

Estimates of Cancer Potency of 2,3,7,8-Tetrachlorodibenzo(p)dioxin Using Linear and Nonlinear Dose-Response Modeling and Toxicokinetics

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Linear and nonlinear toxicity criteria were derived for 2,3,7,8-tetrachlorodibenzo(p)dioxin (TCDD) using the recent National Toxicology Program rat cancer bioassay. Dose-response relationships were assessed for combined liver tumors based on lifetime average liver concentrations (LALCs) estimated with a toxicokinetic model. Rat LALC estimates at the 1% point of departure (POD) were obtained with benchmark dose (BMD) modeling to yield the BMD₀₁ in terms of LALC. The same toxicokinetic model was used to back-extrapolate the human-equivalent external dose (HED). A linear cancer slope factor (CSF) with a value of 1×10^{-4} per pg/kg/day was calculated as the ratio between the benchmark response rate and the HED at the lower confidence limit of the benchmark dose (BMDL)₀₁. A nonlinear reference dose (RfD) with a value of 100 pg/kg/day was developed from the BMD₀₁ value by applying uncertainty factors to rat internal and human external doses. The RfD was 100 times higher than the 10^{-4} risk-specific dose (RSD) based on the linear CSF. For comparison, BMD₀₁ and BMDL₀₁ values were developed for key events in the tumor promotion mode of action (MOA) of TCDD. This MOA involves dysregulation of the normal function of the aryl hydrocarbon receptor and its associated biological processes and results in pathologies that drive tumor promotion and progression. The BMD₀₁ values for key events were consistent with the timing of the key events within the MOA and provided support for the choices of the 1% tumor rate as a POD and dichotomous Hill model for representing receptor-mediated carcinogenicity. Because a threshold toxicity criterion most accurately reflects the MOA, the RfD for TCDD with a value of 100 pg/kg/day is considered appropriate for regulatory purposes, consistent with a 2006 NRC panel's recommendation to develop a threshold-based cancer potency factor for TCDD and with the methodology in U.S. Environmental Protection Agency's Cancer Guidelines.

Key Words: TCDD; dioxin; cancer; risk assessment; slope factor.

The National Toxicology Program (NTP) recently published new cancer bioassays for 2,3,7,8-tetrachlorodibenzo(p)dioxin (TCDD) and other dioxin-like chemicals (DLCs),

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both as single compounds and as mixtures (NTP, 2006a,b,c). These cancer bioassays were conducted using a sophisticated design that incorporated six dose groups, including a stop-exposure group, interim sacrifices, and measures of tissue concentrations and enzyme activity at multiple time points. The NTP cancer bioassay report for TCDD concluded that there was "clear evidence of carcinogenic activity" of TCDD in female Harlan Sprague-Dawley (SD) rats based on increased incidences of cholangiocarcinoma and cellular adenoma of the liver, gingival squamous cell carcinoma of the oral mucosa, and cystic keratinizing epithelioma of the lung (NTP, 2006a). Although the relevance of rodent liver tumors for human risk assessment may be questionable, these tumors have historically been used by U.S. Environmental Protection Agency (USEPA) as the basis of cancer risk assessment of dioxin-like compounds in humans (Kociba *et al.*, 1978; Maruyama and Aoki, 2006; Mayes *et al.*, 1998; USEPA, 1989, 2003; WHO, 1998, 2000, 2001). Therefore, the use of this specific end point from the recent NTP TCDD cancer bioassay, combined with toxicokinetic and toxicodynamic extrapolation, permits comparison of the toxicity values developed here with historical toxicity criteria (USEPA, 1989, 2003). The occurrence of combined liver tumors, hepatocellular adenomas, and cholangiocarcinomas was the strongest carcinogenic response to TCDD observed in the bioassay. Overall, the NTP cancer bioassay for TCDD provides an opportunity to conduct a cancer risk assessment on an internal dose basis and, thus, account for interspecies differences in toxicokinetics and to factor in mode of action (MOA) information for selection of the point of departure (POD) and the dose-response model used for extrapolation (USEPA, 2005; WHO-IPCS, 2005, 2008).

This paper presents two methods of deriving cancer potency estimates: (1) a linear extrapolation method that yields a cancer slope factor (CSF) calculated as the ratio between the chosen benchmark response rate (BMR) or POD and the lower confidence limit of the benchmark dose (BMDL) converted to a human-equivalent external dose (HED); and (2) a nonlinear

approach that yields a reference dose (RfD) calculated as the ratio between the benchmark dose (BMD) at the chosen BMR/POD and the product of several extrapolation/adjustment factors. Linear extrapolation for cancer risk assessment is the default method and is a protective choice when information is lacking (USEPA, 2005; Hattis *et al.*, 2009). However, there is evidence that even proven genotoxic carcinogens exhibit dose thresholds, and thus, biological support for the linear assumption is lacking (Deal *et al.*, 1989; Fukushima *et al.*, 2002; Tsuda *et al.*, 2003; Waddell *et al.*, 2006; Williams *et al.*, 1996, 1999). Nonetheless, USEPA considers linear extrapolation to be an appropriate default method when data regarding the key events in the MOA are judged to be insufficient. However, for TCDD, the data in the scientific literature provide an extensive description of the MOA, and it is appropriate to consider both linear and nonlinear extrapolation as potential regulatory choices.

In this effort, the Carrier/Aylward toxicokinetic model was used to estimate internal dose and corresponding external doses at steady state (Aylward *et al.*, 2005a,b; Carrier *et al.*, 1995a,b). The lifetime average liver concentration (LALC) was chosen as the dose metric most relevant to the liver tumor end point. At all dose levels and times, the concentrations of TCDD observed in the livers of the rats in the NTP bioassay were considerably higher than those observed in adipose tissue (NTP, 2006a). The choice of LALC as the relevant dose metric for liver tumors takes into account these high liver concentrations (WHO-IPCS, 2008).

The most likely hepatocarcinogenic MOA for TCDD and other aryl hydrocarbon receptor (AHR) agonists involves tumor promotion of spontaneously initiated hepatocytes that occurs with threshold-dependent characteristics (Pitot *et al.*, 1987; Viluksela *et al.*, 2000; Waern *et al.*, 1991). An analysis of this and other potential MOAs using a recent operational classification system is presented in the "Materials and Methods" section (Hattis *et al.*, 2009).

The exploration of multiple approaches to cancer risk assessment of dioxin-like compounds will enable a quantitative assessment of uncertainty associated with the carcinogenic MOA. This approach is the best practice, according to USEPA's *Guidelines for Carcinogen Risk Assessment* (USEPA, 2005), and such an approach was recommended both by the USEPA's Science Advisory Board, the National Academy of Sciences expert panel in their review of USEPA's dioxin reassessment, and by other scientists (NRC, 2006; Popp *et al.*, 2006; USEPA-SAB, 2001; Walker, 2007). A newly proposed linear extrapolation method that utilizes interspecies adjustments and low-dose adjustments has been investigated for TCDD and will be the subject of another publication (NRC, 2008; Simon, 2009; White *et al.*, 2009).

MATERIALS AND METHODS

Evaluation of Modes of Action for TCDD Carcinogenesis

A nongenotoxic MOA is the biological foundation for a threshold approach to TCDD cancer dose-response modeling. The proposed mixed MOA for

TCDD-induced liver tumors in female SD rats include (1) tumor promotion via persistent activation of the AHR resulting in key events such as the inhibition of apoptosis within altered hepatic foci and (2) regenerative proliferation due to TCDD-associated cytotoxicity. Promotion via persistent activation of the AHR by TCDD leads to dysregulatory changes in AHR function. Dysregulation is reflected in changes in cytochrome P450 1A (CYP1A) induction, although generally adaptive, inhibition of apoptosis within altered hepatic foci, and increased cell proliferation of altered hepatic foci (Conolly and Andersen, 1997; Graham *et al.*, 1988; Kociba *et al.*, 1978; NTP, 2006a,b,c; Pitot *et al.*, 1987; Schrenk *et al.*, 2004; Schwarz and Appel, 2005; Stinchcombe *et al.*, 1995; Teeguarden *et al.*, 1999; Viluksela *et al.*, 2000; Waern *et al.*, 1991).

Late-stage, high-dose hepatopathy and cytotoxicity was observed in the rats in NTP (2006a; Hailey *et al.*, 2005) and likely resulted in increased regenerative repair-induced DNA synthesis and cell division as part of a tumor progression MOA. Similar to the inhibition of apoptosis due to AHR activation, regenerative repair would also provide a mitogenic stimulus for converting initiated cells within altered hepatic foci into cancer cells. It is not known which is the stronger proliferative stimulus, but the importance of the late-stage events becomes evident when one considers that dose-response of labeling index as a measure of cell proliferation shows a negative association with the tumor response at 14 weeks and a positive association at 31 and 53 weeks (NTP, 2006a).

