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Johansson, Hanna Katarina Lilith; Boberg, Julie; Dybdahl, Marianne; Axelstad, Marta; Vinggaard, Anne Marie

Published in:
Current Opinion in Toxicology

Link to article, DOI:
10.1016/j.cotox.2018.12.001

Publication date:
2019

Document Version
Peer reviewed version

Chemical risk assessment based on *in vitro* and human biomonitoring data: A case study on thyroid toxicants

Hanna KL Johansson, Julie Boberg, Marianne Dybdahl, Marta Axelstad, Anne Marie Vinggaard

Affiliations: *National Food Institute, Technical University of Denmark, Kemitorvet building 202, 2800 Kgs. Lyngby, Denmark*

Corresponding author: *Anne Marie Vinggaard, annv@food.dtu.dk*

Keywords: Risk assessment, *in vitro*, human biomonitoring, thyroid toxicity, environmental chemicals, PFOS

Abstract

Today detailed risk assessment can only be performed for a few percent of the total number of current-use chemicals due to lack of data. Toxicity data is therefore needed for a substantial number of untested chemicals, a task that requires improved and faster chemical risk assessment strategies that are cost-efficient, human relevant and ethically responsible. In this commentary we use a case study on five known thyroid toxic chemicals (perfluorooctanesulfonic acid (PFOS), triclosan, tetrabromobisphenol A (TBBPA), decabromodiphenyl ether (BDE-209), and hexabromocyclododecane (HBCD)) to explore the use of *in vitro* data for hazard assessment together with human biomonitoring (HBM) data for exposure assessment when evaluating human risk. Based on the case study we conclude that *in vitro* and HBM data can be used for risk ranking of chemicals. We envision that an *in vitro*/HBM approach can use data from studies such as the big European initiative HBM4EU together with human relevant *in vitro* data to make alternative risk assessment more valuable to finally be able to 'stand-alone'.

1. Changing the paradigm for chemical risk assessment

Today we only have adequate information to perform detailed risk assessment for a few percent of the total number of current-use chemicals [1]. This reflects a considerable data gap, which is a bottleneck for prediction of human health effects caused by exposure to chemicals. We therefore need to gather or predict more toxicity and exposure data for a substantial number of untested chemicals, a task that – for many reasons – should not be solved by use of traditional animal-based methods only. Hence, improved and faster chemical risk assessment strategies are required to evaluate individual chemicals for which we need knowledge on human safety [2,3]. Here we propose a framework on how to risk rank chemicals based on *in vitro* data and human biomonitoring data (Fig. 1).

1.1 The current paradigm and challenges

Chemical hazard characterization is traditionally based on experimental animal data - often rodent data - for various organ toxicities, reproductive toxicity, carcinogenic effects and mutagenic effects [4]. While such data can be of great value there are several challenges, which complicates chemical risk assessments based solely on this information:

- A scientific challenge exists, as rodent studies do not always predict human responses. Comparison of human and rodent toxicity data for 150 pharmaceuticals showed that rodents predicted 43% of human responses [5].
- A practical challenge exists, as *in vivo* data is lacking for the majority of the industrial chemicals in current use [6].
- An ethical challenge exists, as based on the 3R principles [7], reductions on the use of animals for experimental toxicity studies should be made due to ethical reasons. This has resulted in political and

public pressure as well as EU legislation for test of cosmetics [8] where some *in vitro* approaches already exist.

Human exposure assessment of a specific chemical is often based on data derived separately for various relevant sources. For instance, exposure via food is often derived from data on chemical concentrations in various food items and average data for human food intake patterns, whereas exposure to the same chemical via cosmetics is assessed separately. Assessing aggregate exposures is thus a challenge [9,10] and still large data gaps exists on human exposure to chemicals [11]. Human internal exposure assessed via human biomonitoring (HBM) is a measure of aggregated exposure but are not routinely used in chemical risk assessment [12]. Yet HBM data is increasingly being gathered all over Europe (HBM4EU [13]) and US (NHANES [14]), presenting a great opportunity to evaluate human exposure across sources.

