Subchronic Dose-Response Study of 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin in Female Sprague-Dawley Rats

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Abstract

Toxic and biochemical potencies of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) were studied in a 13-week feeding study in female Sprague-Dawley rats. The diets were supplemented with 0, 0.2, 0.4, 0.7, 5, or 20 µg TCDD/kg diet. The estimated daily intakes were calculated to be 0, 14, 26, 47, 320, or 1024 ng TCDD/kg body weight/day. At the end of the study, TCDD concentrations were measured in liver and adipose tissue. Loss of hepatic retinoids and induction of CYP1A1 and CYP1A2 activities were already found at 14 ng/kg/day, the lowest dose used. Therefore, 95% confidence limits for the no-effect-levels (CNELs) were calculated from the corresponding dose-response relationships by using sigmoidal curve fittings (Hill, Weibull, and a Logistic model) and a probability level of $p < 0.05$. For increases in CYP1A1 and CYP1A2 activities, the right critical values for the CNELs ranged from 0.7 to 4 ng TCDD/kg/day (Hill and Weibull). Based on hepatic TCDD residue levels, these right critical values for the CNELs ranged from 0.06 to 0.4 ng TCDD/g liver (wet weight) (Hill and Weibull). The CNELs in this study agree very well with the no-observed-adverse-effects levels (NOAELs) as reported before in chronic, carcinogenicity, and reproductive studies with rats and TCDD, i.e., 1 ng/kg/day.

Introduction

The 2-year carcinogenicity study with rodents and TCDD of Kociba and co-workers[1] has been extensively used for recommendation of the lifetime human health-based exposure limit of TCDD. A no-observed-adverse-effect level (NOAEL) of 1 ng TCDD/kg/day was found. A lifetime tolerable daily intake ranging from 1 to 10 pg/kg/day of TCDD for humans was derived by using a threshold-based approach. However, risk assessment based on intake dose does not involve kinetic aspects of the compound to be evaluated. Therefore, extrapolations based on intake dose will fail when large pharmacokinetic differences exist between species. The half-life of TCDD in humans was calculated to be 7 years[2]. In rats, half-lifes are reported to range from 17 to 31 days[3-5]. Experiments designed to include target tissue concentrations at steady-state conditions will give a better base for extrapolation. For rats, steady-state conditions will be reached within 13 weeks based on a half-life of 3 weeks for TCDD[6].

In our 13-week feeding study with TCDD, dose-dependent toxic and biochemical responses have been investigated and NOAELs and lowest-observed-adverse-effect levels

(LOAELs) were determined for several parameters. These include a number of recently developed more sensitive biological parameters like hepatic retinoids, next to CYP1A1 and CYP1A2 induction[7]. These parameters were not evaluated in the 13-week feeding study of Kociba and co-workers[8]. In addition, the NOAELs and LOAELs are calculated based on TCDD target tissue concentrations, which should be more pertinent for extrapolation to people.

Methods

Chemicals: TCDD, originating from Dow Chemical (Midland, Mi, USA) and [^{3}H]TCDD were obtained from Givaudan SA (Switzerland) (purities 99%). [^{3}H]TCDD was repurified before use.
Animals and treatment: Female Sprague-Dawley rats [Iva: S/V 50 (SD)], Ivanovas (Kissley, Germany), 8 animals per group, 7 weeks old, starting weight of about 150 grams, were fed on experimental diets for 13 weeks. The diets, in pulverized form, were prepared according to Pluess *et al*[9]. and contained 0, 0.2, 0.4, 0.7, 5, or 20 µg TCDD/kg diet. Water and food were given *ad libitum*. Daily intakes were based on food consumption, diet level, and mean body weight and were estimated to be 0, 14, 26, 47, 320, or 1024 ng TCDD/kg/day.
Cytochrome P450 activity measurements: Microsomal ethoxyresorufin-*O*-deethylase (EROD) activities were fluorimetrically measured according to Burke *et al*[10]. by using a COBAS BIO autoanalyzer. Microsomal CYP1A2 activity was determined as the 4-hydroxylation of acetanilide (4-OH-AA) as a marker according to Liu *et al*[11]. Protein levels were spectrophotometrically determined according to Bradford[12] by using a Biorad Model 3550 microplate reader and BSA as a reference.
Retinoid analyses: Liver retinoids were analyzed according to Brouwer *et al*[13].
Residue analyses: Residues of TCDD in liver and adipose tissue were analyzed for ^{3}H activity by liquid scintillation counting as described previously[14].
Statistics: Data were analyzed for differences to controls with ANOVA (least significant difference, LSD). When the lowest dose used had already an effect, sigmoidal dose-response relationships were fitted to the data by using either the daily dose or the hepatic concentration of TCDD. Calculations were performed with SigmaStat (Jandel Scientific, San Rafael, CA, USA) according to three equations or models. Firstly, a Hill equation was used, secondly a Weibull transformation was applied, and thirdly a Logistic model was used.

