Subject: Trans 1,2-dichloroethylene (Trans) Toxicological Assessment article

A draft technical article entitled "Toxicological Assessment of Industrial Solvents Using Human Cell Bioassays: Assessment of Short-Term Cytotoxocity and Long-Term Genotoxicity Potential" written by B. Hasspieler et al. (2004) is being distributed to some solvent customers.

PPG requested Byron E. Butterworth, Ph.D, to critically review the Hasspieler et al. (2004) manuscript and prepare an Expert Opinion addressing the issues raised. A copy of the Expert Opinion is attached. **This Expert Opinion is to be presented to any customers who may refer to the Hasspieler et al. (2004) manuscript and are asking for PPG's opinion of the conclusions made.**

Among the conclusions drawn by Hasspieler et al. (2004) on the basis of their studies is a prediction that Trans is genotoxic and cytotoxic. Genotoxicity refers to the ability of a chemical to adversely affect the genetic material within a cell. Cytotoxicity refers to the ability of a chemical to kill living cells. Hasspieler et al. (2004) also assert their work shows that nPB (n-propyl bromide) is neither genotoxic or cytotoxic.

Dr. Butterworth was hired by PPG to review this manuscript because he is an internationally recognized expert in the field of genetic toxicology and carcinogenicity research. His critical review of the Hasspieler et al. (2004) manuscript clearly shows that the conclusions drawn by Hasspieler et al. (2004) concerning both Trans and nPB are incorrect, are based on assays that were not validated, use procedures that have fatal technical problems, and are not considered by mainstream toxicologists and regulatory agencies to be suitable procedures to accurately predict genotoxicity. Dr. Butterworth's report reviews the critical flaws point by point in his Executive Summary and further embellishes each point in the body of the report. The Expert Opinion also includes an extensive Reference Section.

This document is geared to be presented to those individuals at a customer location that are familiar with the topics in question. Although not exceedingly technical, a working knowledge of the subject matter by the reader would be needed to fully appreciate Dr. Butterworth's conclusions.

If you have any questions concerning the above, feel free to contact Dr. Jim Barter or myself to discuss.

Expert Opinion

by

Byron E. Butterworth, Ph.D., Fellow ATS

November 19, 2004

Critical Flaws in the Manuscript "Toxicological Assessment of Industrial Solvents Using Human Cell Bioassays: Assessment of Short-Term Cytotoxocity and Long-Term Genotoxicity Potential" by B. Hasspieler et al.

EXECUTIVE SUMMARY

The manuscript "Toxicological Assessment of Industrial Solvents Using Human Cell Bioassays: Assessment of Short-Term Cytotoxocity and Long-Term Genotoxicity Potential" by B. Hasspieler et al.(2004) makes several assertions regarding the genotoxic potential of chemicals of interest to PPG Industries, Inc. Genotoxic refers to the ability of a chemical to adversely affect the genetic material within a cell. This expert opinion was prepared to independently evaluate this body of work. The following critical flaws were found with these studies.

1. Hasspieler et al. (2004) state that: "There is an increasing demand for simple toxicological screening methods to assess human health risk associated with exposure to environmental toxicants. While such a statement may have been true 25 years ago, it is certainly not true today. Numerous, well-validated, predictive assays are commonly in use that are far superior to the versions proposed by Hasspieler et al. (2004).

2. The DNA single-strand break assay as described by Hasspieler et al. (2004) is invalid because it does not measure cytotoxicity (cell killing) or control for the false positive responses that occur under cytotoxic conditions. The Hasspieler et al. (2004) version of this assay has not been validated.

3. The DNA repair assay as described by Hasspieler et al. (2004) is invalid because (a) the metabolic capabilities of the HepG2 cell line in culture is no longer reflective of the metabolic profile in the liver of a person; (b) as a cell line, the HepG2 cells are in the DNA synthesis phase and inhibiting this process has been shown to be difficult and uneven; (c) even a small amount of residual cells in the DNA synthesis phase will give a false positive response relative to the very small amount of unscheduled DNA synthesis (UDS) indicative of DNA repair; (d) DNA synthesis is measured by scintillation counting so that UDS and S-phase synthesis can not be distinguished; and (e) the Hasspieler et al. (2004) version of the assay has not been validated.

4. The actual data are not presented for review. Sixteen chemicals were evaluated in the Hasspieler et al. (2004) paper, but only a summary table giving the "positive" or "negative" judgment call is presented.

5. Hasspieler et al. (2004) predicted that based on their studies *trans*-1,2-dichloroethylene would be genotoxic. An extensive published database shows that this is not the case. *trans*-1,2-Dichloroethylene is clearly nongenotoxic. These results demonstrate the failure of the predictive power of the assays proposed by Hasspieler et al. (2004) for this compound.

