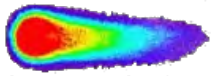


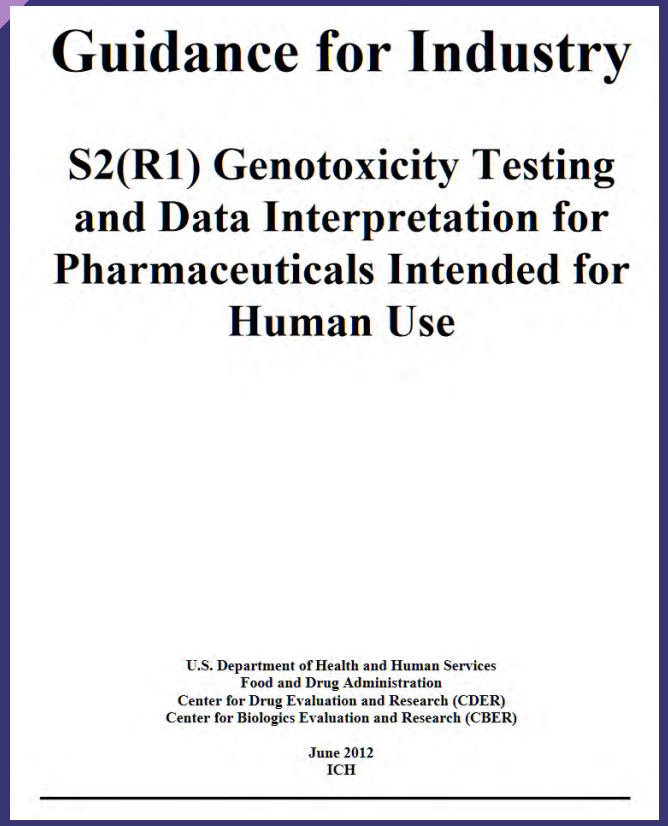
Helix3 Inc.

The Helix3 Difference

Marie Z. Vasquez
Helix3 Inc.

Helix3

Standard Genetox Testing Requirements



Guidance for Industry

S2(R1) Genotoxicity Testing and Data Interpretation for Pharmaceuticals Intended for Human Use

U.S. Department of Health and Human Services
Food and Drug Administration
Center for Drug Evaluation and Research (CDER)
Center for Biologics Evaluation and Research (CBER)

June 2012
ICH

i. Ames bacterial mutation assay
ii. In vitro cytogenetic assay (CA or MN)
iii. In vivo genetox assay in 2 different tissues

BUT:

- investigation of chromosomal aberrations or of gene mutations in endogenous genes is not feasible with standard methods in most tissues.

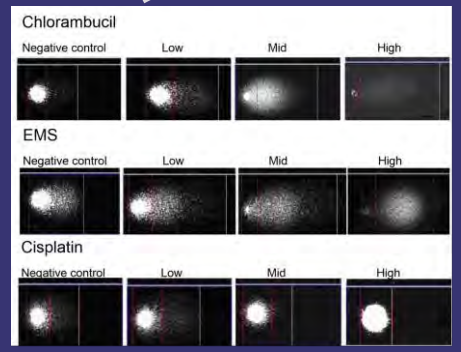
- The TGR assay entails prolonged treatment (e.g., 28 days). Thus the second in vivo assay is typically the **comet assay**

Helix3

The Comet Assay

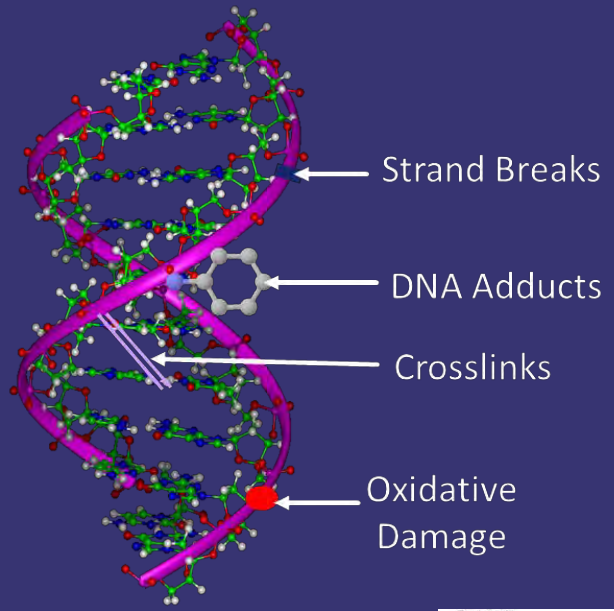
Data Interpretation

Image Analysis



Electrophoresis

Lysis



Exposure

Tissue / Slide Prep

Helix3

Advantages

Flexible

- Any Application / Industry
- Any Test System / Any Organ (no cell division required)
- Any Exposure Method / Route

Helix3

Advantages

Extremely sensitive

- Quantitatively measures individual cell damage requiring few (150) cells per sample
- Detects damage in the absence of any clinical symptoms or lesions
- Low dose concentrations required
- Short exposures (0.5hr - 3 days)

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Why is Comet So Scary?