The mixed MOA proposed here involves tumor promotion via AHR activation with tumor promotion and progression continuing via late-stage hepatotoxicity. The associated key events exhibit dose-response and temporal relationships that are consistent with the body of evidence published on TCDD and other persistent AHR ligands. They are also consistent with the MOA and key events for other receptor-mediated hepatic tumor promoters (Holsapple *et al.*, 2006; Roberts *et al.*, 1997). More information on the MOA and a discussion related to the operational classification scheme for nonmutagenic MOAs for carcinogenesis of Hattis *et al.* (2009) will be provided in the "Discussion" section.

Evaluation of Toxicokinetic Models

The tissue concentration measurements obtained in the bioassay were represented with a toxicokinetic model to facilitate interspecies extrapolation. The LALC values in the SD rats used in the bioassay were estimated with the Carrier/Aylward model (Aylward *et al.*, 2005a,b; Carrier *et al.*, 1995a,b). The Carrier/Aylward model was also used for determination of HEDs based on human liver concentrations corresponding to the rat liver concentrations at the POD (see below). The model was used to estimate target tissue dose in rats and then determine the external human-equivalent dose that would produce a similar target tissue dose in a manner consistent with draft guidance from WHO-IPCS (2008) and guidance from USEPA (2006).

Average liver and adipose concentrations in the rats were estimated using a weekly timescale over 104 weeks, the length of the bioassay. The model estimates for liver and adipose tissue concentrations also matched the measured concentrations quite well (Supplementary Data). For humans, an exposure duration of 75 years, beginning at birth and continuing until age 75, was assumed, and average concentrations were calculated on a monthly basis. Body weight changes were modeled by fitting a variant of the logistic growth equation to data from McDowell *et al.* (2005) (Supplementary Data). The increase in body weight during adolescence is the reason for the inflection in the curves in Figure 1. Carrier/Aylward model parameters for both humans and rats are shown in Supplementary Data.

BMD Modeling

BMD modeling was conducted using USEPA's BMD Software v. 2.0 to obtain dose-response relationships between the poly-3 survival-adjusted incidences of combined liver tumors in rats and LALC (Supplementary Data). A BMR of 1% was used to obtain the liver concentrations for use as the POD (BMC₀₁) along with appropriate 95% lower confidence limits (BMCL₀₁). The best fitting model based on the Akaike information criterion was the multistage model and the best fitting model based on Chi-square *p* values was the dichotomous Hill model (USEPA, 2000). Both of these models are used for nonlinear and linear extrapolation. The fit of the two models in the region of the

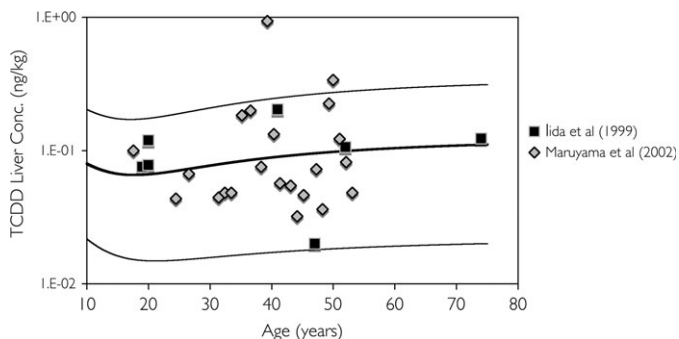


FIG. 1. Comparison of measured and modeled concentrations in human liver. The larger black squares show data from Iida *et al.* (1999) and the smaller gray diamonds show data from figure 5 in Maruyama *et al.* (2002). The thick solid line shows the mean intake of 2.5 pg/day. The thin solid lines show the 10th and 90th intake percentiles of 0.5 and 6 pg/day, respectively (Arisawa *et al.*, 2008)

POD and the low-dose region was indistinguishable (Supplementary Data). The dichotomous Hill model is more consistent with the underlying biology of receptor-mediated toxicity (Fig. 2). The BMDL₀₁ value estimated with the multistage model was considerably lower than that obtained with all other

models (Supplementary Data). Nonetheless, the results from the multistage model are presented alongside that of the dichotomous Hill model for comparison with default USEPA methodology (USEPA, 1989).

A BMR of 1% was chosen because it is near the lower end of the observed range, falling between the second and third doses (Supplementary Data). A BMR of 10% was rejected because it would involve extrapolation across three doses in a region that the data suggest is sublinear; USEPA's *Guidelines for Carcinogen Risk Assessment* cautions against such mid-range extrapolations (USEPA, 2005). The LALC corresponding to a BMR of 1% was higher than that of the lower two doses in the NTP bioassay yet lower than the lowest dose at which tumors were observed.

Choice of End Point and Corresponding Dose Metric

Generally, the occurrence of liver tumors in female SD rats is considered the most sensitive tumor end point for DLCs. Cystic keratinizing epithelioma of the lung appears to be a less sensitive end point than combined liver tumors (NTP, 2006a). The etiology of gingival squamous cell tumors cannot be unequivocally attributed to TCDD. Gingival hyperplasia in the bioassay was between 2 and 40% in controls of seven different bioassays of female SD rats (Yoshizawa *et al.*, 2005), and gingival tumors may result from irritation by impingement of hair shafts or food particles (Garant and Cho, 1979).

The occurrence of liver tumors constitutes the most appropriate end point, in terms of consistency with previous risk assessments and with the MOA, and LALC was chosen as the dose metric. The choice of LALC as the dose metric as opposed to body burden or adipose concentration is consistent with the draft

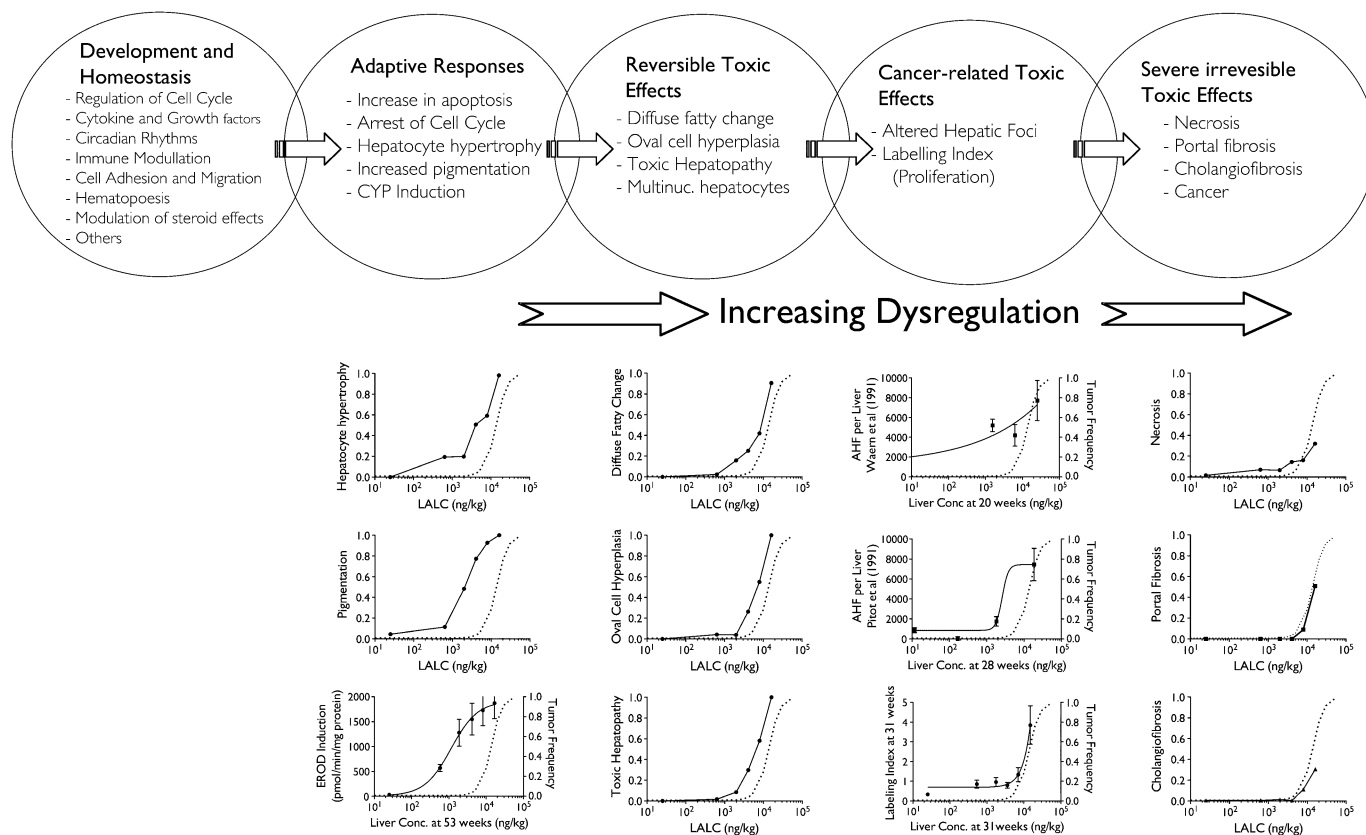


FIG. 2. Schematic of TCDD MOA and dose-response of key events compared to the dose-response of combined liver tumors. The modeled combined tumor response is shown as a dotted line in all plots. As the key events move further from adaptive responses to severe and irreversible toxic effects, their dose-response curves approach that of the combined tumor response. Terminal events in the MOA such as necrosis or portal fibrosis have dose-response curves overlaying or to the right of the combined tumor response. For continuous key events, EROD induction (bottom graph of the first column), AHF occurrence from Waern *et al.* (1991) and Pitot *et al.* (1987), and labeling index (all graphs in the third column), the left-hand y-axis shows the scale of the key event and the right-hand y-axis shows tumor frequency.

