

March 3, 2010

Via E-Mail

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Cancer Research  
615 Chestnut Street  
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Re: “Titanium Dioxide Nanoparticles Induce DNA Damage and Genetic Instability *In vivo* in Mice”, B. Trouiller, *et al.*, *Cancer Research*, 2009; 69:8784

Dear Dr. Prendergast:

The Titanium Dioxide Stewardship Council (TDSC)<sup>1</sup> and the Titanium Dioxide Manufacturers Association (TDMA)<sup>2</sup> submit these comments on the UCLA study published in the journal *Cancer Research* the week of November 15, 2009, titled: “Titanium Dioxide Nanoparticles Induce DNA Damage and Genetic Instability *In vivo* in Mice.”

The TDSC appreciates the scholarship that went into the study, but wishes to note several critically important deficiencies with the study. Importantly, the study does not differentiate clearly between pigmentary TiO<sub>2</sub> (0.25-2.5µm) and TiO<sub>2</sub> nanoparticles (<0.10µm). The study states that “Titanium Dioxide (TiO<sub>2</sub>) nanoparticles are manufactured worldwide in large quantities for use in a wide range of applications...” The study neglects to note, however, that the production of nanoparticle TiO<sub>2</sub> comprises *less than 1%* that of pigment grade TiO<sub>2</sub>.

Importantly also, the end uses are different. Pigment brings opacity and whiteness to paints, plastics, papers, inks, food, and drugs. Nanoparticles of TiO<sub>2</sub> are transparent and their property of UV absorbency is desirable in cosmetics and catalysts.

In the study, TiO<sub>2</sub> nanoparticles were given to mice in drinking water. Contrary to what the study implies, TiO<sub>2</sub> nanoparticles are not used in any applications intended for human or animal consumption, such as the toothpaste and food colorant examples noted in the text. Therefore, ingestion is not a relevant route of exposure for nanoparticle TiO<sub>2</sub>. Nanoparticle TiO<sub>2</sub> is intended

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<sup>1</sup> The members of TDSC include: DuPont, Millennium Inorganic Chemicals -- A Cristal Company, Huntsman Corporation, TRONOX LLC, and Kronos Worldwide, Inc.

<sup>2</sup> TDMA was formed in 1974 to promote the interests of the European TiO<sub>2</sub> industry. TDMA operates under the governance of CEFIC.

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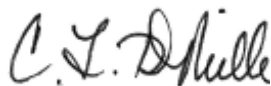
for applications that involve its incorporation into a substrate or matrix. An effective combination of engineering controls and personal protective equipment minimize worker exposure during manufacturing and incorporation. Once in a substrate, there is no or very little exposure to the TiO<sub>2</sub> dust.

Toxicologists from TDSC member company laboratories have reviewed the referenced paper and have expressed concerns about how these studies were designed and conducted. Concerns include the lack of internal particle controls, lack of use of time course evaluations, and, a key concern, the high doses used in the study. A serious concern was that only one type of nanoparticle TiO<sub>2</sub>, a highly active photocatalyst, was used to represent all types of nanoparticle TiO<sub>2</sub>. The TiO<sub>2</sub> photocatalyst used consists largely of anatase, is not surface treated, and has a primary particle size of 20 nm. These properties are not representative of all commercial grades of nanoparticle TiO<sub>2</sub>. A more comprehensive technical response to the study, in preparation by expert resources from both the TDMA and the TDSC, will be forthcoming in the next 1-2 months.

The TDSC is aware of the challenges to environment, health, and safety brought by certain applications of nanotechnology. Our member companies' commitment to product stewardship reflects these challenges and accounts for them accordingly.

Thank you for considering these comments. If you have any questions, please call me at (410) 229-4560 or e-mail at [Curt.DeMille@crystalglobal.com](mailto:Curt.DeMille@crystalglobal.com).

