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Dear Dr. Stokes:

This letter provides written comments on the Guidance Document on Using *In Vitro* Data to Estimate *In Vivo* Starting Doses for Acute Toxicity.

The National Toxicology Program, NICEATM and the Organizing Committee and participants from the International Workshop on *In Vitro* Methods for Assessing Acute Toxicity Breakout Group 1 are to be congratulated for preparing this Guidance Document as a tangible outcome of the work done during the Workshop. We appreciate the opportunity to offer feedback on the document and request that the following comments be seriously considered.

We concur with the ICCVAM recommendation that data from *in vitro* cytotoxicity assays can be potentially useful as one tool for setting a starting dose for an *in vivo* assessment of acute oral toxicity, specifically, when adequate data are not available to estimate a starting dose. We reiterate the ICCVAM conclusion that preliminary information suggests that use of this approach could potentially reduce the number of animals used *in vivo* acute toxicity testing. We suggest that it would be helpful to carry this perspective over into the preface of the guidance document. The preface to the guidance document, as currently written, does not clearly provide the perspective that this method for dose setting has not yet been validated, nor does it clearly provide the context provided in the ICCVAM recommendation that cytotoxicity testing is recommended as one of a number of tools for setting starting doses.

We believe that while the data may support the use of the *in vitro* test data when no other data is available, there are no data presented to suggest that this method is better than the use of data from related chemicals, other experience with the chemical, or data from other animals. On page 10 of the Workshop summary, it is noted that independent of cell type used in the assay, the percentage of data falling within the defined prediction interval (± log 5) is 73-77 %. The flip side of this is that 25 % of the materials fall outside of this range. On page 15 of the Workshop report, 9 chemicals that were part of the UDP validation are discussed. Of these, 7 chemicals fell within the ± log 5 interval, whereas the remaining 2 differed from the *in vivo* values by an order of magnitude. Hence, the prediction will have a significant error rate rendering it less attractive

for use for dose setting for chemicals where other information are available for an expert determination of appropriate starting dose.

It is unfortunate that no attempt was made to compare this degree of accuracy with the result of a similar comparison using currently available QSAR programs or the ability of experienced toxicologists relying on historical data to set starting doses. Instead the document seems to assume that there is no reliable information to set the starting dose for the majority of *in vivo* acute toxicity studies, an assumption that is not supported in the document by any references or even unpublished surveys of contract or industry laboratories.

Particularly in areas where a company or organization has extensive experience with the toxicity of certain classes of chemicals, the reliability of dose setting based on experience with the chemical class can be quite high. Asking toxicologist to ignore their years of experience and extensive in-house and public data bases and instead rely completely on an unvalidated *in vitro* test may well increase rather than decrease the number of animals used in the studies.

This brings me to our second comment. The document spends considerable time discussing the possibility of using any of a large number of *in vitro* assays to set the starting dose, and only appears to recommend two assay systems toward the end of the document. At the same time the document does suggest that the ability of the *in vitro* methods to accurately predict the starting dose should be evaluated. We believe the document should begin with a clearly stated recommendation that one of the two tests for which detailed protocols are provided (neutral red uptake using BALB/c 3T3 cells or human cells, NHK) be used where feasible. These methods are in wide use and not difficult. It will be much easier to evaluate the success of this method, if the testing method is at least somewhat standardized. The discussion on the usefulness of other assay systems should be included at the end of the document or in an appendix rather than being the main body of the document.

Finally, the ICCVAM recommendations do not explicitly address validation of basal cytotoxicity assays for use in dose setting for *in vivo* studies under near term research. We believe such a study should be recommended for a number of reasons. The workshop report presents an opinion that the ad hoc performance of basal cytotoxicity assays for dose setting could be retrospectively evaluated to determine the utility in practice of this method. Specifically, on page 29 of the workshop summary it states “a prospective evaluation in practice (in this case by implementing the use of an *in vitro* cytotoxicity test in the strategy proposed by ZEBET)…. can be made once the necessary guidance document, including worked examples, has been produced. Once a sufficient body of data has been collected, the *in vitro* cytotoxicity tests can be evaluated retrospectively to determine the validity and practical usefulness of the strategy and to assess whether the predicted starting dose for an *in vivo* study is accurate for a sufficiently large enough percentage of test chemicals to continue its use”. We believe this procedure would be very inefficient, has a high probability of not covering all chemical classes of interest, and will be much less accurate than a single formal validation study. A single formal study would not only answer the question of whether the *in vitro* data can be used to set starting doses, but would also provide much needed information on the ability of the *in vitro* test methods to predict rodent LD_{50} values in general.

Secondly, we recommend that the practical usefulness of the proposed basal cytotoxicity tests and the regression relationship between *in vitro* data and *in vivo* data as proposed by Spielmann et al. be further explored. The relationship between the cytotoxicity values and the LD_{50} values was established by the Registry of Cytoxicity (RC) using the mean of available cytotoxicity values available that met inclusion criteria (as stated in the Workshop report.) The use of mean values for comparison ignores the degree of variability in the *in vitro* data and does not allow the determination how accurately a single *in vitro* test would predict the *in vivo* data. The general summary in the workshop report is that the regression function derived from the RC seems to be a reliable description of the general relationship between basal cytotoxicity and rodent oral systemic LD_{50} values. The conclusion could be different if evaluated in the context of specific individual cytotoxicity tests results and *in vivo* acute oral toxicity data.

In addition, a very important consideration both here and for future studies is the use of 1980's RTECs values as the *in vivo* standard to which *in vitro* data are compared. RTECs uses the lowest LD_{50} reported irrespective of the extent of data supporting a different value. This means the RTECs may include values that are significantly different from and completely ignore information on the mean or average values that could be obtained by using all data available from multiple rodent toxicity tests on the same compound. To compound matters, the RC values are based solely on the NIOSH 1984 publication that is, in fact, the 1980 edition. This means that there is good chance that much, if not most, of the data used were obtained from studies run prior to the implementation of Good Laboratory Practices.

We highly recommend that for any future validation studies the *in vivo* data sets used for and generated during the validation of the alternative *in vivo* tests accepted by OECD be used as the standard. The chemicals in these data sets were each tested in multiple *in vivo* acute oral toxicity tests of high quality and the lists were considered to have an appropriate range of toxicities and chemical types for regulatory acceptance of the three previous alternatives to the classical 401 test. There appears to be no scientific justification for insisting upon a different list for validation of an *in vitro* assay, particularly if it is going to be used primarily to set the starting doses for *in vivo* studies. Since the degree of inter and intra laboratory variability has already been determined for the two *in vitro* test recommended, a validation study could be conducted using this standard set of chemicals at a single validated laboratory and then evaluating the data in the context of the already available *in vivo* data.

Thank you again for considering our comments on this document.

Sincerely,

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