Helix3

Disadvantages

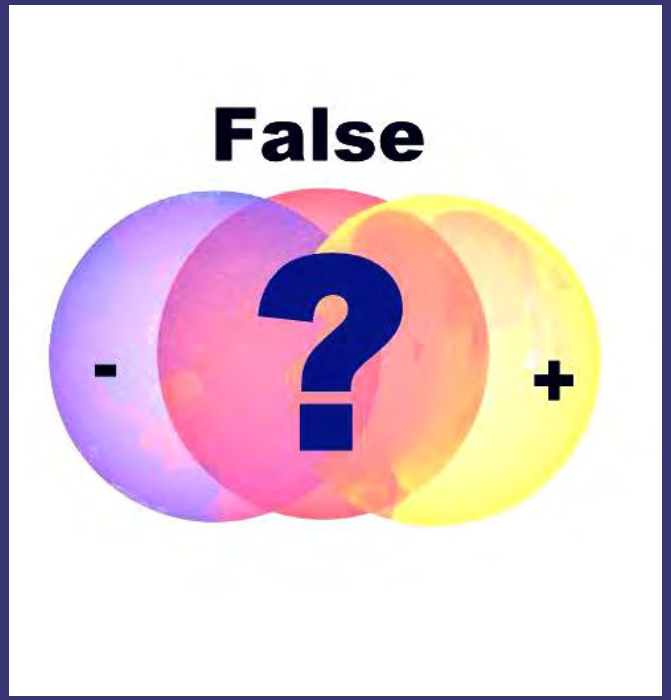
Flexible

- Different tissues, sample times, study design for every test article
- Requires additional homework and non-standard procedures
- Regulators increasingly requiring new / unusual applications

Helix3

Disadvantages

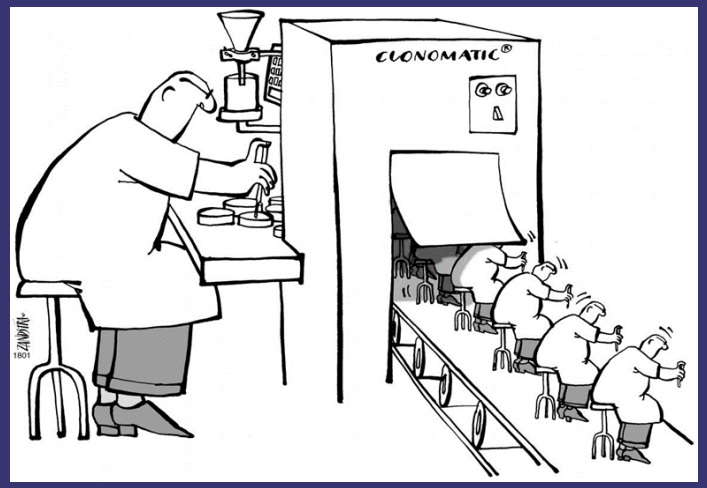
Extremely sensitive



- Susceptible to even minor technical variations and/or methodical bias
- Susceptible to confounding factors (e.g. cytotoxicity)
- Requires substantial training /experience
- Can be difficult to interpret results