Respectfully submitted,



Curt DeMille  
Chair  
Titanium Dioxide Stewardship Council

# COMMENTS ON DNA DAMAGE CAUSED BY NANOPARTICULATE TITANIUM DIOXIDE: TROUILLER ET AL PAPER 2009

## EXECUTIVE SUMMARY

A publication by Trouiller *et al.* (2009) in the journal *Cancer Research* reports that nanoparticulate titanium dioxide (TiO<sub>2</sub>, Aeroxide P25, Evonik, primary particle size of 21 nm) administered to mice in the drinking water produces a uniformly positive response in a number of genotoxicity assays. The technical design of the studies appears to be nonstandard, and the results implicate TiO<sub>2</sub> as the causative agent for DNA damage, resulting from single and double stranded breaks and oxidative damage that is considered secondary to inflammation and/or oxidative stress. The authors claim that “These results represent the first comprehensive *in vivo* genotoxicity study of TiO<sub>2</sub> nanoparticles” and further that “These data suggest that we should be concerned about a potential risk of cancer or genetic disorders especially for people occupationally exposed to high concentrations of TiO<sub>2</sub> nanoparticles...”. For a number of reasons, the current studies fall short of providing adequate support for the claims made. In particular, statistically significant effects were generally only reported or measured at the highest exposure level tested. The magnitude of most reported effects were on the order of 2-fold or less, and thus represented marginal responses. As a result of the non-conventional test methods employed, as well as a lack of more conventional endpoints for comparison, any interpretation of possible human health hazards from this work is questionable.

## BACKGROUND INFORMATION

### Findings from Trouiller *et al.* 2009:

Trouiller *et al.* (2009) administered nanoparticulate TiO<sub>2</sub> as a suspension in the drinking water to mice at concentrations such that the mice received approximate total dosages of 0, 50, 100, 250 or 500 mg/kg body weight (bwt) over 5 days (male mice) or 10 days (pregnant mice, on days 8.5 to 18.5 post coitum, 500 mg/kg bwt). A number of assays were described in this paper that measured DNA damage (breakage), oxidative DNA damage, and inflammation. DNA strand breakage (both single-strand and double strand) was measured in the Comet assay, the micronucleus assay, and the  $\gamma$ -H2AX immunostaining assay. Oxidated DNA damage was assayed by measuring 8-hydroxy-2'-deoxyguanosine (8-OHdG) levels in livers from mice after 5 days of dosing. In addition, DNA deletion (a genetic instability endpoint) was measured as unpigmented retinal pigment epithelium (RPE) in the eyes of the offspring of homozygous (p<sup>un</sup>/p<sup>un</sup>) mice. Inflammatory response was measured as the presence of mRNA levels of inflammatory cytokines in peripheral blood. The results suggest that nanoparticulate TiO<sub>2</sub> administered orally produced a genotoxic response, possibly secondary to inflammation and oxidative stress. All assays for DNA damage (with the exception of the  $\gamma$ -H2AX immunostaining assay) showed significant and positive effects only at the highest dose level tested. The

$\gamma$ -H2AX immunostaining assay, a marker for DNA double-strand breaks, proved to be the most sensitive assay employed and indicated significant increases in  $\gamma$ -H2AX-positive cells in the bone marrow of mice (5 days of treatment) at all treatment levels. At the highest dose level, proinflammatory cytokines (but not anti-inflammatory) were increased indicative of a possible direct effect on circulating effector cells with secondary effects in peripheral organs.

### **Genotoxicity Studies with TiO<sub>2</sub>:**

There is little definitive information available concerning the genotoxic potential of TiO<sub>2</sub> *in vivo*. Micronucleus formation in bone marrow cells and peripheral blood lymphocytes was enhanced following intraperitoneal injection of TiO<sub>2</sub> (form unspecified) in mice, but the effect was not dose-dependent over the range 200 to 1500 mg/kg bwt. (Shelby *et al.*, 1993; Shelby and Witt, 1995). In a companion chromosomal aberration test in mice, no clastogenic response was recorded (Shelby and Witt, 1995). Enhanced mutagenesis in rat alveolar cells, measured at the hypoxanthine-guanine phosphoribosyl transferase (HPRT) locus, was observed after intratracheal administration of 100 mg/kg bwt of TiO<sub>2</sub>, (anatase, median diameter = 0.18  $\mu$ m), a dose that elicited persistent lung inflammation and is suggestive of an effect due to cytotoxicity (Driscoll *et al.*, 1997). Enhanced mutagenesis was not observed at a dose of 10 mg/kg bwt.

There is conflicting information from available studies concerning the *in vitro* genotoxic potential of nanoparticulate TiO<sub>2</sub>. Nanoparticulate TiO<sub>2</sub> has failed to display genotoxic activity in a number of standard *in vitro* assays including: the Ames test using *Salmonella typhimurium* or *Escherichia coli* (Warheit *et al.*, 2007; Theogaraj *et al.*, 2007); chromosomal aberrations in Chinese hamster ovary cells (Warheit *et al.*, 2007); or cytogenetic studies in cultured rat liver epithelial cells (Linnainmaa *et al.*, 1997). In apparent contrast, studies reported by Wang *et al.* (2007) indicate genotoxicity and cytotoxicity of ultrafine TiO<sub>2</sub> produced in cultured human lymphoblastoid cells. Rahman *et al.* (2002) have reported that ultrafine TiO<sub>2</sub> induces micronuclei and apoptosis in Syrian hamster embryo fibroblasts.

