DETERMINATION OF THE DIOXINLIKE ACTIVITY OF SEVERAL POLYHALOGENATED (X=BR, CL) AROMATIC HYDROCARBONS (PHAHS) BY THE MICRO-EROD BIOASSAY

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Objective

The aim of this study was to establish and validate the Micro-EROD bioassay[1,2] with rat H4IIEC3/T hepatoma cells by analysing relative potencies (REPs) of several polyhalogenated (X=Br, Cl) aromatic hydrocarbons (PHAHs) in comparison to 2,3,7,8-TCDD.

Introduction

PHAHs such as polyhalogenated biphenyls (PXBs), polyhalogenated dibenzodioxins (PXDDs) and dibenzofurans (PXDFs) are industrial compounds or by-products that have been widely identified as global environmental contaminants. 2,3,7,8-TCDD is the most toxic member of this class, that are structurally related, have a similar mechanism of action, and cause the same spectrum of biological responses. For several chemicals that fits the criteria of this class of dioxinlike compounds (DLCs) a toxic equivalency factor (TEF) or in a single test relative potencies (REP) have been assigned, which is some fraction of that of TCDD. Several bioassays are based on the binding of the dioxinlike compounds to the cytosolic aryl hydrocarbon (Ah) receptor and the increasing transcription of certain genes, such as cytochrome P450, can be determined. The most important isoenzyme for dioxin and dioxinlike compounds are P4501A1 or 2, which can be finally analysed e.g. by increase in the 7-ethoxyresorufin-*O*-deethylase (EROD). The EROD bioassay is mostly performanced by the wildtype (wt) H4IIE cells (e.g. Micro-EROD assay), by rat hepatocytes, by human hepatoma HepG2, by chicken embryo hepatocyte (CEH) cultures or chicken embryo whole liver. Vamvakas et al (1996)[3] reported that "the H4IIE rat liver cell line remains the standard *in vitro* in biomonitoring programms".

Material and Methods

1. Validation standards

Most of the here tested PHAH were originated from Cambridge Isotope Laboratories. Only 2,2',4,5',6-PBB (Kanto Reagents, Japan), TBBA (Tokyo Kasei Kogyo Co., Ltd., Japan) , p-brominated phenol and 2,4-dibrominated phenol (Wako Pure Chemical Ind., Ltd., Japan) were from other distributors.

2. Micro-EROD bioassay

The Micro-EROD was performed described by Schramm and coworkers (1998)[1,2] and the rat hepatoma cell line H4IIEC3/T was supplied by Dr. F. Wiebel (GSF, Neuherberg-Munich, Germany).The EROD bioassay was continuously improved and down-scaled to the now called Micro-EROD assay (TCDD and the sample were simultaneously analysed in min. 5 doses compared to a blank sample by a 96 well plate-reading spectrofluorimeter; fluorescent-based protein assay; data analysis by complete dose response curve log probit calculation or by the

comparison of the linear range between TCDD and DLC; each concentration was analysed in min. 3 wells/plate in at least 3 independent).

Results and Discussion

1. Bioassay validation quality criteria

Relative potencies (REP) are calculated by comparing the EROD induction from authentic 2,3,7,8-TCDD (0-40 pg TCDD/plate) to the unknown compound according to Hanberg et al. (1991)[4]. The EC_{50} value for 2,3,7,8-TCDD in the here established Micro-EROD assay (1.01 +/- 0.37 pg/ml) was similar to the by Schramm and coworkers published data (0.87 pg/ml)[2].

The coefficient of variation (CV) for TCDD was 37% (n=57), for the here analyzed polychlorinated DLCs between 13 and 37 % (mean 27 %) and for the polybrominated aromatic compounds between 9-52 % (mean 34%). This CV values are similar to the CV values reported for the Macro-EROD assay published by Sanderson et al (1996; CV=29 %)[5], Clemons et al. (1994; CV=25 %)[6], Tillitt et al (1991; CV=34 %)[7] and Hanberg et al (1991; CV=34%)[4] for TCDD and for several DLCs (8-50 %)[4-7].