6. Hasspieler et al. (2004) predicted that based on their studies n-propylbromide (1-bromopropane or n-PB) would be nongenotoxic. In fact, n-propylbromide is mutagenic, and several structural analogues of n-PB are mutagenic as well, including 2-bromopropane, 1-bromobutane, bromoethane, and 1,2-dibromoethane. These results demonstrate the failure of the predictive power of the assays proposed by Hasspieler et al. (2004) for this compound.

BACKGROUND

The manuscript by B. Hasspieler et al. (2004) makes several assertions regarding the genotoxic potential of chemicals of interest to PPG Industries, Inc. PPG provided me with a copy of the Hasspieler et al. (2004) manuscript and asked me to prepare an independent expert opinion on the scientific merit of the paper. PPG was helpful in providing me with supporting reference material that I requested and compensated me for my time. The opinions presented here are mine alone and were not influenced by the sponsor.

By way of introduction, I have been actively involved in research in genetic toxicology and chemical carcinogenesis for over 30 years. I have served as Chief of the Molecular Biology Section at DuPont's Haskell Toxicology Laboratory. I have held various positions at the Chemical Industry Institute of Toxicology including Head of the Department of Genetic Toxicology and Director of the Chemical Carcinogenesis Program. I am currently President of Butterworth Consulting providing expertise on toxicological issues. I have served as President of the Carcinogenesis Specialty Section of the Society of Toxicology, and am a fellow of The Academy of Toxicological Sciences. I have been involved in developing predictive assays for carcinogenic and mutagenic potential for over 25 years (Butterworth, 1979). The *in vivo* hepatocyte DNA repair assay was developed in my laboratory (Mirsalis and Butterworth, 1980). I co-authored the publications establishing the standard techniques for assessing chemically-induced DNA in hepatocytes in vitro and in vivo (Butterworth, 1987; Butterworth et al. 1987a, 1987b). I have directed research studies on the mechanism of action of numerous carcinogens and played a leading role in defining how knowledge of mode of action might guide the risk assessment process (Butterworth and Bogdanffy, 1999).

CRITICAL FLAWS IN THE HASSPIELER ET AL. MANUSCRIPT

<u>Status of Predictive Assays</u>

Hasspieler et al. (2004) state that: “There is an increasing demand for simple toxicological screening methods to assess human health risk associated with exposure

to environmental toxicants. Such screening tools should allow for risk evaluations in terms of both short-term/acute toxicity as well as longer-term genetic damage that may lead to mutagenicity and carcinogenicity." While such a statement may have been a true 25 years ago (Butterworth, 1979), it is certainly not true today. The disciplines of toxicology, genetic toxicology, and chemical carcinogenesis have long since developed excellent and well-validated predictive tools. These screening tools constitute established, required assays by the United States and International regulatory agencies (International Conference on Harmonization (ICH), 1997; Putman et al., 2000). Importantly, the genetic toxicology assays chosen by Hasspieler et al., (2004) are seldom used because they have been shown to be prone to artifacts and there are many superior choices for screening assays.

The DNA Single-Strand Break Assay as Presented is Invalid

The DNA single-strand break assay as described by Hasspieler et al. (2004) utilizes the HepG2 cell line. DNA breakage is evaluated but, compound-induced toxicity is not measured. This is a fatal flaw because all compounds are cytotoxic at some dose, and cytotoxicity results in release of nucleases that break the DNA. It is well known that this assay is prone to this particular artifact. All validated assays that measure DNA breakage also measure the dose-response curve for cytotoxicity. Only compounds that exhibit DNA breakage at non-cytotoxic doses can be considered genotoxic. This technical problem is difficult to control, and such assays are not commonly chosen. Assays that are used must be validated with known genotoxic and known nongenotoxic chemicals to demonstrate that the test can distinguish between them. This was not done with the DNA single-strand break assay described by Hasspieler et al. (2004). The only validation study noted showed a positive response with only three known mutagens, and did not examine any nongenotoxic cytotoxicants at all (Hasspieler et al., 1995).

The UDS-DNA Repair Assay as Presented is Invalid

When a chemical covalently binds to the DNA, the resulting DNA adduct may be removed by a DNA repair process called excision repair. If done in the presence of ^{3}H-

