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1,2,4-THB – Comprehensive Review of Chemical & Toxicological Data
CIR Expert Panel Meeting

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Agenda – 1,2,4-Trihydroxybenzene (THB)

1. Bioavailability of THB
2. Mode of Action of THB
3. Genotoxicity – Key Considerations of Historical Data
4. Genotoxicity – New Alternative Methods (NAM) 3D Skin Models
5. Genotoxicity – NAM *In Vitro* 3D Skin Model Data
6. Genotoxicity – Validation of NAM 3D Skin Models
7. Sensitivity and Specificity of *In Vitro* Methods
8. Genotoxicity – Reassessment
9. Genotoxicity – **NEW *In Vivo*** Data ☆
10. Summary of the OECD/GLP Genotoxicity Data
11. Weight of Evidence/Conclusions

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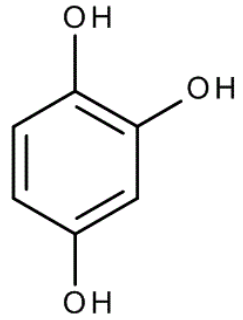
1,2,4-Trihydroxybenzene

1,2,4-Trihydroxybenzene = THB

CAS: 533-73-3

Cosmetic hair dye ingredient



Subject Matter Experts (SMEs)

- Dr. Helmut Greim and Dr. Barry Halliwell
  - Expert analysis of the historical literature in the area of genotoxicity and mode of action of THB (**Exhibit C**)

- Dr. Marilyn Aardema
  - Guidance on the testing battery and a comprehensive review of the weight of evidence (**Exhibit D**)

The body of evidence that will be presented confirms the safe use of THB when used in hair dyes up to 2.5%.

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1. Bioavailability of THB

Dermal absorption of THB was studied using an *ex vivo* skin model (OECD 428/GLP)

- 2 studies: ^{14}C-THB *and* ^{14}C-THB + PTD (p-Toluenediamine as the free base)
  - PTD added to model a conservative and likely use scenario
  - 30 min topical exposure to the skin surface, washed, and returned to water bath for 24hrs
  - Samples were analyzed for radioactivity by Liquid Scintillation Counting (LSC)
  - Systemically available (SA) = Σ epidermis, dermis, receptor fluid
  - Epidermis value includes staining on the surface of the tissue - conservatively elevates the detected epidermal amount (1.85 μg_{eq})
  - One standard deviation (1.76) is added to the calculation (standard practice)

- Results: ^{14}C-THB + PTD study

SA = (1.85 + 0.069 + 0.027) = 1.94 $\mu g_{eq}/cm^2$

SA = 1.94 $\mu g_{eq}/cm^2$ + 1.76 = 3.70 $\mu g_{eq}/cm^2$

- These results correlate with the calculated Log P_{ow} of 0.2 – characteristic of low lipid solubility

Conclusion: THB is poorly absorbed.

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2. Mode of Action of THB

- THB rapidly auto-oxidizes in the presence of oxygen and in aqueous medium which generates H_2O_2 and other Reactive Oxygen Species (ROS)

- The genotoxic effects seen *in vitro* from the generated H_2O_2 and other ROS, as a result of THB reacting are:
  - not seen *in vivo*
  - mitigated by ROS scavenging mechanisms in living organisms

- SCCS has reviewed and approved H_2O_2 for cosmetic use despite significant *in vitro* data showing genotoxic effects (SCCP, 2005)

2. Mode of Action of THB

- Some *in vitro* studies have documented THB to be genotoxic because of the **generation of Reactive Oxygen Species (ROS), such as H_2O_2** (SLR Ref. 27 & 28)

- However, *in vitro* research demonstrated an **attenuation of effect owing to the addition of ROS scavengers** which decomposes ROS to water and oxygen, preventing ROS damage (SLR Ref. 22 & 25)

$$2H_2O_2 \xrightarrow{\text{ROS Scavengers}} 2H_2O + O_2$$

- Publications in the SCCS final opinion of THB also document the **attenuation of effect owing to the addition of scavengers** (SCCS 1598/18 Ref. 22, 26, 32)

The genotoxic effects seen *in vitro* from generated H_2O_2 and other ROS, as a result of THB reacting, is mitigated by ROS scavenging mechanisms in living organisms

