HUMAN RISK ASSESSMENT FOR 2,3,7,8-TETRACHLORODIBENZO-P-DIOXIN (TCDD) BASED ON TOXICITY TESTING IN THE 21 CENTURY APPROACH INVOLVING ARYL HYDROCARBON RECEPTOR (AHR) SIGNALING PATHWAYS

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Introduction

Risk assessment for 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) has been drawing great attention because of its widespread exposure and the long half-life in humans which ranged between 7.1 and 11.3 years[1]. Traditional human health risk assessment for TCDD mainly focused on the health outcome based on the animal studies or population epidemiological studies. However, the critical effects at cellular or molecular level assayed in human originated cell should also be paid attention, which could provide important information for assessing the potential health risk of chemicals.

This study aimed to conduct risk assessment for TCDD based on toxicity testing data involving aryl hydrocarbon receptor (AHR) signaling pathways. We firstly analyzed ToxCast *in vitro* high-throughput screening (HTS) assays related to the AHR pathway to identify the dose-response profile of effects on biological process targets and determine a point of departure (POD) for TCDD. We also reviewed relevant published studies to determine PODs. Then by using the so-called concentration- and age-dependent model (CADM) which was developed by Carrier et al.[2], further optimized by Aylward et al.[3], Ruiz et al.[4] and modified by EFSA[5], we converted in vitro PODs related to the AHR signaling pathways to several human equivalent doses(HEDs) for TCDD and health risk assessment for TCDD could be conducted based on the newly HEDs.

Materials and methods

ToxCast was firstly explored to search the toxicity testing assays for TCDD with active endpoints and relevant to AHR were extracted for further analysis. Furthermore, a review of *in vitro* data for TCDD toxicity on human cells and AHR signaling pathways was performed by searching for indexed articles via Web of Science and PubMed. Given that hepatotoxicity is a major effect of TCDD through AHR signaling pathways, we mainly explored the articles related to human hepatic cell by using the index words included "TCDD", "AHR", "human hepatocytes", "HepG2", e.g., and a total of 492 articles were found. After excluding redundant and repeated data, 34 articles were finally selected to extract data in this study.

As for ToxCast' s *in vitro* data, the activity concentration causing 10% maximum activity(AC10) or 5% maximum activity(AC5) values was calculated for the targeted toxicity testing assay of TCDD and these key points could be set as PODs[6]. Briefly to speaking, TCDD was replaced by chemical ID (CHID) "21315" and then ToxCast pipeline (tcpl) package in R was used to provide normalization, dose–response modeling and visualization solutions for HTS screening efforts. AC10 and AC5 were retrieved from the publicly available ToxCast' s data "InvitroDBv3.3 database" (released September 2020, https://www.epa.gov/chemical-research/exploring-toxcast-data-downloadable-data). The in vitro data for TCDD in targeted assay was evaluated in tcpl with three models: a constant model at zero, a constrained three-parameter Hill model and a constrained five-parameter gain-loss model, whereby the model with the lowest Akaike information criterion value was selected as the 'winning' model. If the Hill or Gain-Loss model won and both of the modeled and observed maximum responses met an efficacy cutoff based on an expandable list of methods, the dose-response series were able to have an active hit- call and the AC10 and AC5 were derived from the winning model parameters. Furthermore, the concentration-response curves of each AHR assay were extracted to acquire AC50 and cytotoxicity limit from the U.S. EPA's Chemistry Dashboard (https://comptox.epa.gov/dashboard)[6]. Since not all responses labeled as hit-call appear to be truly positive results due to the potential confounding effect of cytotoxicity, assays having an AC50 (activity concentration causing 50% maximum activity) value lower than the cytotoxicity limit , where cytotoxicity limit was applying as a threshold[7], were selected for the subsequent HEDs estimation.

As for the selected *in vitro* studies from Web of Science and PubMed, dose-response profile, no-observed-adverse-effect level (NOAEL) or lowest-observed-adverse-effect level (LOAEL) were extracted directly to act as the candidate PODs. These candidate PODs obtained from the published articles were compared with that from ToxCast' s *in vitro* data, and the lower ones were determined as PODs for derivation of HEDs.

The CADM model, which was applied by EFSA to estimate human daily intake of TCDD, was used for converted *in vitro* PODs to HEDs for TCDD in two scenarios: 1) HEDs for women at 35 years were calculated because of the age of childbearing and breastfeeding and 2) we also estimated HEDs for 9-year-old boys since EFSA lately set tolerable weekly intake(TWI)[5] based on the these population for the most critical effect was observed in boys exposed before the age of 10 years, i.e. reduced sperm concentrations, from the Russian

Children's Study. According to EFSA a breastfeeding period of 12 months was modelled[5]. Levels in milk were assumed to be equal to those in the body fat of mothers, resulting from a similar long-term intake as used for the boys after breastfeeding. And after breastfeeding the intake of children was set twice as high as that of adults, because of a higher energy requirement. Finally, the newly HEDs could be directly used in risk assessment for human exposure to TCDD.

