



Permanent Fish Cell Cultures as Important Tools in Ecotoxicology

Karl Fent

University of Applied Sciences, School of Life Sciences, MuttENZ, and Swiss Federal Institute of Technology (ETH),
Department of Environmental Sciences, Zurich, Switzerland

Summary

Permanent fish cell cultures such as hepatoma cells (PLHC-1) and gonadal cells (RTG-2) have successfully been used for acute toxicity assessment of a variety of environmental chemicals such as organotins, substituted phenols and pharmaceuticals. Cytotoxicity significantly correlates with physico-chemical properties such as lipophilicity of the compounds (log Dow). A significant correlation of *in vitro* with *in vivo* acute toxicity in fish

(organotins, substituted phenols) and zooplankton *Daphnia magna* (pharmaceuticals) was found. This indicates the usefulness of fish cell lines for screening and toxicity assessment of chemicals within REACH. Furthermore, a transfection system based on PLHC-1 cells, was developed for the determination of estrogenicity of chemicals and environmental samples.

Keywords: fish cell line, ecotoxicity, REACH, estrogenic activity

Background Information

Environmental assessment of potentially hazardous chemicals

Numerous anthropogenic chemicals from household, industry and agriculture enter aquatic systems directly or via wastewaters. Residues in wastewaters consisting mainly of partially and highly persistent organic chemicals may harm fish, a factor that may contribute to population declines as currently observed in Switzerland. Among the prominent chemicals suspected to be involved are endocrine active chemicals. However, residues of veterinary and human pharmaceutical do also occur in significant concentrations in wastewater contaminated rivers and lakes. For the protection of aquatic systems, in particular of fish, new chemicals should undergo ecotoxicological testing before marketed. The environmental safety assessment of chemicals and pharmaceutical ingredients requires acute, and in many cases, chronic ecotoxicity tests.

In vitro systems such as fish cell lines have become of growing importance in ecotoxicology in the last decade.

The aim of this project was the assessment of the ecotoxicity of chemicals and pharmaceuticals including some mixtures using different *in vitro* cell culture methods.

The pending implementation of the European chemicals regulation REACH will lead to significant additional animal testing, in particular with fish. This project is aimed at reducing and replacing fish tests and demonstrates the advantages of *in vitro* tests which may lead to better acceptance, because there is still a gap in full regulatory acceptance of cell culture systems.

In vitro systems in ecotoxicology

The development of *in vitro* assays in ecotoxicology is needed for scientific, animal welfare, and economical reasons. About 100,000 chemicals (including some 1,000 pesticides) are currently being used, and more than 1,000 enter the market every year. Their ecotoxicological properties should be assessed prior to release into the environment. *In vitro* systems could play a valuable role in such assessments, but they are only recently gaining recognition from governmental bodies and industry. Within the framework of REACH, cell culture systems gain further importance. Toxic effects are often species-specific, and

consequently, toxicity towards fish can only be assessed in fish-specific systems. By using permanent fish cell lines, our goals were (i) to develop novel *in vitro* assays for the assessment of environmental toxicity to fish, thereby reducing and replacing animal testing, and (ii) to demonstrate the general usefulness of this approach in basic and applied ecotoxicology and as a consequence, for application as a screening tool and at the first step in the ecotoxicological assessment within the framework of REACH. After development of basic methods and application for different groups of compounds, we have used this approach recently for further development and analysis of a series of pharmaceuticals present in aquatic systems (Caminada et al., 2006).

Fish cell line PLHC-1

The permanent fish cell line PLHC-1, derived from a hepatocellular carcinoma in the topminnow *Poeciliopsis lucida*, has several advantages over currently used fibroblast-like cells. Firstly they have metabolic activities, necessary to study the metabolism of environmental chemicals acting *via* metabolic activation. Secondly PLHC-1 are easy to cultivate. Thirdly they contain an aryl hydrocarbon receptor and possess inducible and stable cytochrome P450 (CYP) enzymes, which are important for metabolism and detoxification of environmental chemicals. This cell culture system is ideally suited to assess the acute and chronic toxicity of chemicals, pollutants and environmental probes, and to derive structure-activity relationships within chemical classes.

