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Template for AOP update report for selected priority substances

Deliverable Report

D 13.2

WP 13 - Establishing exposure-health relationships

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2 Introduction and Objective

The objectives of the WP13 within the HBM4EU project include establishment of mechanistic links between the adverse health outcomes in human population and exposures to priority chemical stressors. One of the tools that allow to systematically address this objective are Adverse Outcome Pathways (AOP), a concept formalised within AOPKB.org and AOPWIKI.org, which is being developed by joint efforts of international authorities including OECD, EU and US EPA.

Development of AOPs is continuously gaining ground in modern risk assessment; the latter can be viewed as a sequence of events commencing with initial interactions of a stressor with a biomolecule in a target cell or tissue (i.e., a molecular initiating event) progressing through a dependent series of intermediate events, the finally result in a phenotypic change (an adverse outcome). However, the existing available AOPs provide information relevant for limited biological space with toxicological relevance. Hence, it is a major scientific challenge to be able to identify how the various molecular events are interconnected and how the toxicity pathways are activated under current environmental exposure levels. Considering the opportunities provided by HBM4EU regarding collection and analysis of human samples (or re-analysis of existing ones), a well designed analysis plan of integrated exposure biology based on transcriptomics and metabolomics (and the respective bioinformatics analysis), would provide significant insights into the molecular mechanisms that are associated to the 1st set of priority substances. This is also in close collaboration with the work to be done:

a) in WP12, that will provide the necessary information for relating the biologically effective dose that is able to activate toxicity pathways with the environmentally relevant exposure levels

b) in WP14, regarding the identification of molecular signatures that would result in biomarkers of effect

c) in WP15, where the identification of converging pathways of toxicity among the mixture components will allow us to explore the potential synergies at the actual level of chemico-biological interaction

Partners of the WP13 within HBM4EU collect and synthesise relevant information on all HBM4EU priority chemical groups (from exposures, through molecular initiating events and other AOP key events up to the ultimate adverse outcomes). This information is used for validation of AOPs existing within the AOPWIKI (weighting the evidences regarding individual priority chemical groups and specific AOPs) or for the proposals of new individual AOPs or their networks.

To standardise the documentation of AOPs and the relevant workflow, this deliverable provides a template, which will be used in the following periods of HBM4EU and will assure direct compatibility and knowledge sharing with the OECD AOPWIKI.

3 The AOP Template

- To standardise the documentation of an AOP, a workflow in accordance to the OECD template is proposed.
- The author of the AOP should fill every field in the AOP format.

If the field is not pertinent to the proposed pathway, for example, the adverse outcome is localised in the organ or tissue level, so the identification of responses on the higher level–individual or population / ecosystem is not appropriate, then it should be stated as not applicable.

- In addition, instances where information is missing or lacking should be stated clearly.
- Any information added should be properly referenced by links to literature and other information resources & the list of references at the end
- To avoid duplications, assure compatibility and keep updated status, please check whether the information filled into the template (e.g. AOPs, particular Key Events etc.) is not already present in the AOP KB. This can be done by searching for keywords at: http://aopkb.org/search.ashx. Whenever possible, please use references (cross links) to AOP KB.
- General note on Sections No. 1-7 and No. 8-9 in this template: For the first periods of HBM4EU, WP13, Task 13.1., compilation of relevant info to sections 1-7 is essential, and the work should focus initially on these parts of the template. Sections No. 8-9 assess available data and confidence of AOPs, and thorough evaluations (needed for validation, publication and acceptance of the proposed AOP) may eventually be elaborated at later stages.

The AOP template, comprise of the following elements:

3.1 The Adverse Outcome Pathway identifier

- Name the AOP by:
  1) defining a clear and concise adverse outcome
     (example: Early life stage Mortality, Human Neurodevelopmental toxicity, Hepatocellular Carcinomas etc.)
  2) Molecular Initiating Event
     (example: binding of a molecule to a receptor, activation of AhR or PPARalpha)

3.2 Date of publication of AOP

- Not relevant for the initial phase, i.e. information compilation
- Report the: date (day/month/year) of AOP publication.

3.3 Date of updating the AOP

- Not relevant for the initial phase, i.e. information compilation
- Indicate the date (day/month/year) of any update of the AOP
  (clarification: the AOP can be updated/modified for a number of reasons, such as additions of new information or correction of existing information).

3.4 The introduction

- Give short background on the current knowledge about the endpoint of interest

  (example: if a known human neurodevelopmental disorder (e.g. autism) is the endpoint of interest, state the knowledge of the mechanisms that may disrupt the normal development, linking them with the appropriate Molecular Initiating Event).

  (example: if mortality in fishes is the endpoint of interest, state the linkage between Molecular Initiating Event with the Adverse Outcome indicating if it is applicable across taxa).

3.5 Summary of the AOP

- Report briefly the knowledge about the AOP following steps:

3.5.1 Characterisation of the exposure

- Define the route of exposure
  (example: if PAHs are the stressors of the particulate AOP, then the exposure is defined by the pathways of exposure to PAHs e.g., oral and inhalation pathways).

3.5.2 Characterisation of chemical properties

- Identification of properties required to initiate the adverse effect such as:

  1) Bioavailability
     (example: state if the receptor (MIE) is expressed or not in several tissues e.g.: receptor PPARa is expressed in skeletal muscle, intestine, pancreas, lung etc.)
  2) Reactivity
     (example: AhR can be activated by molecules with dimensions of 12 Å x 14 Å x 5 Å)
  3) Metabolism
     (example: the affinity of several polybrominated flame retardants to TTR were found to have affinities 7-10 fold that of T4; however, a microsomal ensyme mediated transformation was needed first (i.e. hydroxylation) for PBDEs).

     (what chemicals trigger and do not trigger the MIE in the AOP; increase or decrease the probability of an association between a chemical and an AOP;)

3.5.3 Identification of the molecular initiating event

- Name and describe the MIE
  (example: MIE name: binding of a xenobiotic to a receptor, description: this event initiate the adverse outcome by displacing a molecule from the binding site resulting to free (x) molecule in a biological fluid).

Place the figure/presentation of the MIE if available.

3.5.4 Identification of the site of action

- Name the site of the chemical (re)action which initiates the adverse pathway
  (example: the binding of a xenobiotic to the T4-binding site of TTR).

3.5.5 Identification of the responses at the macromolecular level

- Describe how the biochemical pathway is affected by the chemical on the molecular target.
  (example of an induced gene expression change: The AHR-ARNT complex then binds to a xenobiotic response element (XRE) found in the promoter of an AHR-regulated gene and recruits co-regulators such as CREB binding protein / p300, steroid receptor co-activator

(SRC) 1, SRC-2, SRC-3 and nuclear receptor interacting protein 1, leading to induction or repression of gene expression).

(example: The hydroxylated PCB metabolites (OHPCBs) are excellent substrates for sulfation (phase II conjugation) via sulfotransferases (SULTs) and thus could represent another mechanism through which clearance can occur).

3.5.6 Identification of the responses on the cellular/tissue level

- Describe the cellular/tissue outcomes, based on available information.
(example: both in vitro and in vivo electrophoretic data showed complete inhibition of radiolabeled T4 binding to TTR).

  (example: This Key Event has as a downstream event an increase of mitogenic cell proliferation of hepatocytes).

3.5.7 Identification of the responses on the organ level

- Describe the organ level responses, based on available information, both physiological and anatomical.
(example of liver tumors/adenocarcinomas: PPARα is necessary for peroxisome proliferator-induced liver cancer in mice).

  (example: In zebrafish embryo hearts, impaired extrusion of Ca2 from the cytosol results in defects in ventricle contraction and heart morphology).

3.5.8 Identification of the responses on the organism level

- Describe the key organism response, based on available information.
(example: Cardiovascular development and function altered by dioxin-like compounds (DLCs) is applicable to birds, teleost and non-teleost fishes).

  (example: In both humans and rodents, the hippocampus undergoes typical stages of neurodevelopment found in most brain regions, including: cell proliferation, migration, differentiation, synapse formation, and the maturation of synaptic function)

3.5.9 Identification of the overall effect on the population or ecosystem

- Describe how the population or ecosystem is affected by the toxic pathway.
(example: Exposure to mixtures of agonists of the AhR during the 1950's, 1960's, and 1970's has been implicated in early life stage mortality of Lake Ontario lake trout (Salvelinus namaycush) leading to population collapse).

  (example: Due to the highly conserved nature of the TTR protein, birds, reptiles, fish and amphibians can also express TTR and be impacted by interference by xeniobiotics).

3.6 Summary of the Key Events of the AOP

- Summarise the qualitative understanding of the AOP

  List them in a table that summarises:

  1) the key events,
  2) documentation of the experimental support for each event, and
  3) a subjective evaluation of the strength of the scientific evidence for that event (e.g. strong, well established, adequate.

- Include also the flow diagram/graphical representation of the intermediate events associated with AOP (The Molecular Initiating Event and the Key Events).

- Check for existing KEs with http://aopkb.org/search.ashx? + provide reference to KE if relevant

3.7 Scientific evidence supporting the AOP

- Include any available information supporting the steps/key events in the AOP.

  This can include any type of data such as: in vivo, in vitro, in silico, in chemico, toxicogenomics.

- Each key event should be considered separately in a single sub-section.

3.8 Assessment of the AOP

3.8.1 Assessment of the Weight-of-Evidence supporting the AOP

- Answer the Hill criteria:

3.8.1.1 Concordance of dose-response relationships

- Report any reference/study giving evidence of dose-response relationship.
  (example: These effects were observed for both the high dose of 2 umol and the low dose of 0.3 umol; however, it was noted that % T4 bound to TTR recovered to almost control levels after 3 hours at the low dose).

  (example: There is an R of 0.84 between relative COX-2 mRNA and altered heart development measured as pericardial area in embryos of Japanese medaka).

3.8.1.2 Temporal concordance among the key events and adverse outcome

- State the agreement between: the sequences of biochemical and physiological events leading to the adverse outcome together with the evidence in the literature.

  (example: Binding of TTR by a xenobiotic (MIE)→ Displacement, Serum thyroxine (T4) from transthyretin (KE 958) → Increased, Free serum thyroxine (T4) (KE 959) → Increased, Uptake of thyroxine into tissue (KE 960) → Increased, Clearance of thyroxine from tissues (KE 961) → Decreased, Thyroxin (T4) in serum (KE 281) → Decreased, Thyroxine (T4) in neuronal tissue (KE 280) → Altered, Hippocampal gene expression (KE 756) → Altered, Hippocampal anatomy (KE 757) → Decreased, Hippocampal function (KE 758) → Adverse Outcome)

3.8.1.3 Strength, consistency, and specificity of association of adverse outcome and initiating event

- Give the scientific evidence on the linkage between initiating event and adverse outcome.
  (example: MIE 957: Binding of TTR by a xenobiotic → AO 402: Cognitive Function, Decreased)

3.8.1.4 Biological plausibility, coherence, and consistency of the experimental evidence

- Explain the: logic, coherence and consistency along with the experimental data supporting the AOP.
  (example: The mechanism of AHR-mediated transcriptional regulation is well understood).

- Describe how the experimental evidence is logical and consistent with the mechanistic plausibility proposed by the theory explaining the initiation of the adverse outcome.

- If possible, describe the coherence of experimental results for multiple chemicals across different species.

(example: The AHR/ARNT complex was confirmed following in vitro exposure to halogenated aromatic hydrocarbons using an electrophoretic mobility shift assay; a dose-dependent supershift in DNA-binding was observed using specific antibodies in chicken and human cell lines).

