P30-65

IN VITRO BLOOD–BRAIN BARRIER MODELS FOR TOXICOLOGICAL ASSESSMENT SUPPORTING SAFE AND SUSTAINABLE BY DESIGN WITHIN THE CHIASMA PROJECT

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INTRODUCTION

CHIASMA PROJECT:

Accessible **I**nnovative **M**ethods for the **S**afety & Sustainability **A**ssessment of **Ch**emicals & Materials

The safety assessment of chemicals and materials has relied strongly on *in vivo* animal testing, forming the basis of current regulatory frameworks. It is shown that *in vivo* animal tests have disadvantages and limitations in various aspects. Consequently, *in vitro* New Approach Methodologies (NAMs) are urgently needed to generate sufficient data addressing regulatory decisions.

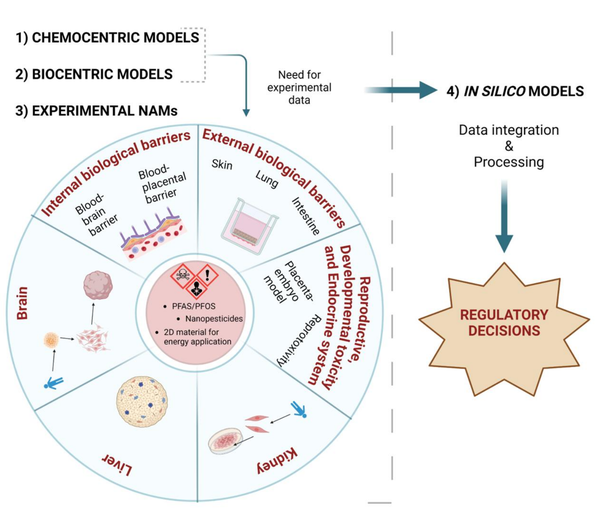


Figure 1: Overview of the CHIASMA project. (The figure is created with BioRender.com.)

In order to assist the regulatory approval process for the next generation of hazard and risk assessment of chemicals, the CHIASMA project aims to establish a novel and improved Safe and Sustainable by Design (SSbD) framework. This platform integrates in-project validated *in vitro* NAMs and *in silico* approaches, with the goal of reducing and eliminating the need for animal testing (*Figure 1*). A key focus of this project is to develop reliable methodologies that meet essential performance criteria, including repeatability, reproducibility, and transferability [1].

The blood-brain barrier (BBB) is a highly selective and protective interface between the bloodstream and the brain. The BBB has been identified as a gap in OECD activities related to developmental neurotoxicity, as its absence in many *in vitro* models may lead to inaccurate risk estimation. For this purpose, a co-culture transwell insert model including the hCMEC/D3 human brain microvascular endothelial cells and differentiated SH-SY5Y neuroblastoma cells has been developed. In the phase I, chemicals indicated as “well-known” toxins have been tested to evaluate the model performance and interlaboratory validity. In the phase II, Per- and polyfluoroalkyl substances (PFAS), nanopesticides and 2D materials for energy applications will be assessed.