1.2 Towards a paradigm shift

A decade ago, the United States National Research Council presented a vision for chemical toxicity testing in the 21st century, in which computational biology and *in vitro* tests based on human biology play a central role [3]. Following this, high-throughput screening (HTS) programs such as ToxCast and Tox21 [2,15,16] were initiated. Such HTS systems, together with computational biology and "omics methods", have generated data on hazard for large numbers of chemicals in a cost-efficient, human relevant and ethical way [2,17]. Several studies have been published on the use of HTS data in reverse dosimetry models for estimation of human exposure and ranking of chemicals [18–21]. In these studies, external intake dose was used as the measure of exposure, whereas we in the present study explore the use of HBM data as the measure of internal exposure. We argue that *in vitro* data for hazard assessment together with HBM data for exposure assessment has a future potential to accommodate some of the scientific, practical and ethical challenges we are presently facing. Our long-term vision is that the use of defined panels of *in vitro* tests combined with human HBM data for the chemical(s) in question can contribute significantly to chemical

regulation. For the purpose of this publication we present a case study with well-known thyroid toxic chemicals.

2. Case study on thyroid toxicants

2.1 Methodology

We decided to focus on chemicals that disturb thyroid hormone levels as this is an emerging endocrine mechanism of action for which new *in vitro* testing strategies are being developed. Our exercise was hypothesized to highlight data gaps that might require future attention. Seventeen compounds were selected for investigation based on their known thyroid toxic effect *in vivo* and relevant human exposure [12]. Out of these seventeen, five were selected as model compounds based on relevance and available literature on *in vitro* and HBM data; perfluorooctanesulfonic acid (PFOS), triclosan, tetrabromobisphenol A (TBBPA), decabromodiphenyl ether (BDE-209), and hexabromocyclododecane (HBCD). A literature search was conducted to extract HBM data (NHANES [22] and PubMed) and thyroid relevant *in vitro* data (PubMed and ToxCast database [23]). For comparison we also included animal *in vivo* studies from which NOAELs for thyroid effects were derived. To enable comparison across HBM, *in vitro* and *in vivo* studies, chemical concentrations in human and animal blood was transformed to nM. The methodology is described in detail in the Supplementary material, but in short: chemical blood levels from epidemiological and *in vivo* studies were re-calculated from the unit g chemical/ g lipid or g chemical/ g wet weight of blood to nM. For triclosan the calculation of human internal concentrations differed, as triclosan has a short half-life and is usually measured in urine. Blood levels were therefore estimated by calculation of daily intake based on concentrations found in urine [24] and hereafter a simplified one-compartment toxicokinetic model was applied [25].

Risk characterization ratios (RCRs) were calculated for each chemical by division of the exposure estimate based on HBM data with a reference value (RV) based on *in vitro* data (RCR=exposure/RV) [26]. The RCR value reflects whether exposures exceed the concentrations considered “safe”. Thus, RCR values >1 indicate that human exposure levels may be associated with a potential risk. RVs were based on *in vitro* data from one experimental study for each chemical that was selected based on expert judgement in terms of relevance

of the mechanism of action and reliability of the study. . From the five selected *in vitro* studies, the 'no observed effect concentration' (NOEC) value was used for RCR calculation. For chemicals with no reported NOEC an extrapolation factor of 10 from LOEC to NOEC was used. Furthermore, we included studies in zebrafish larvae for PFOS, TBBPA, and BDE-209, even though the larvae is not considered an *in vitro* model, only the embryo is [27]. Studies considered to be potential outliers or not showing a mechanism known to be thyroid specific, were excluded.

The *in vitro* studies included are presented in Table 1. The data shows a wide field of tested *in vitro* end points with indications of effects on well-known thyroid endpoints such as antagonism of the thyroid receptor (TR) [28] and transthyretin (TTR) binding [29] by PFOS, activation of the constitutive androstane receptor (CAR) by a triclosan metabolite [30], TTR binding by TBBPA [31], TH reduction of BDE-209 in zebrafish [32–34], as well as some thyroid-specific effect in hepatocytes by HBCD [35].

To take uncertainty of HBM data into account the exposure values were calculated based on an average of means (PFOS, triclosan, TBBPA) or medians (BDE-209, HBCD). Exposure data with values below limit of quantification/limit of detection, measurements in other matrices than blood or urine, studies from Asian countries and occupational exposure studies, were excluded.