3.8.1.5 Alternative mechanism(s) that logically present themselves and the extent to which they may distract from the postulated AOP. It should be noted that alternative mechanism(s) of action, if supported, require a separate AOP.

- Report other possible mechanisms that can lead to the adverse outcome and
- State if they can be covered by this AOP.
  (example: hepatocellular adenomas and carcinomas may occur form other mechanisms except PPARa activation, such as: Constitutive androstane receptor activation, Inhibition of pyruvate dehydrogenase kinase, Androgen receptor activation, Chronic cytotoxicity).

3.8.1.6 Uncertainties, inconsistencies and data gaps

- Include any uncertainties about the experimental details, and
  (such as uncertainties regarding the differences in sensitivity of different biological targets (e.g. cysteine versus lysine, Type I pyrethroid versus Type II)

  (example: There are uncertainties in the precise physiological and toxicological roles of different AhR clades (AhR1, AhR2, AhR3) and isoforms (α, β, δ, γ)).
- the measurements of biological activity in different assays.
  (example: Differences in binding affinity and transactivation of the AhR have been implicated as a key mechanism contributing to differences in sensitivity to agonists of the AhR among species and taxa. However, the precise mechanisms are not fully understood for all taxa).

  (example: In chicken (Gallus gallus), and presumably other species of birds, COX-2 is believed to be up-regulated by the AhR through non-genomic mechanisms that are independent of the AhR/ARNT heterodimer).
- Describe inconsistencies within the reported data, and
  (such as differences between in vivo responses for very similar chemicals)

  (example: All chemicals that cause induction of peroxisome proliferation, induction of fatty acid metabolising enzymes, hepatomegaly, and ultimately liver cancer after prolonged administration are typically able to bind to and activate PPARα).
- report any data gap that cause the weakness of the AOP.
  (example: Roles of ARNTs in other taxa have not been sufficiently investigated to date).

  (example: Nothing is known about differences in altered cardiovascular development and function leading to mortality in invertebrates with open circulatory systems or closed circulatory systems).

3.8.2 Assessment of the quantitative understanding of the AOP

- Include an evaluation of the experimental data and models to quantify the molecular initiating event and other key events.
  (example: Expressions and activities of CYP1A are routinely used as biomarkers of exposure to environmental chemicals that act as agonists of the AhR).

  (example: Strong quantitative relationships are known for exposure to ligands and interaction with DREs on the DNA by use of transfected COS-7 cells and gel shift assays).

- If possible, describe transparent determination of thresholds and response-to-response relationship to scale in vitro and in chemico effects to in vivo outcomes.
  (example: There is a strong quantitative understanding between quantitative structure-activity relationship (QSAR) or binding affinity for the AhR and potency among PCDDs, PCDFs, and planar PCBs).

3.9 Confidence in the AOP

- Discuss the summary of the scientific evidence supporting the AOP by answering the following questions:

3.9.1 How well characterised is the AOP?

- Describe how well the adverse outcome is understood

  1) qualitatively and
     (example: Although limited studies are available (moderated understanding), altered cardiac development and function leading to mortality is acknowledged to be applicable to reptiles, amphibians, and possibly some invertebrates based on presence of a cardiovascular system).
  2) quantitatively
     (example: There is a strong quantitative understanding (strong understanding) between quantitative structure-activity relationship (QSAR) or binding affinity for the AhR and potency among PCDDs, PCDFs, and planar PCBs)

3.9.2 How well are the initiating and other key events causally linked to the outcome?

- Give short statement on the relationship between each key event and adverse outcome.

3.9.3 What are the limitations in the evidence in support of the AOP?

- Indicate any lack or disagreement in the scientific evidence supporting the AOP.
  (example: The strongest results were found for PCP, which was negatively associated with free T4 in neonate cord blood, suggesting PCP reduces the transfer of T4 across the placenta. This confirmed previous findings of Sandau et al 2002, but has conflicted with other study populations and published reports).

  (example: There is no strong evidence that the low-affinity fibrate ligands are associated with cancer in humans, but it still remains a possibility that chronic activation with high-affinity ligands could be carcinogenic in humans).

3.9.4 Is the AOP specific to certain tissues, life stages / age classes?

- Indicate if there are critical life stages, where exposure must occur, to results in the adverse effect. Or specify if there are key events along the pathway, which are dependent on the life stage, although the AOP is known to be initiated regardless of life stage.
  (example: This AOP is only applicable starting from embryonic development. In zebrafish, this critical window extends from fertilisation to approximately 24 hours post fertilisation).
- Indicate also if the AOP is associated also with age- or sex-dependence.
  (example: This AOP is only applicable to early life stages prior to sexual differentiation.)

3.9.5 Are the initiating and key events expected to be conserved across taxa?

- State if the key events for this AOP appear to be conserved across any group of animals (e.g. mammals). Identify the key event(s) which establish species commonalities and differences.

(example: The key events of this AOP appear to be conserved at following species…).

3.10 References

- List the bibliographic references to original papers, books or other documents used to support the AOP.

4 Annexes

4.1 Linking Tetrabromobisphenol A (TBBPA) to AOPs

4.1.1 Objectives

The present document supports WP13, Task 13.1 of HBM4EU project by summarising existing information on toxicity of TBBPA (specific compound from a priority group of Flame Retardants) with the aim to define corresponding Adverse Outcome Pathway(s) (AOP(s)). The information collected is organised in a way, which allows establishment of links between reported biological effects with individual formalised "Key Events" currently present in AOPWiki.

4.1.2 Toxicity of TBBPA (EFSA report from 2013 and update with recent publications)

There is already a large amount of information on TBBPA's toxicity (60 *in vivo* papers including 30 papers in mammals). We therefore decided to summarise the conclusions from a well-documented EFSA report (EFSA, 2013) and update those conclusions with more recent relevant information.

Overall conclusions:
Based on EFSA report, the main toxicological concern was the effect of TBBPA on thyroid hormone homeostasis and this is supported by more recent studies (Cope et al., 2015; Osimitz et al., 2016; Sanders et al., 2016). The report also mentions the risk that TBBPA induces adipogenesis and subsequent obesity, which is also supported by recent studies (Fang et al., 2015; Riu et al., 2014).

Concerning the other toxicological endpoints, the report concludes on either:

- indication for low-moderate toxicity (hepatotoxicity)
- contradictory information (neurotoxicity)
- no indication of toxicity based on available information (reproductive toxicity, teratogenicity, carcinogenicity, genotoxicity and immunotoxicity)

Some of these conclusions (concerning carcinogenicity and reproductive toxicity in particular) may have to be reconsidered on the basis of recent studies.

Thyroid hormone homeostasis

EFSA concludes that this is the main toxicological concern based on several lines of evidence and uses the BMDL10 for decreased circulating T4 in females (16 mg/kg/d) as a reference for risk characterisation.
Another three recent studies in rats confirm the effects of TBBPA on T4 levels (Cope et al., 2015; Osimitz et al., 2016; Sanders et al., 2016). In addition, several studies show that TBBPA inhibits T3-induced amphibian metamorphosis (Mengeling et al., 2017; Wang et al., 2017; Zhang et al., 2014; Zhang et al., 2015).

Adipogenicity and obesity

EFSA reports that TBBPA promotes PPARγ-induced adipogenesis *in vitro* and that it might increase the risk of obesity in mice by affecting Mc4r and Trh expression.

Interestingly, another two studies confirm that TBBPA is a potent PPARγ ligand *in vitro* (IC50 = 1.49μM) (Fang et al., 2015) and activates it *in vivo* in zebrafish at 100nM (Riu et al., 2014). The latter also shows that TBBPA induces weight gain and lipid accumulation *in vivo*.

Carcinogenicity

EFSA concludes that there are no indications of carcinogenicity based on one study with limited number of animals tested (Imai et al., 2009).

However, more recent two-years studies report a significant increase in the incidence of uterine adenocarcinomas and hepatocellular carcinomas after TBBPA exposure (500 mg/kg/d and 250 mg/kg/d, respectively) in rats (Dunnick et al., 2015; Harvey et al., 2015). TBBPA-induced uterine carcinomas share several morphological and molecular features of human endometrial type I carcinomas suggesting that this chemical may increase cancer risk for humans (Harvey et al., 2015). Wikoff and colleagues even propose a mode of action in the form of an AOP involving inhibition of estradiol sulfotransferase as a molecular initiating event (Wikoff et al., 2016). This is supported by another study showing alteration in the expression of genes involved in estrogen homeostasis and cell proliferation after TBBPA exposure (250 mg/kg/d) (Sanders et al., 2016).

It should also be noted that TBBPA is categorised 2A by IARC (probably carcinogenic to humans).

Reproductive toxicity

The report concludes that there are no reproductive effects based on the available studies, and a more recent study comes to the same conclusion (NOAEL=1000 mg/kg/d, highest dose tested) (Cope et al., 2015).

However, this conclusion may be reconsidered based on a two-generation study that shows several alterations of the male reproductive tract on the second generation after long-term exposure to low levels (0.035 mg/kg/d) of TBBPA (Zatecka et al., 2013).

It is also notable that, as mentioned in the EFSA report, TBBPA is a very potent inhibitor of E2 sulfotransferase (IC50=1.6nM) (EFSA, 2013; Hamers et al., 2006), which is expected to enhance E2 activity and could impact the reproductive endpoint.

Neurotoxicity

On the basis of 8 studies in rodents, EFSA concludes that the results are contradictory. 3 additional recent studies in zebrafishes show that TBBPA is potentially highly neurotoxic (Chen et al., 2016; Jarema et al., 2015; Noyes et al., 2015). As shown in Table1 and commented later, neurotoxicity is also linked to changes in thyroid hormones through several AOPs, of which several are affected by TBBPA at the levels of 2 or more KEs (AOPs 42, 65, 134, 152, 8 and 159). This toxicological endpoint might therefore be of concern.

Teratogenicity

EFSA concludes that there is no indication of teratogenic effects of TBBPA, based mostly on two studies in rats. However, more recent studies suggest that it is highly teratogenic in zebrafish (Behl et al., 2015; Chen et al., 2016; Noyes et al., 2015; Wu et al., 2015; Yang et al., 2014). This probably reflects differences in TBBPA-induced teratogenicity among species, and it may be of concern for humans as well.

4.1.3 Search for AOPs underlying TBBPA toxicity

Approach

Following the first step of literature search (see " FR lit search – Procedure"), we collected information from both *in vivo* and *in vitro* original research papers and EFSA report, and we sorted it according to the biological effect (eg, "estrogenic activity", "Decrease plasma levels of T4", "hepatotoxicity"). For each biological effect, we indicated the supporting evidence that it is affected by TBBPA (see Table 1).

We then searched within the AOP wiki (https://aopwiki.org/) to which key events (KEs) those biological effects correspond and then to which AOPs those KEs belong. We also indicated the corresponding molecular initiating event (MIE) and/or adverse outcome (AO) that were not already described in the literature, with the aim of identifying predictive adverse outcomes or potential molecular targets.

All this information and links between reported effects and corresponding AOP-Wiki KEs is collected and organised in the “FRs-AOPs.xlsx” file (See Table1).

We could identify 72 AOPs for which there is evidence that TBBPA affects at least one KE and there are 5 AOPs for which TBBPA has been linked to 3 or more KEs (see Table1).

Results and comments

1. Considering thyroid hormone homeostasis,

One EFSA report (citing 7 independent studies) and 8 additional *in vivo* studies provide evidence that TBBPA affects thyroid hormone homeostasis in rats and frogs (Choi et al., 2011; Cope et al., 2015; EFSA, 2013; Mengeling et al., 2017; Osimitz et al., 2016; Sanders et al., 2016; Wang et al., 2017; Zhang et al., 2014; Zhang et al., 2015).