Helix3

Why Comet is So Scary



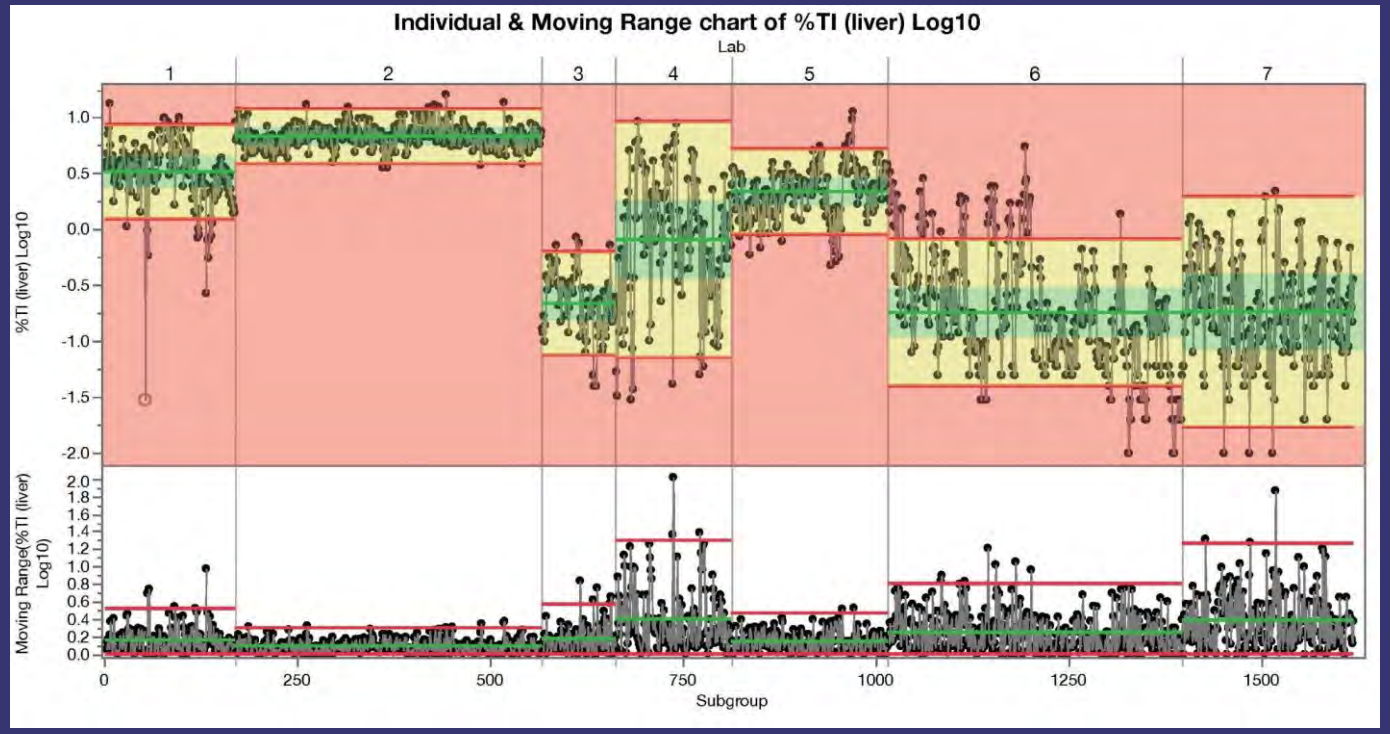
Other CROs focus on volume vs. quality

Comet outcome often determines final regulatory decision

Inadequate training/execution can significantly influence results

Helix3

Poor Quality Control Can Generate False Positives



2022 IWGT Workgroup on Statistical Approaches & Data Interpretation
Presented at EFSA Workshop, May 2, 2022

Stephen Dertinger, Litron, chair Kristine Witt, NIH-NIEHS, co-chair
Carol Beevers, Broughton Group, rapporteur
Andreas Zeller, Roche
Bob Heflich, FDA-NCTR
David Lovell, St. George's University of London
George Douglas & Andrew Williams, Health Canada
Dingzhou (Dean) Li, Pfizer
Daniel Roberts, CRL
Robert Smith, Labcorp
Yoshifumi Uno, MB Medience
Changhui Zhou, InnoStar

Lab 2 = Helix3

- Historical control data (HCD) with high stability and low technical variance
- Low incidence of false positive results
- Successfully refutes and overturns other lab positive results

Other Labs

- "Out of control" HCD with poor stability and high technical variance
- High incidence of false positives
- Unable to refute positives using HCD

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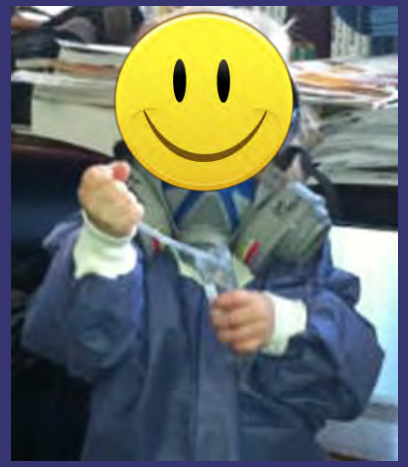
The Comet Assay Experts

Working and learning together for >30 years



Helix3

Why Helix3 Expertise Matters

ECHA
EUROPEAN CHEMICALS AGENCY

https://echa.europa.eu/registration-dossier/-/registered-dossier/13492/7/7/3

Data reported statistical significant increase in mean % Tail DNA in:
- glandular stomach but the registrant claimed that the increase was within historical negative control values (2.67 - 12.74% mean % tail intensity). Therefore was not judged to be biologically relevant.
- non-glandular stomach. The Registrant considers that the increase in percentage tail intensity is caused by the corrosive properties of the substance (ulceration and erosion observed in some animals –not all- at the top dose).
- [No increase was observed in liver]

The Registrant concluded that the test substance is non-genotoxic in liver and glandular and non-glandular stomach tissues.

The explanation of the registrant that the increase in % tail DNA is caused by local toxic effects (as seen in histopath) is not convincing given the data provided (in particular, the mid dose for non-glandular stomach does not show any significant local effect but shows a significant increase in %tail DNA).

EFSA Journal

SCIENTIFIC OPINION

ADOPTED: 4 May 2017

doi: 10.2903/j.efsa.2017.4847

Scientific Opinion on Flavouring Group Evaluation 226 Revision 1 (FGE.226Rev1): consideration of genotoxicity data on one α,β-unsaturated aldehyde from chemical subgroup 1.1.1(b) of FGE.19

Reported results: Statistically significant and dose-dependent increases in %Tail, but all test article dose values were within the range of historical negative controls. Therefore, the test compound is non-genotoxic.

EFSA: "OECD TG 489 is not applicable in this case because the range for [the lab's] historical negative controls is very wide (95% reference range of 0.05-7.14%). Therefore, the Panel concluded that 4,5-epoxydec-2(*trans*)-enal [FL-no: 14.071] is genotoxic in this *in vivo* comet assay in the liver of rats."

Appropriately designing, conducting, and interpreting *in vivo* comet assay studies is critical for successful regulatory submissions

