. This study investigated, among other things, whether there are any gene expression signatures indicative of genotoxicity in female $B_6C_3F_1$ mouse lungs following 13 weeks of inhalation exposure to V_2O_5 particulate aerosols at a tumorigenic concentration of 2 mg/m^3. The authors did not see evidence for enrichment of DNA damage pathways in the tissue, including those associated with oxidative stress, cell cycle disruption, or apoptosis signalling. Further, the observed gene expression pattern differed from that associated with a number of genotoxic carcinogens such as arsenic, formaldehyde, naphthalene and chloroprene. Thus, this study clearly indicated that V_2O_5 likely is not an *in vivo* genotoxicant and that genotoxicity is not an initial key event in the aetiology of the mouse lung tumours.

NTP (2002) – Analysis of Alveolar/Bronchiolar Carcinomas for Mutations in Exons 1 and 2 of K*ras* Gene

In their summary of the *in vivo* testing results, the CLH report noted the presence of K*ras* mutations in the alveolar/bronchiolar carcinomas of mice exposed to V_2O_5. The relevance of these results to the germ cell classification are questionable. First, the mutations were analysed in a tumour tissue (lung) after long-term exposure of V_2O_5. Tumour tissues are known to accumulate a number of genetic defects, including genomic instability, during the promotion and progression stages of tumorigenesis (Vogelstein, 2013), and as such, these events have no relevance to the effects induced by V_2O_5 during the early stages of exposure. Consistent with this notion is the absence of K*ras* mutations in the lung tissues of mice following 4 or 8 weeks of exposure to V_2O_5 (Banda et al., 2015). Multiple other lines of additional evidence also point to the absence of mutagenicity in the lung tissue following inhalation exposure to V_2O_5 (Manjanatha et al., 2015; Schuler et al., 2011), as already discussed in detail above. Thus, it is likely that the K*ras* mutations seen in the tumour tissue are late events during tumorigenesis, driven by inflammation-induced, or other mechanisms of, cell proliferation.

Several Studies Dealing with Clastogenicity and Aneugenicity using the Bone Marrow Micronucleus test

There are two well-conducted erythrocyte micronucleus tests with Klimisch reliability rating of 1 or 2. The first is a GLP-compliant study conducted in accordance with OECD Test Guideline 474 (OECD, 2016) in which micronucleus formation in male Sprague-Dawley rats was evaluated following a single oral gavage dose of V_2O_5 up to the maximum tolerated dose of 120 mg/kg body weight (Beevers, 2011 as reported in ECHA dissemination, 2017). No increases in micronucleated bone marrow polychromatic erythrocytes were observed at either 24 or 48 h after treatment. Bioanalytical evaluation revealed that vanadium levels increased in the bone marrow in a dose-dependent manner, confirming exposure of the target tissue to the test compound. In the second study(NTP, 2002), the ability of V_2O_5 to induce micronuclei in erythrocytes of male and female $B_6C_3F_1$ mice was investigated following 3 months of inhalation exposure to up to 16 mg/m^3 (NTP, 2002). The results of this investigation were again clearly negative; i.e., no increases in micronucleated peripheral blood erythrocytes were observed at any exposure level. Exposure of

bone marrow to vanadium was assured in this study based on the dose-dependent increase in blood vanadium concentrations in female mice when assessed at various time points as part of the two-year cancer bioassay.

Against this backdrop of the clearly negative results, Rojas-Lemus et al. (2014) reported a positive effect in a peripheral blood reticulocyte micronucleus test in male, but not in female, mice exposed to a single concentration of 1.4 mg/m^3 V_2O_5. The authors tried to explain the sex differences based on the protective effects of oestrogens in females. Nevertheless, it is hard to rationalize the lack of a similar effect in the NTP study at a nearly 10-fold higher concentration. Another curious finding in the Rojas-Lemus et al. (2014) study was the steady monotonic increase with time (up to 4 weeks) in the proportion of micronucleated peripheral blood reticulocytes. Since reticulocytes mature into erythrocytes with a half-life of approximately 24 h, it is not expected that micronucleated reticulocytes would accumulate in the peripheral blood with time. Even the possibility of a time-dependent accumulation of vanadium (increased body burden) does not seem to be a plausible explanation since no such accumulation was reported in the NTP (2002) study.