### **Limitations of the Trouiller *et al.* 2009 Study:**

The genotoxicity results reported by the authors appear to be indicative of genotoxicity, and at most provide a basis for confirmatory follow-up studies using more appropriate and robust standardized methodologies. Indeed, from an experimental design perspective, the Trouiller *et al.* study unfortunately has several weaknesses that limit interpretation of the study results. These include but are not limited to the following issues:

1. The authors provided inadequate physicochemical characterization of the nanoscale TiO<sub>2</sub> particle-types used in the study. In particular, more information regarding surface chemistry and reactivity, surface coatings and purity should have been included (Warheit, 2008);

2. Exceedingly high but unconfirmed doses of TiO<sub>2</sub> nanoparticles were used in the study. Given the small group sizes (5 animals/group), and the use of estimated delivered doses, it is possible that certain animals received significantly larger delivered doses, thus providing a possible explanation for some of the minimal effects noted;
3. No attempt was made to accurately measure or to document the biokinetics or dose of ingested nanoscale TiO<sub>2</sub> particles to any target tissues, thus a correlation of response with tissue dose is not possible. Considering the research approach and claims made based on the data, this would have been a necessary inclusion;
4. Benchmark positive control test samples, as well as positive control and historical control data, should have been included. The lack of such information precludes intra-laboratory comparisons of results and calls into question any interpretation of minimally significant effects;
5. No justification was provided for utilizing mice of an age of 4-5 months; younger mice (i.e., 2-month old) are generally used in standardized genetic toxicity assays. This deviation from normal protocol corresponds to a lack of appreciation of the potential age-effects on the erythrocyte population. The reported micronuclei results did not include a measurement of the ratio between immature and mature erythrocytes, and it is not clear which cell population was used for the micronucleus analyses. Therefore it cannot be excluded that the observed positive micronucleus result was caused by an age-related accumulation of micronuclei in the target cell population rather than exposure to TiO<sub>2</sub>.

The claim that is made by the authors that “These results represent the first comprehensive *in vivo* genotoxicity study of TiO<sub>2</sub> nanoparticles” is not supported by the data. In particular, the studies described in Trouiller *et al.* (2009) differ significantly in design from more robust studies conducted under recognized guidelines, thus making an interpretation of the findings difficult. Although the weight of evidence from these studies suggests DNA damage has occurred, there is no indication from the findings whether such damage would have been repaired, caused lethality or induced heritable mutations. An additional major deficiency is the lack of measures of cytotoxicity. DNA damage can result indirectly from such cytotoxicity and it is thus possible that some of the reported effects were observed in the presence of a cytotoxic response. In the Comet assay, the changes reported were < 1.5-fold and were quite minimal in nature. Without comparisons to laboratory control data, such a small increase cannot be evaluated. A 2.1-fold increase in micronuclei in peripheral blood, reported at the highest dose level only, suffers from a similar deficiency. The significance of a 1.27-fold increase in the incidence of pink-eyed unstable locus mutations measured only at the highest dose level is also difficult to interpret. This assay is not widely used and the biological relevance of such a minimal effect is difficult to evaluate. The biological relevance of any of the marginally positive findings can be argued. We have reason to suggest, based on the known photoreactivity of TiO<sub>2</sub>, with possible implications for such endpoints as oxidative DNA damage (8-OHdG) and the Comet assay, that perhaps some of the effects recorded

in the current studies were artifacts of the methods employed and were not a direct consequence of the *in vivo* exposures.

The findings in the  $\gamma$ -H2AX assay were of more significance. The data showed dose-related increases of up to 30% at the highest dose tested and with significant ( $p < 0.001$ ) increases produced at all dose levels. The  $\gamma$ -H2AX assay has proven useful for the detection of DNA double-strand breakage and repair following treatment with ionizing radiation or with a number of DNA damaging chemicals (Löbrich *et al.*, 2010). However,  $\gamma$ -H2AX foci can be produced at lesions other than double-strand breaks, making the assay an indirect rather than direct measure of double-strand breaks. In the current paper, no corroborating assay, such as the Comet assay, was performed on bone marrow samples. In fact, the Comet assay performed on peripheral blood showed only a minimal response and only at the highest dose level tested. The changes noted in the  $\gamma$ -H2AX assay are most likely of biological significance, but without information to indicate such a response would lead to a viable and permanent genetic alteration, it is not possible to ascribe a potential health hazard based on this assay.