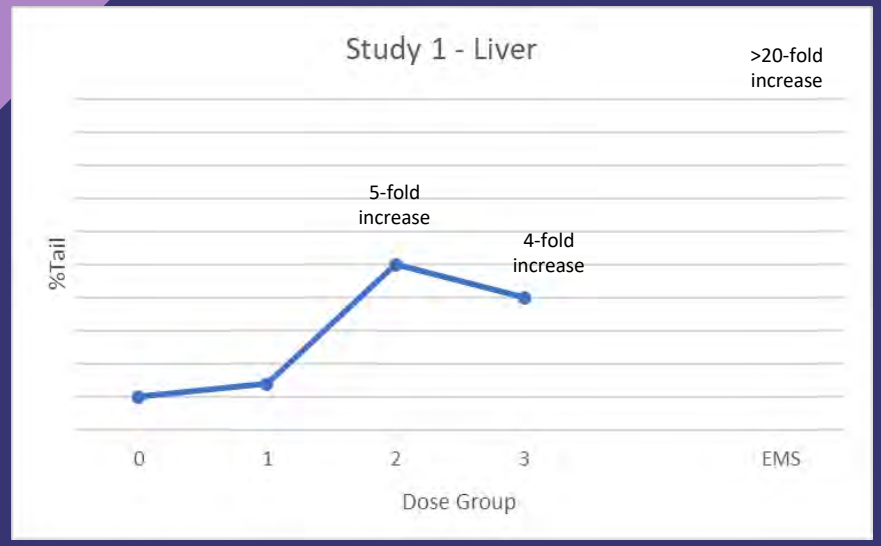
Helix3

Case Studies

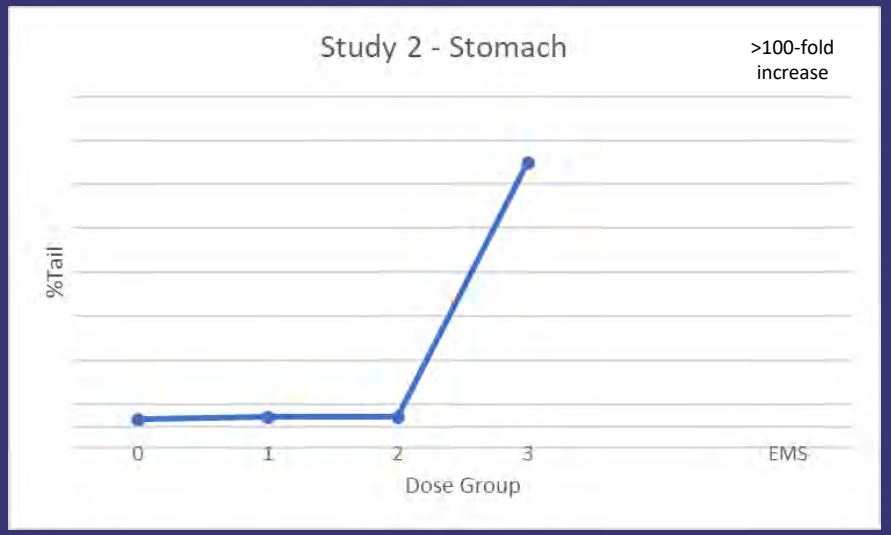
- Regulated studies conducted at other labs generating with false positive comet results
- Technical concerns / issues
- Helix3 technical control methods in repeat studies
- Regulatory conclusions
- Recommendations

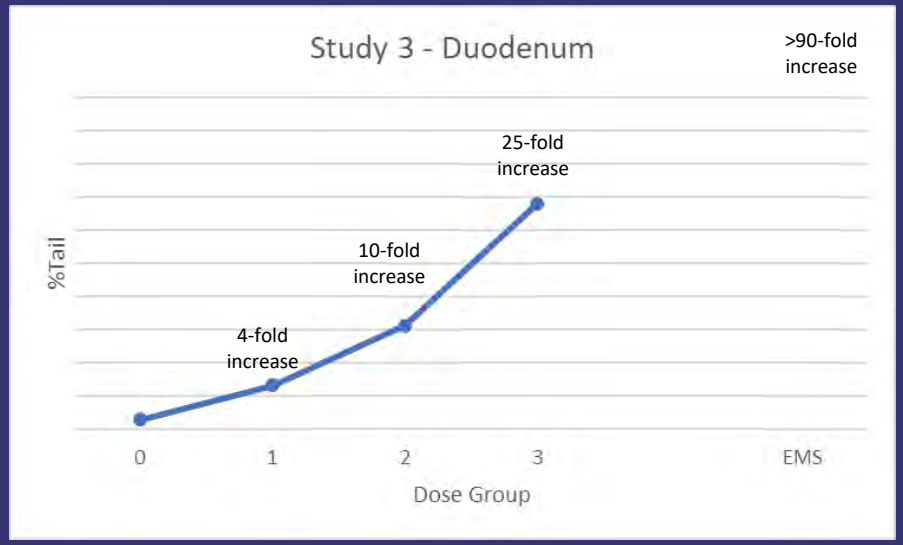
Helix3

3 Studies Conducted at 3 Different Labs

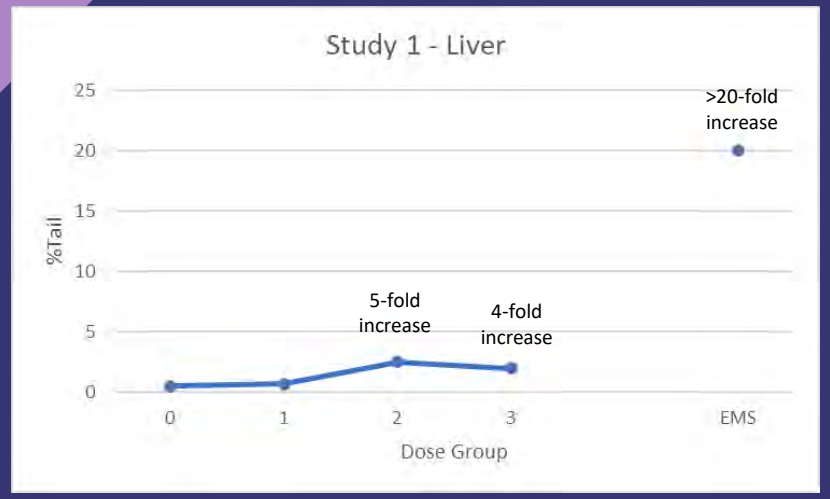


- Statistically significant increases
- Dose-related response
- No histopath findings
- “Hedgehog” increase with dose
- Reproducible in repeat study
- Vehicle within HCD range

