Pharmaceuticals and fish: effects in alternative in vitro cellular and embryonal models

Research centre for toxic compounds in the environment

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Introduction

Fishes are well established and important model species in aquatic toxicology. They represent higher order consumers and respond to the changes in their environment as a whole. Fish embryo acute toxicity test (FET) is an accepted alternative for acute toxicity tests on adult fishes for industrial chemicals. Reflecting the horizontal 3R EU legislation FET is about to be incorporated into the Veterinary Medicinal Products regulations. However, more information is still needed for validation of alternative tests and their applications for environmental risk assessment.

Pharmaceuticals are major micropollutants in water environment and represent a prevailing problem for EU water bodies. Pharmaceuticals (similar to e.g. pesticides or biocides) are special among the other industrial pollutants as they are intentionally produced to have biological effect in the organism and prediction of their side effects in nontarget organisms is very complicated.

Objectives

In the present study, two assays considered as alternatives for short term toxicity test for fish have been used to investigate effects of 8 different pharmaceuticals. The models included *in vitro* assay with a cell line derived from rainbow trout gills (RT Gill W1), which currently undergoes validation by inter-laboratory tests, and extended FET test with zebrafish (*Danio rerio*) embryos. The observed results have been compared with the data on fish toxicity from literature.

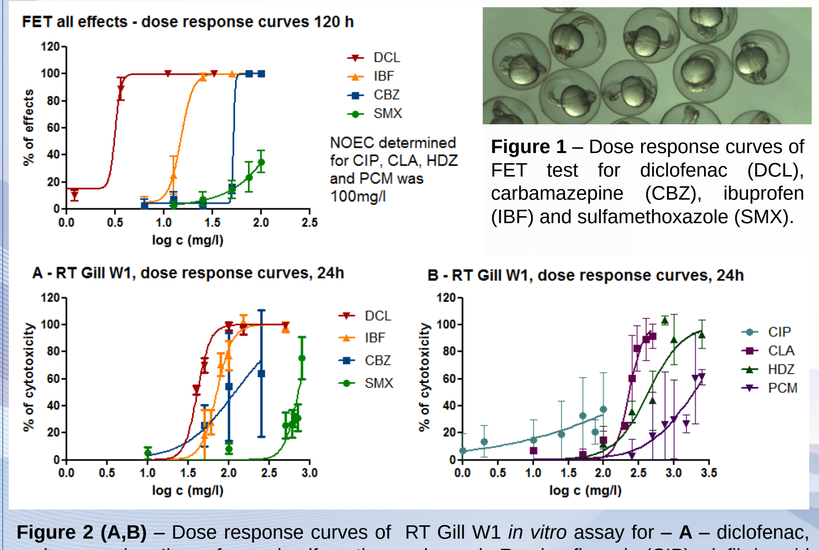

Figure 1 – Dose response curves of FET test for diclofenac (DCL), carbamazepine (CBZ), ibuprofen (IBF) and sulfamethoxazole (SMX).

Figure 2 (A,B) – Dose response curves of RT Gill W1 *in vitro* assay for – **A** – diclofenac, carbamazepine, Ibuprofen and sulfamethoxazole, and –**B –**ciprofloxacin (CIP), clofibric acid (CLA), hydrochlorothiazide (HDZ), and paracetamol (PCM).

Materials & Methods

RT Gill W1 *in vitro* assay was adapted from the procedure for cytotoxicity assay for RT Gill W1 ring study [2]. Cells were exposed in **serum-free media** in dark for 24 hours. Then the cytotoxicity was evaluated as the main endpoint using the combination of three dyes: Alamar Blue (AB), CFDA-AM, and Neutral Red (NR).

FET test was conducted using the modified protocol based on OECD 236 guideline with *Danio rerio* as model species [3]. Embryos were exposed in static or semi-static conditions up to 120 hours post fertilization. Exposures were done in glass crystallization dishes with 20 embryos per well in 40 ml of exposure media. The evaluated endpoints included mortality, malformations and other sub-lethal effects (e.g. heart beat frequency, length).

Correlations of the obtained and literature data were calculated in STATISTICA 12 software using the nonparametric Spearman correlation coefficient.

Conclusions

- ✓ Pharmaceuticals as a broad and diverse group need a simple, reliable and fast assays capable to identify and prioritize their potential environmental adverse effects in cost-effective manner with respect to 3R regulations.
- ✓ Results of FET test from this study correlate very well with available literature data on adult fish acute toxicity as well as with obtained RT Gill W1 data and also with other *in vitro* toxicity values reported for fish.
- ✓ RT Gill W1 cell line stands out among other *in vitro* assays excluding the serum from exposure and the results correlate well with obtained FET effective concentrations.
- ✓ Both assays – FET as well as RT Gill W1 – may be suitable alternatives also for testing of pharmaceuticals, and as they are suitable for high throughput screening, they represent a valuable tool for environmental risk assessment.

FET and RT Gill W1 results

FET test

- ➢ for all substances a limit test with highest concentration 100mg/l was made
- ➢ CBZ, DCL, IBF and SMX which exhibited effects at limit test were tested for full dose-response relationship.
- ➢ DCL was the most toxic and would be categorized as toxic to aquatic organisms according to the EU-Directive 93/67/EEC [1] with EC50=2.1 mg/l.
- ➢ IBF, CBZ and SMX had the EC50 14.8, 51.9 and ~154.9 (extrapolated) mg/l, respectively, which would classify IBF and CBZ as harmful to aquatic organisms.

RT Gill W1 *in vitro assay*

- ➢ full dose response relationship could be derived for all substances except ciprofloxacin where the EC50 value exceeded the limit of solubility.
- ➢ RT Gill W1 results showed generally higher effective concentrations than FET but the toxicity of individual substances followed the same trend.

