DETERMINATION OF RELATIVE POTENCY ESTIMATES FOR PAHS, PBDD/FS AND PXDDS BY MEANS OF THE CALUX ASSAY

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Introduction

The CALUX bioassay is based on the mechanism of action of dioxin-like compounds and involves binding and activation of the aryl hydrocarbon receptor (AhR). Many classes of toxic compounds among which dioxins, PCBs, polycyclic aromatic hydrocarbons (PAHs) are known to show activity in this assay. The present paper focuses on the AhR activity of PAHs, brominated dioxins and furans as well as mixed chlorinated/brominated dibenzodioxins.

PAHs are a group of more than 100 organic compounds composed of two or more aromatic rings. A lot of research has been performed on the 16 priority polycyclic aromatic hydrocarbons (PAHs) of the Environmental Protection Agency (EPA). However, several other PAHs that are not routinely analyzed in various types of environmental matrices are responsible for mutagenic and carcinogenic effects. Among them, the 'heavy PAHs' have been shown to exhibit a considerable mutagenic activity.[1,2]

In 2002, the Scientific Committee on Food of the European Commission established a list of 15 PAHs that are of major concern for human health due to their toxic properties. Therefore, the European Union (EU) recommended to monitor those 15 priority PAHs in food and the environment to enable long-term exposure assessments.[3]

In addition to the new focus on PAHs, the interest for brominated dibenzo-*p*-dioxins and dibenzofurans has increased since the extensive use and occurrence of brominated flame retardants. It is now well recognised that polybrominated dioxins and furans (PBDD/Fs display similar toxic effects as their chlorinated analogues.[4,5] Previously we determined the REP values with the CALUX bioassay for some brominated dioxins and furans.[6] We have now extended this study by determining the REP value of additional brominated compounds among which mixed brominated/chlorinated congeners.

Additionally, we determined the dioxin-like relative potencies (REPs) of 15 EU priority PAHs and of benzo(c)fluoranthene using an *in vitro* luciferase bioassay with the mouse H1L6.1 cell line.

Materials and methods

PAH standards (10 µg/L) were obtained from Dr. Ehrenstorfer GmbH (Germany).

1,2,3,7,8-pentabromodibenzo-p-dioxin (99%) and 2,3,4,7,8-pentabromodibenzofuran (98%) were purchased from Promochem (France), 2,3-dibromo-7,8-dichlorodibenzo-p-dioxin (>98 %) and 2-bromo-3,7,8-triclorodibenzo-p-dioxin (>98 %) were purchased from Wellington Laboratories and 1-bromo-2,3,7,8-tetrachlorodibenzo-p-dioxin (98 %) was purchased from Cambridge Isotope Laboratories. Solutions of brominated compounds and PAHs were kept in amber coloured glass. The 2,3,7,8-TCDD standard solution was purchased from AccuStandard Inc (USA).

The CALUX assay was performed using the mouse H1L6.1 cell line from Xenobiotic Detection Systems, Inc.(USA) as previously described.[6]

The maximum concentration of the PAHs tested in the *in vitro* bioassays was between 50 and 400 µg/mL DMSO. 10 to 14 dilutions of each standard were prepared by twofold dilution of the concentrated stock solution.

The maximum concentration of the brominated compounds tested was 500 ng/mL DMSO. 12 dilutions per compound were prepared by twofold dilution of this solution.

Dose–response curves for the tested compounds were generated and fitted according to the 4 parameter Hill equation (Sigmaplot 2000).[6]

Three replicate dose-response curves were determined for each compound.

REP values were calculated as the ratio of the EC_{50} for TCDD to the EC_{50} for the investigated compound. A range of REP values was determined using the EC_{20} and EC_{80} values of TCDD and the investigated compound.

The efficacy is the maximal response elicited by a compound, expressed as percentage of the maximal response obtained with TCDD.

Results and discussion

- Polycyclic aromatic hydrocarbons

.Table 1: Estimation of CALUX REP values based upon both a weight and molecular basis

