INDUCTION OF CYP1A ACTIVITY IN IN VITRO MODELS AS A BASIS FOR DERIVATION OF SYSTEMIC TEFS

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Introduction

Recent risk assessments of polychlorinated biphenyls (PCBs), dibenzo-*p*-dioxins (PCDDs), and dibenzofurans (PCDFs) have employed the toxic equivalency factor (TEF) concept. In 2005 the World Health Organization (WHO) toxic equivalent (TEQ) approach from 1998 was reevaluated. The current EU-project SYSTEQ aims to develop, validate and implement human systemic TEFs as indicators of toxicity for dioxin-like compounds. Thereby a definite data-set of *in vitro* studies is supposed to function as a fundament for the establishment of novel TEFs. Hence, CYP1A induction measured by EROD activity as a sensitive marker for dioxin-like effects is used to estimate potency and efficacy of selected congeners. For this study, primary rat hepatocytes, the rat hepatoma cell line H4IIE as well as the human hepatocarcinoma cell line HepG2 were used as cell models for the determination of EROD inducing effects after 24 h of treatment with 13 dioxin-like compounds and the non-dioxin like (NDL) PCB 153, respectively. By means of these experiments the relative effective potencies (REPs) of the investigated congeners *in vitro* were established.

Materials and methods

PCDDs (TCDD, 1-PnCDD, 1,6-HxCDD, 1,4,6-HpCDD), PCDFs (TCDF, 4-PnCDF, 1,4-HxCDF, 1,4,6-HpCDF) and PCBs (77, 105, 118, 126, 153, 156) were from Dow, Chemical Company, USA.
All compounds were analyzed and, if necessary, purified by the Department of Chemistry at Umeå University, Sweden. Consumables were obtained from Greiner (Frickenhausen, Germany), chemicals were from Sigma Aldrich (Steinheim, Germany) unless noted otherwise.

Primary rat hepatocytes were obtained from male Sprague Dawley rats (Charles River; Sulzfeld, Germany) weighing 150 – 250 g. Rat livers were perfused as previously described (Schrenk *et al.* 1992). Cells were seeded on rat-tail collagenated 24-well plates at a density of 2 x 10^5 cells/well in seeding medium: DMEM high glucose w/o phenol red (PAA, Coelbe, Germany)/Ham's 12 w/o phenol red (Promocell, Heidelberg, Germany) (1:1) supplemented with FCS 5% v/v (PAA, Coelbe, Germany) HEPES 10 mM (Roth, Karlsruhe, Germany), insulin (100 nM), and Na-selenite (100 nM). After 3 h medium was removed and cells were cultivated in functional medium: DMEM high glucose w/o phenol red/Ham's 12 w/o phenol red (1:1) supplemented with BSA (0.5 mg/mL), HEPES (10 mM), gentamicin (50 µg/mL), linoleic acid (5 µg/mL), transferrin (5 µg/mL), dexamethasone (100 nM), insulin (100 nM), and Na-selenite (100 nM). Test compounds were dissolved in DMSO and added to functional medium. Final DMSO concentration was 0.1% for all treatments. TCDD (1 nM) served as positive control for EROD induction. EROD activity was measured after 24 h of treatment.

H4IIE cells were from ECACC (Salisbury, UK), HepG2 cells from DSMZ (Heidelberg, Germany). H4IIE and HepG2 cells were cultivated in DMEM high glucose w/o phenol red supplemented with 10% v/v FCS, 1% v/v Pen/Strep, and 100 nM dexamethasone. Cells were seeded on 24-well plates at a density of 1.2-1.7 x 10^5 cells/well. After 24 h medium was removed and cells were washed once with 1 mL PBS (137 mM NaCl, 2.7 mM KCl, 6.5 NaH_2PO_4, 1.5 KH_2PO_4, pH 7.4). All compounds were dissolved in DMSO and added to the plates in medium containing 89% v/v DMEM high glucose w/o phenol red, 10% v/v dextran-charcoal stripped FCS (PAA, Coelbe, Germany), 1% v/v Pen/Strep and 100 nM dexamethasone. TCDD (1 nM for H4IIE, 10 nM for HepG2) served as positive control for EROD induction.