Results and discussion

With the increase of *in vitro* data, apart from using for toxicity identification, they could make contribution to human health risk assessment. In the present study, we successfully converted bioactivity concentrations from *in vitro* ToxCast assays into comparable units of doses in humans (i.e., HEDs) using CADM. One major contribution of this study is that we provide *in vitro*-based credible HEDs associated with AHR signaling pathway for TCDD.

In ToxCast *in vitro* data, there were 14 active assays for TCDD of total 235 assays, while one of those assays meet the requirement and selected. The selected assay in ToxCast *in vitro* data was named "TOX21_AhR_LUC_Agonist". As showed in Figure 1, the corresponding best-fit model was Hill model and AC50 was below the cytotoxicity limit which meant the results to be valid. The estimated AC10, AC5 values were 34.78 pM and 17.45 pM for Human liver cell (HepG2).

For published in vitro researches, both HepG2 and human hepatocytes were test and the critical end points mainly focused on the suppression of cell proliferation, significant DNA damage, AHR transactivation and induction of the AHR-dependent target gene. The values of NOAEL extracted from selected articles ranged from 0.1 nM to 1 nM which apparently higher than AC10 calculated based on ToxCast in vitro data. Thus, the AC10, AC5 values from ToxCast in vitro data were set as PODs.

Compared with published *in vitro* studies, the ToxCast *in vitro* data were obtained by applying high-throughput screening technology, which has the characteristics of trace, rapid, sensitive and accurate, and could test the biological activity of a large number of compounds in a short time, and thus provided more sufficient and reliable data. In ToxCast in vitro data, among the 14 active assays for TCDD, the selected assay relevant to AHR signaling pathways was the one with the lowest AC50. This result was accordance with the common concern that TCDD has a higher binding affinity to the AHR and exhibits a greater AHR activation potency[5].

Since PODs obtained from human liver cell, the corresponding HEDs were deduced based on concentration of TCDD in liver estimated by CADM model and AC10 and AC5 were considered equal to 11.2 and 5.6 pg/g fat assuming that one milliliter of fat equal to 1 g. Figure 2 showed the concentration of TCDD changing in boy' s liver exposed for 9 years after a breastfeeding period of 12 months. The high peak at one year old suggested breastfeeding contributes considerably to the children exposure which in line with that EFSA concluded[5]. Therefore, the intake of mothers which make effects both on themselves and boys were viewed as HEDs. Table 1 presents the estimated intake of mothers and boys when TCDD in their liver reaching PODs. The intake corresponded to AC10 for 35-year-old women and boys at the age of 9 were 1.4 and 1.2 pg/kg bw per day, while for AC5 the intakes were 0.85 and 0.7, separately. The intakes for mother of boys were moderately lower than those for women since the intake of TCDD after breastfeeding for children was considered higher than that of adults and the lowest intake of mother, value of 0.7 pg/kg bw per day, was determined as newly HED.

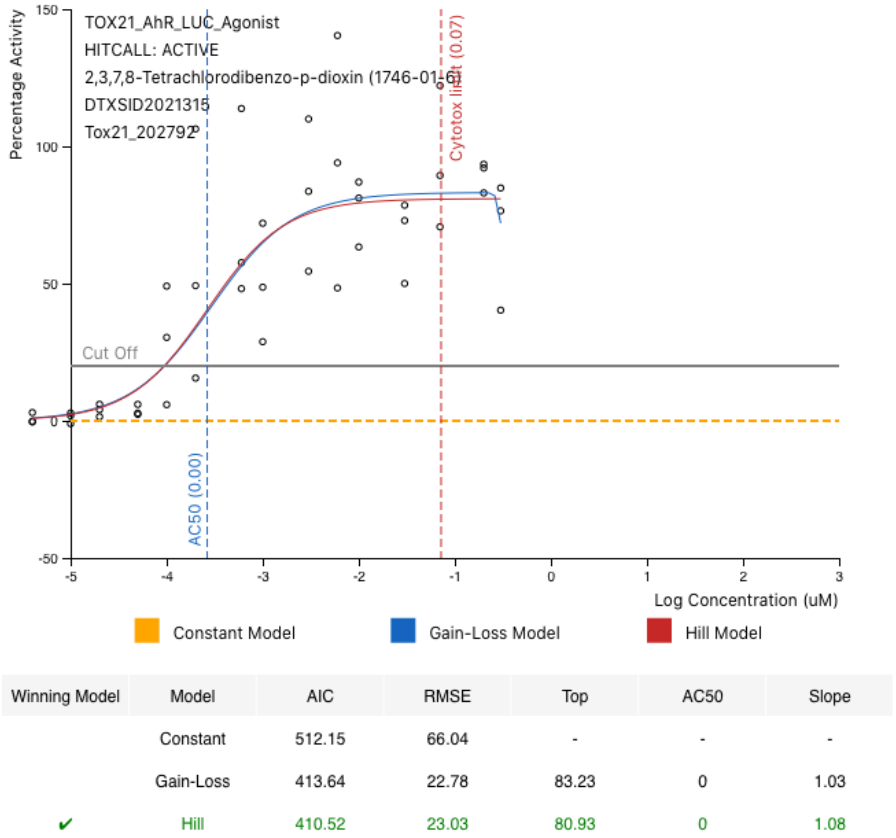
When compared the newly HED deducted from *in vitro* data with the existed health-based guide values (HBGVs), generally the newly HED were much similar with the one determined by United States Environmental Protection Agency(US EPA), which was 0.7 pg/kg bw per day[8]. That value estimated by EFSA based on Russian Children's Study was 0.25 pg/kg bw per day[5] and our HED were of a similar order of magnitude with that one though slightly higher. However, our HED were apparently lower than the one determined by Joint FAO/WHO Expert Committee on Food Additives (JECFA) which considered the tolerable monthly intake (PTMI) for TCDD of 70 pg/kg b.w. (corresponding to 2.3 pg/kg b.w. per day)[8].