The Hill equation:

$y=a+(b-a)*(x^c)/(ED_{50}{}^c+x^c)$, where y is effect at log dose x; a is average minimum; b is average maximum; c is slope; d is $\log(ED_{50})$

The Weibull transformation:

$\log(\log(100/(100-y)))=a+bx$, where y is effect (in % of maximum) at log dose x

The Logistic model:

$y=a/(1+e^{(b*(x-c))})+d$, where y is effect at log dose x; a is the range of the y-value; b is slope; c is y-value at $\log(ED_{50})$; d is average minimum

From these best fit dose-response curves we defined the corresponding calculated no-effect-levels (CNELs) as the measured average control value plus twice the standard deviation (SD), i.e. by using $p < 0.05$ (Student's *t*-test). This y-value was used in the three equations to obtain the corresponding 95% confidence limits for the CNELs. The confidence limits of the CNELs were derived from analysis of variance for (non-) linear regression, or rather, (non-) linear calibration ($p < 0.05$)[15].

The control group was set at log dose -0.5 ng/kg/day (0.32 ng/kg/day; intake dose) or -1.5 ng/g liver (0.032 ng/g liver; internal dose), because of the mathematical impossibility to make a curvefit with the logarithm of zero. The highest correlation coefficients were obtained for these dose-response curves at the corresponding parameters by using log doses (-0.5 ng/kg/day and -1.5 ng/g liver). Moreover, the CNELs were exactly the same for a range of various log doses.

Results and Discussion

Cytochrome P450: Ethoxyresorufin-*O*-deethylase (EROD) activity, a marker for CYP1A1 activity, in liver microsomes was markedly induced by TCDD, up to 49 times control levels as measured in the 1024 ng TCDD/kg/day group (Table 1). At the lowest dose used, 14 ng TCDD/kg/day, EROD activity was still significantly induced, 11 times compared to control

levels. Figure 1A shows the dose-response relationship of the log of the daily dose and EROD activity after TCDD administration using the Hill fit ($r = 0.95$, $p < 0.001$). Sigmoidal curve fitting using the Hill model resulted in a 95% confidence limits for the CNEL ranging from 0.35 to 0.89 ng TCDD/kg day based on intake (dietary dose) (table 2). Figure 1B presents the dose-response relationship of the log of the internal dose, i.e., the EROD activity versus the TCDD liver concentration using the Hill fit ($r = 0.95$, $p < 0.001$). The 95% confidence limits for the CNEL ranged from 0.037 to 0.096 ng TCDD/g liver (wet weight) by curve fitting using the Hill equation. The 95% confidence limits for the CNELs using the Weibull transformation and the Logistic model for EROD activity are shown in table 2.

The 4-hydroxylation of acetanilide (4-OH-AA), a marker for CYP1A2 activity, is presented in table 1. Maximum CYP1A2 activity was measured in the 320 ng TCDD/kg/day group, resulting in a 3.7-fold induction compared to controls. At the lowest dose used, a still significant induction of CYP1A2 activity could be measured compared to the control, namely 1.9 times control levels. The highest dose of TCDD used (1024 ng/kg/day) resulted in a significant decrease in CYP1A2 activity compared to 320 ng TCDD/kg/day. Figure 1C shows the dose-response relationship of the log of the daily dose and CYP1A2 activity after TCDD administration using the Hill fit ($r = 0.92$, $p < 0.001$). The 95% confidence limits for the CNEL ranged from 0.55 to 3.8 ng TCDD/kg/day based on intake (dietary dose) (table 2). Figure 1D presents the dose-response relationship of the log of the internal dose using the Hill fit ($r = 0.82$, $p < 0.001$). The 95% confidence limits for the CNEL ranged from 0.063 to 0.42 ng TCDD/g liver (wet weight) by curve fitting using the Hill equation. The 95% confidence limits for the CNELs using the Weibull transformation and the Logistic model for 4-OH-AA activity are shown in table 2.