2.2 Data availability

Data on exposure and toxicity collected from HBM, *in vitro* and *in vivo* studies for the five chemicals are depicted in Fig. 2 and the references used are shown in Table 1.

Due to limited resources, the HBM data included for PFOS and triclosan were limited to one representative European study and data from the NHANES [22]. For TBBPA, BDE-209, and HBCD a thorough review of the literature was conducted. For TBBPA, three HBM studies were included and several excluded due to several studies with TBBPA levels below limit of quantification/limit of detection. For BDE-209, some studies were also excluded due to measurements below the limit of detection/limit of quantification. Exclusion of these data may somewhat skew the results, leading to an overestimation of the exposure for these compounds.

The included *in vitro* studies have tested thyroid toxicity for a wide range of endpoints (Table 1), but not all chemicals have been tested for all relevant mechanisms of thyroid toxicity. This approach, which includes a range of assays, is analogous to classical risk assessment where several endpoints are evaluated and only the most sensitive endpoint is used in the end.

2.3 Output and ranking

Ranking of the five compounds in terms of calculated RCR values based on *in vitro* NOEC values showed that PFOS was associated with the highest risk (RCR = 8) and HBCD with the lowest (RCR=0.0001) (Table 2):

PFOS >> TBBPA > BDE-209 > Triclosan > HBCD

Based on this analysis, exposure to PFOS alone is highlighted as a potential human health risk. The association is of even greater concern considering that humans are exposed to several perfluorinated compounds [36] that may have the same or similar mode(s) of action, which can cause cumulative effects. The RCR ranking shows that PFOS needs further attention, although its use is restricted within the US [37] and EU [38].

It should be noted, however, that the RCR values are subject to great uncertainties, both for the hazard and exposure data. The RCR values presented here should therefore be regarded as indicative values suggested as examples in this proposed framework, and not as values that should readily be used for risk assessment purposes.

As can be seen in Fig. 2 the *in vitro* active concentrations and the blood levels at LOAELs and/or NOAELs from animal experiments were not that different. However, for PFOS effects are seen at lower concentrations *in vitro* than *in vivo*, whereas for triclosan the situation is opposite. This reflects that toxicity of some perfluorinated chemicals generally seem to be underestimated by animal studies [39] and that human data and physiologically-based kinetic modelling are needed for a proper risk assessment of this group of chemicals. For triclosan the difference may be explained by species differences in CYP induction [40]. In

humans and rodents, increased liver catabolism of thyroid hormones has been identified as one of the primary modes of action of triclosan [40,41]. However, data from nuclear receptor reporter assays show that CAR and PXR activation by triclosan differs between the human and rodent [40], which indicates important species differences in thyroid hormone catabolism.

3. Future application of in vitro and biomonitoring data for risk assessment

3.1 A panel of human-based in vitro assays

We found limited relevant *in vitro* data and data that covers thyroid toxic mode(s) of action and/or key initiating events for the selected chemicals. Furthermore, there is a need for more human-based *in vitro* models as we in this case study only found a few assays based on human biology.

We suggest that defined panels of human-based *in vitro* assays are used to ensure that several important modes of action are covered, which is in line with the vision from the US National Research Council [3]. A defined panel of human-based *in vitro* assays would enable comparison and ranking of chemicals with different potencies, either by use of RCR values or a similar ratio. In terms of the practical challenge with thousands of untested chemicals on the market, *in vitro* assays enable high throughput test strategies such as the ToxCast and Tox21 initiatives in the US [2,15,16].

Toxicity is in some cases caused by metabolites and not the parent compound. The metabolic capacity of the *in vitro* system is therefore important to consider [42] and there are both extra- and intracellular options that can allow evaluation of metabolic capacity in the *in vitro* panel [43–45]. Furthermore, in order to obtain a quantitative link between HBM data and *in vitro* outputs - thereby improving predictions - it would be relevant to correct the *in vitro* output for factors such as protein binding, evaporation, binding to test plates/tubes/pipettes etc. in order to obtain the true intracellular concentration [46,47].