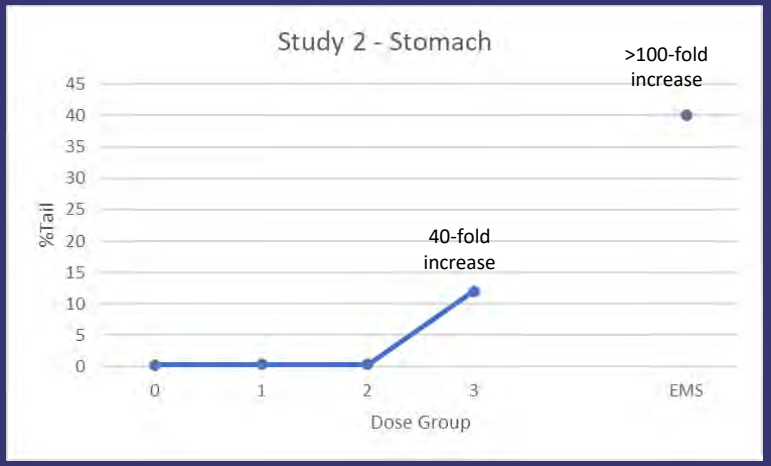


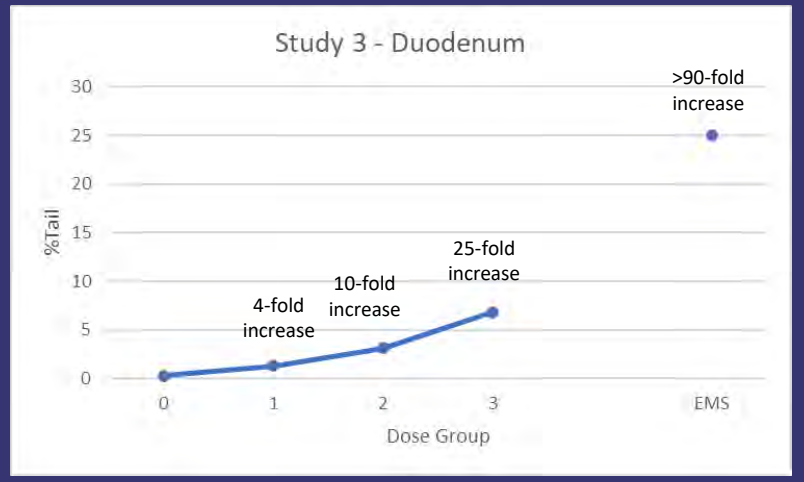


Technical Concerns



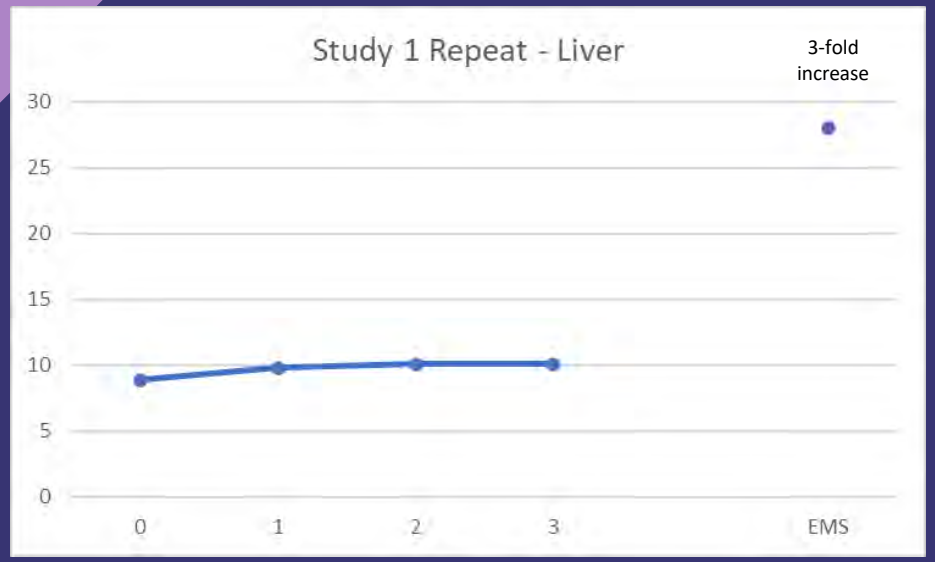
- Mean control %Tail values <1%
- Liver values = GI tract values
- HCD range with >100-fold difference between min and max values
- Overlapping negative and positive control HCD ranges
- TA “hedgehogs” ≥ EMS “hedgehogs”
- Extreme fold-increase detected in positive control



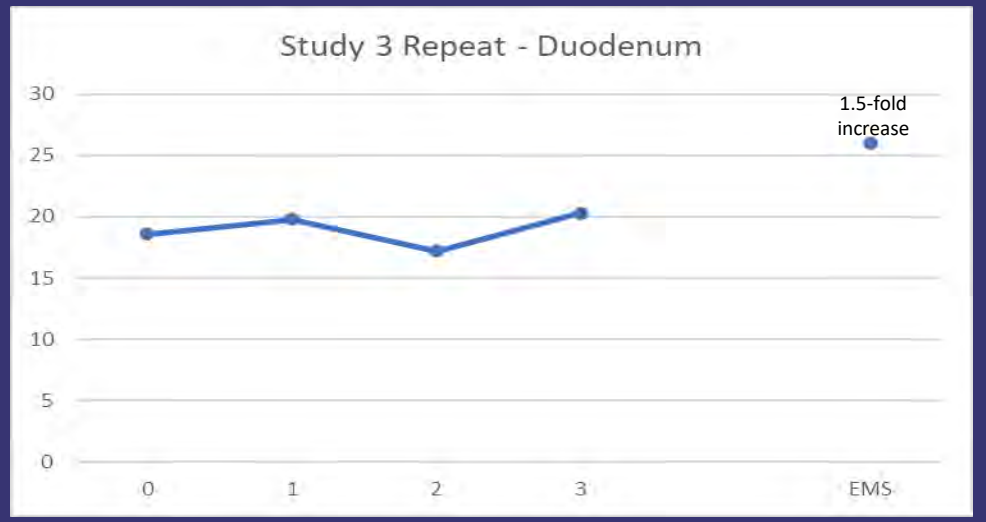


****NOTE: Helix3 recommended AGAINST conducting the repeat studies due to the difficulty of overturning positive in vivo comet results. Sponsors elected to repeat the studies at Helix3 despite our recommendation.**