EROD activity was measured according to van Duursen *et al.* (2005) with modifications. Fluorescence was measured at an excitation wavelength of 544 nm and emission wavelength of 590 nm every 90 s for 30 min. Then, cells were washed with PBS (2 mL/well) and frozen (-80°C) over night. Cells were cracked by thawing three times for 15 min at room temperature and freezing (-80°C) for at least 3 h in between. Protein content was measured by the bicinchoninic acid (BCA) assay (Pierce, Rockford, IL).

Results and discussion:

Primary rat hepatocytes, H4IIE and HepG2 cells were exposed to 13 dioxin-like compounds and the non-dioxin like PCB 153 for 24 h before EROD activity was measured. As an example, results for primary rat hepatocytes, incubated with TCDD, 1-PnCDD, 4-PnCDF, PCB 118, PCB 126, PCB 153 or PCB 156 are presented in Figure 1.

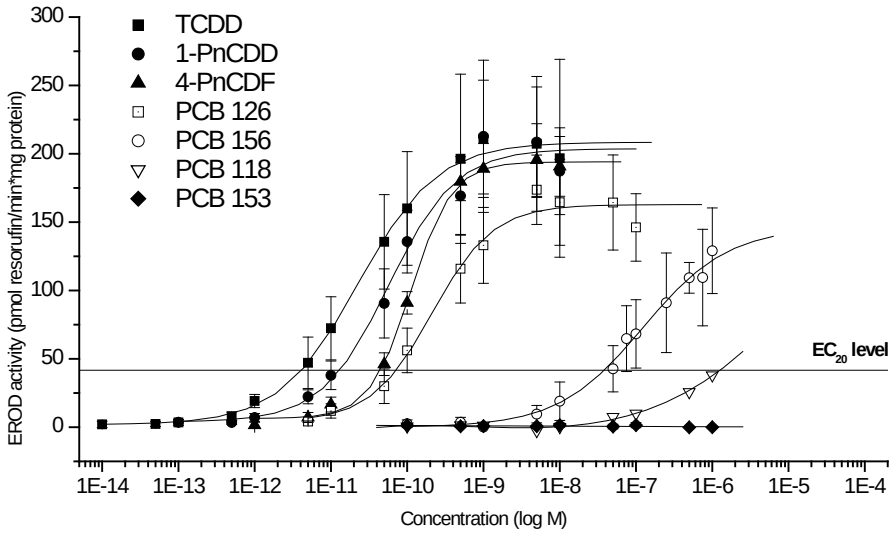

Figure 1: EROD activity (pmol resorufin/min*mg protein) in primary rat hepatocytes after 24 h of treatment with TCDD, 1-PnCDD, 4-PnCDF, PCB 118, PCB 126, PCB 153 or PCB 156.

Values in Figure 1 are means ± SD from at least three independent experiments. Plotting the used concentration in a logarithmic scale against the obtained EROD activities, EC_{50}-values were derived by sigmoidal fitting using Origin software (Microcal Software, Northampton, MA). EROD activity in the respective solvent control was considered as background level and subtracted from the data. For EC_{20}-values, the upper limit of the respective TCDD-derived EROD induction was set 100%, and test compound concentrations attaining its twentieth part were defined as EC_{20}. Respective REPs reveal the compounds potencies relative to the reference compound TCDD.

In Table 1, REPs of the examined compounds for EROD induction in primary rat hepatocytes, H4IIE, and HepG2 cells are compiled and compared with WHO-TEFs (van den Berg *et al.* 2006).

Table 1: Relative effect potencies (REPs) derived from *in vitro* EROD induction. Comparison with WHO-TEFs.

	Primary rat hepatocytes		**H4IIE**		**HepG2**		**WHO-TEF (2005)**
	REP (EC_{50})	REP (EC_{20})	REP (EC_{50})	REP (EC_{20})	REP (EC_{50})	REP (EC_{20})	
TCDD	1.0	1.0	1.0	1.0	1.0	1.0	1
1-PnCDD	0.40	0.32	0.58	0.63	1.7	2.0	1
4-PnCDF	0.20	0.094	0.67	0.64	2.7	2.1	0.3
1,4-HxCDF	0.084	0.034	0.13	0.092	0.79	0.71	0.1
1,6-HxCDD	0.12	0.079	0.12	0.099	0.053	0.065	0.1
TCDF	0.26	0.17	0.023	0.050	0.12	0.12	0.1
PCB 126	0.11	0.058	0.12	0.093	0.013	0.0050	0.1
1,4,6-HpCDD	0.037	0.030	0.067	0.048	0.099	0.14	0.01
1,4,6-HpCDF	0.016	0.0072	0.015	0.010	0.0051	0.0092	0.01
PCB 77	0.0019	0.00042	0.00013	0.000056	-	-	0.0001
PCB 105	-	0.0000049	-	-	-	-	0.00003
PCB 118	-	0.0000032	-	-	-	-	0.00003
PCB 156	0.00018	0.00011	0.000051	0.000047	-	-	0.00003
PCB 153	-	-	-	-	-	-	-