3.2 HBM data for exposure assessment

Use of HBM data for exposure assessment is a promising approach as it measures the sum of chemical contributions from one or more routes of exposure as well as from different sources [48]. HBM data is also valuable for assessment of chemicals with unknown or poorly characterized exposure pathways [49] and thus the integrated internal exposure levels can be used as a better and more relevant measure [50]. Furthermore, external exposure modelling, based on e.g. food consumption patterns and cosmetics use, is likely a greater source of uncertainty than biological measurements [50,51]. On the other hand, HBM data cannot be obtained for all compounds due to e.g. shared metabolites, and an important drawback is that it can only be used for chemicals already on the market [48]. Evaluation of exposure based on HBM data will therefore always be a retrospective rather than preventive approach. Furthermore, HBM data does not contribute with information concerning timing and source of the exposure [48,52], which is central to chemical regulation.

3.3 Other examples of in vitro or HBM based risk assessment

There are other examples of *in vitro* based risk assessment in the literature, however, many of these differ from the present approach by use of external exposure dose (oral intake, mg/kg) [18–21,53], whereas we have used internal exposure dose (blood concentration, nM) based on measured HBM data. Two examples are Campell et al. [53] and Ring et al. [18]:

Campbell et al. [53], used *in vitro* based EC_{10} values for estrogenic activity of parabens as surrogate "safe exposure doses". They used a Margin of Safety approach for risk assessment, which in essence is similar to our approach, except that the inverse ratio was calculated, i.e. division of a no-effect level with an exposure estimate.

Ring et al. [18] used data from ToxCast high-throughput *in vitro* screening assays applied for prioritization. Bioactive *in vitro* concentrations were extrapolated to oral equivalent doses by reverse dosimetry, and these doses were compared to external exposure doses calculated from HBM data. If the estimated exposure is higher than the dose needed to obtain a bioactive concentration in blood, a potential risk is identified. Interestingly, in that study triclosan was identified as one of the compounds with the smallest difference between exposure and activity, i.e. as the most problematic and a priority for further evaluation [18].

HBM data have been applied in studies where their potential as exposure estimates has been investigated. A comprehensive case study on benzene has been conducted [54], however, approaches more similar to the one used in the present case study has been conducted by Hays et al. [51] and Aylward & Hays [50]. In both these studies the authors concluded that internal dose measures from HBM studies are less uncertain than estimated external doses in risk assessment.

4. Conclusion

We used a case study on five thyroid toxic compounds with differential mechanistic profiles to investigate the potential use of human biomonitoring (HBM) together with *in vitro* data for informing human risk assessment. We conclude that calculation of risk characterization ratios based on HBM and *in vitro* data is a helpful tool for ranking chemicals and for designing follow-up studies.

The case study highlighted the pros and cons of informing the risk assessment process with *in vitro* and HBM data and demonstrated that such data can be used for risk ranking of chemicals. Moreover, this approach may be used for pinpointing chemicals for which species differences may play a major role, thereby stressing the importance of basing the risk assessment on human-relevant data. Our vision is that an *in vitro*/HBM approach can use the HBM4EU project - in parallel to the NHANES project in the US - together with more comprehensive human relevant *in vitro* data to make 'alternative' risk assessment much more valuable to finally be able to 'stand-alone'.

Acknowledgements

We would like to thank Dr Terje Svingen for editorial input to the text. This work was financially supported by the Ministry of Environment and Food of Denmark.

Conflict of interest

Declarations of interest: None.

Table 1: Case study literature overview for the five selected chemicals PFOS, triclosan, TBBPA, BDE-209 and HBCD. The first row shows the human biomonitoring (HBM) data with country of origin and references. The second row shows the *in vitro* data with endpoints measured, LOEC/EC_x values, the study used for calculation of risk characterization ratio (RCR) marked in bold, as well as references. The third row shows the *in vivo* data from animal experiments with endpoints and references.