We could identify 19 AOPs for which there is evidence that TBBPA affects at least one KE, of which 12 are affected at the level of 2 KEs and one (AOP 152) at the level of 5 KEs (see Table1):

AOP 152: Interference with thyroid serum binding protein transthyretin and subsequent adverse human neurodevelopmental toxicity

KEs affected :

KE 957 : Binding, Transthyretin in serum (MIE)
KE 958 : Displacement, Serum thyroxine (T4) from transthyretin
KE 959 : Increased, Free serum thyroxine (T4)
KE 281 : Thyroxine (T4) in serum, Decreased
KE 402 : Cognitive Function, Decreased (AO)

TBBPA is listed among the stressors for this AOP, conforting the conclusion and suggesting that our approach is efficient at identifying relevant AOPs for a chemical.

2. Considering estrogenic activity related effects,

3 AOPs involving estrogenic activity are affected by TBBPA at the levels of 3 or more KEs:

AOP 29: Estrogen receptor agonism leading to reproductive dysfunction

KEs affected :

KE 111 : Agonism, estrogen receptor (MIE)
KE 364 : Impaired development of, Reproductive organs (AO)
KE 339 : Altered, Larval development (AO)

We note here that TBBPA has been shown to affect male and female reproductive tracts in rodents whereas this AOP only applies to oviparous animals.

AOP 112: Increased dopaminergic activity leading to endometrial adenocarcinomas (in Wistar rat)

KEs affected :

KE 748 : Increased, Estrogen receptor (ER) activity
KE 772 : Increase, Hyperplasia (glandular epithelial cells of endometrium)
KE 773 : Increase, Endometrial adenocarcinomas (AO)

AOP 167: Early-life estrogen receptor activity leading to endometrial carcinoma in the mouse

KEs affected :

KE 1065 : Activation, estrogen receptor alpha
KE 1069 : Increased, Hyperplasia (glandular epithelial cells of endometrium)
KE 1070 : Increased, adenosquamous carcinomas of endometrium (AO)

3. **Considering hepatotoxicity,**

AOP 220 : Chronic Cyp2E1 Activation Leading to Liver Cancer

KEs affected :

KE 1392 : Oxidative stress
KE 1393 : Hepatocytotoxicity
KE 1395 : Liver Cancer

Conclusion and perspective

1. **AOPs/mechanisms for TBBPA carcinogenicity**

- AOPs 112 & 167 are plausible modes of action for TBBPA-induced uterine carcinomas in rodents, involving increase of estrogenic activity and subsequent endometrium hyperplasia.
- AOP 220 provides a mechanism for the TBBPA-induced hepatocellular carcinomas, involving oxidative stress and hepatotoxicity.

2. **Thyroid-induced avderse outcomes**

Thyroid homeostasis is clearly altered after TBBPA exposure. However, this is not a toxicolocical endpoint *per se* but rather an intermediate step leading to different possible endpoints. Some of these outcomes are reported effects of TBBPA and may be linked to TBBPA-induced effects on thyroid homeostasis through AOPs:

- Neurotoxicity. Effects on neurotoxicity are not entirely clear, with several studies reporting a NOAEL of 1000 mg/kg/d (EFSA, 2013), while other studies do report TBBPA-induced neurotoxicity (Behl et al., 2015; Chen et al., 2016; Jarema et al., 2015; Lilienthal et al., 2008; Nakajima et al., 2009; Noyes et al., 2015; Osimitz et al., 2016). It is linked to effects of TBBPA on thyroid homeostasis through AOPs 42, 65, 134, 152, 8 and 159, further arguing that this toxicological endpoint is of concern. AOP 152 in particular provides a highly plausible mechanism.
- Amphibian metamorphosis (Mengeling et al., 2017; Wang et al., 2017; Zhang et al., 2014; Zhang et al., 2015) is linked to effects of TBBPA on thyroid homeostasis through AOPs 175, 176, 188 and 191-194.
- Obesity (Riu et al., 2014) is not linked to thyroid-related effects of TBBPA through existing AOPs, but Decherf and colleagues propose that Mrc4 and Trh may provide such a link. Indeed, these T3-regulated hypothalamic factors, which control food intake and energy usage, are down-regulated after injection of high doses of TBBPA (Decherf et al., 2010a; Decherf et al., 2010b; EFSA, 2013).

3. **Indication for molecular targets**

There are 3 molecular targets for which direct evidence is supported by AOP search:

- E2 Sulfotransferase.

TBBPA is a very potent inhibitor of E2 sulfotransferase *in vitro*, with an IC50 of 16 nM (Hamers et al., 2006). Wikoff and colleagues propose a mode of action in the form of an AOP where inhibition of sulfotransferase SULT1E1 would be the MIE leading to increase in estrogenic activity, followed by endometrium hyperplasia and subsequent uterine carcinoma (Wikoff et al., 2016). We also identified two existing AOPs (112 and 167) proposing the same chain of KEs leading to uterine carcinomas but we did not find direct evidence that TBBPA affects other? MIEs : „Increase of dopaminergic activity" for the AOP 112 and "prepubertal increase of ER activity" for the AOP 167.

- PPARγ

There are several lines of evidence that TBBPA can efficiently bind and activate PPARγ *in vitro* and in fish (Fang et al., 2015; Riu et al., 2011; Riu et al., 2014; Suzuki et al., 2013). Expected effects on adipogenesis according to AOPs 163 and 72 have also been observed (Riu et al., 2011; Riu et al., 2014).

- Transthyretin (TTR)

There is evidence that TBBPA is a potent TTR ligand *in vitro* (EFSA, 2013; Hamers et al., 2006; Meerts et al., 2000) with an IC50 in the nM range, and this is the MIE of the AOP 152 for which TBBPA affects several other KEs.

In addition, several potential molecular targets arose from the AOP search as MIEs for several different biological effects of TBBPA :

- Aryl hydrocarbon receptor

Teratogenicity (AOP 150) and hepatotoxicity (AOP 41).

- Thyroperoxidase

Neurotoxicity (AOP 42), hearing (AOP 159) and amphibian metamorphosis (AOP 175) through decrease in T4 levels.

- Na+/I+ symporter NIS

Neurotoxicity (AOP 65 and 134), and amphibian metamorphosis (AOP 176) through decrease in T4 levels.

- Deiodinases

Hearing (AOP 155-158) and amphibian metamorphosis (AOP 188-191).

4. Indications for predictive adverse outcome

In addition to the observed adverse outcomes of TBBPA that have been described above, some AO can be predicted as possible consequences of TBBPA's effects according to AOPs.

- Those adverse outcomes include breast cancer and altered reproductive behaviour, as predicted outcomes of the estrogenic activity and oxydative stress (AOPs 200) and follicular cell carcinomas as a consequence of decrease in T4 levels (AOP 110 and 119).
- Obesity is also an AO expected from activation of PPARγ and increase of adipogenesis (AOP 72).

Abbreviations:

AO, Adverse Outcome
AOP, Adverse Outcome Pathway
FR, Flame Retardant
KE, Key event
MIE, Molecular Initiating Event
TBBPA, Tetrabromobisphenol A, CAS 79-74-7

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Table 1:

Molecular Initiating event (MIE) highlighted in green
Adverse Outcome (AO) highlighted in red
AOPs for which the Stressor interacts at the level of **2 or more KEs are indicated in bold.**
AOPS for which the Stressor interacts at the level of **3 or more KEs are indicated using higher case.**