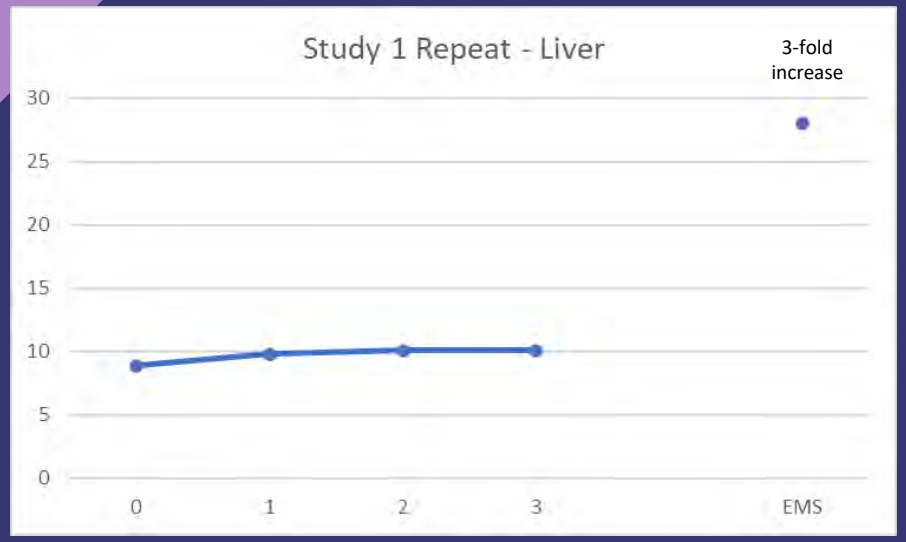
Repeat Studies at Helix3



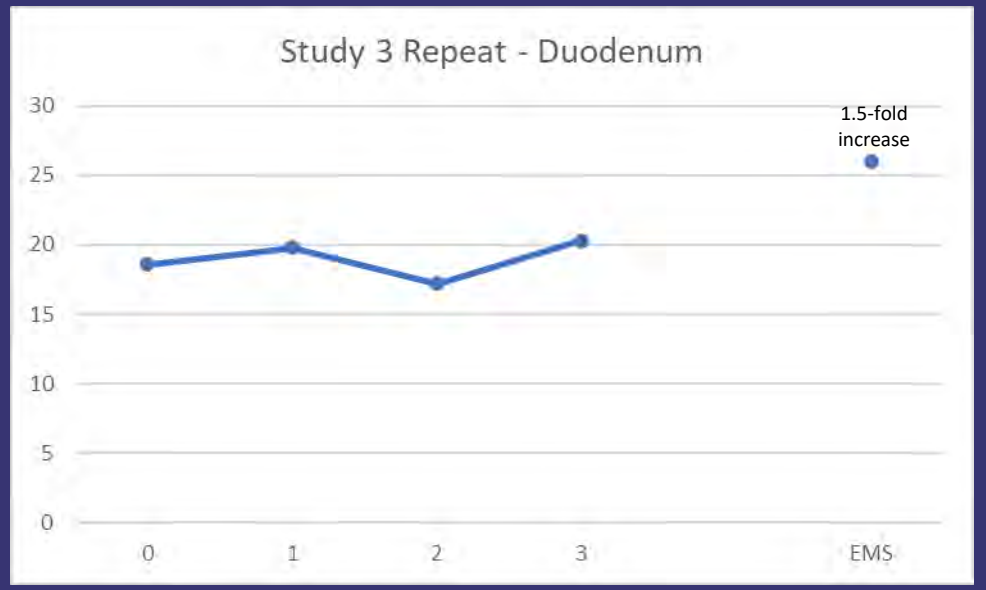
- No increase at any dose
- No dose-related response
- No histopath findings
- No “ghost” increase
- Vehicle within HCD range
- ≥ Plasma concentrations



Helix3 Data Differences



- Mean control %Tail values >5%
- Liver values < GI tract values
- HCD range with ≤3-fold difference between min and max values for all tissues (n=18)
- No overlap between negative and positive control HCD ranges



Helix3

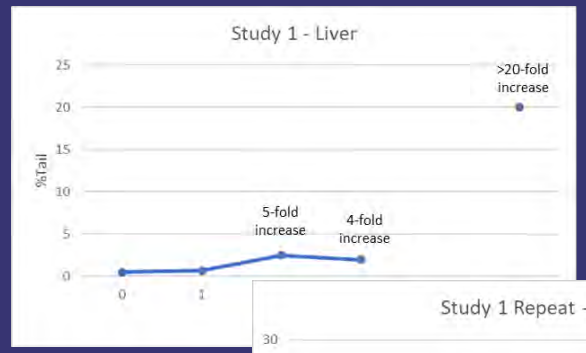
Statistical Analysis

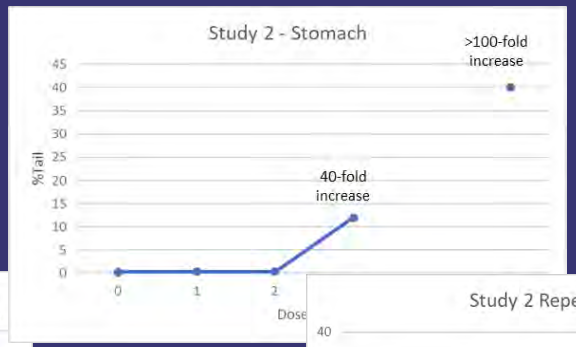
"With %Tail DNA …. There are suggestions that negative control cells should have between 10 and 20% DNA in [the] tail which would obviate statistical problems"