thymidine, this process results in a small amount of incorporation of the ^{3}H-thymidine into the DNA known as unscheduled DNA synthesis (UDS). The widely-used primary hepatocyte in vitro DNA repair assay (Butterworth, 1987; Butterworth et al., 1987a) has the following advantages. First, the cells used are primary cultures of rodent hepatocytes that closely reflect the metabolic capabilities in the whole animal. This is critical because it is often a reactive metabolite that reacts with the DNA. The second advantage is that the primary hepatocyte cultures have few cells that are replicating the DNA in preparation for cell division. Replicative DNA synthesis or cells in the synthesis phase (S-phase) of the cell cycle are incorporating many thousands of times more ^{3}H-thymidine into the DNA compared to UDS, because the entire genome is being duplicated. Even a low level of cells in S-phase can render a DNA repair assay invalid, unless one can distinguish UDS from S-phase synthesis. The third advantage is that incorporation of ^{3}H-thymidine is visualized autoradiographically; it is easy to see which cells are in S-phase and which are undergoing DNA repair. Thus, S-phase cells can be avoided. The fourth advantage is that comparative studies with rat and human primary hepatocyte cultures (prepared fresh from discarded surgical material) show that the primary rat hepatocyte UDS assay and the human primary hepatocyte assay respond almost identically (Butterworth et al., 1989).

In the Hasspieler et al. (2004) version of the UDS-DNA repair assay the cells used are HepG2 cells and has none of the above advantages. In fact, this protocol has the following disadvantages. First, as a permanent cell line, HepG2 cells have long since lost the metabolic profile of human hepatocytes. Second, because HepG2 cells are a constantly growing cell line, most cells are in S-phase. Growth to confluence and addition of hydroxyurea are used to try to stop S-phase synthesis. Autoradiographic analysis has shown, however, that these procedures do not completely block all cells from going into S-phase (Cattley et al., 1986). This results in a very high and uneven baseline. The third, and fatal disadvantage, is that ^{3}H-thymidine is assessed by scintillation counting. That is, the amount of radioactivity is measured without knowing whether the incorporation was UDS or was just somewhat more or fewer cells in S-phase. Assays that are used must be validated with known genotoxic and known

nongenotoxic chemicals to demonstrate that the test can distinguish between them. This was not done with the UDS-DNA repair assay described by Hasspieler et al. (2004).

Actual Data are Not Presented

For scientific publications that present new data, that data must be presented in the publication. In this way, the reader can review the actual numbers to see whether they make a scientifically plausible case. Sixteen chemicals were evaluated in the Hasspieler et al. (2004) paper, but only a summary table giving the "positive" or "negative" judgment call is presented.

Predictions With *trans*-1,2-Dichloroethylene Are Incorrect

Hasspieler et al. (2004) concluded that for the solvents tested "…trans 1,2-dichloroethylene (TDE) appears to have the most potential for genotoxicity…" This is an odd prediction in light of the fact that of the two genetic toxicity assays used, *trans*-1,2-dichloroethylene tested positive in the DNA strand break assay, but not in the DNA repair assay. The Hasspieler et al. (2004) prediction for *trans*-1,2-dichloroethylene turns out to be wrong. The weight of evidence clearly indicates that *trans*-1,2-dichloroethylene is not genotoxic These results are summarized here.

Assays In Which Trans 1,2-dichloroethylene Has Tested Negative

Salmonella mutagenicity assay	NTP, 2002
Salmonella mutagenicity assay	Simmon et al., 1977
Sister chromatid exchange induction in CHO cells	NTP, 2002
Sister chromatid exchange induction in CHL cells	Sawada et al., 1987
Induction of chromosomal aberrations in CHO cells	NTP, 2002
Induction of chromosomal aberrations in CHL cells	Sawada et al., 1987
Sister chromatid exchange induction in mice by gavage	NTP, 2002
Induction of chromosomal aberrations in mice by gavage	NTP, 2002
In vivo mouse peripheral blood micronucleus assay	NTP, 2002
Induction of DNA repair in primary rat hepatocytes	Costa & Ivanetich, 1984

These results clearly demonstrate the failure of the predictive power of the assays proposed by Hasspieler et al. (2004) for this compound.

Predictions for n-propylbromide are Incorrect

Hasspieler et al. (2004) predicted that n-propylbromide (n-PB) and EnSolve® (a formulation of n-propylbromide) would not be genotoxic based on a negative response in the two genetic toxicity assays that they conducted (DNA strand break and DNA repair activity). In contrast, they noted a positive response in both genetic toxicity assays for isopropylbromide. The Hasspieler et al. (2004) prediction for n-propylbromide turns out to be wrong. n-propylbromide (1-bromopropane) is mutagenic (Barber et al., 1981). In addition, several structural analogues of n-PB (2-bromopropane, 1-bromobutane, bromoethane, and 1,2-dibromoethane) are also mutagenic (Barber et al., 1981; Warner et al., 1988). Thus, it is not surprising that n-propylbromide is mutagenic as well. These results clearly demonstrate the failure of the predictive power of the assays proposed by Hasspieler et al. (2004) for this compound.

Summary

The procedures used by Hasspieler et al. (2004) as described in their manuscript have numerous fatal technical problems and fail to predict the well established negative genotoxic potential of trans 1,2-dichloroethylene or the known positive mutagenic potential of n-propylbromide.

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