Genotoxicity - Statement on the safety of Titanium Dioxide (E171) as a Food Additive

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188. The COM reviewed a number of studies to assess the genotoxicity of TiO_2. In addition to papers reviewed by EFSA, a literature search was conducted to find papers published on “genotoxicity” and “titanium dioxide”. Most papers identified used the nano-sized fraction of TiO_2 and not the micro-sized form, nor the specific E171 form.

189. All papers were screened against a series of criteria to assess the characteristics of the material used in the study and the generic study design (tier 1); and the generic experimental details of the genotoxicity study including adherence to Organisation for Economic Co-operation and Development (OECD) test guidelines (tier 2). These criteria were assessed by several members of the Committee through an iterative process. Finally, the experimental details of the study were thoroughly evaluated using expert judgement (tier 3). A number of exclusion criteria were used in the assessment of these studies. Further details of the scoring methodology are described in the COM statements (COM 2024a, b). A RAG rating was used to rank the studies. Papers which achieved a red RAG rating were not assessed any further.

In vitro genotoxicity assessment

190. Overall, for the in vitro assessment, from a total of 191 papers that were initially assessed, 15 papers detailing 16 assays, were categorised as green or amber and were considered to be relevant and of sufficient quality for use in the in vitro genotoxicity assessment of TiO2.

191. Six assays were rated as ‘green’ for the in vitro assessment. These included: 4 assays for the mincronucleus (MN); 1 assay of the mammalian cell hypoxanthine phosphorybosyl transferase (*hprt)*; and 1 assay for chromosome aberration (CA).

192. Ten assays were rated amber and of those: 9 were MN assays; and 1 was an *hprt* assay.

193. All of the assays assessed are described in detail in the ‘Assessment of in vitro studies of TiO_2 genotoxicity’ (COM, 2024a).

In vivo genotoxicity assessment

194. The in vivo assessment initially assessed 53 papers, six papers (detailing 12 assays) were categorised as green or amber and were considered to be relevant and of sufficient quality for use in the *in vivo* genotoxicity assessment of TiO_2.

195. Two assays were rated as ‘green’ for the in vivo assessment. These papers were both for the in vivo MN assay.

196. Ten assays were rated amber and of those: 3 were for the in vivo MN; 1 was for CA; 1 was for the in vivo comet assay; 3 were *Pig-a* mutation studies; 1 was a gpt mutation assay; and 1 was a γ-H2AX assay

197. All of the assays assessed are described in detail in the 'Assessment of in vivo studies of TiO2 genotoxicity' (COM, 2024b).

EFSA review and conclusions on the genotoxicity of TiO_2

198. In EFSA's review of the genotoxicity of TiO_2 in 2016, "The (ANS) Panel concluded that, based on the available genotoxicity database and the Panel's evaluation of the data on absorption, distribution and excretion of micro- and nanosized TiO_2 particles, orally ingested TiO_2 particles (micro- and nanosized) are unlikely to represent a genotoxic hazard in vivo" In their assessment in 2021, EFSA focused particularly on the genotoxicity of TiO_2 nanoparticles, and the vast majority of studies were on this form of the substance.

199. Gene mutation: EFSA considered that some in vitro studies demonstrated that TiO_2 NPs can induce gene mutations in cultured mammalian cells although others did not. Six in vivo studies were considered relevant, one of which indicated the induction of large DNA deletions (also assessed in 2016), however the remaining five studies, that could identify point mutations and small deletions, gave consistently negative results. EFSA concluded that the available experimental data do not confirm the potential of TiO_2 NPs (< 30 nm) to induce gene mutations in vivo.

200. Induction of MN/CA: EFSA noted that the majority of in vitro MN or CA tests gave negative results, independent of particle size. 60% of the tests used NPs as the test material. The majority of in vivo MN and CA tests in vivo were considered positive. All of these studies were with partciles < 60 nm. There were very few studies on larger particles. Taking into account the available

evidence, EFSA considered that - on balance - TiO_2 NPs have the potential to induce MN/ CA. They noted “that a significant portion of the studies was performed using TiO_2 NPs < 30 nm, however some positive results were observed with TiO_2 particles > 30 nm and no clear dependence of the particle size on positive effects in MN/CA assay was observed” (EFSA, 2021).

201. In vitro and in vivo Comet assay: EFSA concluded that based on the results of the in vitro and in vivo comet assays, TiO_2 particles have the potential to induce DNA damage. As noted for MN and CA effects, “a significant portion of the studies were performed using TiO_2 NPs < 30 nm, however some positive results were also observed with TiO_2 particles > 30 nm and no clear dependence of the particle size on positive effects in Comet assay was observed” (EFSA, 2021).

202. DNA Binding: EFSA also concluded that there is evidence, from both in vitro and in vivo studies, for interaction(s) of TiO_2 NPs with DNA. However, due to the techniques employed, it was not possible to determine whether these interactions involved covalent or non-covalent binding.