TABLE 1
Comparison of BMD₀₁ Values for the Nonneoplastic Key Events and Combined Liver Tumors

Effect	Dose-response function	BMD ₀₁ LALC (ng/kg)	BMDL ₀₁ LALC (ng/kg)	POD	BMR	Reversibility
Adaptive responses						
Hepatocyte hypertrophy	Dichotomous Hill	94	37	1%	NA	68%
Pigmentation	Dichotomous Hill	247	125	1%	NA	20%
EROD induction (53 weeks)	Hill	261	195	Average σ of dose groups excluding control	250	NA
PROD induction (53 weeks)	Hill	327	181	+ 1 σ	0.486	NA
A4H induction (53 weeks)	Hill	462	297	+ 1 σ	0.065	NA
Reversible responses						
Diffuse fatty change	Dichotomous Hill	472	247	1%	NA	82%
Oval cell hyperplasia	Dichotomous Hill	1548	986	1%	NA	98%
Toxic hepatopathy	Dichotomous Hill	1048	609	1%	NA	82%
Reversible toxic responses						
Multinucleated hepatocytes	Dichotomous Hill	426	238	1%	NA	46%
Cancer-related responses						
Labeling index at 31 weeks	Hill	4994	3621	+ 1 σ	0.178	NA
Labeling index at 53 weeks	Hill	9157	8233	+ 1 σ	0.537	NA
AHF Waern	Hill	831	435	Lowest response at any dose minus 1 control σ	3800	NA
AHF Pitot	Hill	1854	1104	+ 1 σ	3650	NA
Severe irreversible responses						
Portal fibrosis	Multistage	4010	2293	1%	NA	Irreversible
Cholangiofibrosis	Multistage	3588	1497	1%	NA	Irreversible
Cancer responses						
Combined liver tumors	Dichotomous Hill	2610	1783	1%	NA	Irreversible
Combined liver tumors	Multistage Model	2475	983	1%	NA	Irreversible

Note. NA, not applicable.

guidance from WHO-IPCS (2008). This dose metric was chosen specifically because it was the most plausible based on consistency with the MOA of tumor promotion by AHR activation and progression by hepatopathy.

Human toxicokinetics of DLCs appears to be quite different than that in rodents and is likely due to the greater degree of liver sequestration of DLCs in rodents (Carrier *et al.*, 1995a,b). In addition, measured dioxin concentrations in humans are generally presented as lipid-adjusted blood concentrations. The Carrier/Aylward model has only two compartments—liver and adipose tissue; the adipose tissue compartment is noncontiguous and represents fat throughout the body. The measurement of blood lipid for normalization introduces considerable uncertainty into this measurement (Bernert *et al.*, 2007; Mills *et al.*, 2007; Scott and Kreider, 2009). Because of the relative certainty of measurements of TCDD in rat and human livers and because of interspecies differences in toxicokinetics, LALC is the most plausible and appropriate dose metric for the liver tumor end point.

The human Carrier/Aylward toxicokinetic model was used to calculate HED values in terms of nanograms TCDD per kilogram body weight per day (ng/kg/day). In other words, these values were the modeled human daily doses in ng/kg/day yielding the LALC dose metric values corresponding to the modeled POD tissue concentrations from the rat bioassay.

Choice of the POD

Dose-response evaluations of a number of key events in the MOA provided support for the use of the dichotomous Hill model and for the choice of a POD of 1% for the combined tumor end point. This value is near the lower end of the dose range and is still supported by the data (USEPA, 2005). The lowest two dose levels of 3 and 10 ng/kg/day produce LALC values in the rats that are below the POD. The tumor response appears to begin at a dose level of 22 ng/kg/day, the dose just above the POD, and the survival-adjusted tumor rate at 22 ng/kg/day is 2.9%.

As further support for the choice of a BMR of 1%, the dose responses of a range of potential key events in the MOA were investigated quantitatively and compared to the dose-response for combined liver tumors (Table 1; Fig. 2). These key events include enzyme induction, hepatocyte hypertrophy, increased pigmentation, diffuse fatty change, the occurrence of multinucleated hepatocytes, toxic hepatopathy, cell proliferation, the occurrence of altered hepatic foci, necrosis, portal fibrosis, and cholangiofibrosis. These key events reflect various phases of the MOA as discussed in the "Introduction" section. Dose-response data were obtained from Hailey *et al.* (2005), NTP (2006a), Pitot *et al.* (1987), Toyoshiba *et al.* (2004), and Waern *et al.* (1991). Some of the dichotomous data were presented as both frequency of occurrence and severity. Severity was graded on a scale of 1 to 4. To include both these measures, frequency of occurrence was multiplied by the common logarithm of the average severity plus one and normalized to the highest observed frequency.

$$\text{Adjusted frequency} = \frac{\text{Frequency} \times \log(\text{severity} + 1)}{\text{Maximum frequency}} \quad (1)$$

In this way, the frequency of occurrence data was adjusted to factor in severity. Continuous end points were fit to a Hill model; dichotomous end points were fit to a dichotomous Hill model using USEPA's Benchmark Dose Software v. 2.0.

Linear Extrapolation

The approach used to estimate a cancer potency factor was based on linear extrapolation from the modeled POD value. The lower confidence limit HED (LHED) was back-extrapolated from the BMDL₀₁ tissue concentration in rats using the Carrier/Aylward toxicokinetic model. This methodology is identical to that depicted in Figure 1 of Simon *et al.* (2008). Cancer potency factors were calculated by linear extrapolation from the POD value expressed as LHED₀₁ by

TABLE 2
Extrapolation/Adjustment Factors for Nonlinear Extrapolation

Factor	Abbreviation	Value
Interspecies CSAF for dynamics	AD _{AF}	0.1
Intraspecies CSAF for dynamics	HD _{AF}	3
Interspecies CSAF for kinetics	AK _{AF}	NA
Intraspecies CSAF for kinetics	HK _{AF}	3
LOAEL-to-NOAEL AF	LN _{AF}	1
Subchronic-to-chronic AF	SC _{AF}	1
Database insufficiency AF	DB _{AF}	1

Note. NA, not applicable. The nomenclature in WHO-IPCS (2005) was adopted. Details are provided in Supplementary Data

calculating the ratio between the BMR of 1% and the corresponding POD (Table 3):

$$\text{CSF} = \frac{\text{BMR}}{\text{POD}} \quad (2)$$

Nonlinear Extrapolation and Choice of Extrapolation/Adjustment Factors

The approach to the derivation of cancer RfDs is consistent with a threshold approach to cancer dose-response (USEPA, 2005) and reflects the sustained dysregulation of AHR function by TCDD leading to tumor promotion and progression. Chemical-specific adjustment factors (CSAFs) were used for the animal-to-human toxicodynamic adjustment factor (AD_{AF}) and the human variability toxicodynamic adjustment factor (HD_{AF}) and were developed as detailed in WHO-IPCS (2005). This guidance indicates four areas of consideration of experimental data on which to base AD_{AF} and HD_{AF}—relevance of populations/studies, adequacy of the concentration-response data, adequacy of the numbers of subjects/samples, and additional considerations. These CSAFs are discussed briefly below and in detail in Supplementary Data. Extrapolation factors for adjusting from a lowest observed adverse effect level to a no observed adverse effect level (LN_{AF}), for subchronic-to-chronic dose regimens (SC_{AF}), and for database insufficiencies (DB_{AF}) are shown in Table 2 and also discussed in Supplementary Data.