	Biological effect	Supporting evidence					Key Event (KE)	AOPs	Corresponding MIE (when absent from list of bological effects)	Corresponding AO (when absent from list of bological effects)
		eff. Dose/ conc.	units		biological system	reference				
Reproductive toxicity	estrogenic activity	10	µM	REC5	CALUX Assays	Suzuki et al., 2013	1181, 1064, 111, 1065	**167, 29**,52,**53**,**200, 112**	↗ **dopaminergic activity (112)**	**Breast cancer (200) ; Altered reproductive behaviour (29)**
		19	µM	EC50	MCF-7	Kitamura et al, 2005a				
		0.016	**µM**	**IC50**	**in vitro**	**inhibition of E2 sulfation, Hamers et al., 2006**				
		124	µM	EC20	MCF-7 RPE	Krivoshiev et al, 2016				
	anti-estrogenic activity	10	µM	>		Suzuki et al., 2013	112, 1046			
		451	µM	IC20	6	Krivoshiev et al, 2016				
		50	µM	LEL	MVLN	Song et al., 2014				
	Anti-androgenic activity	10	µM	RIC20	4	Suzuki et al., 2013	742, 27	111, 19		Leydig cell tumors (111) ; Feminisation/incomplete dvpt of male sex organs (19)
		1000	µM	>	NIH3T3	Kitamura et al, 2005a				
	Antogonistic effect Progesterone receptor	10	µM	RIC20	6	Suzuki et al., 2013				
	Malformation male reproductive tract	0.5	mg/kg/d	BMDL5	Rats	testes weight, Van der Ven, 2008	348, 809, 364	18, 124, **29?**	**Act of PPARα (18)** ; Inh of HMG-CoA reductase (124)	
		0.035	mg/kg/d		Mice	Zatecka et al, 2013				
	Malformation female reproductive tract	20	mg/kg/d	LEL	Mice	Uterus rel. weight, Kitamura et al, 2005a	364, 772, 1069	**29?, 112, 167**	↗ **dopaminergic activity (112)**	**Altered reproductive behaviour (29)**
		250	mg/kg/d		Rats	Endometruim hyperplasia, NTP, 2014 ; Wikoff et al, 2016				
	Decrease semen quality	0.9	**nM**	LEL	Sterlet spermatozoa	Linhartova et al, 2015	543, 505, 520	74, 64, 66, 67,68, 69, 70, 71	↗GR activity (64, 71, 66) ; ↘Cholesterol biosynthesis (69) ; hypermethylation in the fetal testis (74) ; proteomic alteration in fetal testis/leydig cells (68, 70)	
	Decreased, impaired fertility	1%		>	Mice	EFSA, 2013				
adipogenesis/obesity	PPARγ binding/activation	0.7	µM	IC50	HGELN-PPARγ cells	Riu et al., 2011	1028	**163, 72**	demethylation PPARγ promoter, chronic high fat diet (72)	**obesity (72)**
		10	µM	LEL	Hela cells	human, zebrafish and xenopus PPARγ Riu et al., 2011				
		1.49	µM	IC50	In vitro assay	Fang et al., 2015				
		0.1	µM	LEL	Zebrafish	Fluo resporter, Riu et al., 2014				
		0.3	µM	EC50	Hela cells	Zebrafish PPARγ, Riu et al., 2014				
		10	µM	REC5	CALUX cells	Suzuki et al., 2013				
		3.9	µM	EC50	Cos7	PPARγ$_1$, Watt and Schlezinger, 2015				
		7.8	µM	EC50	Cos7	PPARγ2, Watt and Schlezinger, 2015				
		2	µM	AC50	ATG_PPARg_TRANS	Tox-Cast				
		0.93-3.72	µM	AC50	ATG_PPRE_CIS	Tox-Cast				
	adipogenesis/lipid accumulation	10	µM		3T3L1	Riu et al., 2011	1449, 1029, 1306	**163, 72,** 213	demethylation PPARγ promoter, chronic high fat diet (72)	**obesity (72)**
		0.01	µM	LEL, ONLY	Zebrafish	Riu et al., 2014				
	Increase Body weight	0.37	µM	>	zebrafish	Huang et al., 2017	N			
		0.1	µM	LEL	Zebrafisf	Riu et al., 2014				
teratogenicity		2.5	g/kg	>	Rats	EFSA, 2013	339, 1001, 947	**29**, 43, 150	**Act. AhR**, Inh. VegfR2	
		0.73	µM	LEL	Zebrafish	Wu et al., 2015				
		0.09	µM	LEL	Zebrafish	Yang et al., 2014				
		6.4	µM	LEL	zebrafish	Noyes et al., 2015				
		4.6	µM	POD	Zebrafish	Behl et al., 2015				
		10	µM	LEL	Zebrafish	Chen et al., 2016a				
		2.95	µM	AC50	NHEERL_144hp	Tox-Cast				
neurotoxicity	behavioral/ locomotor activity tests	0.1	mg/kg/d	LEL/ONLY	Mouse	Nakajima et al., 2009	402	**42, 65, 134, 152**	**Inh Na+/I+ symporter NIS (65, 134) ; Inh. Thyroperoxidase (42)**	
		1000	mg/kg/d		Rats	Osimitz et al., 2016				
		0.0064	µM	LEL	Zebrafish	Noyes et al., 2015				
		1.2	µM		zebrafish	Jarema et al., 2015				
		1000	mg/kg/d	>	Rats	EFSA, 2013				
		1000	mg/kg/d	>	Rats	EFSA, 2013				
		1000	mg/kg/d	>	Rats	EFSA, 2013				
		3000	mg/kg/d	>	Rats	EFSA, 2013				
	Hearing	8	mg/kg/d	BMDL	Rats	Lilenthal et al., 2008	319, 1008	**8**, 155, 156, 157, 158, **159**	**Act. PXR, NR1/2 (8) ; Inh. Thyroperoxidase (159) ; Inh. Deiodinase 1 (157,158) ; Inh. Deiodinase 2 (155,156)**	
	Developmental neurotoxicity	12.2	µM	POD	4	Behl et al., 2015	N			
		5	µM	LEL	Zebrafish	Chen et al., 2016b				
Thyroid	Decrease plasma levels of T4	16	mg/kg/d	BMDL10	Rats	females, one-genration st., Van der Ven, 2008	277, 771, 281, 426, 1093	110, 119, **42, 65, 128, 134,** 54, **159, 175, 176, 188, 192, 193, 8, 152, 194**	**Inh. Thyroperoxidase (119, 42, 159, 175) ; Inh Na+/I+ symporter NIS (65, 134, 54, 176) ; Act. PXR, NR1/2 (8) ;** Decreased, Uptake of inorganic iodide (110) ; **Thyroid hormone synthesis, Decreased (128) ; Inh. Iodotyrosine deiodinase (IYD) (188) ; Inh. Pendrin (192) ; Inh. Dual oxidase (193) ; Act. Hepatic nuclear receptor(s) (194)**	follicular cell adenomas/carcinomas (110, 119)
		31	mg/kg/d	BMDL10	Rats	males, one-genration st., Van der Ven, 2008				
		48	mg/kg/d	BMDL10	Rats	males, sub-acute, Van der Ven, 2008				
		100	mg/kg/d		Rats	EFSA, 2013				
		100	mg/kg/d		Rats	F0 males and F1 ; EFSA, 2013				
		100	mg/kg/d		Rats	Osimitz et al., 2016				
		250	mg/kg/d		Rats	Choi et al., 2011				
		100	mg/kg/d		Rats	Cope et al., 2015				
		250	mg/kg/d		Rats	Sanders et al., 2016				
	Increase T4	11	ng/ml		Eur. Flounder	Kuiper et al., 2007	959	**152**		
	Increased T3	124	mg/kg/d	BMDL10	Rats	males, sub-acute, Van der Ven, 2008	1154	191	**Inh. Deiodinase 3 (191)**	
		2.3	mg/kg/d	BMDL10	Rats	females, one-genration st., Van der Ven, 2008				
	Inhibition of T3-induced amphibian metamorphosis	0.01	µM	LEL	Xenopus	Wang et al., 2016	1101	**175, 176, 188,** 189, 190, **191, 192, 193, 194**	**Inh. Deiodinase 1 (189) ; Inh. Deiodinase 2 (190) ; Inh. Deiodinase 3 (191) ; Inh. Thyroperoxidase (175) ; Inh Na+/I+ symporter NIS (176) ; Inh. Iodotyrosine deiodinase (IYD) (188) ; Inh. Pendrin (192) ; Inh. Dual oxidase (193) ; Act. Hepatic nuclear receptor(s) (194)**	
		0.5-1	µM		Xenopus	Mengeling et al., 2017				
		0.01	µM	LEL	Xenopus	Zhang et al., 2014				
		0.01 !	µM	Opposite	Frog	Zhang et al., 2015				
	Thyroid hormonal activity	1-100	µM		GH3 cells	GH production, Kitamura et al., 2005a				
	Thyroid gland weight	500	mg/kg/d		Rats	decrease, Choi et al., 2011				
		1	%		Rats	increase, Imai et al., 2008				
	TTR binding	0.0077	µM	IC50	*in vitro*	Meerts et al., 2000, EFSA 2013	957	**152**		
	Inh. T4-TTR binding	0.031	µM	IC50	*in vitro*	Hamers et al., 2006, EFSA 2013	958	**152**		

Table 1 (continued)

carcinogenicity	uterine adenomas & carcinomas	500 500	mg/kg/d mg/kg/d		Rats Mice	Harvey et al., 2015 male, Dunnick et al., 2015	773, 1070	**112**, **167**	↗ Binding to 2u (105) ; ↗ cytotoxicity (116) ; ↗ **dopaminergic activity (112)**	
	Hepatocellular and adrenal adenomas and carcinomas	250	mg/kg/d		Mice	male, Dunnick et al., 2015	719, 1395, 378	107, **108**, 117, 118, 37, **220**	Act. CAR ; **Inh. PDK** ; Act. Androgen receptor (117) ; ↗ cytotoxicity ; **Act. PPAR α (37)** ; **Act. Cyp2E1 in liver (220)**	
Immunotoxicity		1700 >300 250	mg/kg/d mg/kg/d mg/kg/d		Mice Rats Rats	EFSA, 2013 EFSA, 2013 Hall et al., 2017	N			
Other chronical toxicities	Hepatotoxicity	350 300 250 1000 16	mg/kg/d mg/kg/d mg/kg/d mg/kg/d µg/m3 (inh)	LEL LEL LEL LEL	Mice Rats Mice Rats Mice	Inflammatory cell infiltration, Tada et al., 2007 increase bilirubin, Osimitz et al., 2016 male, Dunnick et al., 2015 liver weight, Dunnick et al., 2017 Chen et al., 2016c	1291, 270, 139, 902, 1393	**209**, **32**, **41**, **220**, **144**	**long term activation AhR (41)** ; **Act. Cyp2E1 in liver (220)** ; **Disruption Lysosome (144)** ; Pro-mutagenic DNA adduct (46)	
	Kidney toxicity	200 250	mg/kg/d mg/kg/d		Rats Mice	lesions in new born, Fukuda et al., 2004 male, renal tubule, Dunnick et al., 2015	814, 767, 422	**128**, 105, **53**, 33	↗ Binding to 2u (105) ; Act. 5HT2c (33)	
	Forestomach	250	mg/kg/d		Mice	female, Dunnick et al., 2015	1385, 782	217, 227, 228, 229, 115		
	pituitary	0.6	mg/kg/d	BMDL10	Rats	inc. weight, males, one generation st., Van der Ven. 2008	N			
	oxydative stress	200 0.73 0.09 16 0.9	mg/kg (single µM µM µg/m3 (inh) **nM**	 LEL LEL LEL	Rats Zebrafish Zebrafish Mice Sterlet spermatozoa	SOD, Kang et al., 2009 Wu et al., 2015 Yang et al., 2015 Chen et al., 2016c Linhartova et al., 2015	209, 1088, 1392, 210, 211	27, **108**, **144**, 149, 171, 138, 177, 186, **200**, **220**, 17, 31	Inh. Bile Salt Export Pump (27) ; **Inh. PDK (108)** ; **Disruption Lysosome (144)** ; **Act. Cyp2E1 (220)** ; peptide oxydation (149) ; Inh. OAT1 (138) ; Inh. Cyclooxygenase 1 (177) ; Binding SH-/selen- proteins (17)	Cholestasis (27) ; Mortality (138, 177, 186) ; **Breast Cancer (200)** ; Hypertension (149) ; Inc. mesotheliomas (171) ; Neurodegeneration (17) ; Cyanosis (31)

4.2 Linking Tris(1,3-Dichloropropyl) Phosphate (TDCIPP) to AOPs

Objectives

The present document supports WP13, Task 13.1 of HBM4EU project by summarising existing information on toxicity of TDCIPP (specific compound from a priority group of Flame Retardants) with the aim to define corresponding Adverse Outcome Pathway(s) (AOP(s)). The information collected is organised in a way, which allows establishment of links between reported biological effects with individual formalised "Key Events" currently present in AOPWiki.

Approach

Following the first step of literature search (see " FR lit search – Procedure"), we focused on adverse outcome pathways (AOPs) linked to the apical endpoints for which TDCIPP appeared to be highly toxic, that is : reproduction (Baker et al., 2015; Liu et al., 2013a; Wang et al., 2015a; WHO, 1998; Zhu et al., 2015), carcinogenicity (Baker et al., 2015; Freudenthal and Henrich, 2000; WHO, 1998), teratogenicity (Baker et al., 2015; Behl et al., 2015; Du et al., 2015; Farhat et al., 2014a; Fu et al., 2013; McGee et al., 2012; Wang et al., 2015b; Yu et al., 2017), immunotoxicity (Baker et al., 2015) and thyroid hormones (Farhat et al., 2013; Meeker and Stapleton, 2010; Wang et al., 2013; Wang et al., 2015b; Xu et al., 2015). We also considered hepatotoxicity (Baker et al., 2015; Farhat et al., 2014a; Freudenthal and Henrich, 2000; Jacobsen et al., 2017; Liu et al., 2016; WHO, 1998) and nephrotoxicity (Baker et al., 2015; Freudenthal and Henrich, 2000; WHO, 1998).

We collected information from both *in vivo* and *in vitro* original research papers and reports from US-EPA, National Research Council (NRC) and World Health Organization (WHO) (Baker et al., 2015; National Research Council, 2000; WHO, 1998). We then sorted it according to the biological effect (eg, "estrogenic activity", "Decrease of androgens", "hepatotoxicity") and we indicated the supporting evidence that the biological effect is observed following TDCIPP exposure (see Table 1).

We then searched within the AOP wiki (https://aopwiki.org/) to which key events (KEs) those biological effects correspond and then to which AOPs those KEs belong. We also indicated the corresponding molecular initiating event (MIE) and/or adverse outcome (AO), with the aim of identifying predictive adverse outcomes or potential molecular targets.

All this information and links between reported effects and corresponding AOP-Wiki KEs is collected and organised in the "AOPs-TDCIPP.xlsx" file (Table1).

Results and comments

1. **Considering the reproductive endpoint,**

13 original research papers and 3 reports from the NRC, US-EPA and WHO provide supporting evidence that TDCIPP provokes biological effects associated with reproductive toxicity (Baker et al., 2015; Farhat et al., 2014b; Kojima et al., 2013; Krivoshiev et al., 2016; Li et al., 2015; Liu et al., 2012; Liu et al., 2013a; Meeker and Stapleton, 2010; National Research Council, 2000; Porter et al., 2014; Reers et al., 2016; Suzuki et al., 2013; Wang et al., 2015a; WHO, 1998; Zhang et al., 2014; Zhu et al., 2015). Most of the information comes from *in vitro* studies on cell culture or *in vivo* studies in zebrafish. In addition, one study in rat (Baker et al., 2015; Freudenthal and Henrich, 2000; National Research Council, 2000) and two studies in human (Baker et al., 2015; Meeker and Stapleton, 2010) support the conclusion that TDCIPP is an endocrine disruptor and impairs fertility also in mammals (increase of gynecomastia and increased levels of prolactin in humans, malformation of the male reproductive tract in rat and decreased secretory product from the seminal vesicle).