METHODS

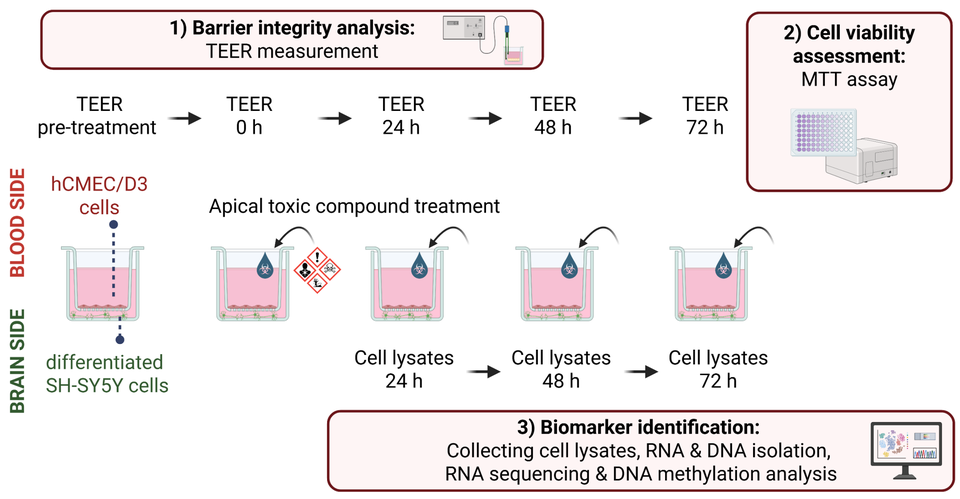
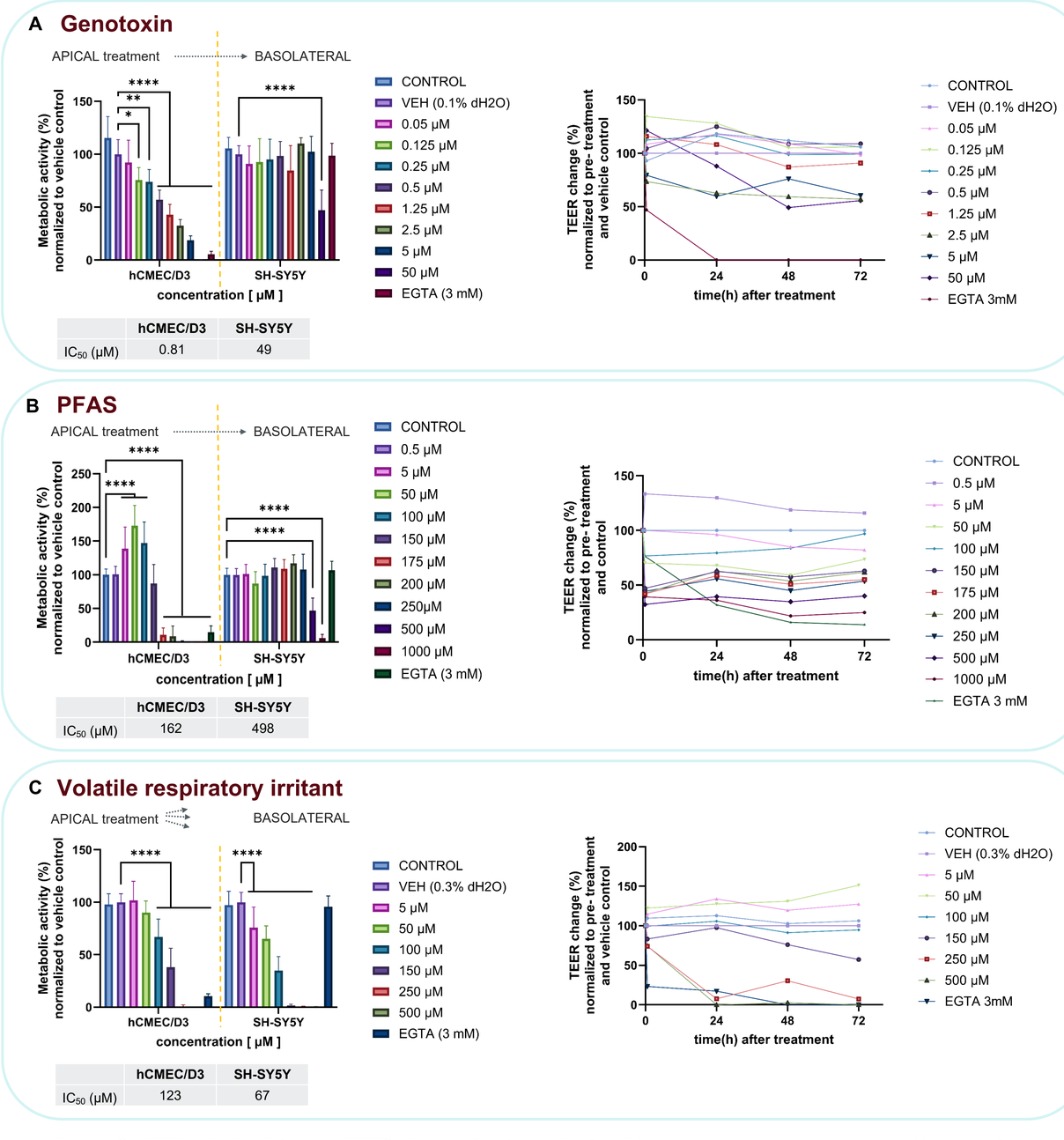


