



Factorial design applied for multiple endpoint toxicity evaluation in Atlantic salmon (*Salmo salar* L.) hepatocytes

Liv Søfteland^{a,*}, Ingvar Eide^b, Pål A. Olsvik^a

^a National Institute of Nutrition and Seafood Research, N-5817 Bergen, Norway

^b StatoilHydro Research Centre, N-7005 Trondheim, Norway

ARTICLE INFO

Article history:

Received 31 October 2008

Accepted 7 July 2009

Available online 14 July 2009

Keywords:

Atlantic salmon hepatocytes

Gene expression

Dioxins

PCB 138

CYP1A

UDPGT

Factorial design

TEQ

ABSTRACT

The toxic equivalent (TEQ) approach is traditionally used in risk evaluation of dioxins. Non-dioxin-like PCBs are not included in this approach and TEQ can therefore underestimate toxicity. In this study, a factorial design and multiple endpoint strategy have been used to evaluate the combined toxicity and possible interactions between the non-dioxin-like PCB 138 and the potent AhR agonists 2,3,7,8-TCDF (TCDF) and 1,2,3,7,8-PeCDD (PCDD). Primary hepatocyte cultures from Atlantic salmon were exposed for 24 h and qPCR was employed to create CYP1A dose–response curves and to quantify the transcriptional levels of eight genes (CYP1A, UDPGT, HSP70, GR, GPX, MnSOD, GST and p53). Principal component analysis (PCA) was used to evaluate response similarities between genes. PLS regression was used to model CYP1A and UDPGT responses to the three chemicals. The contour plot examinations of the CYP1A model indicated an antagonism between PCDD and TCDF and in the UDPGT model a possibly synergistic interaction between PCB 138 and PCDD. The results indicate that PCB 138, in combination with TCDF and PCDD, can contribute to the measured CYP1A and UDPGT responses. Using primary cell cultures, multivariate data analysis of qPCR data is shown to be a useful tool in toxicological studies. A multiple endpoints strategy can enhance the quality of risk evaluation of chemical compounds.

© 2009 Elsevier Ltd. All rights reserved.

1. Introduction

Fish can be exposed to a complex mixture of interacting chemicals in the environment. The levels of the pollutants polychlorinated dibenzo-*p*-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs) and polychlorinated biphenyls (PCBs) have been reduced in the marine environment (AMAP, 1998), though, they are still of great concern regarding to seafood safety (Berntssen et al., 2005). Relative high concentrations of these chemicals are measured in fatty farmed fish like salmon. Salmonid fishes are among the most sensitive vertebrates to these chemicals (Elonen et al., 1998). Despite extensive research on the effects of dioxin and dioxin-like PCBs (dioxins), the mechanisms for the broad range of toxicity are still not fully known. The toxicity is mainly thought to be exerted through a common mechanism, the aryl hydrocarbon receptor (AhR) (Mandal, 2005; Walker, 2007). Binding of a ligand to the AhR results in subsequent transcriptional activation of the CYP1A gene (Denison et al., 2004) and a number of phase I and II enzymes, which belongs to the AhR gene battery e.g. UDP glucuronyltransferase (UDPGT) (Carpenter et al., 2002). The AhR gene battery is conserved in all vertebrates (Nebert et al., 2000) and plants (Baerson et al., 2005), and is apparently important in cell control, in

cells defense against oxidative stress (Nebert et al., 2000) and are involved in the development of the liver, cardiovascular system and immune system (Hahn, 2002a; Mimura and Fujii-Kuriyama, 2003). Further, there are several cross-talks between AhR and other signaling pathways (Bemanin et al., 2004; Bock and Köhle, 2006).