203. Overall, the FAF Panel concluded that “a concern for genotoxicity of TiO_2 particles that may be present in E 171 cannot be ruled out. A cut-off value for TiO_2 particle size with respect to genotoxicity could not be identified.”

Health Canada review and conclusions on the genotoxicity of TiO_2

204. Health Canada’s Food Directorate noted that, based on the currently available studies including the EOGRT study (Leuschner, 2020), food grade TiO_2 is not genotoxic in-vivo. However, they recognise that the studies available on this

endpoint are limited and additional OECD guideline-compliant studies are recommended to confirm this. They conclude that any adverse effects found related to oral exposure were based on findings from non-standard studies in which the form of TiO_2 administered was homogenized suspensions of particles with ultrasonic dispersion and that these do not fully represent exposure to food grade TiO_2.

FSANZ review and conclusions on the genotoxicity of TiO_2

205. No in-vivo genotoxicity studies in which food-grade TiO_2 with dietary administration was used could be identified. FSANZ identified four in-vivo genotoxicity studies, two studies using food-grade TiO_2 administered by oral gavage (Bettini et al., 2017 and Jensen et al., 2019) and two studies using intraperitoneal administration of Unitane (0-220) (Shelby et al., 1993 and Shelby and Witt, 1995). These latter two studies assessing genotoxicity by the NCI/NTP were identified by the US National Toxicology Program and were assumed to use Unitane 0-220, as the test item was not described (Shelby et al., 1993 and Shelby and Witt, 1995)

206. FSANZ noted that DNA damage was not observed either in the two comet assays (food-grade TiO_2, oral gavage) or the micronucleus and chromosomal aberration studies (Unitane 0-220, intraperitoneal injection). In vitro studies (GLP- and OECD test guideline-compliant, food-grade TiO_2) found no evidence of gene mutation in mammalian cells nor clastogenicity or aneugenicity or cellular uptake (micronucleus assay using human peripheral blood lymphocytes). There was some evidence of uptake and internalisation by A549 cells however no particles were detected in the nucleus. FSANZ noted that “the absence of confirmed cellular uptake in the in vitro genotoxicity studies may limit confidence in the negative results, although alternatively the absence of direct exposure of the

nucleus to food-grade TiO_2 in these studies may indicate a low intrinsic hazard from a direct genotoxicity perspective (OECD, 2014)".

JECFA 2024 review and conclusions on the genotoxicity of TiO_2

207. JECFA noted that overall, the available data did not provide convincing evidence of genotoxicity for food grade TiO_2 E171 (INS 171). However, the Committee recognised the limitations of the current methodology with respect to the testing of poorly soluble particulate materials.

COM review and conclusions on the genotoxicity of TiO_2

208. The COM have provided conclusions on the in vitro and the in vivo studies separately and these are presented below.

COM Opinion of the in vitro genotoxicity studies reviewed

209. "After reviewing the in vitro genotoxicity studies performed to date on TiO_2, we note the following points:

i. There were four in vitro studies of the highest quality (labelled "green" here) that used TiO_2 nanoparticles of different sizes and forms in the micronucleus assay. Only one study tested micro-sized (anatase)TiO_2 that was more representative of E171 (Demir et al. 2015) which was negative in the micronucleus assay. All four "green" studies that used anatase TiO_2

nanoparticles reported negative results for the MN endpoint. Of the two green studies that used rutile TiO_2 nanoparticles, one was negative and the other was weakly positive for MN induction in a non-standard cell line but only at the two lowest doses used (1 and 5 mg/ml) (Di Bucchianico et al 2017). Two green studies used TiO_2 nanoparticles of mixed anatase/rutile form and both were negative for MN induction.

ii. There were two green studies that both used anatase/rutile TiO_2 nanoparticles in either the hprt gene mutation assay or CA assay. The TiO_2 nanoparticles were negative in the hprt assay. In the CA assay, the TiO_2 nanoparticles were positive, but the CA frequency decreased with increasing TiO_2 concentration, and despite the significant induction of CA, this study was negative with the micronucleus assay.

iii. There were eight amber studies (i.e., ones that contained some suboptimal aspects) that used TiO_2 nanoparticles of different sizes and forms in the micronucleus assay. Four studies used anatase TiO_2 nanoparticles and three of these were negative for micronuclei induction. The one positive study reported a dose-dependent increase in micronuclei induction in lymphocytes from healthy individuals. All three studies that used nanoparticles of mixed anatase/rutile TiO_2 were negative for micronuclei induction. Two studies that used anatase/brookite TiO_2 nanoparticles reported positive results for micronuclei induction.

iv. The one amber study on hprt mutations was positive at low anatase TiO_2 nanoparticle doses but not at higher doses (Vital et al. 2022).

v. Some “green” studies included other assays (e.g. Comet assay) to provide mechanistic information but results were inconsistent, showing either no increase (Demir et al., 2015), or an increase in oxidative DNA damage (Di Bucchianico et al., 2017) but only at the highest dose (Unal et al., 2021). Andreoli et al., 2018 and Stoccoro et al., 2017 showed ROS involvement.