2. Relative potencies (REPs) for several PCDD, PCDF and PCB congeners

Table 1 reviews most of the validation studies reported from the EROD- and CALUX-bioassay with H4IIE cells compared with the in this study obtained REP- and CV-values.

Table 1: REP values for PCDD/PCDF- and PCB-congeners analyzed with H4IIE cells by EROD or CALUX-assay compared to our study ([a]: Micro-EROD; [b]: Macro-EROD; [c]CALUX)

Studies	2,3,7,8-TCDD (REP 1)	1,2,3,7,8-PCDD	2,3,7,8-TCDF	2,3,4,7,8-PCDF	PCB-126	PCB-156	PCB-157
This study[a]: REP (n/ CV in %)	(45/37)	0.57 (4/28)	0.078 (5/28)	0.47 (9/26)	0.044 (3/37)	2.3E-5 (4/13)	4.5E-5 (4/29)
Li[a;2];1999	(-/-)	-	0.150	-	0.050	-	-
Schrenk[b;8]; 1996	(-/-)	0.18	-	-	0.20	6.0E-5	5.0E-5
Sanderson[b/c;5]; 1996	(11/29)	0.30[b]/0.79[c]	0.090	0.28[b]/0.69[c]	0.047[b]/ 0.017[c]	-	-
Clemons[b;6]; 1994	(31/25)	1.1	0.03-	0.40	0.100	5.2E-5	-
Hanberg[b;4]; 1991	(30/34)	-	-	-	0.100	1.0E-4	4.0E-5
Tillitt[b;7]; 1991	(54/34)	-	6.4E-3	-	0.022	5.4E-5	-
Bovee[c;21]; 1998	(-/-)	0.49	-	0.34	0.065	3.8E-5	-
Safe[b;9]; 1991	(-/-)	0.011	0.092	1.4	0.32-0.75	2.1E-4-9.0E-5	2.1-6.0E-5
Safe[10]; 1998 (in vitro and in vivo)	(-/-)	0.07-0.64	0.006-0.43	0.11-0.67	0.003-0.77	1.3E-5 - 1.1E-3	6.0E-4 - 6.0E-5
WHO (1993/1998)	1.0	0.5/1.0	0.1	0.5	0.1	0.0005	0.0005

3. Relative potencies (REP) for several polybrominated aromatic hydrocarbons

Several reviews are published about polybrominated diphenylethers (PBDEs)[11,12], dibenzo-p-dioxins (PBDDs)/ dibenzofurans (PBDFs)[13-16] and PBBs[12,17]. PBDDs/PBDFs occur as trace contaminants in brominated flame retardants and are produced during combustion of these chemicals.

The biological effects of PBDDs and PBDFs are similar, if not identical, to those of PCDDs and PCDFs. There are seven 2,3,7,8-substituated PBDDs and ten 2,3,7,8-substituated PBDFs, as well as 337 possible 2,3,7,8-substituated PXDDs and 647 possible 2,3,7,8-substituted PXDFs. Safe and coworkers (1998)[9,10] reported REP values *in vivo/in vitro* of the AHH/EROD activity in the immature male rat for several brominated and mixed brominated dioxins, demonstrating similar toxic response of these brominated dioxin congeners. Table 2 summarises several literature studies about PBDDs/PBDFs.

Table 2. REP values for several polybrominated PXDDs/PXDFs compared to 2,3,7,8-TCDD analyzed by a) *in vivo* EROD activity of rat liver (Weber, 1999)[16], b) *in vitro* EROD activity of rat hepatoma H4IIE cells (Safe and coworkers; 1987-1991)[9] b) *in vivo* by AHH induction in rat liver (Safe, 1991)[9] , c) *in vitro* AHH activity of rat hepatoma H4IIE cells (Bradlaw, 1980)[19] and d) in a rainbow trout early life stage mortality bioassay (RTELSM bioassay; Hornung et al, 1996)[18] in comparison to e) the data from this study by the Micro-EROD.