Correlation of *in vitro* and *in vivo* toxicity

The cytotoxicity of more than 50 important environmental chemicals with various modes of action, including organotin (organic tin) compounds (Brüschweiler et al., 1995), chloro- and nitrophenols, sulfonic acids, and hormone-disrupting estrogenic chemicals (alkylphenols) have been assessed (Fent and Hunn, 1996; Brüschweiler et al., 1996). Inhibition of neutral red (NR) uptake into cells based on the accumulation of neutral red in lysosomes of viable cells and the tetrazolium salt reduction (MTT) assay based on the mitochondrial metabolic function were found to be most suitable tests, allowing rapid and reliable assessment. We have recently shown that these assays are very useful for the determination of cytotoxicity of 34 pharmaceuticals to which fish may potentially be exposed in wastewater-contaminated aquatic systems (Caminada et al., 2006).

An excellent quantitative correlation was found between the two assays (Caminada et al., 2006, Brüschweiler et al., 1995). Organotin compounds were the most toxic, followed by higher substituted phenols including estrogenic nonylphenol, lower

substituted phenols, and sulfonic acids. The *in vitro* results showed a trend similar to the *in vivo* acute toxicity in fish. Hence, acute fish toxicity of chemicals acting via different modes of action can be estimated *in vitro*. Recently, we have also demonstrated that cytotoxicity was perfectly correlated with the lipophilicity of pharmaceuticals, defined by their log Dow in case of generally acting or narcotic compounds (Caminada et al., 2006). In addition, the cytotoxicity of pharmaceuticals having a general mode of action was clearly correlated with *in vivo* acute toxicity in the zooplankton organism *Daphnia magna*, for which acute data were available. PLHC-1 cells are therefore a promising tool in the toxicity screening and in the evaluation of chemicals prone to contaminate aquatic systems.

Detection of hormone-disrupting chemicals

Considerable public and scientific concern has arisen over chemicals that act on hormone systems, because of their negative effects on reproduction. *In vitro* systems to determine the estrogenic activity of chemicals (e.g. permanent fish cell culture systems) are urgently needed. We have developed a transfection assay based on transient transfection of PLHC-1 cells using plasmids containing an estrogen receptor and a reporter gene (Fent 2001) for assessing estrogenic chemicals and estrogenic activity in environmental samples (Ackermann et al., 2002). Estrogenic chemicals that act via binding to the estrogenic receptor showed clear dose-response curves.

Relevance regarding the 3Rs

Fish cell culture systems using permanent hepatoma (PLHC-1) or RTG-2 gonadal cells (Caminada et al., 2006) are a promising tool for basic and applied research, and for routine ecotoxicology tests, namely for screening purposes, but also as a basic step in the assessment of chemicals within REACH. Our studies

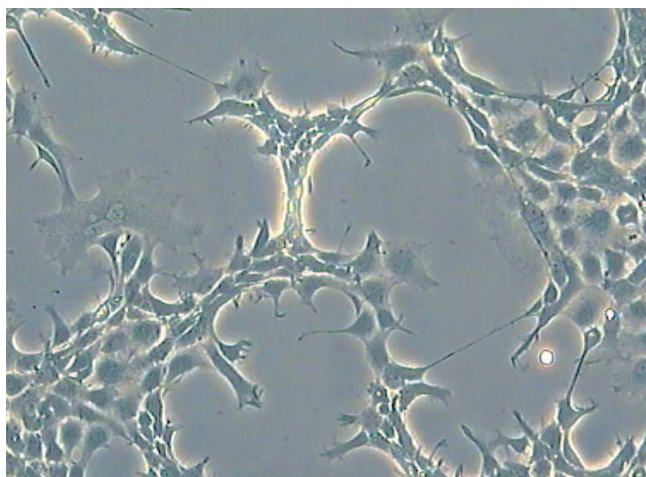


Fig. 1: Permanent fish hepatoma cells PLHC-1.