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3. Genotoxicity – Key Considerations of Historical Data

- The historical literature is a result of academic research and may not be reliable in identifying hazard for regulatory risk assessment purposes

- Many of the historical *in vitro* studies of THB have deficiencies that are acknowledged in Table 2 of the Draft SLR (09/28/23)
  - THB test article not well characterized (purity/impurity)
  - Unvalidated methodologies
  - Lack of solvent information
  - Lack of dosing solution analysis
  - Non-GLP

4. Genotoxicity - New Alternative Methods (NAMs) 3D Skin Models

NAMs needed to assess genotoxicity endpoints **reliably**:

- 3D skin models proposed as non-animal alternative by the Cosmetics Europe Genotoxicity Taskforce (status update by Pfuler *et.al*., 2014)

- This taskforce project was undertaken in part, to **minimize potential "misleading positives" seen *in vitro* that were not observed *in vivo*** (e.g., OECD Tier 1 testing methods like the Ames Assay)

- Significant **advantages of new 3D skin models**:
  - Primary human keratinocytes used as source
  - Metabolically competent and mimic *in vivo* assay
  - Dermal exposure is relevant for cosmetic ingredients
  - Minimize unnecessary *in vivo* follow-up testing on misleading positives in non-cosmetic uses

5. Genotoxicity – NAM *In Vitro* 3D Skin Model Data

3D Comet Assay - Phenion® Full-Thickness Human Skin Assay of THB (GLP)

- Due to potential interference of colorimetric measurements multiple measures of cytotoxicity were employed:
  - Adenylate kinase (AK)
  - Lactate dehydrogenase (LDH)
  - Intracellular adenosine triphosphate (ATP)

- Topical application conducted after range finding study
  - Experiments: 48hr exposures with and without aphidicolin
  - Aphidicolin - a DNA repair inhibitor added to improve the sensitivity of the assay
  - Negative and positive controls met acceptance criteria for valid experiment

Conclusion: THB did not induce DNA damage at any dose in either experiment (Dossier Section 3.7).

5. Genotoxicity – NAM *In Vitro* 3D Skin Model Data

3D Reconstructed Skin Micronucleus (RSMN) Assay of THB (GLP)

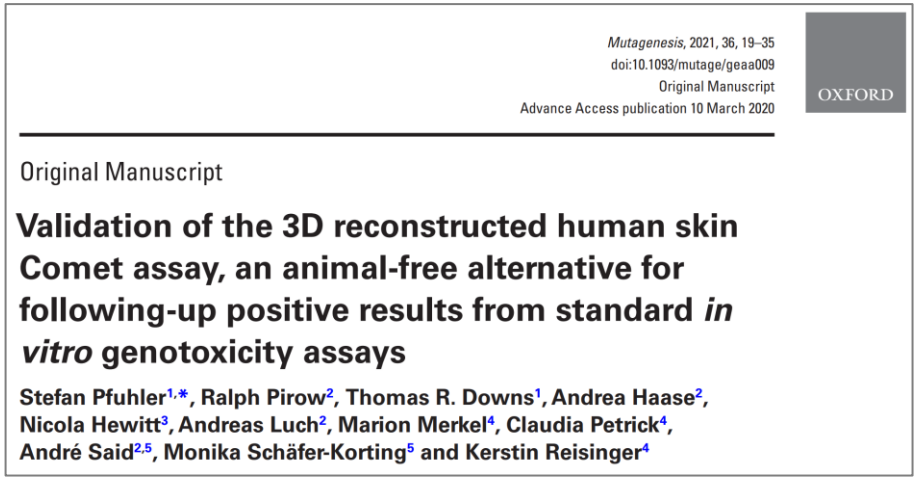
- Cytotoxicity measured by the method of relative viable cell count (RVCC)
- Topical application conducted after dose range finding study
  - Experiment 1: 48hr exposure 12- 224 µg/cm^2; Additional daily applications at 24, 48hr
  - Experiment 2: 72hr exposure 12- 224 µg/cm^2; Additional daily applications at 24, 48, 72hr
- Negative and positive controls met acceptance criteria for valid experiment

Conclusion: No statistically significant increases in micronucleated polychromatic erythrocytes (MnPCEs) was observed at any dose in either experiment (Dossier Section 3.7).