Taken together, data from reliable erythrocyte micronucleus tests inform that V_2O_5 is not an *in vivo* clastogen or aneugen. These data, along with the previously discussed negative findings for *in vivo* mutagenicity (Banda et al., 2015; Manjanatha et al., 2015) and DNA strand breakage (Schuler et al., 2011) in tumour target tissues, provides a persuasive argument that V_2O_5 is not an *in vivo* genotoxicant at either the gene or chromosome level or at the level of the whole genome.

The CLP report also cites a number of other studies dealing with micronucleus induction in rodent erythrocytes. Several of these studies have limitations and render them unsuitable for inclusion in drawing weight- of- evidence conclusions. For example, no details on either the study's methodology, nor the actual data were available from the study of Sun et al. (1987, as cited in WHO 1988). This study was cited from a secondary source, and as such, this citation and its accompanying positive and negative calls should not be used for drawing any conclusions vis-à-vis CLP classification. Second, the current OECD test guidelines for genetic toxicology testing clearly state that the intraperitoneal route of administration is not a physiologically relevant route for testing (OECD, 2016). Given this authoritative guidance, the most logical approach would be to exclude data from such studies for regulatory purposes, including in the CLH classification.

Such an approach is akin to excluding studies in which the test material is directly injected into a tissue to investigate its genotoxicity – a methodology sometimes practiced during the early days of genetic toxicology testing, but no longer considered appropriate. Accordingly, the study reported by Garcia-Rodriguez et al. (2016) should be also excluded from consideration.

Additional Studies Examining DNA Damage

The CLH report lists a number of studies that examined DNA damage either by the comet assay or by other non- or poorly-validated methods. Altamirano-Lozano et al. (1999) investigated DNA damage in a number of tissues from male mice using the comet assay following intraperitoneal injection of up to 23 mg V_2O_5/kg body weight. Altamirano-Lozano et al. (1996) also examined DNA damage in testicular cells of male mice following intraperitoneal administration of V_2O_5 (up to 23 mg/kg body weight). As discussed previously, data from intraperitoneal administration studies should not be used for regulatory decision-making because intraperitoneal injection is not a physiologically relevant route (OECD, 2016). For V_2O_5, intraperitoneal injection is a further issue as V_2O_5 will dissociate in aqueous solution and the valence state of the vanadium will depend on solution pH (Assem and Levy, 2009). When arriving at a decision on the genotoxicity of a substance, this consideration is especially important when there are studies of higher quality and relevance already available, which is the case for V_2O_5. In addition, the comet assay is recognized as not being adequately validated in terms of methodology and reproducibility for routine use in assessing genotoxic hazard or risk in germ cells (Speit et al., 2009).

Paramanik and Rajalakshmi (2013) examined differences in the migration on an agarose gel of DNA extracted from various tissues following oral gavage administration of Wistar rats with 70 mg/kg V_2O_5. The methodology, however, is neither standard nor been validated for measuring DNA damage. As such, results from this study should not be given any weight in the overall assessment of the *in vivo* genotoxicity of V_2O_5.

Other Miscellaneous *In Vivo* Studies in the CLH Report

The CLH report lists a number of other endpoints in Table 6 under the header "Summary table of mutagenicity/genotoxicity tests in mammalian somatic or germ cells in vivo". Most, if not all, of

these studies are neither validated to assess genotoxicity, nor relevant because of the methodology used. The sister chromatid exchange (SCE) in the bone marrow of mice by Altamirano-Lozano et al. (1993) is irrelevant not only because of the intraperitoneal route of administration, but also because SCEs are no longer considered as *bona fide* endpoints to assess genotoxicity, as reflected in the deletion by the OECD of the guideline.[1]

The immunohistochemical methodology used by Rodriguez-Lara et al. (2016) to assess genotoxicity in testicular cells of mice exposed by inhalation to V_2O_5 is not a standard method; furthermore, its specificity and sensitivity have not been properly validated. Similarly, the chromatin and nuclear changes observed by Rodriguez-Lara et al. (2013) through transmission electron microscopy of the spleen cells from male mice following inhalation exposure have never been validated as representing *bona fide* genotoxicity outcomes. As such, these findings should not be used as part of the weight of evidence to assess *in vivo* genotoxicity of V_2O_5 – particularly when data for other validated endpoints already exist, as is the case with V_2O_5 as discussed previously. The report by Cano-Gutierrez et al. (2012), in which oxidative stress damage (inflammation and lipid peroxidation) was measured in the livers of mice exposed by inhalation to 1.4 mg/m^3 of V_2O_5, should be similarly excluded for lack of validation. In addition, the purported findings of oxidative damage in the liver in this study is at odds with the lack of any liver histopathology in the NTP carcinogenicity bioassays conducted in mice and rats.

