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Dear Dr. Lieder:

The expert review, described in this letter, of the mouse lymphoma cell mutation testing performed by NOTOX on 3M product, T-6906, was conducted at my request by Dr. Brian Myhr, Associate Director of Genetic and Cellular Toxicology at Covance's Vienna, VA facility. Dr. Myhr has had over 20 years of experience conducting and evaluating data from Mouse Lymphoma assays. Data from a dose range-finder, two trials of the mutation assay with and without rat liver S9 activation, and the description of the technical performance of the mutation assay, were reviewed as reported in a draft report signed April 25, 1998, by the NOTOX study director.

The test article, T-6906, was evaluated by the NOTOX study director as nonmutagenic in the absence of S9 activation and mutagenic with S9 activation. Our assessment of the study is that it was technically inadequate, and the reported data are insufficient to reach a valid conclusion regarding the mutagenic activity of the test article in this assay. The study should be repeated under currently acceptable test conditions to draw any valid conclusions regarding the mutagenic activity of T-6906.

The specific deficiencies of the study (or reporting) are listed as follows:

1. The mouse lymphoma assay has been validated specifically for clone 3.7.2C of L5178Y cells. The report does not specify the clone, and the issue is raised if NOTOX has any karyotypic documentation that 3.7.2C cells were used. This is very important to establish because the mutagenic response of a different clone may be greatly diminished.
2. The S9 was prepared in-house from Wistar rats. S9 is usually prepared from Fischer 344 or Sprague-Dawley rats, so one wonders about the efficacy of the S9 preparation and any documentation of its enzymatic activity. In fact, 8% v/v S9 (80 µl/ml) was used in the assay; this contrasts with the use of 20 to 30 µl/ml S9 concentrations used routinely with commercially available S9 with documented high enzyme activity. At 80 µl/ml, this S9 would be highly toxic to mouse lymphoma cells. While DMN gave a good response in the assay, the report does not give its concentration in the cell cultures, so no comparison with literature results is possible.
3. EMS should not be used as the positive control for nonactivation test conditions. This compound induces very few small mutant colonies and thus provides no assurance that the laboratory is able to detect this major class of mutants. Positive controls like MMS, which induce many small mutant colonies, are currently highly recommended. (Note: the concentration of EMS in the cultures was not reported.)
4. The laboratory appears to have poor detection of small mutant colonies, which raises the issue of lack of definitive negative evaluations of test compounds. The historical range of negative control mutant frequencies was given as 7 to 58×10^{-6} , which is much too low, especially for the microwell method. Mutant frequencies below about 30×10^{-6} just aren't acceptable. (By the way, the report gives positive

exponents rather than negative exponents for mutant frequencies.)

In the data tables, DMN should be showing many more small colonies than large colonies. In Table 3, in particular, DMA is shown inducing primarily large colonies. There are very few small colonies and almost none in the concurrent negative control. This describes poor experimental conditions for detecting induced mutants.

5. The dose range-finding test using 3-hour treatments is described in Table 1. The toxicity of the test article should be measured by multiplying the two columns of responses given (RCC and RCE - see our notations in Table 1) to calculate the "survival" (RS) relative to the solvent control. When this is done, the survival decreases steadily with increasing dose down to 23% at 333 µg/ml without S9. (The increase in survival at the top dose of 1000 µg/ml is strange and suggests some adsorption of soluble test article to precipitated material.) One needs only to take the assay to 20% survival, so there just was no need to shift to 24-hour treatments for the mutation assay as indicated in the range finding results section on page 14 of the report. Furthermore, the most common measure of toxicity (RTG) includes the decrease in growth rate over the expression period, so the toxicity would be even greater than indicated in Table 1. The same would be true for the +S9 portion of the testing, so that the mutation assay with S9 was very likely carried to higher test article concentrations than needed. The precipitating range could probably have been avoided altogether.

