

HELIX3 INC.

Regulatory Safety Testing with the *In Vivo* Comet Assay: Recommendations for Data Interpretation

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ABSTRACT

As the *in vivo* comet assay is increasingly used for the evaluation of target organ genotoxicity in regulated safety testing, concerns and questions about appropriate study designs and data interpretation continue. And while several published recommendations note that issues such as cytotoxicity should be addressed, guidance for how those issues should be addressed and interpreted is often neglected. To advance a better understanding of the *in vivo* comet assay and to demonstrate how it can be used effectively in safety testing, the results and interpretation of several studies evaluating pharmaceuticals are presented and discussed. Topics of discussion include explicit details for assessing the effects of cytotoxicity, the determination of a positive genotoxic response, and recommendations for defending a negative response.

INTRODUCTION

The *in vivo* comet assay is the most powerful tool for detecting genotoxicity in target organs and at the optimal sample time based on pharmacokinetics. To demonstrate how the comet assay can be used in safety testing to effectively detect different classes of genotoxins, data from multiple pre-clinical safety tests conducted by Helix3 are presented. All of the compounds tested and presented are pharmaceutical products that were in development at the time of testing. In every study conducted, compounds were tested without knowledge of the compound, its mechanism of action, pharmacokinetics, intended use, and/or target organ(s). Dose concentrations, target organs, and sample times evaluated were at least initially selected by the Sponsor. All studies were conducted in accordance with US FDA (21 CFR Part 58) and OECD (ENV/MC/CHEM [98]17) GLP regulations.

Positive response criteria, the decision tree for conducting repeat studies, and recommendations for identifying and defending a negative response are presented. Where cytotoxicity as determined by the low molecular weight (LMW) DNA diffusion assay is presented with DNA migration data, the percentage of cells with LMW DNA diffusion (%LMW) is presented with the Olive Tail Moment (OTM) multiplied by 3 or 4 for combined side-by-side viewing.

METHODS & MATERIALS

Test Animals

Non-fasted virus anti-body free male or female Sprague Dawley or Wistar Han rats 8-9 weeks of age and with a mean body weight variation of $\leq 10\%$ at the start of dosing were used for each study. Uniquely identified animals were single housed in polysulfone cages with absorbent bedding and maintained at 18-23 C with a relative humidity of 40-60% and an air exchange rate of 70 1 exchanges per hour. Lighting was controlled to maintain 12 h of light and 12 h of dark and animals were provided Purina Certified Rodent Chow 5002 (Purina Mills, NC) and water *ad libitum*. All animal procedures were in compliance with the National Research Council Guide for the Care and Use of Laboratory Animals (1996) and the US Animal Welfare Act Regulations (9 CFR 1-4). All protocols and procedures were reviewed and approved by the Helix3 Institutional Animal Care and Use Committee.

Test Compounds and Dose Selection

All test compounds were pharmaceuticals, the metabolites of pharmaceuticals, or impurities generated by the synthesis of a pharmaceutical. They were provided to Helix3 as coded substances. Proprietary information about these compounds (e.g. structures, chemical class, intended uses and/or sources) are not disclosed. Since necrosis as classically detected by histopathology can decrease DNA migration and early stages of cytotoxicity (e.g. glycogen depletion) can increase DNA migration (1), dose ranges were selected to ensure that there were at least 2 non-cytotoxic dose concentrations tested.

METHODS & MATERIALS (CONT.)

Comet Assay

The standard procedure as described by Hartmann *et al.*(2) was used for the comet assay. Multiple tissues were evaluated in each study. However, only the tissue with a detected effect is presented. Since background migration levels can vary depending on the tissue type and the mechanism (strand breaks versus crosslinks) of genotoxicity (1), electrophoresis conditions were adjusted as necessary to optimize sensitivity and specificity.