Animal-to-human adjustment factor for toxicodynamics (AD_{AF}). Van den Berg *et al.* (2006) and Connor and Aylward (2006) suggest that humans are

approximately 1/10 as sensitive as rodents on a toxicodynamic basis. Numerous studies have examined induction of CYP enzymes in hepatocytes as a sensitive marker of TCDD binding to the AHR and activation of AHR pathways. Silkworth *et al.* (2005) and Xu *et al.* (2000) are the most relevant studies because they compare hepatocytes from the same rat strain used in the NTP bioassay to hepatocytes from humans. Xu *et al.* (2000) compared the expression of CYP1A1 and CYP1A2 mRNA and 7-ethoxyresorufin-O-deethylase (EROD) induction as a measure of CYP1A1 in hepatocytes in primary culture from male SD rats and human donors. Silkworth *et al.* (2005) measured the expression of CYP1A1 and CYP1A2 mRNA in hepatocytes in primary culture from female SD rats, a single female Rhesus monkey, and from five human donors. Recent work on hepatocytes in primary culture from female SD rats and human donors are consistent with the published data (Budinsky *et al.*, 2009; Rowlands *et al.*, 2009). In addition, the AHR gene polymorphisms occurring in the nonresponsive DBA/2 mouse strain are present at analogous positions in the human AHR consensus sequence and result in a less accessible ligand-binding pocket and lower TCDD-binding affinity (Ema *et al.*, 1994; Moriguchi *et al.*, 2003; Procopio *et al.*, 2002; Rowlands *et al.*, 2008a,b). All these data sets indicate that for this fundamental early signal response to TCDD, human hepatocytes are 10-fold or more less sensitive than those from the SD rat strain, consistent with the presence of a low-affinity binding site on the human AHR congruent with that observed in DBA/2 mice (reviewed in Connor and Aylward, 2006).

Human variation adjustment factor for toxicodynamics (HD_{AF}). The methodology in WHO-IPCS (2005) was also used to estimate a CSAF to account for differences in toxicodynamic sensitivity within humans to include the most sensitive individuals. The range of HD_{AF} values was from 2 to 7 (Silkworth *et al.*, 2005; Xu *et al.*, 2000).

One can use *in vivo* data on caffeine metabolism in humans to provide an upper bound on HD_{AF}. Lambert *et al.* (2006) observed an approximate doubling of CYP1A2 activity between Yucheng patients and controls and a sevenfold variation in CYP1A2 activity among all subjects. These values were also confirmed by data from Abraham *et al.* (2002). Guzelian *et al.* (2006) provide a review and analysis of these findings.

The work on nucleotide sequences in the human AHR indicates that the core ligand-binding domain is not polymorphic across six ethnic groups, although other regions of the AHR gene do show some polymorphisms (Rowlands *et al.*, 2008a). Some of these polymorphisms in the nonbinding region are synonymous and result in no change in the amino acid sequence of the protein; others appear to impart a lower sensitivity (Rowlands *et al.*, 2008a). In addition, functional evaluations suggest that human polymorphisms in the AHR locus will result in either no change or decreased binding affinity for TCDD and similar effects for TCDD-initiated downstream events (Harper *et al.*, 2002; Wong *et al.*, 2001).

Overall combined adjustment factor. The values of all adjustment/extrapolation factors are shown in Table 2 and discussed in detail in Supplementary Data. CSAFs and other adjustment/extrapolation factors were applied on an internal or an external dose basis, as appropriate in a fashion identical to that depicted in Figure 2 of Simon *et al.* (2008) (Table 4). The net combined adjustment/extrapolation factor is 1. The low net adjustment factor (AF) is a result of the choice of an adjustment factor of 0.1 for animal-to-human adjustment for toxicodynamics (AF_{AD}) and the choice of 10 for the human variability adjustment factor (AF_H). This low value for AF_{AD} is justified by the extensive literature on the difference in sensitivity between humans and laboratory animals regarding effects mediated by the AHR (Van den Berg *et al.*, 2006; Connor and Aylward, 2006).

Using Nonneoplastic Sentinel Key Events as Tumor Precursors

Further insight on the relationship between nonneoplastic events and liver tumors can be obtained by assessing the observed liver tumor incidence as a function of the continuous non-neoplastic responses of enzyme induction or increased labeling index or as a function of event frequency for the dichotomous responses of increased pigmentation, hepatocyte hypertrophy, diffuse fatty change, and toxic hepatopathy (Fig. 3). Dose-response

TABLE 3
Low-Dose Linear Extrapolation with CSFs and RSDs

Derivation step	Liver tissue	
	1% POD (dichotomous Hill model)	1% POD (multistage model)
Modeled LECx in rats (tissue concentration, ng/kg)	1783	983
Modeled LHEDx (external dose, ng/kg/day)	0.1	0.05
Estimated CSF (per ng/kg/day)	0.1	0.2
Estimated CSF (per mg/kg/day), commonly used units	100,000	200,000
RSD (mg/kg/day), risk level = 10 ⁻⁶	1 × 10 ⁻¹¹	5 × 10 ⁻¹²
RSD (mg/kg/day), risk level of 10 ⁻⁴	1 × 10 ⁻⁹	5 × 10 ⁻¹⁰

TABLE 4
Nonlinear Estimates of Cancer Potency to Obtain RfDs based on Combined Liver Tumors and Four Key Events

Derivation step	Combined liver tumors					
	1% POD (dichotomous Hill model)	1% POD (multistage model)	Toxic hepatopathy (15% POD)	EROD (6 × BMD)	AHF (3 × BMD)	Labeling index (0.5 × BMD)
Modeled EC _x in rats (tissue concentration, ng/kg)	2610	2475	3085	1566	2493	2497
After application of LN _{AF} (1)	2610	2475	3085	1566	2493	2497
After application of AD _{AF} (0.1)	26,100	24,750	30,850	15,660	24,930	24,970
Modeled HED from Carrier model (external dose, ng/kg/day)	1.3	1.1	1.5	0.8	1.2	1.2
RfD (ng/kg/day) after application of HK _{AF} (3) and HD _{AF} (3)	0.1	0.1	0.15	0.08	0.1	0.1
RfD (mg/kg/day) (commonly used units)	1 × 10 ⁻⁷	1 × 10 ⁻⁷	1 × 10 ⁻⁷	8 × 10 ⁻⁸	1 × 10 ⁻⁷	1 × 10 ⁻⁷

relationships and dose thresholds were determined for several of these key events for consideration in the nonlinear evaluation based on MOA (Fig. 2; Tables 1 and 4).

RESULTS

Evaluation of the Toxicokinetic Model

The Carrier model with inputs appropriate for rats provided a good fit to the NTP bioassay data for both liver and adipose tissue (Supplementary Data). The Carrier model with inputs

appropriate for humans from birth to age 75 also provided a good fit for liver concentrations measured in humans experiencing background exposures (Iida *et al.*, 1999; Maruyama *et al.*, 2002) (Fig. 1).

Maruyama *et al.* (2002) use a daily human intake rate for TCDD of 12.8 pg/day obtained from an untranslated Japanese government report. This value was likely based on the use of detection limits *in lieu* of zero for non-detect analyses and, as a result, overestimated TCDD intake. Hence, the data of Arisawa *et al.* (2008) with a mean intake rate of 2.5 pg/day and

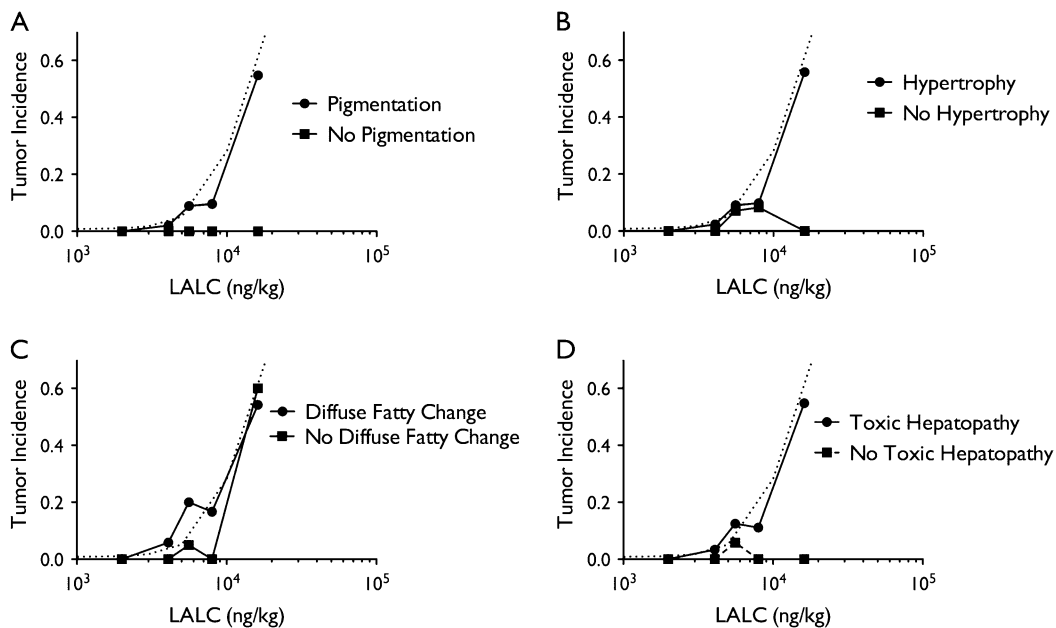


FIG. 3. Comparison of unadjusted tumor incidence rates with the incidence of four key events. (A) Plots of the dose-response of unadjusted tumor incidence for rats with and without an increase in pigmentation. (B) Plots of the dose-response of unadjusted tumor incidence for rats with and without hepatocyte hypertrophy. (C) Plots of the dose-response of unadjusted tumor incidence in rats with and without diffuse fatty change; 3/5 rats (60%) without diffuse fatty change had tumors. Hence, the necessity of this key event for tumor formation is not so clear as for the other key events. Please see text for additional details. (D) Plots of the dose-response of unadjusted tumor incidence with and without toxic hepatopathy. In all plots, the dotted line shows the survival-adjusted combined tumor response.