We could identify 31 AOPs for which there is evidence that TDCIPP affects at least one KE and there are 8 AOPs for which TDCIPP has been linked to 3 or more KEs (see Table1):

AOP 18: PPARα activation in utero leading to impaired fertility in males

KEs affected :

- KE 446 : Reduction, testosterone levels
- KE 348 : Malformation, male reproductive tract (AO)
- KE 406 : Impaired fertility (AO)

AOP 29: Estrogen receptor agonism leading to reproductive dysfunction

KEs affected :

- KE 111 : Agonism, estrogen receptor (MIE)
- KE 307 : Increase, Vitellogenin synthesis in liver
- KE 220 : Increase, Plasma vitellogenin concentrations
- KE 78 : Reduction, cumulative fecundity and spawning
- KE 339 : Altered, Larval development (AO)

This AOP provides a plausible mechanism for the reproductive toxicity of TDCIPP in fishes. Because it involves regulation of levels of Vitellogenin, it only applies to oviparous animals.

AOP 67 : Modulation of adult Leydig cell function subsequent to estradiol activation in fetal testis

KEs affected :

- KE 658 : Decreased testosterone by the fetal Leydig cells, Increased estradiol (MIE)
- KE 659 : Decreased testosterone by the fetal Leydig cells, Activation by other estraidiol agonists
- KE 505 : Decreased sperm quantity / quality in the adult, Decreased fertility (AO)

AOP 69: Modulation of Adult Leydig Cell Function Subsequent to Decreased Cholesterol Synthesis or Transport in the Adult Leydig Cell

KEs affected :

- KE 642 : Decreased Cholesterol, Decreased De Novo Biosynthesis of Choleseterol (MIE)
- KE 644 : Decreased Cholesterol, Decreased Transport of Cholesterol to the Inner Mitochondrial Membrane
- KE 645 : Decreased Cholesterol, Decreased Testosterone Production by Adult Leydig Cells
- KE 646 : Decreased Cholesterol, Decreased sperm quantity and/or quality in the adult testis (AO)

AOP 66: Modulation of Adult Leydig Cell Function Subsequent Glucocorticoid Activation in the Fetal Testis

KEs affected :

- KE 654 : Decreased testosterone by the fetal Leydig cells, Activation by other glucocorticoid receptor agonists (MIE)
- KE 505 : Decreased sperm quantity / quality in the adult, Decreased fertility (AO)

AOP 64: Glucocorticoid Receptor (GR) Mediated Adult Leydig Cell Dysfunction Leading to Decreased Male Fertility

KEs affected :

- KE 494 : Glucocorticoid Receptor Agonist, Activation (MIE)
- KE 446 : Reduction, testosterone level
- KE 520 : Decreased sperm quantity or quality in the adult, Decreased fertility
- KE 406 : impaired, Fertility (AO)

AOP 124: HMG-CoA reductase inhibition leading to decreased fertility

KEs affected :

KE 807 : Decreased, cholesterol
KE 808 : Decreased, Testosterone
KE 809 : malformed, male reproductive tract
KE 330 : Decreased, Fertility (AO)

This AOP is applicable to rats and could therefore provide one possible mechanism for the observed toxicity of TDCIPP on the male reproductive tract in rats (Baker et al., 2015).

AOP 71: Modulation of Adult Leydig Cell Function Subsequent to Glucocorticoid Activation

KE 525: Apoptosis of adult Leydig cells, Decreased testosterone by adult Leydig cells
KE 651: Glucocorticoid Receptor mediated alterations in steriodogenic enzymes, Decreased testosterone by adult Leydig cells
KE 520 : Decreased sperm quantity or quality in the adult, Decreased fertility (AO)

2. Considering other toxicological endpoints of TDCIPP,

We could link this flame retardant (FR) to another 45 AOPs, 4 of them being affected at the levels of 3 or more KEs:

AOP 53 : ER agonism leading to reduced survival due to renal failure

KEs affected :

KE 111 : Agonism, estrogen receptor (MIE)
KE 418 : Increased, Vitellogenin synthesis
KE 220 : Increase, Plasma vitellogenin concentrations
KE 422 : Increased, nephropathy

This AOP provides a mechanism for nephrotoxicity of TDCIPP that involves renal deposition of Vitellogenin. It is therefore limited to oviparous animals and does not provide a mechanism for the nephrotoxicity described in rats (Baker et al., 2015; Freudenthal and Henrich, 2000).

AOP 220 : Chronic Cyp2E1 Activation Leading to Liver Cancer

KEs affected :

KE 1392 : Oxidative stress
KE 1393 : Hepatocytotoxicity
KE 1395 : Liver Cancer (AO)

AOP 200 : Estrogen receptor activation leading to breast cancer

KEs affected :

KE 1181 : Activation, Estrogen receptor (MIE)
KE 177 : N/A, Mitochondrial dysfunction 1
KE 1088 : Increased, Oxidative Stress

AOP 144 : Lysosomal damage leading to liver inflammation

KEs affected :

KE 209 : Peptide Oxidation
KE 177 : N/A, Mitochondrial dysfunction 1
KE 902 : Inflammation, Liver

Conclusion and perspective

1. AOPs/mechanisms for reproductive toxicity of TDICPP

TDCIPP provokes biological effects that are linking it to many AOPs for the reproductive endpoint. Some of these AOPs are potentially affected by TDCIPP at the level of 3 or more KEs and could therefore provide mechanisms for TDCIPP-induced reproductive toxicity.

- AOPs 29 is a highly plausible mode of action for reproductive toxicity of TDCIPP observed in fish, involving agonism of estrogen receptor and increase of vitellogenin.
- AOPs 18 & 124 are applicable to rats (potentially also to humans) and could therefore provide mechanisms for the observed toxicity of TDCIPP on the male reproductive tract in rats (Baker et al., 2015).
- AOPs 64, 66, 67, 69 & 71 provide related mechanisms for the decrease in semen quality observed in rats at low doses of TDCIPP (Baker et al., 2015; National Research Council, 2000). All AOPs involve reduced level of testosterone produced by Leydig cells in rats. Although decrease in testosterone levels has been reported following TDCIPP exposure, studies were performed in zebrafish (Liu et al., 2012; Liu et al., 2013a) and it remains to be tested in Leydig cells.

There may also be links between biological effects of TDCIPP that are not described in an existing AOPWiki databases, leading to the generation of new AOP(s) in the future.

2. Indication for molecular targets

The outcomes of this literature research on AOPs could help directing future research for the molecular targets of TDCIPP.

- There is good evidence that TDCIPP affects several KEs of AOPs 18 and 124, but is not clear whether it affects MIEs : „Activation of PPARα“ for the AOP 18 and “Inhibition of HMG-CoA reductase“ for the AOP 124. The AOPs 51 and 37 also links activation of PPARα to biological effects of TDCIPP (decrease of testosterone and impaired fertility (AOP 51), and hepatocellular adenomas for (AOP 37)), further suggesting that PPARα could be a molecular target. However, one study has tested agonistic activity of TDCIPP on PPARα and found no effect (highest concentration tested 100µM) (Kojima et al., 2013). The following step in the AOP 18 involves Steroidogenic Acute Regulatory Protein (STAR), which may consitute another potential target.
  We could not find published studies of the effects of TDCIPP on HMG-CoA.
- Decrease in Testestosterone and fecundity or hepatocellular adenomas are linked to activation of androgen receptor through AOPs 23 and 117, whereas *in vitro* studies rather show a strong anti-androgenic activity (Kojima et al., 2013; Reers et al., 2016; Suzuki et al., 2013).

- AOPs 64, 66 and 71 involve Glucocorticoid receptor (GR) activation as a molecular event leading to decrease in sperm quality/quantity. However, evidence that TDCIPP activates GR is weak (Liu et al., 2013b).

3. Indications for predictive adverse outcome

Our literature search links TDCIPP to AOPs leading to advserse outcomes that have not been observed after TDCIPP exposure so far, but that might be of concern.

- Those adverse outcomes include endometrial carcinomas and breast cancer as possible outcomes of the estrogenic activity (AOPs 167, 112 and 200) and Leydig cell tumors as a consequence of anti-androgenic activity (AOP 111). Indeed, there is evidence that TDCIPP is carcogenic and it is included on the Proposition 65 list of chemicals known to cause cancer (Baker et al., 2015; Freudenthal and Henrich, 2000; Kammerer, 2017; National Research Council, 2000).
- Feminisation is another expected adverse outcome of anti-androgenic activity (AOP 19). Interestingly, gynecomastia has been associated with exposure to TDCIPP in men (Baker et al., 2015) and would support the conclusion that feminisation is one possible adverse outcome of TDCIPP.

4. An approach for searching AOPs for other priority compounds

This general procedure for AOPs search as presented here for TDCIPP will be applied to other flame retardants (FRs) for which there is enough literature data available, starting with the other two selected FRs (see document “ FR lit search – Procedure”), i.e. :

- Triphenyl phosphate
- Tricresyl phosphate

It would be interesting to see whether they may affect similar AOPs and maybe share some molecular targets. One *in vitro* study concluded that TDCIPP with these other 2 FRs cluster together when considering their endocrine properties (Suzuki et al., 2013).

Abbreviations:

AO, Adverse Outcome
AOP, Adverse Outcome Pathway
FR, Flame Retardant
KE, Key event
MIE, Molecular Initiating Event
TDCIPP, Tris (1,3-dichloropropyl) phosphate, CAS 13674-87-8

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Table 1:

Molecular Initiating event (MIE) highlighted in green
Adverse Outcome (AO) highlighted in red
AOPs for which the Stressor interacts at the level of **2 or more KEs are indicated in bold.**
AOPS for which the Stressor interacts at the level of **3 or more KEs are indicated using higher case.**