Compound	Weber 1997[a]	Safe 1991[b]	Safe 1991[c]	Mason 1987[b]	Bradlaw (1980)[d]	Hornung (1996)[e]	This study[f] REP (n=3; CV in %)
2,3,7,8-TBrDD	> 1.0	2.3	5.3	0.34	0.62	1.1-2.5	0.65 +/- 0.06 (9)
2,3-diBr-7,8-diCDD	< 1.0	3.4	8.2	1.4	-	-	0.69 +/- 0.35 (38)
3,7-diBr-2,8-diCDD		-	-	-	-	0.68	-
8-Br-2,3,7-triCDD	> 1.0	-	-	-	-	0.65	-
2-Br-3,7,8-triCDD	0.50	0.23	1.6	-	-	-	0.94 +/- 0.36 (38)
1,2,3,7,8-PBrDD	-	0.27	0.16	0.12	-	0.09	0.30 +/- 0.13 (43)
1,2,4,7,8-PBrDD	-	0.024	0.02	0.010	-	-	-
1,3,7,8-TBrDD	-	0.0031	0.0006	1.3E-3	-	0.013	-
2,3,7,8-TBrDF	-	-	-	-	-	0.25	0.62 +/- 0.27 (44)
2,3,4,7,8-PBrDF	-	-	-	-	-	0.06-0.1	0.21 +/- 0.03 (14)
1,2,3,4,7,8-HxBrDF	-	-	-	-	-	0.002	-
1-MBr-2,3,7,8-TCDD	-	-	-	-	-	-	0.60 +/- 0.31 (52)

[[a]EROD in vivo; [b]Macro-EROD in vitro; [c]AHH in vivo; ; [d]AHH in vitro; [e]RTELSM bioassay; [f]Micro-EROD]

The results in Table 2 documented mostly similar relative response of the brominated dioxin/furan congeners with their chlorinated analogues (except 2,3,7,8-TBDF which showed in the RTELSM bioassay and our study a 8-9 fold more potency than 2,3,7,8-TCDF) comparable results between the here listed toxicological studies with different bioassays.
In this study also several other polyhalogenated aromatic hydrocarbons (PHAH) have been tested with the Micro-EROD test, but failed to show any EROD induction in the here available concentrations:

2,2',4,4'-TBDE, 2,2',4,4',5-PBDE, 2,3,3',4,4',5,6-HBDE, 2,2',4,5',6-PBB, TBBA, p-brominated phenol, 2,4-dibrominated phenol all with a REP under $< 4.0 \times 10^{-5}$ (available starting concentration: 25 ng/ml; EC_{50}-TCDD: 1.0 pg/ml).

This results could maybe proved correct by Murk et al. (1996)[20] which analysed by CALUX bioassay lower REP values for 2,2',4,4'-TBDE (7.1 x 10^{-7}), 2,2',4,4',5-PBDE (5.9 x 10^{-6}) and 2,2',4,4',5,5'-HBDE (4.3 x 10^{-6}) indicating that a higher starting concentration would be necessary to obtain results by the Micro-EROD bioassay.

Conclusion: The present study demonstrated the utility of the Micro-EROD bioassay with rat H4IIE/C3 rat hepatoma cells for analzing and calculating REP values for several polyhalogenated (X=Br, Cl) aromatic hydrocarbons (PHAHs) such as PCDDs/PCDFs, PCBs and PBDD/PBDFs.

Acknowledgments: The authors wish to thank Dr. F. Wiebel and Dr. KW. Schramm (GSF, Neuherberg-Muenchen, Germany) for providing the H4IIEC3/T hepatoma cells and all their support throughout the establishment of the Micro-EROD bioassay.

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