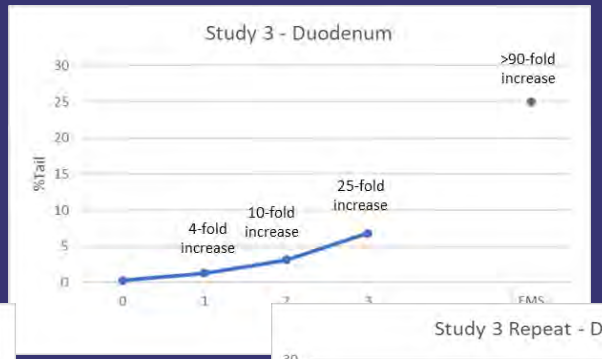
Lovell, David P., and Takashi Omori. (2008) Statistical Issues in the use of the comet assay. Mutagenesis Vol 23 (3) 171-182

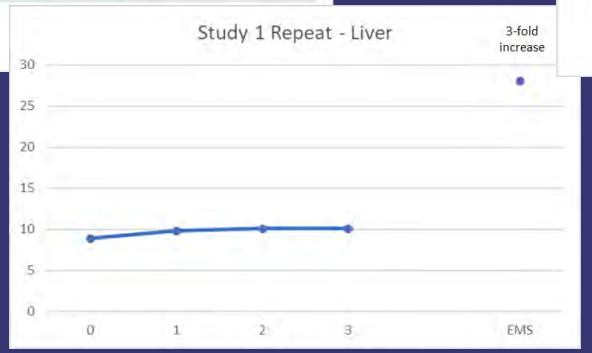
"The best test of whether cells are in a satisfactory condition for comet assay analysis is that control, untreated cells should give comets with a background level of breaks (i.e., ~10% of DNA in the tail [for image analysis scored cells])."

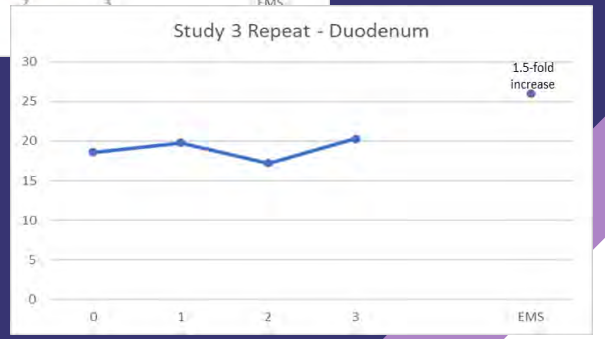
Collins, Andrew (2004) The comet assay for DNA damage and repair. Molecular Biotechnology Vol 26: 249-257









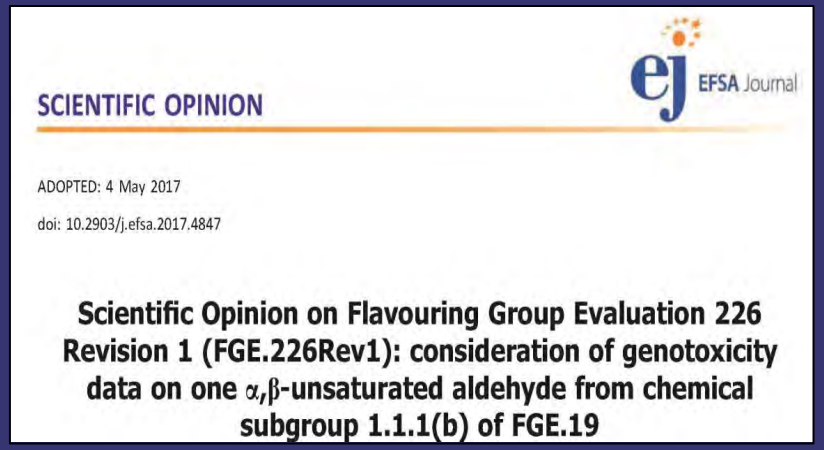


Helix3

Helix3 Study Execution Differences

- Methods and procedures balanced across dose groups
- Consistent timing (<7 minutes) between animals and samples
- Optimal laboratory and procedural conditions
- Minimal technical/scorer bias