	Biological effect	Supporting evidence					Key Event (KE)	AOPs	Corresponding MIE (when absent from list of bological effects)	Corresponding AO (when absent from list of bological effects)
		eff. Dose/ conc.	units		biological system	reference				
Reproductive toxicity	estrogenic activity	6.4	µM	REC20	CHO-K1 Dual	(Luc reporter ERα, Zhang et al., 2014)	1181, 1064, 111, 1065	167, **29**,52,**53**,**200**,112	↗ dopaminergic activity (112)	Endometrial carcinomas (167, 112) ; Breast cancer (200)
		7.8	µM	REC20	Yeast 2 hyb	(2 Hyb ERα, Zhang et al., 2014)				
		10	µM	>	CHO-K1	(Luc reporter ERα, Kojima et al., 2013)				
		10	µM	>	CHO-K1	(Luc reporter ERβ, Kojima et al., 2013)				
		3	µM	REC5	CALUX Assays	(Erα CALUX, Suzuki 2013)				
		20.6	µM	EC20	MCF-7 RPE	(RPE of MCF-7 cells, Krivoshiev et al., 2016)				
		0.046	µM	LEL	zebrafish	ER mRNA and target genes, Liu C et al., 2013				
	anti-estrogenic activity	38	µM	IC20	1	(RIE of MCF-7 cells, Krivoshiev et al., 2016)	112, 1046	**30**, 165		ovarian granular cell tumors (165)
		23	µM	LEL	3	(Inh. of E2-Luc reporter, Liu et al., 2012)				
		ND		RIC20		(Luc reporter ERα, Zhang et al., 2014)				
		ND		RIC20		(2 Hyb ERα, Zhang et al., 2014)				
		NC		RIC20		(Erα CALUX, Suzuki 2013)				
	Anti-androgenic activity	10	µM		1	(inh of R1881-induced effects, Reers et al., 2016)	742, 27	111, **19**		Leydig cell tumors (111) ; Feminisation/incomplete dvpt of male sex organs (19)
		1.9	µM	RIC20	1	(Luc reporter AR, Kojima et al., 2013)				
		1.9	µM	IC50	1	(AR CALUX, Suzuki 2013)				
	Antagonistic effect Progesterone receptor	0.85	µM	IC50	1	(PR CALUX, Suzuki 2013)				
	Glucocorticoid receptor	>10	µM			Suzuki et al., 2013	122, 494, 1396, 651, 654	14, 214, **64**, 221, 222, **66**, **71**		Increased disease susceptibility (14), seizure/hypertension (214), depression (214, 221), agitation (214, 222)
		16	µM	RIC20		Inh., Kojima et al., 2012				
		0.46	µM	LEL	zebrafish	Inc. mRNA, Liu, C et al., 2013				
	PPARα activation	>10	µM			Kojima et al., 2013	227	18, 51, 61, 37, 213	Act. NRF2, NR1H4 (61)	**Inc., liver steatosis (61)**
		4.6	µM	LEL	zebrafish	mRNA, Liu, C et al., 2013				
	Increase of Vitellogenin	2.3	µM	LEL	zebrafish	*vtg* mRNA in males, Liu et al 2012	220, 307, 419, 418	**29, 53**		
		0.09	µM	LEL	zebrafish	plasma VTG in males, Liu X et al., 2013				
		0.46	µM	LEL	zebrafish	plasma VTG in females, Liu X et al., 2013				
		10	µM	LEL		*vtg* mRNA in CEH, Porter et al 2014				
	Increase CYP19a levels	2.3	µM	LEL	zebrafish	*cyp19a* mRNA, Liu et al., 2012	(decrease only)			
		2.3	µM	LEL	1	*cyp19a* mRNA, Liu et al., 2012				
	Decrease, Cholesterol	45	µg/g		chicken	embryo, Farhat et al., 2014	642, 646, 645, 807	**69**, **124**		
	Increase of 17beta-estradiol (E2)	0.023	µM	LEL	1	(H295R, Liu et al., 2012)	658	**67**		
		2.3	µM	LEL	zebrafish	Liu et al., 2012				
		2.3	µM	LEL	zebrafish	Liu X et al., 2013				
		0.046	µM	LEL	zebrafish	females, Wang et al., 2015b				
	Decrease of androgens (Testosterone (T) or 11-Ketotestosterone (11-KT))	0.09	µM	LEL	zebrafish	11-KT, males, Liu et al., 2012	446, 413, 495, 525, 645, 658, 808, 274	**51**, **18**, **64**, **71**, **66**, **69**, **67**, **124**, 23	**Act of PPARα (18, 51) ; Inh of HMG-CoA reductase (124) ; ↗GR activity (64, 71) ; ↘Cholesterol biosynthesis (69) ; Agonism androgen receptor (23)**	
		0.46	µM	LEL	zebrafish	T, female, Liu X et al., 2013				
		0.09	µM	LEL	zebrafish	T, male, Liu X et al., 2013				
		0.46	µM	ONLY	zebrafish	11-KT, female, Liu X et al., 2013				
		0.09	µM	ONLY	zebrafish	11-KT, male, Liu X et al., 2013				
	Increase of Testosterone (T)	0.023	µM	LEL-	1	(H295R, Liu et al., 2012)	N			
		2.3	µM	LEL	zebrafish	Liu et al., 2012				
		0.046	µM	LEL	zebrafish	females, Wang et al., 2015b				
	Increase of E2/T or E2/11-KT	0.023	µM	LEL	1	(H295R, Liu et al., 2012)	N			
		2.3	µM	LEL	zebrafish	E2/11-KT, females Liu et al., 2012				
		2.3	µM	LEL	zebrafish	E2/T, males Liu et al., 2012				
		0.09	µM	LEL	zebrafish	E2/11-KT males, Liu et al., 2012				
		0.46	µM	LEL	zebrafish	E2/T, female, Liu X et al., 2013				
		0.09	µM	LEL	zebrafish	E2/T male, Liu X et al., 2013				
		0.46	µM	ONLY	zebrafish	E2/11-KT, female, Liu X et al., 2013				
		0.09	µM	ONLY	zebrafish	E2/11-KT, male, Liu X et al., 2013				
	Increased prolactin	Human				(Meeker and Stapelton, 2010)	1084, 1077	170, 168	↘ Dopaminergic activity (170) ; ↘ GnRH release in hypothalamus (168)	Mammary adenomas/carcinomas (170, 168)
	Malformation male reproductive tract	5	mg/kg/d	LEL	Rat	Reported as second source, US-EPA 2015	348, 330, 809	**18, 124, 29?**	**Act of PPARα (18) ; Inh of HMG-CoA reductase (124)**	
		5	mg/kg/d	LEL	Rat	WHO EHC209, 1998				
	Decrease semen quality	5	mg/kg/d	LEL	Rat	NRC 2000 ; US-EPA, 2015	543, 497, 505, 520	74, **64**, **66**, **67**,68, **69**, 70, **71**	**↗GR activity (64, 71, 66) ; ↘Cholesterol biosynthesis (69) ;** hypermethylation in the fetal testis (74) ; proteomic alteration in fetal testis/leydig cells (68, 70)	
		not significant			Human	(Meeker and Stapelton, 2010)				
		5	mg/kg/d	LEL	Rat	WHO EHC209, 1998				
	Increased gynecomastia	Human				(Murphy et al., 1981, cohort study reported in second source in US-EPA 2015)	?266?	**?19?**		
	Reduction, Cumulative fecundity and spawning	0.015	µM	LEL	zebrafish	Zhu et al., 2015	78, 330, 972	**29**, 25, **23**, **30**, 122, 123, 153	**Inh Aromatase (25, 153) ; Agonism androgen receptor (23) ;** Inh prolyl hydroxylase (122) ;	
		0.09	µM	LEL	zebrafish	Liu X et al., 2013				
		0.046	µM	LEL	zebrafish	Wang et al., 2015b				
		0.015	µM	LEL	aphnia Magna	Li et al. 2015				
	Decreased, impaired fertility	400	mg/kg/d	LEL	Rats	WHO EHC209, 1998	406, 330	7, **51**, **18**, **64**, **124**, 216, 238	**Inh Aromatase (7) ; Act of PPARα (18, 51) ; Inh of HMG-CoA reductase (124) ;** ↗ reactive oxygen species (216, 238)	
	Mitochondrial function/transport						644, 1261, 177	**69**, 205, 48, 3, **144**, **200**, **17**	Binding of agonist, Ionotropic glutamate receptors, Binding of inhibitor, NADH-ubiquinone oxidoreductase, Disruption, Lysosome, Binding, SH/seleno proteins	
teratogenicity		3.83	µM	EC50	zebrafish	Pericardial edema, Du et al., 2015	339, 1001, 947	**29**, 43, 150	Act. AhR, Inh. VegfR2	
		0.23	µM	LEL	zebrafish	Wang et al., 2015c				
		3	µM	LEL	zebrafish	McGee et al., 2012				
		50	µg/g		chicken	Farhat et al., 2014				
		1	µM	LEL	zebrafish	Fu et al., 2013				
		8.9	µM	POD	zebrafish	Behl et al., 2015				
		0.0013	µM	≤	zebrafish	Yu et al., 2017				
		400	mg/kg/d	LEL	Rats	US-EPA, 2015				
carcinogenicity	renal adenomas & testicular carcinomas	20	mg/kg/d	LEL	Rat	Freudenthal and Henrich, 2000 ; US-EPA, 2015	713	105, **116**	↗ Binding to 2u (105) ; ↗ cytotoxicity (116)	
		20	mg/kg/d	LEL	Rat	WHO EHC209, 1998				
	Hepatocellular and adrenal adenomas and carcinomas	80	mg/kg/d	LEL	Rat	Freudenthal and Henrich, 2000 ; US-EPA, 2015	719, 1395, 378	107, **108**, 117, 118, **37**, **220**	Act. CAR ; Inh. PDK ; **Act. Androgen receptor (117)** ; ↗ cytotoxicity ; **Act. PPAR α (37) ; Act. Cyp2E1 in liver (220)**	
		5	mg/kg/d	LEL	Rat	WHO EHC209, 1998				
	Thyroid adenomas	80	mg/kg/d	LEL	Rat	WHO EHC209, 1998	N			

Table 1 (continued):

Immunotoxicity	Decrease B-cell antigen	2.5	mg/kg/d	LEL	Mouse	US-EPA, 2015	N			
	Atopic dermatitis	human				Araki et al., 2014	312, 985	N		
	Decrease T-cell antigen	25	mg/kg/d	LEL	Mouse	US-EPA, 2015	N			
Thyroid	Decrease plasma levels of T4	0.046	µM	LEL	zebrafish	female and F1, Wang et al., 2015c	277, 771, 281, 426, 1093	110, 119, 42, 65, **128**, 134, 54, 159, 175, 176, 188, 192, 193, 8, 152, 194	Inh. Thyroperoxidase (119, 42, 159, 175) ; Inh Na+/I+ symporter NIS (65, 134, 54, 176) ; Act. PXR, NR1/2 (8)	follecular cell adenomas/carcinomas (110, 119) ; Cognitive function (42, 65, 134, 152) ; Altered amphibian metamorphosis (175, 176, 188, 192, 193, 194) ; Loss cochlear function (8) ...
		0.23	µM	LEL	zebrafish	female, Xu et al., 2015				
		Human				Meeker and Stapelton, 2010				
		0.12	µM	LEL	zebrafish	larvae, Wang et al., 2013				
		7.64	µg/g		chicken	embryo, Farhat et al., 2013				
		> 150	mg/kg/d		Rat	Pregnant female (from GD10 to weaning), Moser et al., 2015				
Other chronical toxicities	PXR Activation	1.4	µM	REC20		Kojima et al., 2013	245	60, 11		**hepatic steatosis (60)**
		0.39	µM	AC50	ATG_PXRE_CI	ToxCast, 2017				
		0.35	µM	AC50	ATG_PXR_TRA	ToxCast, 2017				
	Hepatotoxicity	2.3		LEL	zebrafish	(Liu et al., 2016)	1291, 270, 139, 902, 1393	209, 32, 41, **220**, **144**	long term activation AhR (41) ; **Act. Cyp2E1 in liver (220)** ; Disruption Lysosome (144) ; Pro-mutagenic DNA adduct (46)	
		45	µg/g		chicken	embryo, Farhat et al., 2014				
		62.5	mg/kg/d	LEL	Mouse	WHO EHC209, 1998 ; US-EPA, 2015				
		500	ng/µl		Quail	hepatic sinusoidal dilatation, Jacobsen et al., 2017				
		200	mg/kg/d	LEL	Rat	WHO EHC209, 1998				
		80	mg/kg/d	LEL	Rat	WHO EHC209, 1998				
	Kidney toxicity	20	mg/kg/d	LEL	Rat	Freudenthal and Henrich, 2000 ; US-EPA, 2015	814, 767, 422, 784	**128**, 105, **53**, 33, **116**	↗ Binding to 2u (105) ; Act. 5HT2c (33)	
		200	mg/kg/d	LEL	Rat	WHO EHC209, 1998				
		5	mg/kg/d		Rat	hyperplasia convoluted tubules, WHO EHC209, 1998				
		80	mg/kg/d		Rat	chronic nephropathy, WHO RHC209, 1998				
	oxydative stress	30	µM	LEL	PC12	Li et al., 2017	209, 1088, 1392, 210, 211	27, **108**, **144**, 149, 171, 138, 177, 186, **200**, **220**, **17**, 31	Inh. Bile Salt Export Pump (27) ; Inh. PDK (108) ; Disruption Lysosome (144) ; **Act. Cyp2E1 (220)** ...	Cholestasis (27) ; heptaocellular adenomas (108) ; Liver inflamation (144) ; Mortality (138, 177, 186) ; **Breast Cancer (200) ; Liver Cancer (220)** ...
		100	µM	LEL	E.Coli	Krivoshiev et al., 2015				
		0.23	µM	LEL	zebrafish	Wang et al., 2015c				

4.3 Linking Triphenyl Phosphate (TPhP) to AOPs

Objectives

The present document supports WP13, Task 13.1 of HBM4EU project by summarising existing information on toxicity of TPhP (specific compound from a priority group of Flame Retardants) with the aim to define corresponding Adverse Outcome Pathway(s) (AOP(s)). The information collected is organised in a way, which allows establishment of links between reported biological effects with individual formalised "Key Events" currently present in AOPWiki.

