The comet assay with standard aquatic test organisms as an alternative test system for environmental risk assessment of human pharmaceuticals

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Background

Human pharmaceuticals find their way into aquatic ecosystems e.g. via municipal wastewater, where they pose a potential threat to aquatic organisms. A comprehensive environmental risk assessment (ERA) is necessary to minimize their risks. In accordance to the guidance on human pharmaceuticals (EMA, 2006) for an ERA, effects on aquatic organisms are determined based on standardized guidelines including (OECD 201, 210 and 211). In a previous project (German Environmental Agency, FKZ 3718 65 420 1) alternative test systems for an ERA were identified. One of the identified test systems was the Comet Assay, a genotoxicity test that quantifies DNA damage by measuring the fraction of DNA that migrates out of a nucleus during gel electrophoresis (Tail intensity or TI%), with environmentally relevant organisms. The aim of this study was to establish the comet assay as a genotoxicity assay for the environmental risk assessment of human pharmaceuticals, both *in vivo* with *Daphnia magna* and *in vitro* with a cell line of *Danio rerio* liver cells (ZF-L).

Comet assay

The comet assay is a genotoxicity assay applicable to practically all eukaryotic cells, capable of visualizing the frequency of DNA strand breaks [1].

- Cells are mixed with agarose
- Agarose gels are placed onto a microscopy slide
- Lysis solution removes membranes
- An alkaline solution causes DNA to unwind and separate into single strands
- Electrophoresis draws loose DNA fragments out of the nucleus
- Endpoint: Tail Intensity in percent, fraction of DNA in the comet tail

Low frequency of strand breaks: **Low Tail Intensity**, more DNA in Comet "Head"

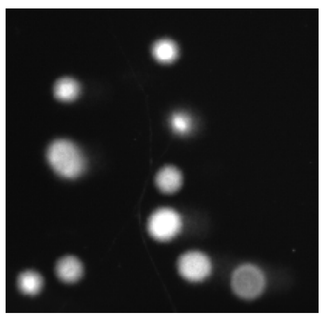

Figure 1: Comet Assay with ZF-L cells (Control)

High frequency of strand breaks: **High Tail Intensity**, more DNA in Comet "Tail"

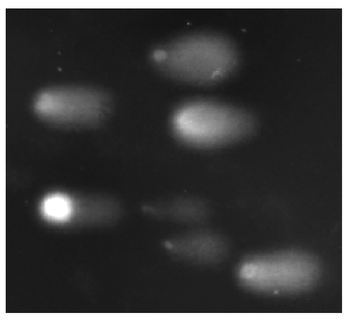

Figure 2: Comet Assay with FZ-L cells 48 h exposition with 50 mg/L Cyclophosphamid)

Method

	Daphnia magna	*Danio rerio* liver cell line ZF-L
Exposition	• 15 individuals aged <24h per replicate • Exposition in 50 mL beakers • 48 hours of exposition • Triplicates	• approx. 100 000 cells per replicate • Exposition in 24 well plate • 48 hours of exposition • Triplicates
Tissue preparation	• Mechanical homogenization with glass microbeads • Filtration with a 50 µm PET filter	• Trypsination
Comet Assay	• Mixing of cell suspension with low melting point agarose • Placement of 6 gels per replicate on agarose coated microscopy slides • 60 minutes lysis • 20 min incubation in alkaline gel electrophoresis solution • 20 min gel electrophoresis at 24 Volt	