	PFOS	**Triclosan**	**TBBPA**	**BDE-209**	**HBCD**
HBM data (country of origin)	US [22] Denmark [36]	US [22] Denmark [55]	France [56,57] Belgium [58]	US [59,60] UK [61–63] Greece [64] Spain [63,65–68] Netherlands [63] Germany [69] Denmark [70,71] Sweden [72–74] Norway [63]	Canada [75] Australia [76] Greece [64] Netherlands [77] Belgium [49,78] Germany [69] Sweden [79,80] Norway [81]
***In vitro* data** (endpoints ranked according to potency. The study used for RCR calculation in bold)	**Antagonism of TR LOEC 100 nM** [28] Competitive binding to TTR IC_{50} 130 nM [29] T4 reduction and changed gene expression in zebrafish embryos LOEC 200 nM [82], LOEC 200 nM [83], LOEC 400 nM [84] Binding to TRα-LBD IC_{50} 16000 nM [85] Inhibition of iodide uptake in hNIS assay LOEC 17000 nM [86]	**Metabolite activated CAR EC_{50} 900 nM** [30], EC_{50} 9800 nM [40] Inhibition of sulfotransferase IC_{50} 1410 nM [87] Decreased NIS in FRTL-5 cells LOEC 10000 nM [88] Reduced activity of iodotyrosine deiodinase LOEC 60000 nM [89] TPO inhibition LOEC 253 000 nM [90]	**TTR binding IC_{50} 31 nM** [31], IC_{50} 3070 nM [91] Gene expression in zebra fish embryos LOEC 184 nM [92], LOEC 202 nM [93] Gene expression in zebra fish liver cells LOEC 400 nM [94] Growth hormone production in GH3 rat pituitary cells LOEC 1000 nM [95,96] Inhibition of rat disulfide isomerase IC_{50} 1180 nM [97] TR antagonism and TR-related effects LOEC 1000 nM [98], LOEC 3000 nM [99], IC_{50} 4600 nM [100], LOEC 10000 nM [101], IC_{50} 29500 nM [102] TRα transcriptional regulation IC_{75} 24000 nM [103] T-screen LOEC 10000 nM [104] Translocation of TRβ LOEC 25000 nM [105] Cell cycle regulation in human thyroid cells LOEC 75000 nM [106]	**TH reduction, gene and protein expression in zebra fish embryos LOEC 83 nM** [32], LOEC 83 nM [33] LOEC 104 nM [34] PXR activation LOEC 100000 nM [107]	**Effects on TH-inducible hepatic protein and TTR in chicken embryonic hepatocytes LOEC 1000 nM** [35] *Ex vivo Xenopus laevis* tadpole tail tip length regression LOEC 1000 nM [108] Increased TR-mediated gene expression LOEC 3120 nM [109] TTR binding IC_{50} 12000 nM [31] T-screen LOEC 21000 nM [31]
***In vivo* animal data** (endpoints)	Reduced T4 levels in monkeys at estimated blood concentrations of 26,000 nM (NOAEL) and 76,000 nM (LOAEL) [110,111]	Reduced T4 levels in rats at estimated blood concentrations of 21 nM (NOAEL) and 214 nM (LOAEL) [112,113]	Reduced thyroid hormone levels in rats at blood levels estimated to 919 nM (NOAEL) [114,115]	Reduced T4 in male rodents at blood levels estimated to 4800 nM (BMDL) [116–118]	Reduced T4 in female rats with a NOAEL of 200 µg/g lipid in liver [119]. According to Szabo et al. [120] HBCD blood levels are approximately 33% of the hepatic adipose tissue levels after 10 days of exposure in mice. Based on this the blood level was estimated to 395 nM (NOAEL)

Table 2: Human biomonitoring (HBM) data, *in vitro* data and calculated risk characterization ratios (RCRs) for the five chemicals (RCR = exposure (HBM data) /reference value (*in vitro*)).

Data type	PFOS	Triclosan	TBBPA	BDE-209	HBCD
Human biomonitoring data *Mean (nM)*	25	0.04	0.2	0.05	0.01
***In vitro* data** *NOEC (nM)*	3	90	3.1	8.3	100
Risk Characterization Ratio	8	0.0004	0.06	0.006	0.0001