Since the TEF concept is grounded on TCDD being the reference compound, REPs for treatment with TCDD are 1.0 constantly. PCB 153, a non dioxin-like congener, did not show any EROD induction in all cell models as expected.

REPs gained from primary rat hepatocytes, and H4IIE cells, respectively, widely confirmed the current TEFs. Nevertheless, some deviations occured. REPs for 1-PnCDD, 4-PnCDF, and 1,4,6-HpCDD obtained from primary rat hepatocytes were about twice lower in comparison to the respective REPs derived from H4IIE cells, meaning H4IIE cells being more sensitive towards these compounds. In case of 1,4,6-HpCDD, REP values from rodent cells tended to be slightly above the current TEF. For 1-PnCDD, REP values in primary rat hepatocytes, and H4IIE cells were below the WHO-TEF by half, approximately. Also REPs from PCB 105 and 118 were lower than the current TEF, 10-fold in particular. Remarkably, in case of these PCBs, no complete sigmoidal curves were obtained. Thus, the assessment regarding PCB 105 and 118 turns out to be less reliable. 4-PnCDF REPs from primary rat hepatocytes were lower in comparison to the WHO-TEF, while the H4IIE REPs tended to be higher. In contrast, primary rat hepatocytes were more sensitive than H4IIE cells towards TCDF.

As shown in Table 1, most of the REPs (EC_{50}, EC_{20}) in human HepG2 cells deviated from the current WHO-TEFs. Comparing the WHO-TEF for 1-PnCDD (TEF=1) with the determined REPs in HepG2 cells, a 2-fold higher REP was achieved. This was also observed with 4-PnCDF and 1,4-HxCDF, where REPs were about 7-10 times higher compared to the WHO-TEF. In case of 1,6-HxCDD, the REP values were twice lower than the current WHO-TEF. In HepG2 cells, minor REPs also were derived for PCB 126, and 1,4,6-HpCDF. REP values for TCDF, 1,4,6-HpCDD and 1,4,6-HpCDF were within the same range as the corresponding WHO-TEFs. It was not possible to derive any REPs for the PCBs (77, 105, 118, and 156) due to a lack of EROD induction in HepG2 cells. In contrast to these findings, Zeiger *et al.* (2001) established an EC_{50} for PCB 77 of 2.7E-6 M, derived for 48 h of treatment.

The most obvious differences between REPs in the rodent cell models (primary rat hepatocytes and H4IIE) and the human hepatocarcinoma cell line were observed with 1-PnCDD, 4-PnCDF and 1,4-HxCDF. For these compounds, the REP in HepG2 was higher than the corresponding TEF, whereas H4IIE tended to have lower REPs, followed by primary rat hepatocytes with the lowest REPs. Comparing (absolute) EC_{50} and EC_{20} values for H4IIE as well as for primary rat heptocytes to the respective HepG2 parameters, rodent cell models were more sensitive in general. For example, the EC_{50} for TCDD in HepG2 cells (2.43E-10 M) was more than 25 times higher than in H4IIE cells (9.49E-12 M).

It was the aim of this study to quantify CYP1A induction measured by EROD activity as a sensitive marker for dioxin-like effects to estimate potency and efficacy of selected congeners. In conclusion, sensitivities of the used cell models were obtained in the following order: H4IIE > primary rat hepatocytes > HepG2, which was revealed previously (Lipp *et al*. 1992). Further studies with primary human hepatocytes are in progress, which will provide substantial data within the SYSTEQ project. In this cell model, inter-individual variations are expected to be demanding, though (Schrenk *et al*. 1995). Furthermore, *in vivo* experiments are going to add important information in this regard. Consideration of the range of variations of several REPs will be challenging for the establishment of novel TEFs.

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