Approach

Following the first step of literature search (see " FR lit search – Procedure"), we focused on adverse outcome pathways (AOPs) linked to the apical endpoints for which TPhP appeared to be highly toxic, that is : reproduction (Liu et al., 2013a; Meeker and Stapleton, 2010), thyroid hormones (Kim et al., 2015; Liu et al., 2016), hepatotoxicity (Baker et al., 2015; Chen et al., 2015a; Du et al., 2016), teratogenicity (Behl et al., 2015; Du et al., 2015; Isales et al., 2015; Kim et al., 2015; McGee et al., 2013; Sun et al., 2016) and adipogenicity (Morris et al., 2014).

We collected information from both *in vivo* and *in vitro* original research papers and US-EPA report (Baker et al., 2015), and we sorted it according to the biological effect (eg, "estrogenic activity", "Decrease of androgens", "Pericardial edema"). For each biological effect, we indicated the supporting evidence that it is affected by TPhP (see Table 1).

We then searched within the AOP wiki (https://aopwiki.org/) to which key events (KEs) those biological effects correspond and then to which AOPs those KEs belong. We also indicated the corresponding molecular initiating event (MIE) and/or adverse outcome (AO), with the aim of identifying predictive adverse outcomes or potential molecular targets.

All this information and links between reported effects and corresponding AOP-Wiki KEs is collected and organised in the "AOPs-TPhP.xlsx" file (Table1).

Results and comments

1. **Considering the reproductive endpoint,**

11 original research papers provide supporting evidence that TPhP provokes biological effects associated with reproductive toxicity. Most of the information comes from *in vitro* studies on cell culture (Chen et al., 2015b; Hu et al., 2017; Kojima et al., 2013; Krivoshiev et al., 2016; Suzuki et al., 2013; Zhang et al., 2014) or *in vivo* studies in zebrafish (Liu et al., 2012; Liu et al., 2013a; Liu et al., 2016). Studies in rodents and rabbit concluded that TPhP has rather low (Baker et al., 2015) or moderate (Chen et al., 2015a) toxicity regarding the reproductive endpoint, but one study in human shows association between TPhP exposure and decrease of semen quality (Meeker and Stapleton, 2010). In addition, *in vitro* studies that have tested several organophosphorus flame retardants in parallel found that TPhP has the highest estrogenic activity (Kojima et al., 2013; Suzuki et al., 2013; Zhang et al., 2014), greater than the tested BDEs (Suzuki et al., 2013).

We could identify 36 AOPs for which there is evidence that TPhP affects at least one KE and there are 6 AOPs for which TPhP has been linked to 3 or more KEs (see Table1):

AOP 18: PPARα activation in utero leading to impaired fertility in males

KEs affected :

KE 266 : Decrease, Steroidogenic acute regulatory protein (STAR)
KE 446 : Reduction, testosterone levels
KE 348 : Malformation, male reproductive tract (AO)

AOP 29: Estrogen receptor agonism leading to reproductive dysfunction

KEs affected :

KE 111 : Agonism, estrogen receptor (MIE)
KE 307 : Increase, Vitellogenin synthesis in liver
KE 220 : Increase, Plasma vitellogenin concentrations
KE 78 : Reduction, cumulative fecundity and spawning
KE 339 : Altered, Larval development (AO)

This AOP provides a plausible mechanism for the reproductive toxicity of TDCIPP in fishes. Because it involves regulation of levels of Vitellogenin, it applies to oviparous animals.

AOP 67 : Modulation of adult Leydig cell function subsequent to estradiol activation in fetal testis

KEs affected :

KE 658 : Decreased testosterone by the fetal Leydig cells, Increased estradiol
KE 659 : Decreased testosterone by the fetal Leydig cells, Activation by other estraidiol agonists
KE 505 : Decreased sperm quantity / quality in the adult, Decreased fertility

AOP 66: Modulation of Adult Leydig Cell Function Subsequent Glucocorticoid Activation in the Fetal Testis

KEs affected :

KE 654 : Decreased testosterone by the fetal Leydig cells, Activation by other glucocorticoid receptor agonists (MIE)
KE 505 : Decreased sperm quantity / quality in the adult, Decreased fertility (AO)

AOP 64: Glucocorticoid Receptor (GR) Mediated Adult Leydig Cell Dysfunction Leading to Decreased Male Fertility

KEs affected :

KE 494 : Glucocorticoid Receptor Agonist, Activation (MIE)
KE 446 : Reduction, testosterone level
KE 520 : Decreased sperm quantity or quality in the adult, Decreased fertility

AOP 71: Modulation of Adult Leydig Cell Function Subsequent to Glucocorticoid Activation

KE 525: Apoptosis of adult Leydig cells, Decreased testosterone by adult Leydig cells
KE 651: Glucocorticoid Receptor mediated alterations in steriodogenic enzymes, Decreased testosterone by adult Leydig cells
KE 520 : Decreased sperm quantity or quality in the adult, Decreased fertility (AO)

2. Considering the other toxicological endpoints,

We could identify another 40 AOPs including 16 AOPs associated with changes in thyroid hormones, 5 AOPs associated with hepatotoxicity, 4 AOPs associated with developmental (cardio)toxicity and 7 AOPs associated with adipogenicity. All of these AOPs are linked to TPhP at the level of less than 3 KEs.

Additional lines of evidence may arise in future research that will further support some of these AOPs as plausible mechanisms for TPhP toxicity.

Conclusion and perspective

1. AOPs/mechanisms for reproductive toxicity of TPhP

TPhP provokes biological effects that are linking it to many AOPs for the reproduction endpoint. Some of these AOPs are potentially affected by TPhP at the level of 3 or more key events and could therefore provide mechanisms for TPhP-induced reproductive toxicity.

- AOP 29 is a plausible mode of action for reproductive toxicity of TPhP observed in fish, involving agonism of estrogen receptor and increase of vitellogenin.
- AOPs 64, 66, 67 & 71 provide related mechanisms for the decrease in semen quantity/quality associated with TPhP exposure in men (Meeker and Stapleton, 2010). All AOPs involve reduced level of testosterone produced by Leydig cells in rats. Decrease in testosterone levels has indeed been reported following TPhP exposure in mouse, in TM3 Leydig cells and in zebrafish (Chen et al., 2015a; Chen et al., 2015b; Liu et al., 2012; Liu et al., 2013a; Liu et al., 2016).
- AOP 18 leads to defects in the male reproductive tract, through activation of PPARα, decrease in STAR protein and decrease in testosterone levels. Data available suggests that TPhP is only moderately toxic in the mouse for two of these KEs (decrease in STAR levels and defects in the male reproductive tract), with a LOAEL of 300mg/kg/d and a NOAEL of 100 mg/KG/d (Chen et al., 2015a; Chen et al., 2015b). Furthermore, one study has tested agonistic activity of TPhP on PPARα (MIE for this AOP) using transactivation assay on CHO cells and found only very weak effect (15% activation at 30µM) (Kojima et al., 2013).

2. Indication for potential molecular targets

The outcomes of this literature research on AOPs could help directing future research for the molecular target of TPhP.

- Several AOPs link decrease in Testosterone and decrease in sperm quantity/quality with an increase in Glucocorticoid receptor activity (AOPs 64, 71 and 66). It might therefore constitute one possible molecular target. However, evidence that TDCIPP activates GR is weak (Liu et al., 2013b).
- Both hepatotoxicty and developmental cardiotoxicity, which are observed following sub-acute or acute treatment with TPhP in mouse and fish (Baker et al., 2015; Du et al., 2015; Du et al., 2016; Isales et al., 2015; Kim et al., 2015; McGee et al., 2013; Morris et al., 2014; Sun et al., 2016), are linked through AOPs 21, 41 and 150 to the activation of the Aryl hydrocarbon receptor (AhR). This could therefore also consitute another molecular target, although one study in zebrafish strongly suggests that TPhP-induced cardiotoxicity is not mediate through AhR (McGee et al., 2013).
- Carboxylesterases Ces1f, Ces1e, Ces1c, Ces2a and Ces1e have been described as potential molecular targets with IC50 ranging from 5 to 2300 nM (Morris et al., 2014). Authors associate Ces1 inhibition to hypertrigliceridemia, consistent with the phenotype of obesity, hepatic steatosis and hyperlipidemia observed in Ces1g mutant mice (Quiroga et al., 2012). Carboxylesterases may also play an important role in tumor cell killing/surveillance and is a major player in drug metabolism,making some drugs rather ineffective while increasing toxicity of others (Markey, 2011). Carboxylesterases are unfortunately not linked to any AOPs so far. Decreased hepatic carboxylases activity has been observed after exposure to FM550 (1mg/kg/d) - a mixture that contains TPhP – supporting the link between TPhP and carboxylases (Patisaul et al., 2013). TPhP is also a

very potent inhibitor of human monocyte carboxylesterase (hCES1?) with a Ki of 8nM (Saboori et al., 1991).

3. Indications for predictive adverse outcome

Our literature search clearly links TPhP to AOPs leading to advserse outcomes that have not been observed after TPhP exposure so far, but that might be of concern.

Those adverse outcomes include endometrial carcinomas and breast cancer as possible outcomes of the estrogenic activity (AOPs 167, 112 and 200), and liver cancer as an outcome of hepatotoxicity (AOP 220). In particular, the AOPs 200 and 220 leading to breast cancer and liver cancer also involve an increase in oxydative stress that has been observed following TPhP exposure *in vitro* (An et al., 2016; Chen et al., 2015b; Hendriks et al., 2014; Krivoshiev et al., 2015; Schang et al., 2016) and in mouse (100 mg/kg/day oral exposure for 35 days) (Chen et al., 2015a).

Liver steatosis and obesity are another two expected outcomes of the adopogenesis/lipogenesis that have been observed after TPhP exposure *in vitro* and in mouse (Cano-Sancho et al., 2017; Morris et al., 2014; Pillai et al., 2014; Tung et al., 2017a; Tung et al., 2017b).