Determination of a Positive Response

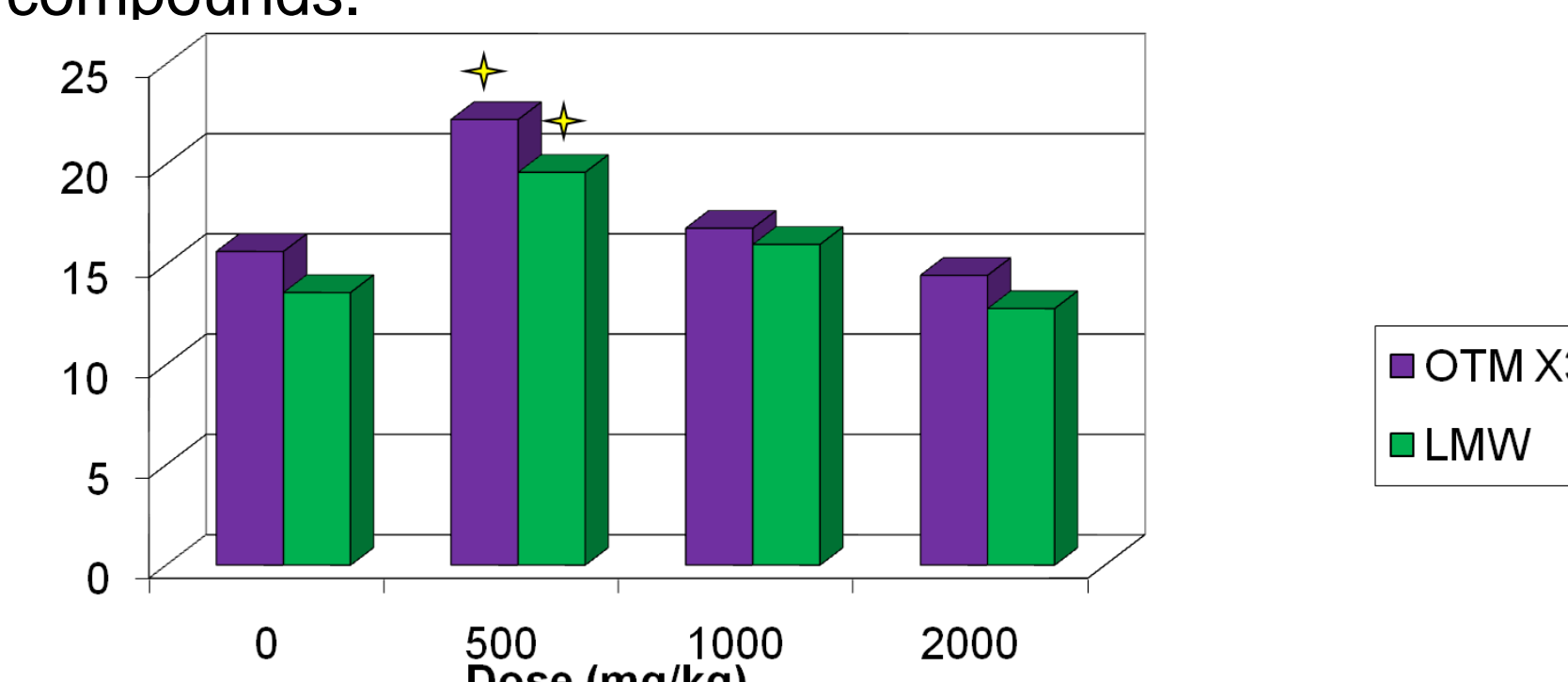
Although a mean difference in effect of ≥ 2 -fold and/or a mean difference in $\geq 5\%$ in %Tail DNA has been suggested as possible criteria for a positive response, these criteria are more appropriate for binomial or count data rather than the continuous data generated by the comet assay. As such, they do not address or account for the distribution of the data, inter-animal variability or the equality of variances between the compared dose groups and may thus be misleading when attempting to make conclusions about genotoxicity. Therefore, it is more appropriate and objective to use pre-determined criteria and statistical analysis with the appropriate adjustments for distribution and variability.

Statistical analysis with Analyse-It Software (Leeds, UK) using individual animal data and 95% confidence levels was conducted on the extent of DNA migration as determined by the OTM and the %LMW. The Shapiro-Wilk test was conducted on the concurrent vehicle control data to determine the normality of the baseline level distribution. Based on the normality of the distribution, the appropriate parametric or non-parametric tests with the appropriate adjustments for homogeneity of variances (if applicable) were used to compare dose groups and/or to determine the presence of a dose response. Log transformation ($\log[n]$) of data that was not normally distributed (3), and/or the use of one-way ANOVA and a Dunnett's post hoc test was also conducted with no change in the results between the different statistical analysis (data not shown).