Summarily, our newly HED confirmed that risk assessment for TCDD based on in vitro data involved AHR signaling pathway and using models to convert *in vitro* PODs to HEDs was valid and could provide critical evidence. In addition, our results supported current HBGVs recommended by EFSA and EPA that population daily intake of TCDD lower than HBGVs were mostly safe, even concerned at cellular or molecular level.

The present study has several limitations. Firstly, this study lack of evaluating the uncertainty of CADM model and variability within individuals. Further consideration of the distribution of physiological parameters of targeted population might help to solve the problem. Additionally, the new HED were specific to TCDD. Further study is needed to confirm whether these values can be generalized to other dioxins.

Table 1. Expected liver levels of women at 35 years old and boys at age 9 years for durations of breastfeeding 12 months, and subsequent dietary exposure up to 9 years of age being double that of the mothers

PODs	Intake of mothers (pg/kg bw per day)	Human milk level (pg/g fat)	Breast feeding duration (months)	Intake by boys (pg/kg bw per day)	Liver level (pg/g fat)
Women at 35 years old					
AC10	1.4	25.0	12	-	11.2
AC5	0.85	16.8	12	-	5.6
Boys at 9 years old					
AC10	1.2	22.0	12	2.4	11.2
AC5	0.7	14.4	12	1.4	5.6



Winning Model	Model	AIC	RMSE	Top	AC50	Slope
	Constant	512.15	66.04	-	-	-
	Gain-Loss	413.64	22.78	83.23	0	1.03
✔	Hill	410.52	23.03	80.93	0	1.08

Figure 1. The concentration-response curve of the selected ToxCast high-throughput screening in vitro assay used in our analysis. The curve was extracted from the U.S. EPA's Chemistry Dashboard.

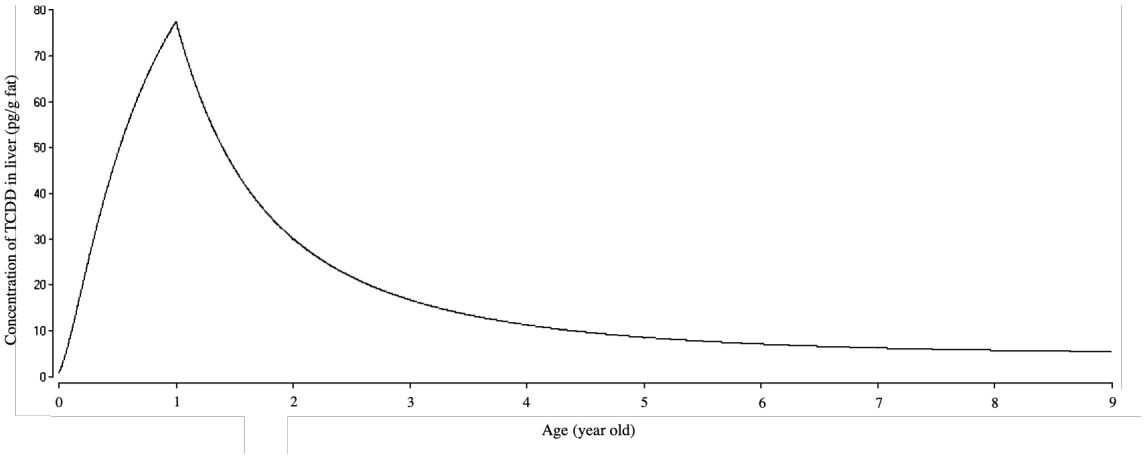


Figure 2. Concentrations in liver tissue (pg/g fat) calculated for boys exposed for 9 years to 1.4 pg/kg bw per day after a breastfeeding period of 12 months with levels in milk of 14.4 pg/g fat (the level resulting from exposure of mothers for 35 years to 0.7 pg/kg bw per day)

Acknowledgements
This work was supported by grants from the National Key R&D Program of China [2017YFC1600500].

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