The proposal by Trouiller *et al.* (2009) that the measured genotoxicity of nanoparticulate TiO<sub>2</sub> may result from inflammation and oxidative stress is supported by evidence presented. Increases in pro-inflammatory cytokines are in line with an induced oxidative stress from TiO<sub>2</sub> exposure. It is also reasonable to postulate that such oxidative stress could lead to the other genotoxic results presented including single- and double-stranded DNA breaks, deletions and oxidized DNA. However, levels of 8-hydroxy-2'-deoxyguanosine (8-OHdG), indicative of an oxidative DNA lesion, were only increased 1.5-fold at the highest administered dose. With no data from other dose levels (dose-response) and without concurrent cytotoxicity measures, the biological significance of this small increase cannot be adequately assessed. The current study is further limited by a lack of a single assay performed in all affected tissues, thus impeding any continuity of interpretation.

In summary, the Trouiller *et al.* genotoxicity results with nanoscale TiO<sub>2</sub> particulates appear to be intriguing as a preliminary finding. However, the data would be more compelling if the authors had generated results using a standardized *in vivo* genotoxicity assay, along with adequate material characterization, appropriate assessments of doses to target tissues, dose response and time course experimental designs, and corresponding benchmark positive control test substances. Accordingly, it seems clear that standardized models and reproducibility of the results are necessary prerequisites for the establishment of verifiable genotoxicity assay results.

## **Discussion and Proposed Study Protocols**

In the current studies reported by Trouiller *et al.* (2009), a mutagenic response in mice treated orally with nanoparticulate TiO<sub>2</sub> was reported and suggested to be secondary to an inflammatory response and oxidative stress. In the absence of measures of cytotoxicity, and with predominantly minimal effects observed at only the highest dose level tested, the relevance of these findings for the assessment of potential adverse health effects is

questionable. Oral absorption of nanoparticulate TiO<sub>2</sub> with systemic distribution is suggested from the results; however, the lack of tissue measurements of TiO<sub>2</sub> as well as the lack of a consistent bioassay system applied to all potential target tissue does not allow a meaningful correlation of tissue response with dose to be made.

The authors contend “These data suggest that we should be concerned about a potential risk of cancer or genetic disorders especially for people occupationally exposed to high concentrations of TiO<sub>2</sub> nanoparticles...”, with the implication that this applies to the oral route of exposure. However, more conventional 2-year feeding studies in rats and mice with pigment-grade TiO<sub>2</sub> at levels in feed up to 50,000 ppm (5%) were without effect (NTP, 1979). And although lung tumors in rats, under conditions of lung-overload, are produced after chronic inhalation exposure to either pigment-grade or nanoparticulate TiO<sub>2</sub> (Lee *et al.*, 1985; Heinrich *et al.*, 1995), tumors in other organs are absent. In addition, epidemiological evidence from well-conducted investigations has shown no causal link between TiO<sub>2</sub> exposure and the risk of cancer in humans (Chen and Fayerweather, 1988; Fryzek *et al.*, 2003; Boffetta *et al.*, 2001 and Boffetta *et al.*, 2004).

In order to further investigate the preliminary findings of genotoxicity of nanoscale TiO<sub>2</sub> particles reported by Trouiller *et al.*, the following testing strategy is proposed:

- Prior to commencing the study, the physicochemical characteristics of the nanoparticle test substance should be rigorously characterized, in a manner as previously described (Warheit, 2008). Some of these characterization elements include – crystal structure, particle size distribution, purity, surface area, and shape, as well as additional particle surface characteristics such as reactivity, coatings, and agglomeration.
- Following adequate material characterization of the TiO<sub>2</sub> nanoparticle-type, a standardized *in vivo* micronucleus assay (OECD 474) which detects chromosomal breakage and disturbance of mitotic spindles) (rat or mouse species) should be implemented, concomitant with a Comet assay (DNA damage). It is suggested that oral gavage should be the route of administration/exposure.
- Positive control groups should include cyclophosphamide, as traditionally used for these studies, concomitant with a metal nanoparticulate positive control particle such as Chromium (Cr VI) or a Nickel compound. The inclusion of cyclophosphamide demonstrates the reproducibility of the assay. Moreover, the addition of Ni or Cr provides an attempt to establish a metal nanoparticulate positive control sample for this assay.

The results obtained from a study employing this proposed testing strategy (Warheit and Donner, 2010) may produce useful insights and would serve to confirm or challenge the findings of Trouiller and coworkers.

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