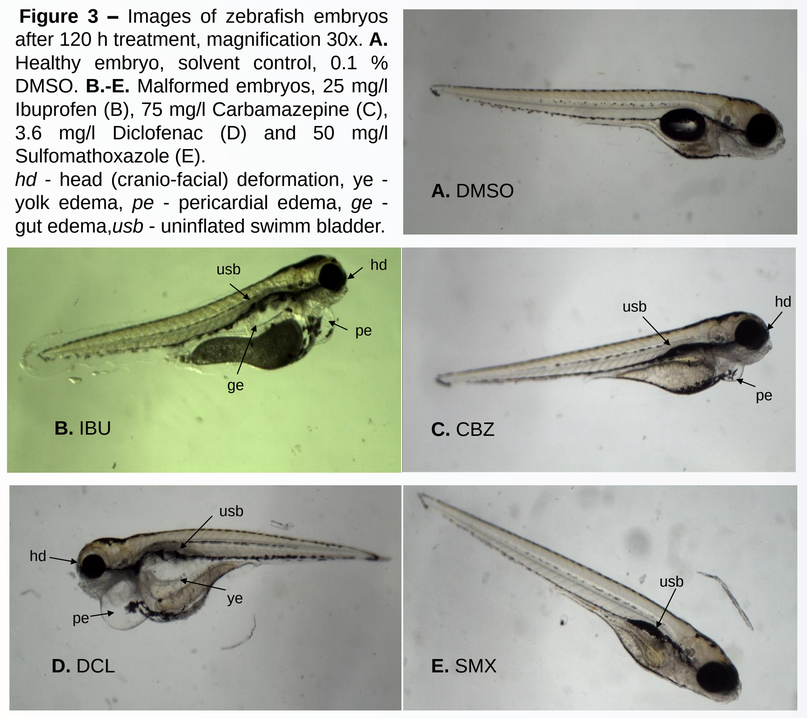

Figure 3 – Images of zebrafish embryos after 120 h treatment, magnification 30x. **A.** Healthy embryo, solvent control, 0.1 % DMSO. **B.-E.** Malformed embryos, 25 mg/l Ibuprofen (B), 75 mg/l Carbamazepine (C), 3.6 mg/l Diclofenac (D) and 50 mg/l Sulfomathoxazole (E).
hd - head (cranio-facial) deformation, ye - yolk edema, *pe* - pericardial edema, *ge* - gut edema,*usb* - uninflated swimm bladder.

	Obtained results			Literature data	
Substance	**FET all effects EC50 (mg/l)**	**RT Gill W1 - EC50 minimal (mg/l)**	**Acute toxicity on adult fish LC50 (mg/l)**	***In vitro* fish toxicity EC50 (mg/l)**	**Chronic fish toxicity (mg/l)**
Carbamazepine	**51.9**	**131.9**	38.2 ± 16,5 [3],[4],[5]	117 [9]	2.5 ± 2.6 [14],[15],[16],[5],[17]
Ciprofloxacin	>100	>100	>100 [33]	850 [34]	
Clofibric acid	>100	**231.6**	526.5 [6]	345 ± 331 [10],[11]	25.3 ± 38,6 [18],[19],[20],[21]
Diclofenac	**2.1**	**28.48**	8 [7]	53.9 ± 34.2 [12],[13],[11]	1.84 ± 3.4 [22],[23],[24],[25],[17],[26]
Hydrochlorothiazide	>100	**431.2**		459 [11]	
Ibuprofen	**14.8**	**70.97**	142[8]	107.4 ± 64.7 [13],[11]	2.5 ± 4.9 [27],[28],[29],[30],[31],[8]
Paracetamol	>100	**1908**	>160 [4]	19† [10]	95 [32]
Sulfamethoxazole	**154.9***	**720.3**	562.5 [4]	27.4† [12]	1.3 ‡ [35]

Table 1 – Results obtained in FET and RT Gill W1 assay and literature data on fish toxicity. FET results – EC50 was derived for DCL, IBF, CBZ and SMX; other substances had no effect in highest tested concentration 100 mg/l. **RT Gill W1 results** – EC50 was derived for all substances except CIP for which no effect was observable in 100 mg/l (solubility limit). For literature data, the given value represents an average from literature values ± standard deviation, if more than one reference was found. * - extrapolated value, † - the value was excluded from correlation analysis of the data due to the high bias from other reported values, ‡ - value was derived by PBT profiling, not by toxicity assay. Values which are indicated > (higher than) were for purposes of correlation calculations replaced by 2.5 times higher value.

	FET all effects EC50	**RT Gill W1 - EC50 minimal**
FET all effects EC50	✖	0.873
Acute toxicity on adult fish LC50	0.867	0.786
***In vitro* fish toxicity EC50**	0.941	0.943
Chronic fish toxicity	0.395	0.429

Table 2 – Correlations of obtained results with data from literature – non-parametric Spearman correlation was used (indicated in red are Spearman coeficients with $p < 0.5$)

Correlation analysis

Correlation analysis shows the relevant relationships between the results of FET test and literature data on acute toxicity for fish and also for the average chronic effective concentrations. Moreover there is correlation between FET and RT Gill W1 effective concentrations.

Discussion on alternative assays

Prolonged **FET test represent a highly sensitive embryo-larval life** stage with many advantages as: visual observation of malformations in transparent embryos, rapid development covering whole organogenesis in only 5 days, as well as possibility of multiple repetitions, and fast toxicity screening.

RT Gill W1 assay with **serum-free exposure** simulates the natural aquatic exposure with gills as organ directly exposed to contaminants in water. Cytotoxicity evaluation combining three endpoints gives the information on the cell metabolism (functionality of mitochondria), integrity of cell membrane, and the functionality of lysozomes.

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