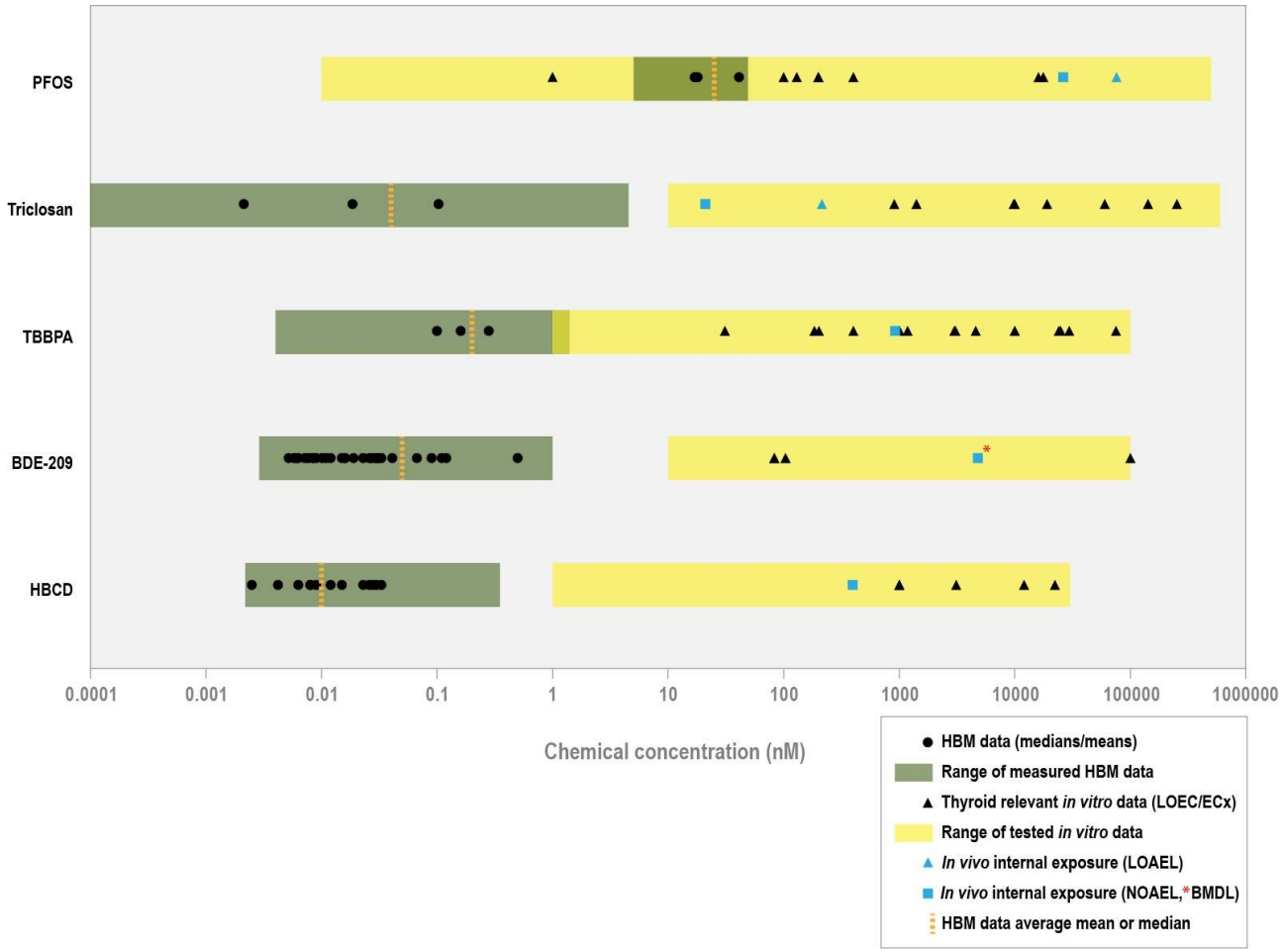

Figure 1 . HBM data (green bars and dots), *in vitro* data (yellow bars and triangles), and *in vivo* data from animal experiments (blue triangles and squares) are depicted in this figure. The measured chemical levels in humans are generally lower than the effect concentrations found *in vitro* and *in vivo.* The exception is PFOS where the measured human levels and the effective concentrations *in vitro* are relatively close. Furthermore, average HBM values (orange, stippled lines) are relatively similar for all five chemicals except PFOS, where the internal human concentrations are higher.

(LOEC = lowest observed effect concentration; ECx = Effect Concentration at X percent of effect; LOAEL = lowest observed adverse effect level, NOAEL = no observed adverse effect level; BMDL = bench mark dose level)

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