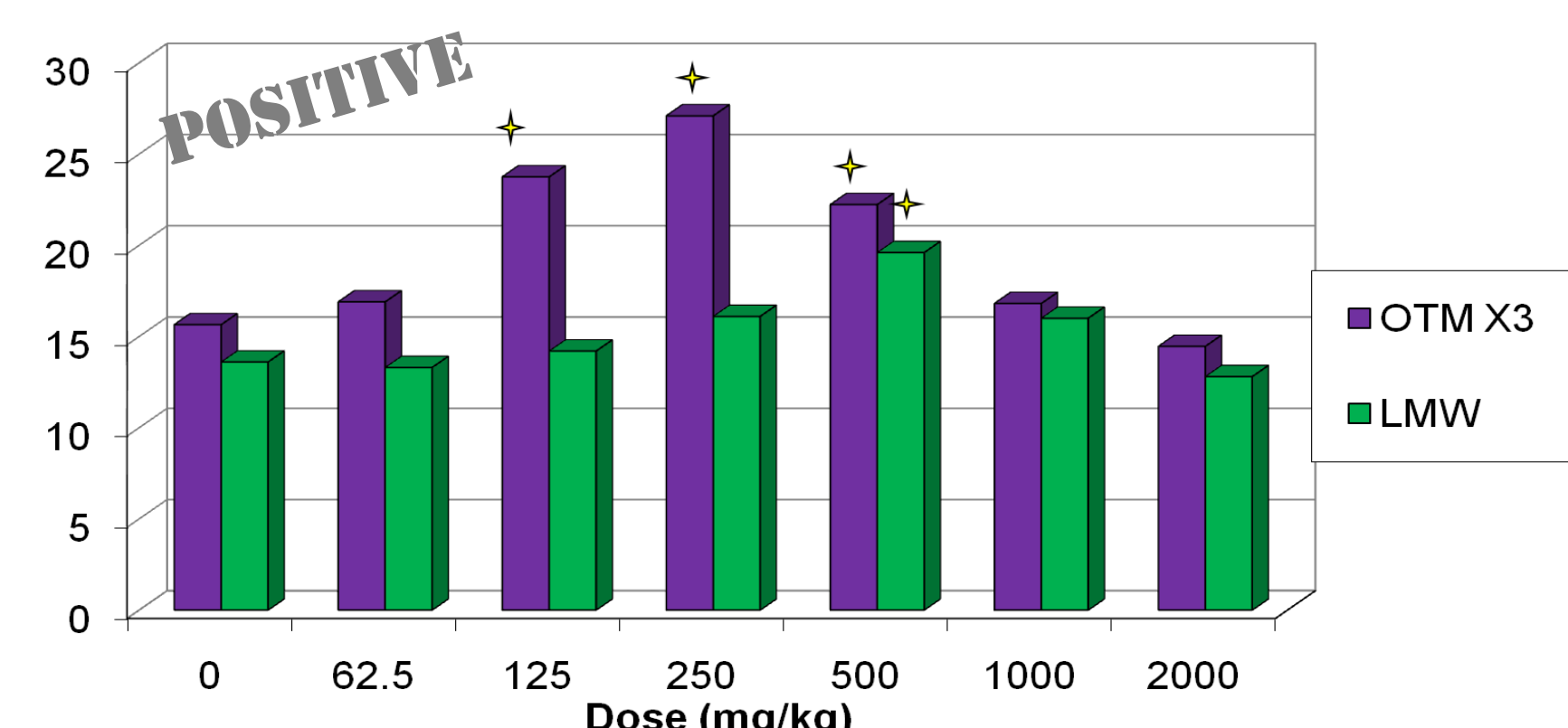
RESULTS

Test Compound 1

Test Compound 1 was initially tested to the maximum dose for non-toxic (i.e. non-lethal) compounds.



A significant and dose-related decrease in DNA migration and %LMW indicated cytotoxicity in the target organ. Therefore, a repeat experiment was conducted at lower doses.