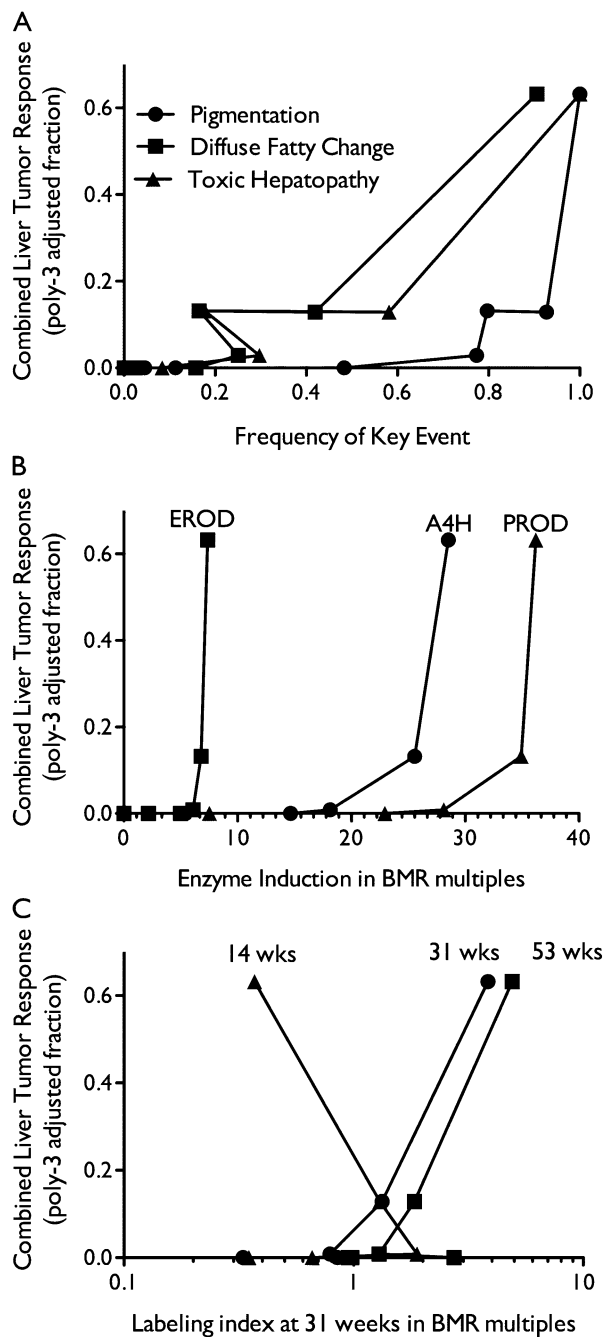


FIG. 4. Tumor response vs. key events. (A) Plot of the frequency of three key events versus the adjusted tumor response showing that for no tumor response occurs at an incidence of toxic hepatopathy or diffuse fatty change less than about 25% and no tumor response occurs at a pigmentation incidence of less than 80%. (B) Enzyme induction at 53 weeks showing that, for EROD, tumor response does not occur until over 7 times the EROD BMR (Table 4), 15 times the A4H BMR, and almost 30 times the PROD BMR. (C) Cell proliferation at 14, 31, and 53 weeks indicating tumor response does not occur until at least twice the BMR at 31 weeks and almost 10-fold the BMR at 53 weeks; at 14 weeks, it is likely that cell proliferation is reduced in the higher dose groups because of cytotoxicity.

an 80% CI from 0.5 to 6 pg/day were used (Fig. 1). The intake value from Focant *et al.* (2002) of 6 pg/day for Europeans is at the 90th percentile of the intakes measured by Arisawa *et al.* (2008).

The Carrier toxicokinetic model provides a good fit to both rat tissue concentrations from the NTP bioassay and human tissue concentrations resulting from background, low-dose exposure. For the rat external doses ranging from 3 to 46 ng/kg/day, the model under-predicts the liver concentrations at 14 weeks. From the measured concentrations in rats, the concentrations in both liver and fat at 104 weeks are underpredicted by the model for doses of 3 through 22 ng/kg/day, whereas the concentrations are overpredicted for doses of 46 and 100 ng/kg/day. This overprediction is the most obvious at a dose of 100 ng/kg/day where the modeled concentration is more than 1 SD above the average measured concentration in both liver and adipose tissue (Supplementary Data).

What is perhaps most interesting is the apparent switch from underprediction to over-prediction between daily doses of 22 and 46 ng/kg/day. If binding to CYP1A2 or other proteins in the liver reaches saturation at this point, more of the TCDD in the liver may be available for elimination, distribution to body lipids other than those sampled in NTP (2006a), or increased in free cytosolic TCDD available for binding to the AHR. An increase in elimination is not accounted for in the Carrier/Aylward model because the model uses a constant hepatic elimination rate. Between doses of 22 and 46 ng/kg/day, the proportion of rats with tumors increases about fourfold, and this observation may suggest the occurrence of a dose-dependent transition.

BMD Modeling

BMDS software v. 2.0 from USEPA was used to obtain the both the numerical coefficients of the dichotomous Hill model, those of the multistage model, and to predict the rat liver BMD_{01} and $BMDL_{01}$ values for both models (Table 1; Supplementary Data). The frequency of combined liver tumors was plotted versus the rat liver concentrations obtained with the Carrier/Aylward model along with the fitted curves from the dichotomous Hill model and the multistage model (Supplementary Data). The fit of the two models in the low-dose region is almost identical.

Choice of End Point and POD

The relevance of rodent liver tumors to humans remains an area of uncertainty. Nonetheless, the use of this tumor type in female SD rats was chosen for consistency with previous TCDD risk assessments (USEPA, 1988, 2003).

The dose-response data for the various noncancer end points support the use of combined liver tumors, the choice of the 1% BMR, and the choice of the dichotomous Hill model (Table 1; Fig. 2). The noncancer end points that are identified as being

within the portion of the MOA that are adaptive or represent reversible toxic responses are the increases in enzyme induction, hepatocyte hypertrophy, pigmentation increase, and the occurrence of multinucleated hepatocytes. The BMD_{01} values for these end points are considerably below that for the combined tumor end point (Table 1; Fig. 2). The noncancer end points within the portion of the MOA that reflect cytotoxicity are diffuse fatty change and toxic hepatopathy. The BMD_{01} values for these end points are higher but still less than that for combined liver tumors. The BMD_{01} values for portal fibrosis and cholangiofibrosis are greater than that for combined tumors and are likely terminal events that occur in association with hepatopathy (Fig. 2).

A similar observation is noted when the BMD_{01} values were calculated for the occurrence of altered hepatic foci (AHFs) from Pitot *et al.* (1987) and Waern *et al.* (1991). The occurrence of AHF is considered to be reflective of potential tumor growth, and BMD_{01} values based on AHF were up to twofold higher than the BMD_{01} values for diffuse fatty change and toxic hepatopathy that reflect cytotoxicity (Table 1; Fig. 2). What is also striking is the visual similarity between the dose-response curve for labeling index at 31 weeks and the combined tumor response (NTP, 2006a) (Fig. 2); however, the BMD for labeling index at 31 weeks obtained using a BMR of one control SD above the control mean is over twofold greater than the BMD_{01} for combined tumor response, and the BMD for the labeling index at 53 weeks is almost fourfold greater (Table 1). This suggests that either tumor growth or regenerative hyperplasia (rather than tumor formation) is reflected by the dose-response for labeling index. Labeling index data from 14 weeks were not modeled because it did not rise monotonically with dose; instead, it reached a peak at an external dose of 22 ng/kg/day (NTP, 2006a) (Fig. 3C).

Reversibility of the various end points was calculated as follows using the severity-adjusted frequency of an effect observed in the animals from the 100 ng/kg/day stop-exposure study and the severity-adjusted frequency of that same effect in the 100 ng/kg/day full-term dose group (Table 1):

$$\text{Reversibility} = (1 - f_{\text{stop-exp}})f_{100}. \quad (3)$$

Considering all these noncancer end points in light of the established MOA provides support for the choice of a 1% response as a POD. The MOA and the various end points are reflective of receptor-mediated toxicity; this point becomes abundantly clear from an inspection of Figure 2 revealing the sigmoid nature of dose-response curves of all key events shown. Hence, it was appropriate to use the 1% tumor response as a POD and to use the dichotomous Hill model for the combined tumor response.