Figure 2: Schematic workflow of the BBB model for toxicity evaluation. Lysates were drawn after TEER measurement without toxin renewal. (The figure is created with BioRender.com.)

1) **Differentiation of SH-SY5Y cells:** SH-SY5Y cells were cultured in DMEM/F12 supplemented with 10% fetal bovine serum (FBS), 1% L-glutamine, and 1% penicillin/streptomycin (P/S). Differentiation was performed in two phases: (i) 10 µM retinoic acid with 15% FBS, and (ii) serum-free medium supplemented with 50 ng/mL BDNF, with L-glutamine and P/S maintained in both phases.
2) **Culturing hCMEC/D3 cells:** hCMEC/D3 cells were cultured in EBM-2 medium supplemented with ascorbic acid, FBS, human Fibroblast Growth Factor (hFGF), HEPES, and P/S. Inserts were pre-coated with Collagen IV and Fibronectin overnight at 37°C prior to cell seeding. The FBS content was reduced from 5% to 0.25% on day 5 of the culture.
3) **Development of co-culture system:** The differentiated SH-SY5Y cells were combined with the apically seeded hCMEC/D3 cells at day 11 of differentiation and for the basolateral side the differentiated SH-SY5Y cells were supplemented with DMEM/F12 without BDNF during the co-culturing.
4) **Transendothelial electrical resistance (TEER) measurement:** TEER was measured using chopstick electrodes before treatment, and at 0 h, 24 h, 48 h, 72 h after exposure with toxic compounds. (Results in *Figure 3*)
5) **Toxin treatment:** Toxins were treated apically and the apical media renewed after 24h, 48 h and 72 h before respective TEER measurements.
6) **MTT cell viability analysis:** Following TEER measurement at 72 h, cell viability was assessed by MTT assay based on mitochondrial dehydrogenase activity. (Results in *Figure 3*)
7) **RNA sequencing:** Cell lysates were collected after 72 h of compound exposure. Total RNA and DNA isolation was performed using the AllPrep DNA/RNA/Protein Mini kit®. RNA quantity and purity were assessed using a NanoDrop spectrophotometer. mRNA library preparation and RNA sequencing were performed by Novogene on an Illumina NovaSeq X Plus platform. (Results in *Figure 4*)

RESULTS



	hCMEC/D3	SH-SY5Y
IC_{50} (µM)	0.81	49

	hCMEC/D3	SH-SY5Y
IC_{50} (µM)	162	498

	hCMEC/D3	SH-SY5Y
IC_{50} (µM)	123	67

Figure 3: MTT cell viability and TEER change for the selected Phase I chemical compounds: A) a genotoxin, B) a PFAS, C) a volatile respiratory irritant. MTT cell viability was assessed after 72 h exposure in hCMEC/D3 and SH-SY5Y cells (*left panels*). Cell viability (%) was normalized to either untreated or vehicle controls. TEER measurements [Ω*cm^2] were normalized to pre-treatment measurement (0 h) and either untreated or vehicle controls and presented as TEER change (%) (*right panels*). Metabolic activity (%) data represented as mean ± standard deviation, N= 2-7 independent experiments, n=3-5 replicates per experiment. Statistical significance was assessed using two-way ANOVA followed by Tukey’s multiple comparisons test. A p value < 0.05 was considered statistically significant.