Based on diet dose, the right critical value, which is the CNEL of interest, ranged from 0.7 to 13 ng TCDD/kg/day. However, the 95% confidence limits using the Logistic model were very broad, which suggests that this model is not applicable for interpolation at low-dose levels using this data-set. Based on the two other fit procedures, Hill and Weibull, the right critical value ranged from 0.7 to 4 ng TCDD/kg/day using EROD and 4-OH-AA as parameters. In a 30-week experiment study of Tritscher and co-workers[16] with female Sprague-Dawley rats, a NOAEL and LOAEL of 0.1 and 0.3 ng/kg/day, respectively, was reported for CYP1A1 (both mRNA and protein). However, the rats were dosed once every two weeks with TCDD which does not imply that a steady-state was reached at the end of the study. In addition, DeVito *et al*[17]. reported in a 90-day gavage study with mice and TCDD an increase in EROD activity in microsomal fractions of liver, lung, and skin at the lowest dose used, i.e., 1.5 ng/kg/day.

The 95% confidence limits for the CNELs based on TCDD liver concentration gave again a broad range using the Logistic model. Based on the Hill and Weibull fits, the right critical value ranged from 0.06 to 0.4 ng TCDD/g liver (wet weight) using EROD and 4-OH-AA as parameters.

The sigmoidal dose-response relationships as found in our study were also found after single-dose exposure to TCDD in rats after 4 days using EROD activity, CYP1A1 mRNA, and UGT1 mRNA as parameters[18]. The sigmoidal dose-response relationships as reported in these studies, give a clear basis for calculating a threshold for TCDD exposure.

Hepatic retinoids: Dose-dependent reductions in liver retinoid levels by TCDD were observed after 13 weeks on the diets (Table 1). Hepatic retinol and retinylpalmitate levels were reduced to 56 and 20% of control values, respectively, at 0.2 μg TCDD/kg diet. Since these large reductions occurred at the lowest dose used, no good sigmoidal dose-response curves could be fitted. Håkansson *et al*[19]. also reported on hepatic vitamin A reductions after a 13-week

feeding period of 0.2 μg TCDD/kg diet. However, they found a reduction to 76% of control levels by using a hydrolysis method for total hepatic retinoid analyses.

The loss in hepatic retinoid stores as observed in our study may be influenced by induction of cytochrome P450 and presumably further stimulated by the co-induction of several UDPGTs. Leo and Lieber[20] observed oxidation of retinol *in vitro* to polar metabolites, including 4-hydroxyretinol, in a system requiring oxygen and NADPH. Roberts *et al*[21]. reported that the purified rabbit CYP1A2 as well as the phenobarbital-inducible CYP2B4 showed high activities in the 4-hydroxylation of retinoic acid, retinol, and retinal.

The sensitivity of hepatic retinoid depletion clearly demonstrates an urgent need for the investigation at lower dose-exposure ranges for calculating CNELs for this parameter.

Table 1. Ethoxyresorufin-*O*-deethylase (EROD) and acetanilide-4-hydroxylase (4-OH-AA) activities in hepatic microsomes, and hepatic retinoid levels in rats fed on diets containing TCDD for 13 weeks.

Dose of TCDD (ng/kg/day)	EROD (nmol/mg.min)	4-OH-AA (μg/mg.min)	Hepatic retinol (mg/g liver)	Hepatic retinyl-palmitate (mg/g liver)
0	0.19 ± 0.02	0.65 ± 0.071	14.9 ± 3.1	472 ± 96
14	2.16 ± 0.17[a]	1.24 ± 0.15[a]	8.4 ± 1.2[a]	94 ± 24[a]
26	4.10 ± 0.40[a]	1.61 ± 0.12[a]	8.2 ± 0.8[a]	107 ± 27[a]
47	5.02 ± 0.30[a]	2.05 ± 0.09[a]	5.1 ± 0.3[a]	74 ± 14[a]
320	9.95 ± 0.37[a]	2.39 ± 0.19[a]	2.2 ± 0.3[a]	22 ± 8[a]
1024	9.26 ± 0.43[a]	1.94 ± 0.18[ab]	0.6 ± 0.2[a]	3 ± 1[a]

Note. Means ± SE, $n = 8$.
[a] Significant to control (LSD test, $p < 0.05$).
[b] Significant to 320 ng TCDD/kg/day (LSD test, $p < 0.05$).