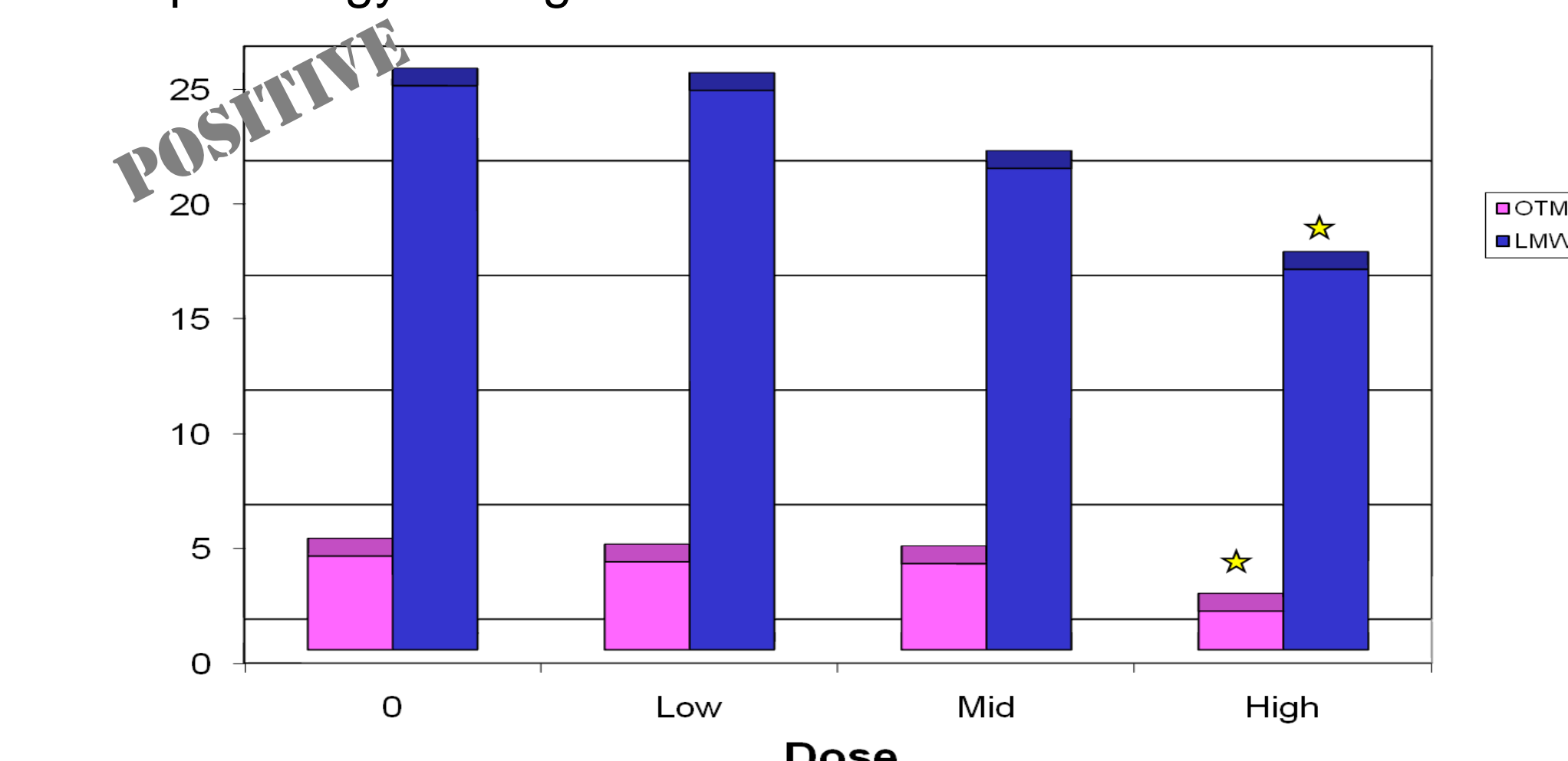


A statistically significant and dose-related increase in OTM but not %LMW was induced at 125 and 250 mg/kg. Therefore, Test Compound 1 was classified as positive for inducing genotoxicity.

RESULTS (CONT.)