The DNA synthesis inhibition assay (Sun, 1987, as cited in WHO 1988) is not considered to be a test for genotoxicity. Additionally, very little primary information on the methodology and results are available for this study, which is cited from the secondary source.

Finally, the dominant lethal test reported by Altamirano-Lazano et al. (1996) suffers from the methodological deficiency of using an irrelevant route of administration (i.e., intraperitoneal), which makes the study unsuitable for using in a weight of evidence decision-making for regulatory purposes. Similarly, a lack of methodological details and access to primary data renders

[1] [1] OECD Test 479 Genetic Toxicology: In vitro Sister Chromatid Exchange Assay in Mammalian Cells was deleted on 2 April 2014 following an OECD Council decision. www.oecd-library.org.

the negative results reported by Sun (1987, as cited from WHO 1988) in a dominant lethal test in male mice exposed subcutaneously to V_2O_5 unsuitable in a weight of evidence evaluation.

Weight of Evidence Narrative on the *In Vivo* Genotoxicity of V_2O_5

In reviewing the available *in vivo* genotoxicity studies on V_2O_5, a clear picture emerged. In the most stringent and relevant studies in which the genotoxicity of V_2O_5 was investigated using high, tumorigenic concentrations, no evidence for either mutagenicity or chromosomal damage or aneugenicity was shown. These studies include Manjanatha et al. (2015), Banda et al. (2015), Black et al. (2015), Schuler et al. (2011) and NTP (2002). A number of other studies claimed positive findings for *in vivo* genotoxicity. In general, several of these latter studies suffer from methodological issues. The important question to ask in this context is whether to give any weight to these deficient studies, especially when much higher quality studies are already available from which to make an independent assessment. In the opinion of this reviewer, given the preponderance of weight available from those studies that either followed a validated protocol or were conducted according to the state-of-the-art methodology, it is reasonable to conclude that V_2O_5 is not likely to be an *in vivo* genotoxicant. Consequently, the Muta 1B classification is neither warranted nor justified. In this case, the most appropriate, worst-case scenario, classification for V_2O_5 is Category 2.

This review's conclusion that V_2O_5 is not likely an in vivo genotoxicant strengthens the argument that this substance is not a genotoxic carcinogen and DNA reactivity can be excluded in the mode of action for mouse lung tumours. This conclusion was based on the absence of genotoxicity in the tumour target tissue (i.e., lung) using multiple, well-established endpoints.

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Appendix

Bhaskar Gollapudi, Ph.D. – Short Biographic Sketch

B. Bhaskar Gollapudi, Ph.D.

Dr. Bhaskar Gollapudi is a Senior Managing Scientist in Exponent's Health Sciences Center located in [redacted]. Dr. Gollapudi specializes in Genetic and Molecular Toxicology/Chemical Carcinogenesis with emphasis on the identification of mode of action and human relevance of toxicity findings. He has almost 40 years of research and issue management experience at a multinational (agro)chemical company dealing with the safety of a diverse portfolio of substances. He has published more than 100 papers in peer-reviewed journals. Dr. Gollapudi is the Editor-in-Chief of Environmental and Molecular Mutagenesis, the official journal of the US Environmental Mutagenesis and Genomics Society. He serves on a number of scientific committees, including the Committee on Toxicology of the U.S. National Academies of Sciences (NAS) and the International Workshops on Genotoxicity Testing (IWGT), an organization involved in developing consensus on genotoxicity testing strategies and data interpretation. Dr. Gollapudi is the recipient of the 2014 Arnold J. Lehman Award from the Society of Toxicology in recognition of his contributions to the field of risk assessment and chemical regulation.

Dr. Gollapudi's Recent (2018-2019) Publications

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