Dose thresholds were determined for several key events (Table 1). The frequency of three dichotomous events was plotted against the combined tumor response to determine an incidence threshold that could serve as a POD (Table 1;

Fig. 4A). Continuous end points were expressed in units of BMR multiples. For enzyme induction at 53 weeks, the control response was subtracted from the responses at all dose levels, and this result was divided by the BMR; for all three enzymes, the BMR was a measure of the SD. The survival-adjusted tumor incidence was plotted against BMR multiples for enzymatic activities measured by EROD reflecting CYP1A1 induction, 7-pentoxoresorufin-*O*-deethylase (PROD) reflecting CYP2B induction, and acetanilide-4-hydroxylase (A4H) reflecting CYP1A2 induction (Table 1; Fig. 4B). Labeling indices at 14, 31, and 53 weeks were also expressed as BMR multiples in the same way and plotted versus the survival-adjusted tumor response (Table 1; Fig. 4C). For the occurrence of AHF, a BMR multiple value of 3 for AHF from Waern *et al.* (1991) was estimated from the data (Tables 1 and 4). Rat liver tumor response appears to increase as a function of non-neoplastic liver effects in a nonlinear manner that is consistent with a threshold. Hence, these key events can either be used as tumor precursors for dose-response evaluation or to support other choices related to their role within the MOA.

Linear Estimates of Potency—CSFs

For the linear estimate of potency, the Carrier/Aylward human model was also used to back-extrapolate to obtain an external HED corresponding to the $BMCL_{01}$ (Table 3). The POD value was then divided by this HED_{01} value to obtain the linear cancer potency factor.

The 1% POD yielded a CSF of 0.1 per ng/kg/day. The RSD at a risk level of 10^{-4} (the upper end of USEPA's regulatory risk range) would be 1 pg/kg/day. The value of the RSD is between 20% and sixfold greater than the current TCDD-toxic equivalents (TEQs) intake in the United States (USFDA, 2006).

Nonlinear Estimates of Potency—RfDs/Tolerable Daily Intakes

For the nonlinear estimate of potency, the identified adjustment factors were applied to the benchmark concentrations in rats described in the "Materials and Methods" section to estimate a human RfD based on the cancer end point. The value of the RfD was 0.1 ng/kg/day or 100 pg/kg/day or $1E-07$ mg/kg/day. Nonlinear dose-response adjustment was also performed for key events in the MOA, and these results support the RfD developed from the combined tumor response (Table 4).

Sentinel Nonneoplastic Key Events as Tumor Precursors

One or more key events may be used as sentinel or precursor key events for tumor formation, providing flexibility, especially for nonlinear dose-response evaluation (USEPA, 2005). To decide which of the key events was most appropriate, the relationship of various key events to tumors was assessed. Pigmentation is closely associated with tumor incidence; none

of the rats without increased pigmentation had liver tumors (Fig. 3A). Hepatocyte hypertrophy and toxic hepatopathy were similarly associated with tumor formation (Figs. 3B and 3D). For diffuse fatty change, the rats with and without this key event showed similar tumor rates at a dose of 100 ng/kg/day; however, only 5/53 rats did not have diffuse fatty change in the high-dose group; three of these five rats (60%) without diffuse fatty change developed liver tumors (Fig. 3C).

When the survival-adjusted tumor incidence is plotted against the frequencies of pigmentation, diffuse fatty change, or toxic hepatopathy, it becomes clear that all three of these key event measures are associated with tumor incidence (Fig. 4A). Regarding enzyme induction, tumor response does not begin until EROD induction has reached a level sixfold greater than the BMR; it does not begin until A4H induction is 15-fold greater than the BMR and it does not begin until PROD induction is 28-fold greater than the BMR (Fig. 4B).

The plots for labeling index at 31 and 53 weeks are very similar whereas that for labeling index at 14 weeks shows a negative slope (Fig. 4C). Cytotoxicity would tend to reduce cell proliferation and reduce the labeling index. This suggests that at 14 weeks, cytotoxicity has a greater effect than tumor progression and, at later times, the growth of tumors predominates. This is consistent with the MOA of high-dose hepatopathy resulting in increased regenerative repair-induced DNA synthesis and cell division (Conolly and Andersen, 1997; Graham *et al.*, 1988; Hailey *et al.*, 2005; Kociba *et al.*, 1978; Pitot *et al.*, 1987; Schrenk *et al.*, 2004; Schwarz and Appel, 2005; Stinchcombe *et al.*, 1995; Teeguarden *et al.*, 1999; Viluksela *et al.*, 2000; NTP, 2006a; Waern *et al.*, 1991).

Hence, for three of four key events showing dichotomous responses, the association with the combined tumor response was striking and strongly supports the threshold nature of the cancer response within the MOA. Basically, the animals without increased pigmentation, without hepatocyte hypertrophy, or without toxic hepatopathy did not develop cancer.

The POD was adjusted for the four key events of toxic hepatopathy, occurrence of AHF, cell proliferation, and EROD induction. For the dichotomous event, toxic hepatopathy, its adjusted frequency was plotted against the combined tumor response to determine an event threshold that could serve as a POD (Fig. 4A).

The latter two continuous end points were in units of BMR multiples. For enzyme induction at 53 weeks, the control response was subtracted from the responses at all dose levels, and this result was divided by the BMR; for all three enzymes, the BMR was a measure of the SD. The survival-adjusted tumor incidence was plotted against EROD, A4H, and PROD induction at 53 weeks expressed as BMR multiples (Table 4, Fig. 4B). Labeling indices at 14, 31, and 53 weeks were also expressed as BMR multiples in the same way and plotted versus the survival-adjusted tumor response (Fig. 4C). A BMR multiple value of 3 for AHF from Waern *et al.* (1991) was estimated from the data in Table 1. These adjusted PODs for key events were

used to estimate nonlinear RfDs, and the values were very similar to that for the combined tumor response (Table 4).

Comparison of Potency Estimates

The values of RSDs at risk levels of 10^{-6} and 10^{-4} and based on linear extrapolation and the resulting CSF are 1×10^{-11} and 1×10^{-9} mg/kg/day, respectively. The value of the RfD is 1×10^{-7} mg/kg/d, which is 100 to 300 times greater than the current TEQ intake in the United States (USFDA, 2006).

DISCUSSION

The combined liver tumor end point in female SD rats was chosen here for consistency with previous risk assessments of DLCs and its consistency with the MOA (Hattis *et al.*, 2009; Boobis *et al.* 2008; USEPA, 2005; Meek *et al.*, 2003; Sonich-Mullin *et al.*, 2001). It is important to note, however, that cancer is not the only risk assessment end point that can be selected for dioxin human health risk assessment (e.g., WHO, 1998, 2000, 2001); currently, in the United States, female rat liver tumor dose-response relationships are used (NTP, 2006a,b,c; USEPA, 1989, 2003). The quantitative risk assessment results presented here based on cancer as the end point highlights several key factors that are considered in risk assessment decisions, namely the modeling approach, the internal dose metric, and species-specific adjustment/extrapolation factors.

The Choice of Linear versus Nonlinear Approach Based on the MOA

The decision with the greatest quantitative impact is the choice of a linear approach versus a nonlinear approach. This decision hinges upon the carcinogenic MOA for DLCs of tumor promotion.

USEPA *Guidelines for Carcinogen Risk Assessment* indicate that linear, low-dose extrapolation is recommended for agents that are DNA reactive with mutagenic activity or for "agents for which human exposures or body burdens are high and near doses associated with key precursor events in the carcinogenic process, so that background exposures to this and other agents operating through a common MOA are in the increasing, approximately linear, portion of the dose-response curve" (USEPA, 2005). As will be discussed below, a mutagenic or genotoxic MOA for TCDD cannot be supported, and neither are current human exposures or body burdens close to those associated with thresholds for key events; hence, linear extrapolation is not appropriate or applicable.

The MOA supports nonlinear extrapolation. The most likely carcinogenic MOA for TCDD and other AHR agonists in the liver is a mixed MOA of tumor promotion and regenerative repair (Hattis *et al.*, 2009). The underlying early

tumor promotional key events consist of AHR activation, inhibition of intrafocal apoptosis, and increased cell proliferation (Graham *et al.*, 1988; Grasl-Kraupp *et al.*, 2000; Luebeck *et al.*, 2000; Moennikes *et al.*, 2004; Stinchcombe *et al.*, 1995; Teeguarden *et al.*, 1999; Worner and Schrenk, 1996). Via AHR activation, TCDD stimulates the early clonal growth of altered hepatic foci (Pitot *et al.*, 1987; Teeguarden *et al.*, 1999; Viluksela *et al.*, 2000). Subsequently, the regenerative repair portion of the MOA includes a toxic hepatopathy that results in increased cell division and growth, further promoting altered hepatic foci. This finding is consistent with the late-developing liver tumors observed in female rats that occur after 53 weeks of TCDD administration (Hailey *et al.* 2005; NTP, 2006a). Precancerous lesions such as eosinophilic foci occur as early as 53 weeks, but tumors do not occur until later (Hailey *et al.* 2005). Ever since the initial observations of liver tumors in female SD rats by Kociba *et al.* (1978), it has been known that sustained liver injury is a significant and necessary factor in the MOA of tumor promotion/progression for hepatocellular carcinoma (Goodman and Sauer, 1992).