The importance of AhR in the toxicity of dioxins resulted in the development of the toxic equivalent (TEQ) concept, which is used in risk assessment of mixtures with such chemicals. The TEQ method utilizes toxicological data from single chemical exposures, and it relies on a number of assumptions including that different compounds have identical dose–response curves (Safe, 1998; Toyoshima et al., 2004) and that the combined effects of chemicals are additive (Safe, 1994; Clemons et al., 1998). The majority of toxicological studies on dioxins have evaluated one endpoint using the TEQ method (Seed et al., 1995; Boström et al., 2002). The few mixture studies that have been performed have utilized pure PCB mixtures like Arochlor 1254 or a simple mixture composed of compounds with similar properties (e.g. potent AhR agonists) (Schmitz et al., 1995; Clemons et al., 1998). Occurrence of interaction between chemicals in mixtures is set by comparing the theoretical TEQ and experimental TEQ (Schmitz et al., 1995, 1996).

PCB 138 is among the non-dioxin-like PCBs that are found in high concentrations in fish (Corsolini et al., 2005; Moon et al., 2006). Toxic effects of these PCBs are less studied than the effects

* Corresponding author. Tel.: +47 99630173.

E-mail address: lso@nifes.no (L. Søfteland).

of dioxins, but their toxicity is thought to be induced by other mechanisms than AhR, and is therefore not included in the TEQ method. However, there is evidence that the effects of non-dioxin-like PCBs are more severe than first expected (Fischer et al., 1998) and it appears that these PCBs can interact with potent AhR inducers (de Jongh et al., 1993; Schmitz et al., 1995). Consequently, there is a need for more knowledge concerning the toxicity of non-dioxin-like compounds and their use within the context of risk assessment (Fischer et al., 1998; Henry and DeVito, 2003). Since dioxins can affect numerous cellular targets, it is a great challenge to develop multiple endpoint strategies, which can enhance the risk assessment of chemicals (Risso-de Faverney et al., 2001). Experimental design has increasingly been employed in toxicity testing of mixtures (Eide, 1996) and the advantage to use design has been emphasized by many scientists (Eide and Johnsen, 1998; Simmons and Gennings, 1996). Factorial design is a commonly used experimental design, which is used for screening and can identify responses of individual variables and interactions between chemicals (Myers and Montgomery, 2002).

In this study with primary cultures of Atlantic salmon hepatocytes, utilizing factorial design and multiple endpoints, the aim was to evaluate how the combined effects of PCB 138, PCDD and TCDF would affect the transcription of a set of genes encoding biomarker proteins known to respond to these chemicals.

2. Materials and methods

2.1. Chemicals

1,2,3,7,8-pentachlorodibenzo-*p*-dioxin (PeCDD) and 2,3,7,8-tetrachlorodibenzofuran (TCDF) were purchased from Fluka Chemicals (Fluka Chemicals, Sigma-Aldrich, Oslo, Norway) and 2,2',3,4,4',5'-hexachlorobiphenyl (PCB 138) was purchased at Promochem (Wesel, Germany). The different stock solution were prepared in dimethyl sulfoxide, DMSO (>99.9% pure, Sigma-Aldrich, Oslo, Norway).

2.2. Isolation of primary cultures of hepatocytes

Juvenile Atlantic salmon (*Salmo salar*) were obtained from Havbruksstasjonen at Matre and kept in 1500 liters tank at the animal holding facility at the Institute of Marine Research, Bergen, Norway. The fish was fed once a day regular commercial feed from Skretting, Norway (Spirit 400–50A HH, 6.0 mm). All glassware, instruments and solutions were autoclaved prior to liver perfusion. Hepatocytes were isolated from five approximately 19 months old female salmon (289–665 g) with a two-step perfusion method earlier described by Seglen (1976) and Dannevig and Berg (1985). The fish was under metacaine anaesthesia (0.07 g/ml) during the surgical intervention. Concisely, a peristaltic pump (Miniplus 3, Gilson, USA) was used to clear the liver for blood (4 ml/min) via the hepatic vein followed by digestion with collagenase VIII (1 mg/ml) (Sigma-Aldrich, Oslo, Norway). When the digestion of the liver was complete, the organ was removed from the fish. The hepatocytes were gently shaken in PBS by using tweezers and homogenized by carefully pipetting up and down with a 50 ml disposable pipette. Subsequently, the cell suspensions were filtered (100 µm nylon gauze) and the cells collected by centrifugation (50 × *g* for 5 min). The cells were washed twice with PBS (Bio-Whittaker, Verviers, Belgium) and centrifugated at 50 × *g* for 5 min. The supernatant was decanted and the cell pellet resuspended in L-15 medium containing 10% FBS, 1% glutamax (Invitrogen, Norway) and 1% penicillin–streptomycin–amphotericin (10,000 units/ml potassium penicillin 10000 mcg/ml streptomycin sulfate and 25 mcg/ml amphotericin B.) (Lonzo, Medprobe, Oslo,

