The HET-MN (Hen`s Egg Test for MicroNucleus induction) - An *in vitro* genotoxicity assay reflecting systemic availability of test compounds

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Introduction

- Genotoxicity hazard identification starts with *in vitro* testing across different sector industries. These assays are based on two-dimensional cell cultures, which are limited in reflecting routes of exposure and intrinsic metabolic capacity.
- In contrast, the HET-MN is characterized by a high intrinsic metabolic capacity enabling testing of compounds under conditions which mirror ADME without adding S9 mix.
- HET-MN combines developing hen´s egg, as used for vaccine production or HET-CAM studies, with the analysis of micronucleus frequency in erythrocytes.
- Physiologically and legally considered a non-animal test method.
- Intended as an addition to the *in vitro* toolbox to follow-up on positive findings from initial 2D *in vitro* studies.
- We report here on the HET-MN validation and the test design, as well as the metabolic capacity of the test system.

Test design

Study design
(1) Solubility study
(2) Dose range finder
- Top dose 100 mg/egg, i.e., in line with max. dose of *in vivo* studies.
- Maybe reduced due to low solubility/ viability.

Experimental design
- Solvent control: distilled water, isopropyl myristate, 10% DMSO, 10% ethanol
- Positive control: cyclophosphamide (pro-mutagen)
- At least three concentrations of test compound

Read-out parameter
- Evaluation of 6 eggs per dose groups with 1000 cells per egg, i.e., 6000 cells per dose/control group
- Micronucleus frequency in erythrocytes
- Viability of eggs
- Change of PCE/NCE ratio considered not sufficiently sensitive.

Data evaluation
(1) Validity check
- Quality criteria for SC and PC
- Viability of dose group ≥40%
- Bioavailability shown by

(i) increase of MN-rate or
(ii) decrease of viability.

(2) Statistical analysis
- Lab-specific threshold + Umbrella-Williams-Test

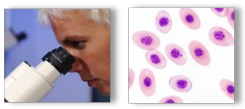
(3) Consideration of biological relevance

ADME

Application on day 8 of egg development

Sampling on day 11

Microscopical analysis of erythrocytes



HET-MN allows mirroring systemic availability of compounds, i.e., ADME

- **A**bsorption
- **D**istribution via the vessel system
- **M**etabolism in the liver and the yolk sac membrane
- **E**xcretion into bladder equivalent (allantois)

Validation results

Parameter	Lab 1	Lab 2	Lab 3	Overall
Sensitivity	67	89	67	84
Specificity	100	100	95	98
Accuracy	83	94	82	91

- > 30 chemicals tested double-blinded
- Phase I: each chemical tested in 3 labs, Phase II + III: each chemical tested in 1 lab.
- Independent chemical selection and statistical analysis

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Metabolism results

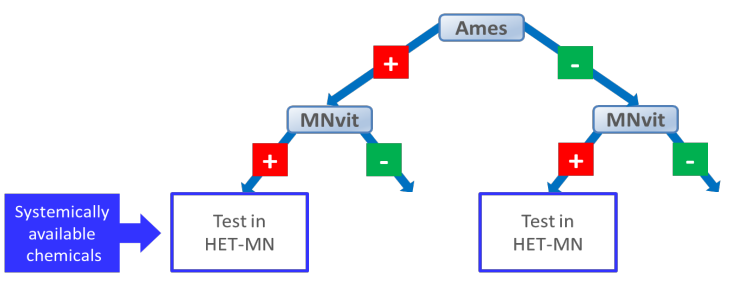
Phase I

Substance	HET-MN result	Involved CYP P450 according to literature
2-Acetylaminofluorene (2AAF)	+	**1A1/1A2**
2-Aminoanthracene (2AA)	+	**1A2, 4B1, 1B1**
2,4-Diaminotoluene (2,4-DAT)	+	**1A2**
4-nitroquinoline 1 oxide (4-NQO)	+	**NQO1**
Benzo[*a*]pyrene	+	**1A1, 1B1**
Cyclophosphamide	+	**2B6, 2C9, 3A4**
Dimethylnitrosamine (DMN)	+	**2B4, 2E1, 2A6**
Diethylnitrosamine (NDEA)	+	**2A6**
7,12- Dimethylbenz*[a]*anthracene (DMBA)	+	**1B1, 1A1**
Ifosfamide	+	**2B1, 2B4, 2B5**
Acrylamide	+	**2E1**
Cytarabine	+	Only **3A4**-mediated drug interactions

Phase II (quantitative data)
- Clear enzyme activity of glutathione S-transferase, UDP-glucuronosyltransferase, sulfotransferase.
- Enzyme activity [pmol enzyme activity/min/mg protein] of the developing hen´s egg (own data) lies within the ranges reported for mouse/rat (literature) and human liver (literature).

The >10 references used for comparison available upon request.

Follow-up strategy of orally exposed chemicals



For orally exposed ingredients with positive results in the MNvit the suggested follow up assay is the HET-MN assay.

Conclusions

This assay has a number of advantages:

- ✓ Mirrors systemic availability of compounds.
- ✓ Not an animal experiment.
- ✓ Clear intrinsic metabolic activity negates the need to add rat liver S9 mix.
- ✓ Excellent predictivity (91% accuracy).
- ✓ Chicken eggs (Specific Pathogen Free eggs used for vaccine production or HET-CAM) globally available, with no difference observed between suppliers so far.
- ✓ The HET-MN has gained regulatory acceptance from the EU Scientific Committee on Consumer Safety (SCCS), which suggests the assay as a follow-up to help address positive findings from initial testing with the classical *in vitro* test battery.

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References: (1) Reisinger K et al., Hen's Egg Test for Micronucleus Induction (HET-MN). Methods Mol Biol. 2019. doi: 10.1007/978-1-4939-9646-9_10. (2) Reisinger K, et al., The Hen's Egg Test for Micronucleus-Induction (HETMN): Validation data set. 2021. geab016. doi: 10.1093/mutage/geab016. Online ahead of print. (3) Maul et al., Validation of the hen's egg test for micronucleus induction (HETMN): Detailed protocol including scoring atlas, historical control data and statistical analysis. Mutagenesis. 2021. geab026. doi: 10.1093/mutage/geab026. Online ahead of print.