PAH		CALUX REP weight derived	REP range EC_{20}-EC_{80}	CALUX REP Molar derived	Efficacy % of TCDD
benzo(c)fluorene	B[c]F	NI	NI	NI	9
cyclopenta(c,d)pyrene	CPP	NI	NI	NI	15
benzo(a)antracene	B[a]A	1 E-5 ± 2 E-6	8 E-5 - 3 E-6	7 E-6 ± 1 E-6	52
chrysene	Chry	8 E-5 ± 6 E-6	4 E-4 - 8 E-5	6 E-5 ± 4 E-6	51
5-methylchrysene	5MeChry	1 E-4 ± 1 E-5	3 E-4 - 1 E-4	1 E-4 ± 1 E-5	64
benzo(a)pyrene	B[a]P	6 E-5 ± 9 E-6	6 E-5 - 6 E-5	5 E-5 ± 7 E-6	88
benzo(b)fluoranthene	B[b]F	2 E-4 ± 2 E-5	4 E-4 - 1 E-4	1 E-4 ± 1 E-5	95
benzo(k)fluoranthene	B[k]F	1 E-3 ± 2 E-4	4 E-3 - 1 E-3	1 E-3 ± 2 E-4	110
benzo(j)fluoranthene	B[j]F	8 E-5 ± 1 E-5	2 E-4 – 6 E-5	6 E-5 ± 1 E-5	120
indeno(1,2,3-cd)pyrene	I[123-cd]P	3 E-5 ± 3 E-6	9 E-5 – 8 E-6	2 E-5 ± 3 E-6	143
benzo(g,h,i)perylene	B[ghi]Pe	NI	NI	NI	23
dibenzo(a,h)antracene	DB[ah]A	2 E-5 ± 3 E-6	4 E-4 – 2 E-6	2 E-5 ± 3 E-6	125
dibenzo(a,e)pyrene	DB[ae]P	3 E-4 ± 3 E-5	5 E-4 – 4 E-4	3 E-4 ± 3 E-5	210
dibenzo(a,h)pyrene	DB[ah]P	NC	NC	NC	117
dibenzo(a,i)pyrene	DB[ai]P	6 E-4 ± 7 E-5	6 E-3 – 6 E-4	6 E-4 ± 6 E-5	116
dibenzo(a,l)pyrene	DB[al]P	NI	NI	NI	24

NI= no significant induction ; NC = REP could not be calculated, dose-response relationship insufficient for estimate; REP values are expressed as the mean ± the standard error of three separate determinations.

Table 2: Comparison of relative potency (REP) values for PAHs obtained with an *in vitro* luciferase bioassay. Only the 15 EU priority PAHs and B[c]F are included. REPs were calculated relative to a TCDD standard, on a molar concentrations basis, based on the EC_{50} estimates for the PAHs and for TCDD.

PAH	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)
B[c]F	NI	-	-	-	-	-	-	-
CPP	NI	-	-	-	2 E-7	-	-	-
B[a]A	7 E-6	2 E-3	NI	1 E-5	7 E-6	1 E-6	-	3 E-6
Chry	6 E-5	-	1 E-4	1 E-2	1 E-4	2 E-6	1 E-4	3 E-6
5Me-chry	1 E-4	-	-	-	9 E-5	-	-	-
B[a]P	5 E-5	4 E-3	8 E-5	1 E-5	9 E-5	1 E-6	3 E-4	8 E-6
B[b]F	1 E-4	-	2 E-4	-	3 E-5	4 E-6	9 E-4	2 E-5
B[k]F	1 E-3	4 E-1	2 E-2	5 E-2	2 E-3	1 E-4	5 E-4	2 E-4
B[j]F	6 E-5	-	-	-	-	-	-	-
I[123-cd]P	2 E-5	-	3 E-3	-	3 E-4	1 E-5	8 E-4	2 E-5
B[ghi]Pe	NI	-	NI	-	NI	-	-	<2 E-7
DB[ah]A	2 E-5	-	2 E-3	5 E-2	1 E-3	4 E-6	1 E-3	4 E-5
DB[ae]P	3 E-4	-		-	2 E-5	-	-	-
DB[ah]P	NC	-		-	7 E-5	-	-	-
DB[ai]P	6 E-4	-		-	2 E-4	-	-	-
DB[al]P	NI	-		-	5E-6	-	-	-

(1) This study, (2) Ziccardi et al.[7],(3) Ziccardi et al.[8], (4) Clemons et al.[9], (5) Machala et al.[10], (6) Villeneuve et al.[11], (7) Behnish et al.[12], (8) Jones and Anderson [13]

cell lines: (1) mouse H1L6.1 cell line
(2) and (3) mouse hepatoma H1L1.1c2 cells
(4) mouse Hepa1c1c7 cells
(5), (6) and (7) rat hepatoma H4IIE cell line
(8) human hepatoma cell line