An early key event in the tumor promotion portion of the MOA is the inhibition of apoptosis in spontaneously initiated altered hepatic foci. Inhibition of apoptosis by TCDD via AHR activation has been observed in chemically initiated hepatic foci in rats as well as in primary rat hepatocytes initiated by UV irradiation (Schrenk *et al.*, 2004; Stinchcombe *et al.*, 1995). The molecular biology of activated AHR has confirmed that activated AHR interacts directly with the E2F protein, the main transcription factor responsible for the induction of S-phase-specific genes and one of the critical proteins that controls cell entry into the cell cycle and apoptosis (Mitchell and Elferink, 2009; Puga *et al.*, 2009). Through this AHR-E2F cross talk, the cell cycle is inhibited and apoptosis is prevented, and this allows spontaneously initiated hepatocytes to escape normal cell cycle control eventually resulting in increased cell proliferation (Huang and Elferink, 2005; Hushka and Greenlee, 1995; Mitchell and Elferink, 2009; Mitchell *et al.*, 2006; Roberts *et al.*, 1997). The AHR plays a complex role in normal cell cycle control, and its dysregulation results in the apical end point of tumors (Barouki *et al.*, 2007; Beischlag *et al.*, 2008; Bock and Kohle, 2006; Gasiewicz *et al.*, 2008; Hushka and Greenlee, 1995; Ma *et al.*, 2009; Mimura and Fujii-Kuriyama, 2003; Mitchell and Elferink, 2009).

As part of the tumor progression portion of the MOA, sustained AHR activation causes liver injury requiring new cell division-related repair; the accompanying mitogenic signals stimulate initiated cells to divide and proliferate, further promoting the development of tumors (Mitchell *et al.*, 2006; Tijet *et al.*, 2006). Thus, through TCDD activation of the AHR, spontaneously initiated hepatocytes are prevented from dying by inhibition of apoptosis and are also stimulated to divide in the enhanced mitogenic environment resulting from regenerative proliferation in the late stages. These key events

associated with tumor promotion and progression reflect sustained high-level AHR activation resulting in the eventual disruption of normal AHR-mediated transcription circuits and the eventual development of hepatopathy that drives tumor formation; dose thresholds are evident for these key events (Figs. 2 and 3), and the combination of events will also display a dose threshold.

Finally, an important consideration is the recognition that TCDD and other DLCs are not direct-acting mutagens or initiators (NTP, 2006a,b,c; Turteltaub *et al.*, 1990; Whysner and Williams, 1996). TCDD actually decreases the occurrence of age-dependent DNA adducts in the livers of female SD rats (Randerath *et al.*, 1990). Although genotoxicity is the default basis for a linear approach to cancer risk assessment, TCDD is not a genotoxic carcinogen and it is appropriate to use a nonlinear approach with sufficient scientific basis (USEPA, 2005).

Overall, when examined with the human relevance framework in mind, the key events and the MOA provide a strong foundation for the application of a threshold toxicity RfD to the cancer risk assessment of TCDD (Boobis *et al.*, 2008; Meek *et al.*, 2003; Sonich-Mullin *et al.*, 2001). The use of a threshold for TCDD is consistent with USEPA's Cancer Guidelines and the recommendations made by the NRC in their review of the USEPA's dioxin risk assessment (NRC, 2006; USEPA, 2005).

The human relevance framework requires examination of other potential MOAs (Meek *et al.*, 2003). A recent classification scheme provides categorical examples of potential MOAs for consideration (Hattis *et al.*, 2009). For example TCDD's tumor progression could be mediated via suppression of cellular and humoral immunity (Stevens *et al.*, 2009; Sulentic *et al.*, 1998; Kerkvliet, 2009; Kerkvliet *et al.*, 1996; Pande *et al.*, 2005). However, there is no direct evidence of TCDD-induced immune suppression in the development of liver tumors, whereas immune cell infiltration contributing to hepatopathy is observed in the livers of rats in the NTP bioassay (Hailey *et al.* 2005).

An indirect genotoxic MOA involving TCDD-induced CYP1A enzymes and metabolism of estradiol to DNA-reactive compounds has been proposed (Graham *et al.*, 1988; Wyde *et al.*, 2001). However, since an increase in DNA adducts was not observed in female rats administered TCDD, and no dose-dependent increase in adducts occurred in mice in response to TCDD, experimental support for the hypothesis of the formation of DNA adducts from reactive estradiol species is not well supported (Randerath *et al.*, 1990; Turteltaub *et al.*, 1990).

CYP1B1 catalyzes the formation of 4-hydroxyestradiol from estrogen, and this metabolite has been associated with oxidative DNA damage and increased cancer risk (Cavalieri *et al.*, 2000; Jefcoate *et al.*, 2000; Rifkind, 2006). In contrast, CYP1A enzymes catalyze the formation of 2-hydroxyestradiol, and this compound is not associated with either DNA damage or cancer risk (Cavalieri *et al.*, 2000). In the livers of female SD rats, CYP1A1 and CYP1A2 are induced to a greater extent than

is CYP1B1 and 2-hydroxylation would be the predominant reaction (Badawi *et al.*, 2000; Reichard *et al.*, 2005; Walker *et al.*, 1999). Therefore, CYP1A-mediated metabolism of estradiol would theoretically occur to a much greater extent than CYP1B1 metabolism of estradiol, and indirect genotoxicity due to estrogen metabolites seems an unlikely MOA for TCDD (Reichard *et al.*, 2005; Jefcoate *et al.*, 2000).

In addition, estrogen is a tumor promoter itself, and the promotional effects of estrogen cannot be readily separated from those of TCDD; hence, estrogen could potentially add to tumor promotional MOA for TCDD (Lucier *et al.*, 1991; Vickers and Lucier, 1996).

Finally, a role for reactive oxygen species (ROS) in the MOA for TCDD has been proposed possibly resulting from the futile cycling of the induced CYP1A and CYP1B1 enzymes, estradiol quinone formation, or disruption of mitochondrial function. After 20 weeks of dosing with 100 ng/kg/day of TCDD, there was no evidence of an increase in 8-oxo-dG adducts that are presumably due to ROS (Wyde *et al.*, 2001). Initiation with diethyl nitrosamine (DEN) appeared to be necessary for adduct formation (Wyde *et al.*, 2001). Furthermore, 8-oxo-dG adducts induced by TCDD in DEN-initiated rats are dose dependent and require TCDD dosages of 36 ng/kg/day or higher (Wyde *et al.*, 2001).

The role of ROS in tumor promotion and cytotoxicity, for example, development of hepatopathy, is well recognized (Roy *et al.*, 2007; Goetz and Luch, 2008). There is strong concordance of the dose-response of hepatopathy and tumor development (Figs. 2–4). However, ROS formation would be mitigated by induction of oxidative stress response mediated by nuclear regulatory factor 2 (Nrf2), and this phenomenon may provide the biological basis of reduced cancer risk from the consumption of cruciferous vegetables (Kohle and Bock, 2006, 2007). In addition, ROS resulting from AHR activation may have important physiological effects on gene signaling and gene activation (Dalton *et al.*, 2002). Therefore, it seems unlikely that indirect genotoxicity from estrogen metabolites or ROS contributes significantly to tumor formation in rat livers.

In summary, it is recognized that key events contributors could include estrogen-induced tumor promotion or the enhancement of hepatopathy from immune-mediated or ROS-induced tissue damage (Reichard *et al.*, 2005). Quantitative dose-response relationship data for these effects could possibly add to the analyses presented here. However, the limited dose-response information for these factors precludes their examination in this paper.

Overall, when examined with the human relevance framework in mind, the key events and the MOA provide a strong foundation for the application of a threshold toxicity RfD to the cancer risk assessment of TCDD (Boobis *et al.*, 2008; Meek *et al.*, 2003; Sonich-Mullin *et al.*, 2001). The use of a threshold for TCDD is consistent with USEPA's Cancer Guidelines and the recommendations made by the NRC in their review of the USEPA's dioxin risk assessment (NRC, 2006; USEPA, 2005).

Current human exposures are below those associated with key events. As discussed in the previous section, hepatic injury is necessary for tumor formation, and currently, there is no evidence of hepatic injury at background dioxin exposures in laboratory animals and humans, suggesting no increased risk of cancer at these background dioxin exposures.