There was consensus agreement among mouse lymphoma assay experts at the 1999 IWGTP workshop that RTG or RS should be used for the toxicity measurement. Work is ongoing to choose one measure, probably RTG. The choice of toxicity measurement is critical to the choice of proper test article concentration range and thus to the evaluation of test compounds in this assay. The method used in the NOTOX study will lead to inappropriately high concentrations of test article being tested.

6. In both mutation assay trials, the expression period was 3 days after the 3-hour treatment with T-6906 with S9 metabolic activation. This is just not acceptable. It is well known that the maximum mutant detection occurs 2 days after treatment, and the mutant frequency falls rapidly thereafter. By waiting for 3 days, the sensitivity of the assay is seriously compromised. The detection of mutagenic activity of T-6906 (if real, and I doubt it is) would become much enhanced at 2 days of expression.

7. The conditions for determining mutant frequencies were not optimal for controlling variability. For the microwell method, it is recommended to use 1.6 cells/well for CE and 2000 cells/well for mutant selection x 4 plates to give 0.768×10^6 cells for analysis. The NOTOX study used 1 cell/well for CE and 2000 cells/well x 3 plates for only 0.576×10^6 cells for analysis. The soft agar method of the assay analyzes 3×10^6 cells for mutants. The smaller numbers used by NOTOX are detrimental to precision, and consequent assay fluctuations could well be responsible for spurious 2-fold and 3-fold increases in mutant frequency, especially when relative to low negative control mutant frequencies. This is likely what occurred for the Table 3 results with S9 that were evaluated as showing mutagenicity.

8. It is not clear how the mutant frequency was calculated. The tables show total number of wells with mutants, but the Poisson calculation should come from the proportion of wells with no mutants. The text indicates this was done correctly, but no verification is possible from the data provided. Total wells evaluated and total empty wells should be given for each culture.

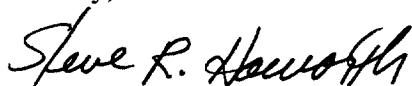
9. In addition to the reasons given above for not even making an evaluation at this point, the S9 activation results should never have been evaluated as positive for mutagenicity. Table 2 results are just not interpretable. The test material precipitated at 1000 µg/ml, and the sudden and large increase in mutant frequency is symptomatic of some technical problem. There was no substantial increase in small mutant colonies relative to large mutants colonies, which is what one would expect nearly all the time. And the toxicity, if measured by RTG, would probably have been excessive (less than 10% RTG). If RTG measurements had been used, the highest selected dose probably would not have exceeded about 300 µg/ml.

Table 3 results with S9 activation are probably just demonstrating assay variability. At 175 $\mu\text{g/ml}$, where there was a very toxic response at 17% survival, there was no increase in mutant frequency. This was apparently the most toxic treatment in the table and should have shown the largest mutant frequency. The so-called responses at 250 and 750 $\mu\text{g/ml}$ are therefore not believable without confirmation under better assay conditions. These were probably excessively toxic treatments by the RTG measurement of toxicity.

As an added note, there is no reason by updated OECD guidelines for mouse lymphoma assay, adopted in July, 1997, to perform 24-hour treatments. Short-term treatments of 3-5 hours are sufficient with and without S9 activation. If negative results are obtained and if the test material is a pharmaceutical, then 24-hour treatments in a second trial without S9 are currently used to meet ICH guidelines for FDA submission. Other submissions to FDA, to CFSAN for example, for food additives or food contact materials, will follow the updated OECD guidelines; there is no need for 24-hour treatments. This information was just confirmed by an FDA staffer involved with the revision of the genotoxicity guidelines in the FDA Redbook.

Thank you for the opportunity to review this work. Should you have any additional questions, please do not hesitate to contact us.

Sincerely,

A handwritten signature in black ink, reading "Steve R. Haworth". The signature is fluid and cursive, with the first name "Steve" being more prominent.

Steve R. Haworth, Ph.D.
Vice President
Genetic and Cellular Toxicology