6. Genotoxicity - Validation of NAM 3D Skin Models

NAM 3D Skin models validated and accepted into the OECD Tier 2 Test Guideline development program for dermally exposed compounds

- 3D Comet Assay Validation*
  - Overall accuracy of 83%
    - sensitivity of 77%
    - specificity of 88%
- RSMN Validation**
  - Overall accuracy of 80%
    - sensitivity of 75%
    - specificity of 84%



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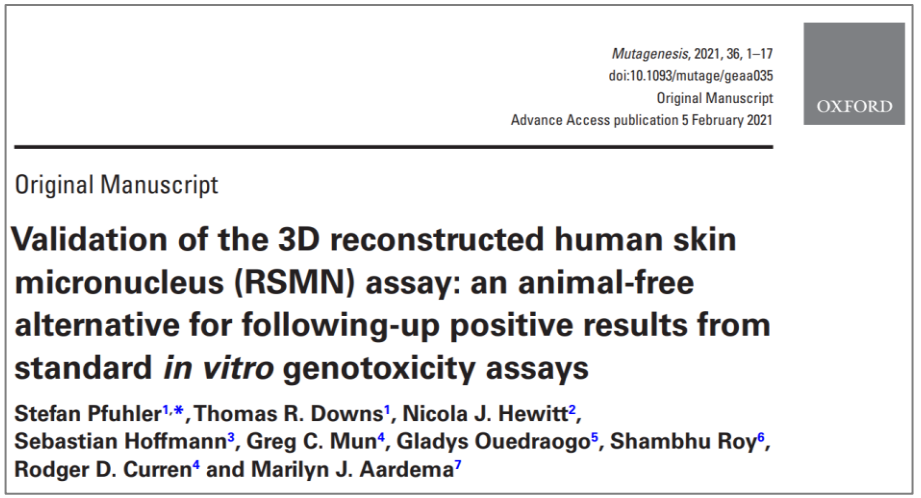
Advance Access publication 10 March 2020

OXFORD

Original Manuscript

Validation of the 3D reconstructed human skin Comet assay, an animal-free alternative for following-up positive results from standard *in vitro* genotoxicity assays

Stefan Pfuhler[1,*], Ralph Pirow[2], Thomas R. Downs[1], Andrea Haase[2], Nicola Hewitt[3], Andreas Luch[2], Marion Merkel[4], Claudia Petrick[4], André Said[2,5], Monika Schäfer-Korting[5] and Kerstin Reisinger[4]



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Stefan Pfuhler[1,*], Thomas R. Downs[1], Nicola J. Hewitt[2], Sebastian Hoffmann[3], Greg C. Mun[4], Gladys Ouedraogo[5], Shambhu Roy[6], Rodger D. Curren[4] and Marilyn J. Aardema[7]

*Pfluer et.al., Validation of the 3D reconstructed human skin Comet assay, an animal-free alternative for following-up positive results from standard in vitro genotoxicity assays. Mutagenesis Mutagenesis. 2021 Jan; 36(1): 19–35

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7. Sensitivity and Specificity of *In Vitro* Methods

	Ames	Micronucleus (MN)	3D Comet	3D RSMN
Sensitivity	0.6	0.81	0.77	0.75
Specificity	0.77	0.48	0.88	0.84
Total	**1.37**	**1.29**	**1.65**	**1.59**

- The sum of *Sensitivity* and *Specificity* should be between 1.5 - 2.0* for data to be considered high-quality and high-value
- Ames & Micronucleus (MN) *In Vitro* assays can be helpful when considered as part of the weight of evidence but may not conclusive**
- The NAM methods for assessing potential for genotoxicity are superior to the Ames & MN *In Vitro* for dermally exposed chemicals like hair dye ingredients

Conclusion: The data we collected under GLP with these models can be considered to contribute valuable information to the overall weight of evidence.