The body burden basis for choosing linear extrapolation mentioned in USEPA's Cancer Guidelines is also not applicable based upon a consideration of background tissue concentrations of TCDD in humans. Measured concentrations of TCDD in human liver tissue due to background exposure range from approximately 0.02 to 0.9 ng/kg, with an arithmetic mean of 0.1 ng/kg (Iida *et al.* 1999; Maruyama *et al.*, 2002), with concentration generally increasing with age. More recent data indicate an average value of 0.5 ng/kg with values ranging from non-detect to 2 ng/kg (Iida *et al.* 2007). These human liver concentrations are several orders of magnitude lower than the rat liver concentrations associated with an increased liver tumor incidence (4200–14,000 ng/kg, Table 2). It is also worth noting that a liver concentration of 2 ng/kg is 50-fold less than the lowest BMD₀₁ for any of the key events (Table 1). As discussed in the previous section, hepatic injury is necessary for tumor formation, and currently, there is no evidence of hepatic injury at background dioxin exposures in laboratory animals and humans, suggesting no increased risk of cancer at these background dioxin exposures.

Upper percentile values of TCDD blood concentrations in the general population are around 30 pg/g lipid TEQ (Connor *et al.*, 2008; LaKind *et al.*, 2009). Biomonitoring equivalents corresponding to regulatory values from agencies in the European Union range from 30 to 70 pg/g lipid (Aylward *et al.*, 2008). This range would correspond a long-term intake of 2–6 pg/kg/day, 20- to 50-fold lower than the nonlinear RfD. Hence, human background exposures are not close to threshold doses for key precursor events and linear extrapolation is not applicable.

Sentinel Key Events as Tumor Precursors

As indicated, the knowledge of the MOA including AHR activation as a central mechanistic factor suggested the use of one or more key events or adaptive responses as a sentinel for tumor formation, providing flexibility especially for nonlinear extrapolation (USEPA, 2005).

Three key events and one adaptive response were chosen as four possible sentinel events: (1) toxic hepatopathy, (2) the occurrence of altered hepatic foci positive for gamma-glutamyl transpeptidase (γ GT+-AHFs), (Pitot *et al.*, 1987; Waern *et al.* 1991), (3) labeling index at 31 weeks (NTP, 2006a), and (4) adaptive induction of AHR activation-dependent CYP1A1 measured by the dose-related increase in EROD at 53 weeks of the NTP bioassay (NTP, 2006a).

The tumor response does not occur until the incidence of toxic hepatopathy reaches 15% (Fig. 4A); it does not occur

until approximately three times the BMD for the occurrence of AHF based on the lower BMD of 831 from Waern *et al.* (1991) (Table 1), and it does not occur until EROD induction reaches six times the BMD (Fig. 4B). Although the labeling index at 31 weeks provided a close fit to the adjusted tumor response, its BMD value was almost twofold greater than that of the combined tumor response (Table 1). Hence, appropriate multiplying factors were used as BMD adjustments to enable these four responses to be consistent with the combined tumor response (Table 4). The nonlinear RfDs resulting from these sentinel key events are indistinguishable from that based on the combined tumor end point. The threshold nature of all these end points and the accumulated knowledge regarding the MOA of DLCs all support the involvement of binding to the AHR and existence of a threshold that is both biologically plausible and experimentally observable.

Reconciling Biological Homeostasis with Linear Extrapolation

Recently, the National Research Council released a report entitled "Science and Decisions: Advancing Risk Assessment," and the same material has been published elsewhere (NRC, 2008; White *et al.*, 2009). This report suggests three conceptual models and associated linear extrapolation methods for developing toxicity criteria for both cancer and noncancer end points. The first model is for nonlinear individual responses and linear population responses. The second model is for nonlinear individual and nonlinear population responses. The third model is for linear individual and linear population responses. The implication is that linear extrapolation will be the preferred extrapolation method for the most cancer and noncancer effects.

Presently, this methodology is novel with a need for further guidance, case-study examples, and further discussion on how to apply the adjustment approach. The approach to extrapolation may have profound effects upon risk management decisions and a full discussion of the biological basis for low-dose adjustment, and potential outcomes of adopting the general use of this methodology have not yet occurred (Burke *et al.*, 2009; Rhomberg, 2009; White *et al.*, 2009). However, the majority of biological data on dose-response suggests that most chemicals exhibit a dose threshold (e.g., Deal *et al.*, 1989; Fukushima *et al.*, 2002; Waddell *et al.*, 2006; Williams *et al.*, 1996, 1999; Tsuda *et al.*, 2003). Indeed, linear and nonlinear dose-response functions for TCDD are considered equally likely (Walker and Yang, 2005).

Continued existence for any organism is a matter of maintaining homeostasis in the face of an unremitting array of a variety of stressors. Organisms have the capacities to deal with many different stressors, but these capacities are finite. When one or more of these capacities are exceeded, a departure from homeostasis, usually in the form of disease or death, occurs. Specific aspects of an organism's biology determine these capacities and the associated thresholds (Mayr, 1982).

Comparison with Existing Toxicity Criteria

The RfD value of 100 pg/kg/day is over two orders of magnitude higher than the RSD at 10^{-4} risk based on the USEPA 1988 CSF and three orders magnitude higher than the RSD at 10^{-4} risk based on the USEPA 2003 CSF. The RfD value of 100 pg/kg/day is two orders of magnitude higher than the RSD at 10^{-4} risk based on the linear CSF derived here.

The RfD value of 100 pg/kg/day is about 50-fold higher than estimated tolerable daily intakes (TDIs) of TEQs from WHO-JECFA of 2.3 pg TEQ/kg/day (WHO, 1998, 2000, 2001). The WHO-JECFA TDI value of 2.3 pg/kg/day is based on decreased sperm counts, suggesting that cancer may not be the only sensitive end point for risk assessment purposes (WHO, 1998, 2000, 2001). The WHO rationale includes recognition of a common MOA, that is, AHR activation, as a central feature of both cancer and noncancer effects of TCDD, thereby allowing the most sensitive response, in this case, epididymal sperm decrements in young rat pups, to establish a TDI. However, recent work brings this male reproductive end point into question as a basis for risk assessment (Bell *et al.*, 2007a,b,c). The laboratory animals considered by WHO-JECFA are between 10 and 1000 times more sensitive than humans with regard to AHR activation (Connor and Aylward, 2006; Silkworth *et al.*, 2005). This difference is partially accounted for by the use of HD_{AF} of 0.1 in RfD development in contrast to RSDs based on linear extrapolation.

The conclusion of WHO-JECFA (WHO, 2001) that cancer may not be the most sensitive end point observed in laboratory animals that also has relevance for humans is supported by the fact that the cancer-based RfD derived here is approximately 40-fold higher than the TDI of 2.3 pg/kg/day (WHO, 1998, 2000, 2001).

Recommendations for Toxicity Criteria

The consistency between the nonlinear RfD values at the 1% POD of 100 pg/kg/day based on the combined liver tumor response and the RfDs based on the four key events support the use of this value for regulatory purposes. The use of key events as support for a nonlinear RfD based on a tumor end point is consistent with the MOA of TCDD, the framework for human relevance and USEPA's Cancer Guidelines (Boobis *et al.*, 2008; Cohen *et al.*, 2004; Meek *et al.*, 2003; Seed *et al.*, 2005; Sonich-Mullin *et al.*, 2001; USEPA, 2005). In general, the use of linear extrapolation for all end points ignores the biological fact that most toxic responses represent a departure from the range of homeostatic control and, thus, do indeed exhibit thresholds—the dose makes the poison.

CONCLUSIONS

A comprehensive cancer bioassay was recently published for TCDD (NTP, 2006a). From these data, linear and nonlinear

cancer potency factors were derived. Dose-response relationships were assessed for combined liver tumors based on LALCs in rats estimated with a toxicokinetic model. Rat liver concentration estimates at the 1% POD were then obtained with BMD modeling. The same toxicokinetic model with inputs appropriate for humans was used to back-extrapolate HEDs corresponding to the POD values. Nonlinear RfDs for cancer were calculated as the ratio between the rat BMD₀₁ and corresponding HED and various extrapolation/uncertainty factors. Linear cancer potency factor or CSFs was calculated as the ratio between the POD and the HED corresponding to the rat BMDL₀₁. The values of the nonlinear RfDs were orders of magnitude higher than the RSDs from the linear potency factor calculated here or those developed by USEPA in the 1988 and 2003 dioxin risk assessments.

The risk assessment presented here incorporates elements recommended by both the NRC (2006) and the USEPA's Science Advisory Board (USEPA-SAB, 2001) in their recent reviews of the USEPA dioxin reassessment drafts (USEPA, 1989, 2003): (1) use of a nonlinear approach, (2) reliance on internal dose metrics, (3) accounting for toxicokinetic differences in absorption, distribution, and elimination between humans and rats, and (4) a quantitative assessment of the uncertainties associated with various choices in the risk assessment process. This quantitative uncertainty analysis demonstrated that choice of approach (linear vs. nonlinear) had the largest impact on estimated tolerable doses, with estimated tolerable doses 100- to 1000-fold higher (less risky) from the nonlinear approach depending on the chosen target risk. The significant advantage of nonlinear RfDs over linear CSFs is their ease of application for regulatory purposes and that the explicit inclusion of a threshold reflects the underlying biology and MOA more faithfully.

SUPPLEMENTARY DATA

Supplementary data are available online at <http://toxsci.oxfordjournals.org/>.

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