Regulators Reject Negative Conclusions Based on Poor Quality Data



SCIENTIFIC OPINION

EFSA Journal

ADOPTED: 4 May 2017

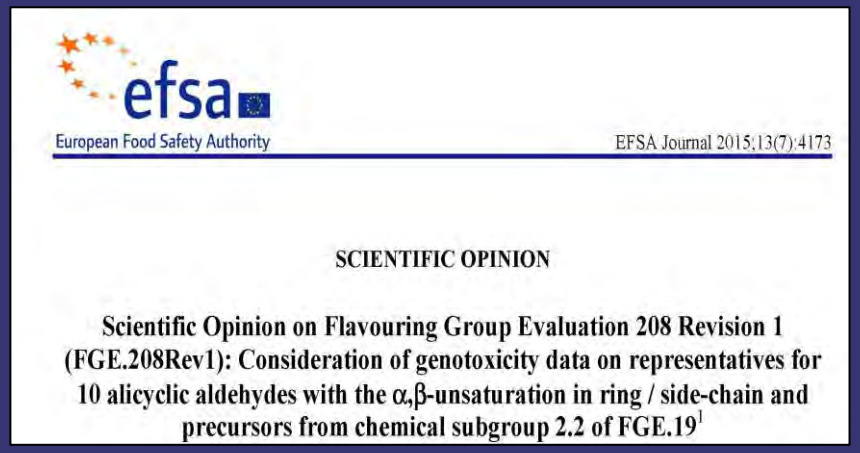
doi: 10.2903/j.efsa.2017.4847

Scientific Opinion on Flavouring Group Evaluation 226 Revision 1 (FGE.226Rev1): consideration of genotoxicity data on one α,β-unsaturated aldehyde from chemical subgroup 1.1.1(b) of FGE.19

- Statistically significant and dose-dependent increases in %Tail
- Lab conclusion: Negative because all test article dose values were within range of historical negative controls

OECD TG 489 is not applicable in this case because the range for historical negative controls is very wide (95% reference range of 0.05–7.14%).

Therefore, the Panel concluded that 4,5-epoxydec-2(*trans*)-enal [FL-no: 16.071] is genotoxic in this *in vivo* comet assay in the liver of rats.



efsa

European Food Safety Authority

EFSA Journal 2015;13(7):4173

SCIENTIFIC OPINION

Scientific Opinion on Flavouring Group Evaluation 208 Revision 1 (FGE.208Rev1): Consideration of genotoxicity data on representatives for 10 alicyclic aldehydes with the α,β-unsaturation in ring / side-chain and precursors from chemical subgroup 2.2 of FGE.19[1]

- 3-4-fold and statistically significant dose-dependent increases in %Tail
- Lab conclusion: Negative because all test article dose values were within range of historical negative controls

The Panel noted that the range for both the negative and positive historical control values were extremely wide for this test laboratory. In addition, there was an overlap of the negative (95 % range: 0.02–11.39) and positive (95 % range: 7.15–65.07) control values.

The Comet arm of this study indicates that *p*-mentha-1,8-dien-7-al induces DNA damage in liver.

Helix3

Regulators Reject Poorly Supported Positive Disqualification Attempts

Table 1: Summary Table Comet Assay – Glandular Stomach

Dose Level	Group Mean % Hedgehogs	Group Mean % Tail Intensity	Group Mean of Mean of Median % Tail Intensity per Animal
Vehicle	3.84 ± 1.44	2.05 ± 0.62	0.69 ± 0.42
480 mg/kg bw	2.65 ± 1.03	3.98 ± 1.71[a]	2.52 ± 1.61[b]
240 mg/kg bw	4.18 ± 1.33	2.92 ± 0.79[b]	1.22 ± 0.63[c]
120 mg/kg bw	4.00 ± 1.40	2.72 ± 0.99	1.18 ± 0.64[c]
Positive (MNU)	6.04 ± 1.01	21.09 ± 1.81[a]	19.28 ± 1.88[a]

[a]= P < 0.001

[b]= P < 0.01

[c]= P < 0.05

Table 2: Summary Table Comet Assay – Non-Glandular Stomach

Dose Level	Group Mean % Hedgehogs	Group Mean % Tail Intensity	Group Mean of Mean of Median % Tail Intensity per Animal
Vehicle	6.12 ± 2.32	6.68 ± 1.88	4.35 ± 1.74
480 mg/kg bw	6.41 ± 1.07	11.42 ± 3.16[a]	9.30 ± 3.87[a]
240 mg/kg bw	3.93 ± 1.04	11.92 ± 3.58[a]	10.29 ± 3.97[a]
120 mg/kg bw	4.83 ± 1.28	7.92 ± 2.42	5.92 ± 2.42
Positive (MNU)	7.78 ± 1.71	41.68 ± 3.60[a]	41.90 ± 4.21[a]

[a]= P < 0.001

ECHA
EUROPEAN CHEMICALS AGENCY

https://echa.europa.eu/registration-dossier/-/registered-dossier/13492/7/7/3

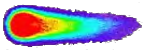
Data reported statistical significant increase in mean % Tail DNA in:

- glandular stomach but the registrant claimed that the increase was within historical negative control values (2.67 - 12.74% mean % tail intensity). Therefore was not judged to be biologically relevant.
- non-glandular stomach. The Registrant considers that the increase in percentage tail intensity is caused by the corrosive properties of the substance (ulceration and erosion observed in some animals –not all- at the top dose).
- [No increase was observed in liver]

The Registrant concluded that the test substance is non-genotoxic in liver and glandular and non-glandular stomach tissues.

The explanation of the registrant that the increase in % tail DNA is caused by local toxic effects (as seen in histopath) is not convincing given the data provided (in particular, the mid dose for non-glandular stomach does not show any significant local effect but shows a significant increase in %tail DNA).

Data provided by Frank Le Curieux, ECHA



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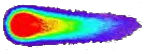
Regulatory Decisions

In all three case studies:

- The quality of the previous studies were considered unreliable
- Regulators accepted the Helix3 negative results over the previous positive results
- The FDA specifically inspected the Helix3 studies and reported no findings
- The test compounds were progressed to clinical trials with at least one progressing to Phase III



Conclusions

1. Helix3 procedural and quality control methods minimize the risk of false positive results

2. Helix3 Background levels of >5% with low variability provides statistical strength and is more resistant to statistical artifacts that can trigger false positive results

3. Comet studies conducted at Helix3 are the most reliable and therefore the least expensive and risky path forward