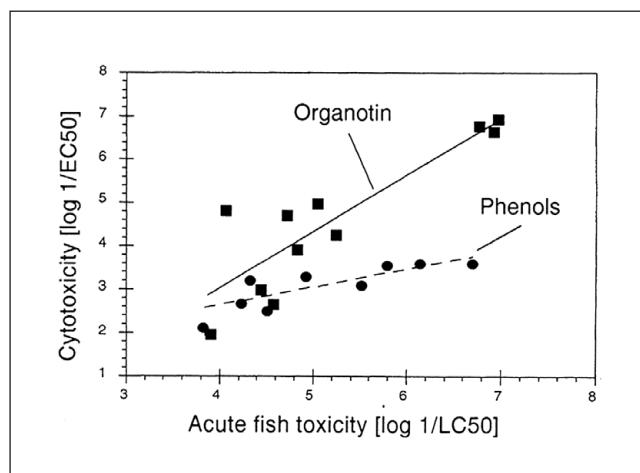


Fig. 2: Relationship between *in vitro* cytotoxicity and acute *in vivo* toxicity in fish demonstrated with two compound groups.

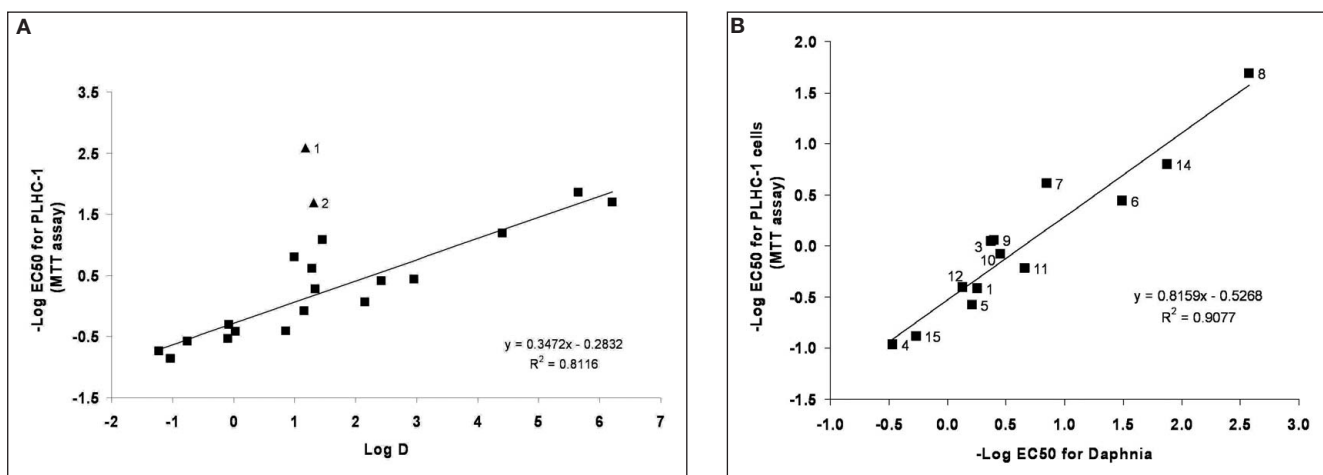


Fig. 3: A) Significant correlation between cytotoxicity of pharmaceuticals acting by a general (narcotic) mode of action and physico-chemical properties of the compounds. B) Significant correlation between cytotoxicity and *in vivo* acute toxicity in *Daphnia magna*.

clearly demonstrate their usefulness for ecotoxicological research, rapid initial ecotoxicity screening and for environmental risk assessment of chemicals and environmental samples for cytotoxicity and estrogenicity. Cytotoxicity was correlated both with physico-chemical properties and *in vivo* acute toxicity. We are convinced that this helps in a reduction of animal testing in aquatic ecotoxicology.

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Correspondence to

Prof. Dr. Karl Fent
University of Applied Sciences
School of Life Sciences
Gründenstrasse 40
4132 Muttenz
Switzerland
e-mail: karl.fent@bluewin.ch