Test Compound 2

Test Compound 2 was tested to the MTD based on lethality and histopathology findings.



A statistically significant and dose-related decrease in OTM and %LMW was induced at the highest dose. Test Compound 2 was classified as positive for inducing crosslinks. Histopathology findings and additional experiments (data not shown) confirmed the results.

Test Compound 3

Test Compound 3 was tested to the MTD based on clinical signs of stress.

Dose (mg/kg)	TL		% Tail		OTM		% LMW	
	MEAN	SEM	MEAN	SEM	MEAN	SEM	MEAN	SEM
EMS	64.2	± 2.60	41.8	± 1.49	14.9	± 0.77	**	18.8 ± 4.78
Vehicle	57.7	± 1.07	28.3	± 1.03	9.6	± 0.46		21.8 ± 2.68
15	61.7	± 2.47	30.1	± 1.95	10.3	± 0.93		20.2 ± 3.36
30	65.5	± 2.49	33.1	± 1.49	11.9	± 0.87	*	23.8 ± 5.00
60	59.9	± 1.40	27.6	± 0.56	9.4	± 0.43		19.2 ± 4.11
Trend Test P-Value					0.433			0.353
Trend Test Excluding High Dose P-Value					+0.023*			0.355

Data based on 100 cells scored per animal; 6 animals per dose group. Statistically significant at *p<0.05; **p<0.01.

A statistically significant and dose-related increase in OTM but not %LMW was induced at the mid-dose of 30 mg/kg. The decrease in OTM at 60 mg/kg may have been a result of cytotoxicity. By nature, epithelial-based tissues (e.g. GI tract) are more prone to cytotoxicity and higher variability. **To determine the reproducibility of the results and to determine the shape of the dose response curve, a repeat experiment with an inclusive dose range was conducted.**

Dose (mg/kg)	TL		% Tail		OTM		%LMW	
	MEAN	SEM	MEAN	SEM	MEAN	SEM	MEAN	SEM
EMS	66.8	± 3.05	43.2	± 2.80	14.9	± 1.40	**	14.82.37
Vehicle	56.1	± 2.65	31.0	± 2.80	9.6	± 1.14		14.80.48
10	57.2	± 2.47	30.5	± 3.85	10.0	± 1.59		19.03.97
20	61.2	± 3.02	32.2	± 3.20	10.7	± 1.11		20.86.10
30	54.3	± 3.40	32.6	± 3.85	10.3	± 1.49		17.02.41
40	58.9	± 2.06	35.8	± 4.34	12.0	± 1.88		13.52.38
50	57.8	± 2.94	34.6	± 4.85	11.2	± 1.66		16.02.83
Trend Test P-Value					0.125			0.324

Data based on 100 cells scored per animal; 6 animals per dose group. Statistically significant at *p<0.05; **p<0.01.