Since the dose-response curves for the TCDD standards and PAH standards were not always parallel and did not display the same efficacy, the responses from EC_{20} and EC_{80} were calculated to derive a range of relative potencies (REP_{20-80} range).[14]
Among the PAH's tested, benzo(c)fluorene, cyclopentapyrene, benzo(g,h,i,)perylene and dibenzo(a,l)pyrene did not induce a significant response in the CALUX bioassay after a 24-h incubation time (Table 1). Even at concentrations of 400 µg/mL, the response was less than 25% of the maximal response of the TCDD standard. A weak induction potency of cyclopentapyrene, benzo(g,h,i)perylene, and dibenzo(a,l)pyrene after 24 h of exposure was also mentioned by Machala et al.[10] who used the rat hepatoma H4IIE cell line. Dibenzo(a,i)pyrene yielded the greatest maximal response, but the dose-response curve did not display a sigmoidal shape. The highest EC_{50} value was found for benzo(k)fluoranthene, which is consistent with the results of other studies that used an *in vitro* luciferase bioassay.[7-11,13] Although the dioxin-like relative potencies (REP) can vary considerably, depending on bioassay techniques and test conditions, the ranking of REP values for different PAHs obtained in the present study is comparable with the results from other researchers using an *in vitro* luciferase bioassay (Table 2). Additionally, the CALUX response was investigated for the EU priority PAHs B[c]F and B[j]F, for which no REP-values were known until now.

- Brominated dioxins

An overview of the obtained results is given in Table 3. REP values were determined both on a weight and on a molar basis. The dose-response curves of all tested compounds displayed parallelism with the TCDD dose-response curve and attained the same maximal response as for 2,3,7,8-TCDD.

Table3: CALUX REP values for PBDD/Fs and mixed Br/Cl PXDDs

	CALUX REP Weight derived (wt) (EC_{50})	REP range (wt) (EC_{20}-EC_{80})	CALUX REP Molar derived (M) (EC_{50})	Efficacy % TCDD	REP(M) DR-CALUX[12] (EC_{50})	REP (wt) DR-CALUX [16] (EC_{50})	WHO TEF*	CALUX REP* [(2)] (wt)
12378-PBDD	0.12± 0.02	0.11-0.17	0.25±0.05	112	0.21	0.49	1	0.73
23478-PBDF	0.25 ± 0.06	0.25-0.31	0.52±0.14	110	0.094		0.5	0.58
23-B-78-CDD	0.63 ± 0.09	0.54-1.10	0.90±0.14	111	0.86	0.88	1	1
2-B-378-CDD	0.63 ± 0.10	0.52-1.04	0.83±0.15	118	0.67	2; 0.88	1	1
1-B-2378-TCDD	0.49 ± 0.08	0.43-0.75	0.71±0.13	116	0.28		1	0.73
2378-TBDD	0.49 ± 0.07[(1)]	0.38-0.60[(1)]	0.76[(1)]	96[(1)]	0.77	0.62	1	1
2378-TBDF	0.11 ± 0.01[(1)]	0.11-0.12[(1)]	0.17[(1)]	101[(1)]	0.6		0.1	0.067
12378-PBDF	0.08 ± 0.01[(1)]	0.080-0.097[(1)]	0.14[(1)]	99[(1)]	0.14		0.05	0.14

REP values are expressed as the mean ± the standard error of three separate determinations.
[(1)] data from Brown et al.[6], [(2)] data from Brown et al.[15]
*REP/TEF for the chlorinated analogue congener.

The difference between weight derived and molar derived REPs is due to the large difference in molecular weights from the brominated compounds and from TCDD.
The REP values on a weight basis are for almost all brominated compounds lower than the corresponding WHO TEF and CALUX REP values for their chlorinated analogue.
The highest REP values were found for 23-B-78-CDD and 2-B-378-TCDD for which the REP values exceed the REP for TBDD. The REP value of another mixed chloro/bromo substituted dibenzo-*p*-dioxin that we investigated (1B-2378-TCDD) approaches the REP for TBDD while fully brominated analogue 12378-PBDD is characterised by a much smaller REP value.

Our data can be compared with limited literature data on REP values that were determined with other CALUX assays (Table 3). Behnisch et al. published REP values on a molar base.[12] The REPs that we obtained in our study are in general somewhat higher except for 2378-PBDF. For 2 1B-2378-TCDD and for 23478-PBDF the REP values deviate more. Olsman et al. obtained higher REP values than our data for PBDDs and PXDDs when calculated on a weight basis.[16] The data from these two studies were obtained by applying the DR-CALUX bioassay in which a rat cell line is used. In the current study a mouse cell line was used. The differences between the REP values for some congeners obtained by using different CALUX assays must be ascribed to the different assay conditions.
REP values must therefore be considered as 'assay specific' values and when such values are compared, care must be taken to account for the calculation methods.

Despite the growing evidence of *in vivo* and *in vitro* toxicity effects of brominated and mixed halogenated dibenzo-*p*-dioxins and dibenzofurans[4,5], data on environmental occurrence and exposure to different brominated and mixed chlorinated/brominated congeners is still limited.

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