*Power, M et.al., Principles for high-quality, high-value testing, Evid Based Med. 2013 Feb;18(1):5-10.
**Walmsley, RW, Billington, N., How Accurate is *in vitro* prediction of carcinogenicity? Br J Pharmacol. 2011 Mar; 162(6): 1250–1258

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8. Genotoxicity – Reassessment

- Despite providing SCCS our negative result from the repeat Ames Assay with ROS scavengers and the additional NAM studies, SCCS concluded that genotoxicity could not be ruled out on the strength of the historical literature data alone
- SCCS commented that due to the conflicting historical *in vitro* data, an *in vivo* study would be the only way to generate confirmatory evidence that THB is non-genotoxic (as was done for the approval of H_2O_2 in cosmetics) – data, that even if generated, SCCS would not review due to the animal testing ban on cosmetics
- Combe desired to objectively demonstrate that THB is safe for use
- In order to accomplish this, Combe undertook an *in vivo* Micronucleus(MN) study in mice (Exhibit B)
- SCCS is unable to review this **new *in vivo* data** due to the animal testing ban on cosmetics

Mouse Bone Marrow Micronucleus (MN) *in vivo* assay in 2019 (GLP)

An ***in vivo*** study is a definitive way to confirm the absence of genotoxic effect

Objective: to repeat the MN study in mice that was completed in 1993 in compliance with the current OECD 474

Method:

- Route of Exposure was Intra-peritoneal injection
- Maximum Tolerated Dose (MTD) was selected by the study Principal Investigator after the dose range-finding assay in male and female mice
- Pivotal study was conducted male mice as the most sensitive based on clinical observations and mortality
- Dosing included 50% and 25% of the selected MTD

9. Genotoxicity – NEW *In Vivo* Data

Mouse Bone Marrow Micronucleus (MN) *in vivo* assay in 2019 (GLP)

Results:

- MTD of 25 mg/kg was selected by the Principal Investigator
  - 50% MTD = 12.5 mg/kg
  - 25% MTD = 6.25 mg/kg

- Positive and negative controls met acceptance criteria for valid experiment

Conclusion: No statistically significant increase in micronucleated polychromatic erythrocytes (MnPCEs) was observed at any dose. This supports the historical in vivo data collected at 50 mg/kg.

10. Summary of OECD/GLP Genotoxicity Studies

OECD #	Study Date	Type	Description	Result
471 (1997)	Mar. 2004	*In Vitro*	Ames Bacterial Gene Mutation Assay	**Weak Positive (TA98, TA100)**
471 (1997)	Aug. 2015	*In Vitro*	Ames Bacterial Gene Mutation Assay	**Weak Positive (TA1537)**
471 (1997) *mod*	Dec. 2018	*In Vitro*	Ames Bacterial Gene Mutation Assay **with ROS Scavengers**	**Negative**
473 (1983)	1995	*In Vitro*	Mammalian Chromosome Aberration Assay	**Negative**
476 (1997)	Nov. 2004	*In Vitro*	Mammalian Cell Gene Mutation Assay (hprt locus)	**Negative**
487 (2014)	Aug. 2015	*In Vitro*	Micronucleus Assay (HPBL)	**Negative**
OECD # Pending	Jan. 2017	*In Vitro*	3D Skin Comet Assay	**Negative**
OECD # Pending	Jan. 2019	*In Vitro*	3D Reconstructed Skin Micronucleus Assay	**Negative**
474 (1983)	Jan. 1993	*In Vivo*	Micronucleus Assay in Mice	**Negative**
474 (2016)	Dec. 2019	*In Vivo*	Micronucleus Assay in Mice	**Negative**

11. Weight of Evidence/Conclusions

- THB demonstrates genotoxic effects in some *in vitro* studies
- Inclusion of **ROS scavengers** in our Ames assays **eliminated the genotoxic effect** – concordant with other in vitro ROS scavenger studies in the literature.
- Many historical literature data may be **unreliable** for regulatory risk assessment due to deficiencies (chemical characterization, dosing solution analysis, unvalidated methods, non-GLP)
- SME assessments included in Combe dossier **conclude that THB does not pose a genotoxic risk** and like H_2O_2, triggers the formation of ROS via identical mechanisms
- Four *in vitro* GLP studies in Combe dossier confirm THB is non-mutagenic/non-genotoxic (OECD)
- *In vitro* GLP NAM models, 3D Comet and 3D RSMN show THB is non-mutagenic/non-genotoxic
- These 3D NAM methods have now been **accepted for OECD Guideline development**
- **THB confirmed to be non-mutagenic/non-genotoxic in two *in vivo* GLP studies** (OECD)

Weight of evidence indicates that THB does not pose a genotoxic risk to the consumer when used as a hair dye ingredient up to 2.5%.

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Thank you for your time

Questions?

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