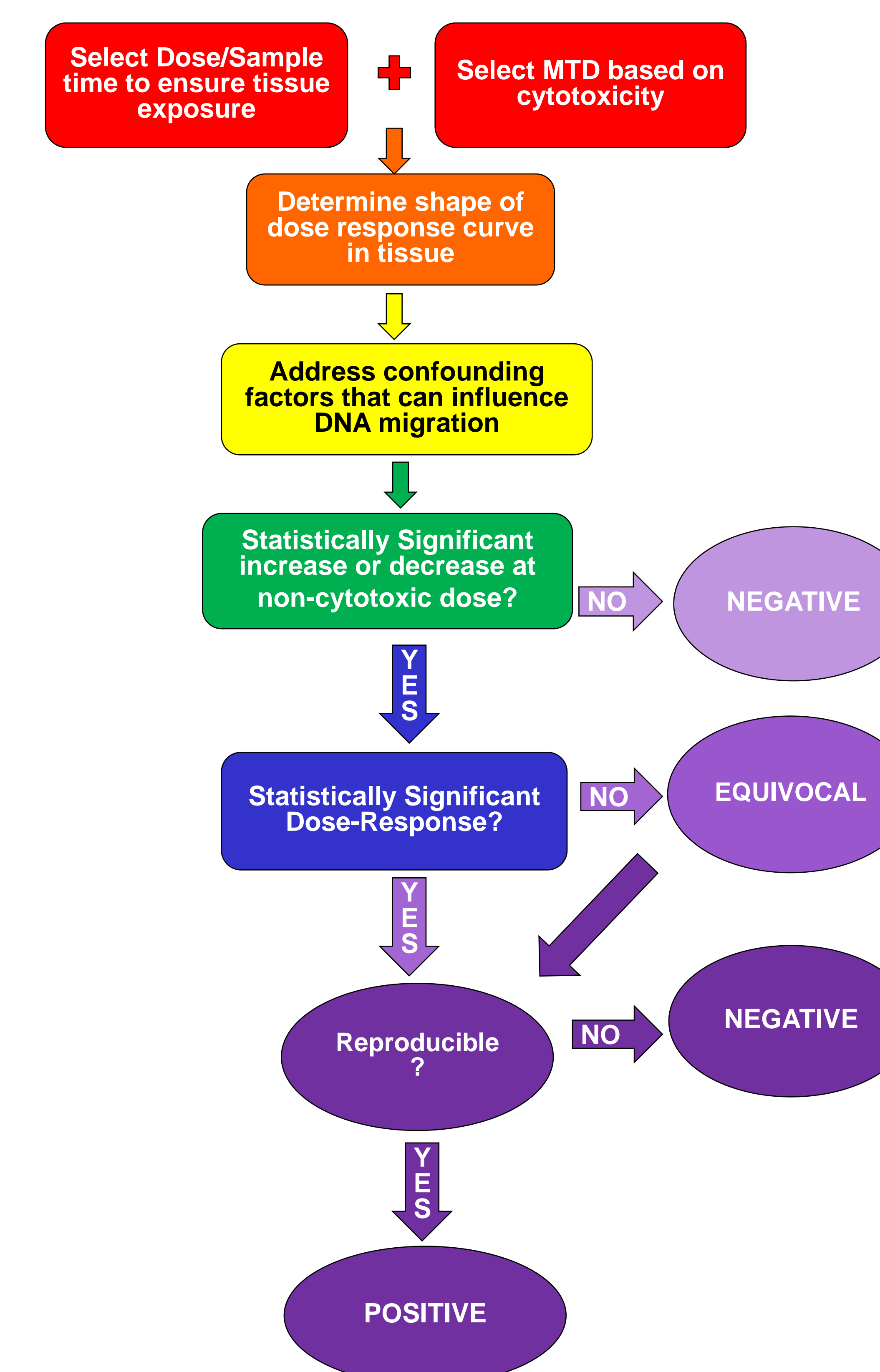
Although the values between the two experiments were similar, Experiment 2 had slightly more variability as noted by the SEMs. As a result, **neither statistical significance nor the general dose response was reproducible.** Therefore, Test Compound 3 was classified as negative for inducing genotoxicity.

RESULTS (CONT.)

Conclusions

Based on multiple GLP *in vivo* comet assay studies conducted and interpreted blind without prior knowledge of the test compounds or mechanisms, the following recommendations for interpreting comet assay data for safety testing are provided:

- Ensure target organ and/or systemic exposure was achieved.
- Use standard statistical analysis methods with pre-determined criteria (e.g. 95% confidence levels) and the appropriate adjustments for distribution and variability.
- Determine dose response curve accounting for confounding factors (e.g. cytotoxicity, crosslink induction) than can influence DNA migration.
- Classify genotoxicity and/or biological relevance based on the reproducibility of a statistically significant response.



References

1. Vasquez, M.Z. (2009) Combining the *in vivo* comet and micronucleus assays: a practical approach to genotoxicity testing and data interpretation. *Mutagenesis* doi:10.1093/mutage/geb060.
2. Hartmann, A., E. Agurell, C. Beevers, S. Brendler-Schwaab, P. Clay, A. Collins, A. Smith, G. Speit, V. Thybaud, and R.R. Tice (2003) Recommendations for conducting the *in vivo* alkaline comet assay. *Mutagenesis* 18(1): 45-51.
3. Wiklund, S.J. and Agurell, E. (2003) Aspects of design and statistical analysis in the comet assay. *Mutagenesis*, 18 pp 167-175.