Results

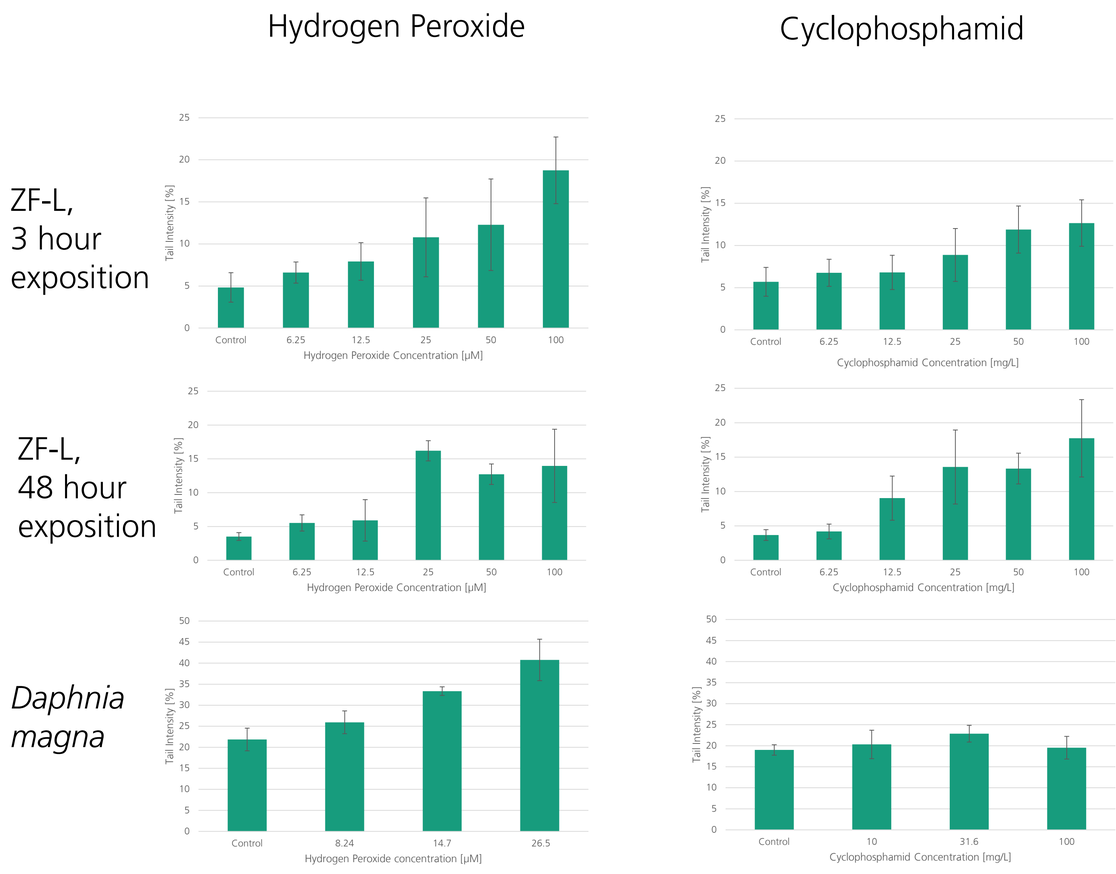

Figure 3: Tail Intensity over concentration of reference genotoxicants Hydrogen Peroxide (left) and Cyclophosphamid (right) after the comet assay with ZF-L (top and middle) and Daphnia cells (bottom)

Table 1: Lowest observable effect concentrations (LOEC) for human pharmaceuticals determined via the comet assay with *D. magna cells* and *D. rerio* liver cells (LOEC was determined as the lowest concentration showing a statistically significant difference to control)

Substance name	Use	LOEC in D. magna comet assay	LOEC in ZF-L in vitro comet assay
Abamaciclib	Chemotherapy	> 10 mg/L	> 10 mg/L
Cyclophosphamid	Chemotherapy	> 100 mg/L	**12.5 mg/L**
Dabrafenib	Chemotherapy	> 50 mg/L	> 15 mg/L
Edoxaban	Cardiology	> 50 mg/L	> 25 mg/L
Imatinib Mesylate	Chemotherapy	> 50 mg/L	**100 mg/L**
Palbociclib	Chemotherapy	> 10 mg/L	> 10 mg/L
Pitavastatin	Cardiology	**50 mg/L**	> 50 mg/L
Ribociclib	Chemotherapy	> 40 mg/L	Not determined yet
Rosuvastatin	Cardiology	> 5 mg/L	Not determined yet

Conclusions

- **Both the in vivo and in vitro method were successfully established and confirmed by testing cyclophosphamide (DNA alkylating agent) and hydrogen peroxide (reactive oxygen species) as reference substances.**
- **The in vivo method showed a low sensitivity, while the in vitro method appeared to be more sensitive.**
- **Test concentrations (mg/L range) were above the predicted environmentally relevant concentrations (ng/L range) and above the data of chronic daphnia and fish tests.**
- **The comet assay appears to be appropriate to assess the genotoxicity of a test substance, but due to the low sensitivity compared to the established test systems (e.g. OECD 210/211) it is not necessarily useful for an ERA of human pharmaceuticals.**

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References :

[1] Ostling, O.; Johanson, K. J. (1984): Microelectrophoretic study of radiation-induced DNA damages in individual mammalian cells. In Biochemical and Biophysical Research Communications 123 (1), pp. 291–298. DOI: 10.1016/0006-291x(84)90411-x.