Norway). The Trypan Blue exclusion method was performed in accordance with the manufacturer's protocol (Lonzo, Medprobe, Oslo, Norway) and was used to determine cells viability. For further use, the cell viability had to be >90%. 1.83×10^6 cells per well (in 2 ml complete L-15 medium) were plated on laminin (1.8 µg/cm²; Sigma-Aldrich, Oslo, Norway) coated 12 well culture plates (TPP, Trasadingen, Switzerland) and the hepatocytes were kept at 10 °C in a sterile incubator without additional O₂/CO₂ (Sanyo, CFC FREE, Etten Leur, Netherland).

2.3. Chemical exposure

The cells were cultured for 36–40 h prior to chemical exposure with an exchange of medium after 18–20 h. The cells were exposed for 24 h to single chemicals ((PCDD, TCDF (0.0001–10 nM) or PCB138 (0.0001–10 µM)) or to simple mixtures of PCB 138, TCDF and PCDD according to the experimental design (Table 1), using environmentally relevant concentrations. The concentrations used for the factorial design were obtained from the linear part of the different dose–response curves. A full factorial design was used with two levels (low and high concentrations), a zero (control) concentration, and two center points as replicates and in order to evaluate linearity (Table 1). Cells from one fish were used to make the dose–response curves. The cells were exposed in triplicates using 12-wells culture plates. Cells from four fish were employed for the factorial design experiment.

2.4. In vitro toxicology

MTT-based *in vitro* toxicology assay kits were used to evaluate the toxicity of the chemical mixture and were performed in accordance with the manufacturer's protocol (Sigma-Aldrich, Oslo, Norway). The MTT test is based on spectrophotometrically determination of the cell number as a function of mitochondrial activity in living cells. The 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) solution was dissolved in PBS (yellow in color). The absorbance was measured after 4 h incubation at 570 nm using an iEMS reader (LabSystems I EMS Reader MF, Helsinki, Finland). Cells from three fish were used and 0.16×10^6 hepatocytes per well were plated out in 96-well plates and exposed as explained earlier.

2.5. RNA extraction

The RNeasy Plus mini kit was used to extract total RNA according to the manufacturer's protocol. RNA was eluted in 50 µl RNase-free MilliQ H₂O and stored at –80 °C (Qiagen, Crawley, UK). The RNA quality was assessed with the NanoDrop® ND-1000 UV–Vis Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo

Table 1

Overview over the different concentration combinations used for the various chemicals (PCDD, TCDF and PCB 138) in the factorial design model.

Ex. No.	PCDD (nM)	TCDF (nM)	PCB 138 (µM)
1	0.03	0.1	0.05
2	0.1	0.1	0.05
3	0.03	1	0.05
4	0.1	1	0.05
5	0.03	0.1	1
6	0.1	0.1	1
7	0.03	1	1
8	0.1	1	1
9	0.065	0.55	0.525
10	0.065	0.55	0.525
11	0	0	0

Download English Version:

<https://daneshyari.com/en/article/2602633>

Download Persian Version:

<https://daneshyari.com/article/2602633>

[Daneshyari.com](https://daneshyari.com)