Table 2. 95% confidence limits for the calculated no-effect-levels using Hill, Weibull, and Logistic fits.

Parameter	CNEL[a] (95% confidence limits)		
	Hill	Weibull	Logistic
EROD (diet dose)	[0.350; **0.893**]	[0.287; **0.688**]	[0.0; **5.42**]
4-OH AA (diet dose)	[0.546; **3.79**]	[0.721; **3.99**]	[0.742; **13.1**]
EROD (liver dose)	[0.0374; **0.096**]	[0.0297; **0.0845**]	[0.0; **0.516**]
4-OH AA (liver dose)	[0.0625; **0.418**]	[0.0735; **0.406**]	[0.056; **1.39**]

[a] For diet dose: ng TCDD/kg/day; for liver dose: ng TCDD/g liver (wet weight).

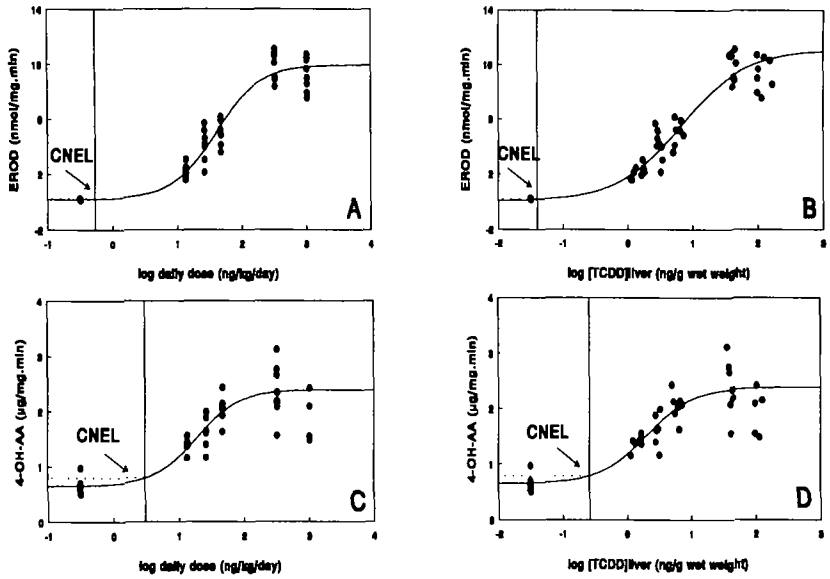

Figure 1(**A-D**). Sigmoidal dose-response relationship between ethoxyresorufin-*O*-deethylase (EROD) activity and dietary TCDD levels (**A**) or hepatic TCDD levels (**B**); 4-hydroxylase (4-OH-AA) activity and dietary TCDD levels (**C**) or hepatic TCDD levels (**D**). The calculated-no-effect levels (CNELs) were 0.9 ng TCDD/kg/day (**A**), 0.1 ng TCDD/g liver (wet weight) (**B**), 4 ng TCDD/kg/day (**C**), and 0.4 ng TCDD/g liver (wet weight) (**D**).

In conclusion, sensitive biochemical effects like CYP1A1 and CYP1A2 induction and loss of hepatic retinoids occurred at the lowest estimated daily intake in this study (14 ng TCDD/kg/day). The dose-response relationships as determined by non-linear curve fits used CYP1A1 and CYP1A2 activities as parameters. The right critical values for the 95% confidence limits ranged from 0.7 to 4 ng TCDD/kg/day (Hill and Weibull). Based on hepatic TCDD residue levels, these right critical values ranged from 0.06 to 0.4 ng TCDD/g liver (wet weight) (Hill and Weibull). At the 14 ng TCDD/kg/day dose level, strong reductions in hepatic retinoids were observed. This indicates that reductions in hepatic retinoid levels are at least as sensitive as inductions in CYP1A1 or CYP1A2 activities are. The CNELs in this study agree very well with the NOAELs as reported before in chronic, carcinogenicity, and reproductive studies with rats and TCDD, i.e., 1 ng/kg/day[1,22].

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Literature

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