210. Overall, the COM opinion is that there is little evidence that TiO_2 micro-sized or nanoparticles are genotoxic in vitro based on data from well conducted studies. The limited number of positive studies all report no dose-response effects, with significant effects being observed at the lowest doses used, although it is acknowledged this may be due to differences in dispersion and agglomeration at low and high doses. There is also a lack of replication of study outcomes using the same nanoparticle in different labs.

211. Currently a definitive assessment of the safety of food grade E171 is difficult when there are no high-quality OECD-compliant studies that adequately incorporate the study design considerations and characterisation of the nanoparticulate fraction present in E171. With the exception of one study, the studies identified in this report are not representative of E171, where the fraction of nanoparticulate is <50% and according to the recent "Guidance on the implementation of the Commission Recommendation 2022/C 229/01 on the definition of nanomaterial" (https://data.europa.eu/doi/10.2760/143118), E171 would not fall under the definition of a NM, hence we need GLP studies with E171 that also include robust physicochemical characterisation and nano-specific adaptations to the TG protocol to definitively assess the hazard. Nanoparticles of TiO_2 are considered worst-case scenario for E171, as E171 is anticipated to be less reactive.

212. We also note that there is a dearth of high-quality datasets available with well documented nanomaterial characteristics where the relevant OECD test guidelines (using suitably adapted protocol designs for the testing of nanomaterials) have been followed." (COM, 2024a. Not yet published)

COM Opinion of the in vivo genotoxicity studies reviewed

213. “After reviewing the *in vivo* genotoxicity studies performed to date (up to 2023) on TiO_2, we note the following points:

i. The highest quality *in vivo* studies labelled here as “green” (n=2), both show negative results for the micronucleus endpoint (Donner et al., 2016; Sadiq et al., 2012). There were no “green” studies for other endpoints.

ii. Only Donner et al., (2016) used pigment grade TiO_2 (including micro-sized anatase that was most similar to E171) and therefore was most relevant to the concern for human health in this case. This study showed no micronucleus induction.

iii. The Donner et al., (2016) paper also used a physiologically relevant oral route, which is most appropriate for the assessment of dietary exposure of food grade TiO_2. The authors acknowledge that absorption from the GI tract is low, meaning poor bone marrow exposure. This is important for risk assessment purposes where the oral bioavailability of E171 in humans is very low (≤0.0013% - refer to COT opinion/)

iv. The Sadiq et al., (2012) study, that used an i.v. route (a route that is most likely to achieve bone marrow exposure), also showed a negative micronucleus response and confirmed bone marrow exposure to titanium.

v. The studies labelled as “amber” (i.e., contained some suboptimal aspects) showed a mixture of positive (4/9) and negative (5/9) results for the genotoxicity endpoints studied.

vi. The positive studies included chromosomal and DNA damage endpoints and were all associated with cytotoxicity and/or indirect mechanisms of genotoxicity, such as oxidative damage and inflammation. There was no evidence of gene mutations, however no definitive conclusion can be made

due to the deficiencies in the study designs and limited number of available studies.

vii. The route of administration of nano-sized TiO_2 in these “amber” studies was often not via the most relevant oral route (only 2/9 studies) when considering the use of E171 as a food grade material. The less relevant endotracheal route was employed in 3/9 studies and the i.v. route and i.p. route were employed in 3/9 and 1/9 studies, respectively. Often the dosing regimens employed in these studies were suboptimal and did not follow the recommendations of the OECD test guidelines, which also makes interpretation difficult.

viii. All these “amber” studies used a nano-sized TiO_2 material which is less relevant to the E171 material.

214. Overall, we conclude that there is little evidence in the literature to suggest that there is a health concern related to genotoxicity induction by TiO_2, particularly via the oral route and especially the micro sized TiO_2 fraction (most studies used the nano-sized material).

215. Currently a definitive assessment of the safety of food grade E171 is difficult when there are no high-quality OECD-compliant studies that adequately incorporate the study design considerations and characterisation of the nanoparticulate fraction present in E171. We also note that there is a dearth of high-quality data sets that are OECD compliant, and this has led to a lot of conflicting data and uncertainty in the risk assessment for TiO_2.” (COM, 2024b. Not yet published).

COT review and conclusions

216. The COT agree with the COM conclusions. While there is a lack of information specifically on the genotoxicity of food grade E171, there is sufficient information on the different particle types present to reach an overall conclusion.