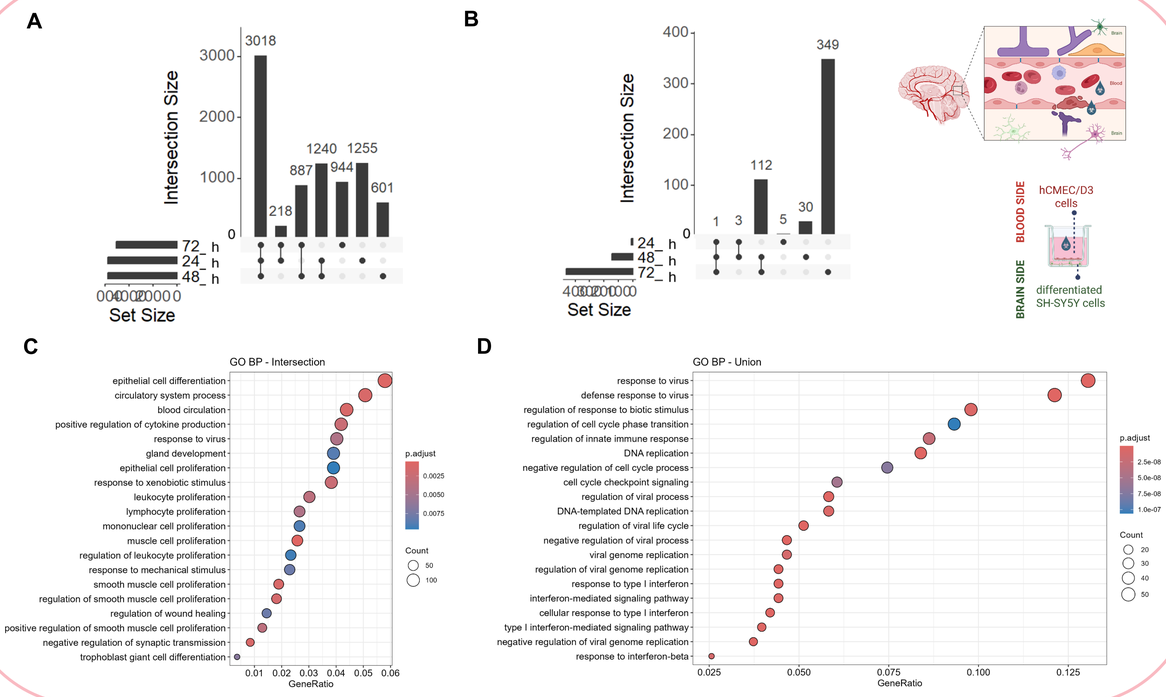


Figure 4: Biomarker identification by RNA sequencing following genotoxin exposure. Quantification of differentially expressed genes (DEGs) in A) hCMEC/D3 and B) differentiated SH-SY5Y cells. Enrichment plots of DEGs showing the preliminary functions in C) hCMEC/D3 and D) differentiated SH-SY5Y cell lines, which will be contributing to the AOP fingerprint. Interestingly, more targets were identified in hCMEC/D3 cells than in differentiated SH-SY5Y cells, which indicates that the apically applied toxin may need to permeate the endothelial cell barrier to exert effects on the neuronal cells on the brain side.

OUTLOOK

- Testing phase 2 chemicals (including nanopesticides, PFAS, 2D materials for energy applications).
- Evaluating genotoxicity using the γH2AX assay to compare the applicability of different NAMs for toxicological assessment.
- *In silico* analysis of molecular data (RNA sequencing, DNA-methylation) to identify toxin specific biomarkers & develop assays for toxicological assessments and regulatory applications.

REFERENCES

[1] Weber, P., Arnesdotter, E., Attar, N.,(…) Turan Sorhun, D. Neuhaus, W., Serchi, T. (2025). CHIASMA: Advancing chemicals and materials safety and sustainability assessments through innovative integration of in vitro and in silico (new approach) methodologies. *Computational and structural biotechnology journal*. https://doi.org/10.1016/j.csbj.2025.11.032.

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