Abbreviations:

AO, Adverse Outcome
AOP, Adverse Outcome Pathway
FR, Flame Retardant
KE, Key event
MIE, Molecular Initiating Event
TPhP, Triphenyl phosphate CAS 115-86-6

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Table 1:

Molecular Initiating event (MIE) highlighted in green
Adverse Outcome (AO) highlighted in red
AOPs for which the Stressor interacts at the level of **2 or more KEs are indicated in bold.**
AOPS for which the Stressor interacts at the level of **3 or more KEs are indicated using higher case.**

	Biological effect	Supporting evidence					Key Event (KE)	AOPs	Corresponding MIE (when absent from list of biological effects)	Corresponding AO (when absent from list of bological effects)
		eff. Dose/ conc.	units		biological system	reference				
Reproductive toxicity	estrogenic activity	0.27	µM	REC20	CHO-K1 Dual-luc	(Luc reporter ERα, Zhang et al, 2014)	1181, 1064, 111, 1065	167, **29**,52,**53**,**200**,112	↗ dopaminergic activity (112)	Endometrial carcinomas (167, 112) ; Breast cancer (200)
		0.65	µM	REC20	Yeast 2 hyb	(2 Hyb ERα, Zhang et al, 2014)				
		4.9	µM	REC20	CHO-K1	(Luc reporter ERα, Kojima et al, 2013)				
		6.5	µM	REC20	CHO-K1	(Luc reporter ERβ, Kojima et al, 2013)				
		3.3	µM	EC20	CALUX Assays	(Erα CALUX, Suzuki 2013)				
		88	µM	EC20	MCF-7 RPE	(RPE of MCF-7 cells, Krivoshiev et al, 2016)				
		6	µM	LEL	zebrafish	ER2b mRNA, Liu C et al, 2013				
	anti-estrogenic activity	ND		IC20		(RIE of MCF-7 cells, Krivoshiev et al, 2016)	112, 1046	**30**, 165		
		0.003	µM	LEL	1	(Inh. of E2-Luc reporter, Liu et al, 2012)				
		ND		RIC20		(Luc reporter ERα, Zhang et al, 2014)				
		ND		RIC20		(2 Hyb ERα, Zhang et al, 2014)				
		NC		RIC20		(Erα CALUX, Suzuki 2013)				
	Anti-androgenic activity	17	µM	RIC20	2	(Luc reporter AR, Kojima et al, 2013)	742, 27	111, 19		Leydig cell tumors (111) ; Feminisation/incomplete dvpt of male sex organs (19)
		5.8	µM	IC50	3	(AR CALUX, Suzuki 2013)				
	Antagonistic effect Progesterone receptor	1.9	µM	IC50	3	(PR CALUX, Suzuki 2013)				
	Increase Progesterone	10	µM	>		Schang et al, 2016				
		20	µM	LEL		Hu et al, 2017				
	Glucocorticoid receptor	>10	µM			Suzuki et al, 2013	122, 494, 1396, 651, 654	14, 214, **64, 71**, 221, 222, **66,**		Increased disease susceptibility (14), seizure/hypertension (214), depression (214, 221), agitation (214, 222)
		15	µM	RIC20		Inh., Kojima et al, 2013				
		0.06	µM	ONLY	zebrafish	Inc. mRNA, Liu, C et al, 2013				
	PPARα activation	>10	µM			Kojima et al, 2013	227	18, 51, 61, 37, 213	Act. NRF2, NR1H4 (61)	Impaired fertility (18, 51), **Inc., liver steatosis (61)**, **hepatocellular adenomas/carcinomas (37)**
		>10	µM			Honakoski et al, 2004				
		6	µM	LEL	zebrafish	mRNA, Liu, C et al, 2013				
	Increase of Vitellogenin	0.12	µM	LEL	zebrafish	*vtg* mRNA in males, Liu et al 2012	220, 307, 419, 418	**29, 53**		
		3	µM	LEL	zebrafish	plasma VTG in males, Liu X et al, 2013				
		0.61	µM	LEL	zebrafish	plasma VTG in females, Liu X et al, 2013				
	Increase CYP19a levels	3	µM	LEL	zebrafish	*cyp19a* mRNA, Liu et al, 2012	(decrease only)			
		3	µM	LEL	3	*cyp19a* mRNA, Liu et al, 2012				
		1.53	µM	LEL	zebrafish	female, *cyp19a* mRNA, Liu et al, 2016				
	Increase STAR levels	0.15	µM	LEL	zebrafish	female, *star* mRNA, Liu et al, 2016	(decrease only)			
		184	µM	LEL	TM3 Leydig cell	*star* mRNA, Chen et al., 2015a				
	Decrease STAR levels	300	mg/kg	LOAEL	Mouse	*star* mRNA, Chen et al., 2015b	266	**18**		
	Increase of 17beta-estradiol (E2)	3	µM	LEL	5	(H295R, Liu et al, 2012)	658	**67**		
		3	µM	LEL	zebrafish	Liu et al, 2012				
		3	µM	LEL	zebrafish	female, Liu X et al, 2013				
		0.61	µM	ONLY	zebrafish	male, Liu X et al, 2013				
		0.015	µM	LEL	zebrafish	female, Liu X et al, 2016				
		0.015	µM	ONLY	zebrafish	male, Liu X et al, 2016				
	Decrease of androgens (Testosterone (T) or 11-Ketotestosterone (11-KT))	3	µM	LEL	zebrafish	11-KT, males, Liu et al, 2012	446, 413, 495, 525, 645, 658, 808, 274	51, **18**, **64**, **66**, **71**, 69, **67**, **124**, **23**	Act of PPARα (18, 51) ; Inh of HMG-CoA reductase (124) ; ↗GR activity (64, 71, 66) ; ↘Cholesterol biosynthesis (69) ; Agonism androgen receptor (23)	
		3	µM	LEL	zebrafish	T, males, Liu et al, 2012				
		3	µM	LEL	zebrafish	T, female, Liu X et al, 2013				
		3	µM	>	zebrafish	T, male, Liu X et al, 2013				
		3	µM	LEL	zebrafish	11-KT, female, Liu X et al, 2013				
		3	µM	>	zebrafish	11-KT, male, Liu X et al, 2013				
		1.53	µM	LEL	zebrafish	11-KT, female, Liu X et al, 2016				
		0.015	µM	LEL	zebrafish	11-KT, male, Liu X et al, 2016				
		184	µM	LEL	TM3 Leydig cell	T, Chen et al., 2015a				
		300	mg/kg	LOAEL	Mouse	Chen et al., 2015b				
	Increase of Testosterone (T)	3	µM	LEL	5	(H295R, Liu et al, 2012)	N			
	Increase of E2/T or E2/11-KT	0.3	µM	LEL	3	(H295R, Liu et al, 2012)	N			
		3	µM	LEL	zebrafish	E2/11-KT, females Liu et al, 2012				
		3	µM	LEL	zebrafish	E2/T, males Liu et al, 2012				
		3	µM	LEL	zebrafish	E2/11-KT males, Liu et al, 2012				
		0.61	µM	LEL	zebrafish	E2/T, female, Liu X et al, 2013				
		0.12	µM	LEL-only	zebrafish	E2/T male, Liu X et al, 2013				
		3	µM	LEL	zebrafish	E2/11-KT, female, Liu X et al, 2013				
		0.61	µM	ONLY	zebrafish	E2/11-KT, male, Liu X et al, 2013				
		1.53	µM	LEL	zebrafish	E2/11-KT, female, Liu X et al, 2016				
		0.015	µM	LEL	zebrafish	E2/11-KT, male, Liu X et al, 2016				
	Increased prolactin	Human				(Meeker and Stapelton, 2010)	1084, 1077	170, 168	↘ Dopaminergic activity (170) ; ↘ GnRH release in hypothalamus (168)	Mammary adenomas/carcinomas (170, 168)
	Malformation male reproductive tract	300	mg/kg	LOAEL	Mouse	seminiferous tubules number, Chen et al., 2015b	348, 330, 809	**18**, **124**, **29?**		
	Decrease sperm quantity	Human				(Meeker and Stapelton, 2010)	543, 497, 505, 520	74, **64**, **66**, **67**,68, **69**, 70, **71**	↗GR activity (64, 71, 66) ; ↘Cholesterol biosynthesis (69) ; hypermethylation in the fetal testis (74) ; proteomic alteration in fetal testis/leydig cells (68, 70)	
	Reduction, Cumulative fecundity and spawning	0.61	µM	LEL	zebrafish	Liu X et al, 2013	78, 330, 972	**29**, 25, **23**, 30, 122, 123, 216, 238, 153	Inh Aromatase (25, 153) ; Agonism androgen receptor (23) ; Inh prolyl hysdroxylase (122) ;	
	Decreased, impaired fertility	690	mg/kg/d	>	Rat	oral, US-EPA 2015	406, 330	7, 51, **18**, **124**, 216, 238		
		1000	mg/kg/d	>	Rabbit	dermal, US-EPA 2015				
Thyroid	Decrease plasma levels of T4	1.5	µM	LEL	zebrafish	female, Liu et al, 2016	277, 771, 281, 426, 1093	110, 119, 42, 65, 128, 134, 54, 159, 175, 176, 188, 192, 193, 8, 152, 194	Inh. Thyroperoxidase (119, 42, 159, 175) ; Inh Na+/I+ symporter NIS (65, 134, 54, 176) ; Act. PXR, NR1/2 (8) ...	follecular cell adenomas/carcinomas (110, 119) ; Cognitive function (42, 65, 134, 152) ; Altered amphibian metamorphosis (175, 176, 188, 192, 193, 194) ; Loss cochlear function (8) ...
	Increase T3 and T4	0.12	µM	LEL	zebrafish	Kim et al., 2015	959	152		
Adipogenicity	adipogenesis and lipogenesis	50	mg/kg/d	LEL	mouse	hypertriglyceridemia, Morris et al, 2014	1449, 1029, 1306, 327, 454	**72**, **163**, **213**, 36, 57, 58, **60**	demethylation PPARγ promoter	**hepatic steatosis (36, 57, 58, 60), Obesity**
		10	µM	LEL	3T3-L1	adipogenesis, Tung et al, 2017				
		10	µM	LEL	3T3-L1	adipogenesis, Cano-Sancho et al, 2017				
		5	µM	LEL	Cos-7	lipid accumulation, Pillai et al, 2014				
		20	µM	LEL	human pre-	lipid accumulation, Tung et al., 2017b				
	PXR Activation	2.8	µM	REC20		Kojima et al, 2013	245	**60**, 11		**hepatic steatosis (60)**
		0.71	µM	AC50	ATG_PXRE_CIS_up	ToxCast, 2017				
	PPARγ Activation/binding	30	µM	15% max		Kojima et al, 2013	1028	**163**, **72**	demethylation PPARγ promoter, chronic high fat diet (72)	**obesity (72)**
		38	µM	IC50		Pilai				
		0.9	µM	LEL	zebrafish	transcriptomic PPAR signaling, Du et al, 2016				
		30	µM	LEL		Belcher et al, 2014				
		3.27	µM	EC20		Hu et al, 2017				
		40	µM	IC50		Fang et al, 2015				
		6	µM	LEL	zebrafish	mRNA, Liu, C. et al, 2013				
		4.6	µM	AC50	ATG_PPARg_TRAN	ToxCast, 2017				

Table 1 (continued):

Hepatotoxicity		Inhibition of carboxylesterases	0.005-2.3	µM	IC50	HEK293T cells	Morris et al., 2014	N			
		Hepatotoxicity	350	mg/kg/d	LOAEL	Rat	liver weight, US-EPA, 2015	902, 1291, 270, 139, 1393	209, 32, 41, **220, 144**	**Long term activation AhR ; Act. Cyp2E1 (220)**	**Liver Cancer (220)**
			0.15	µM	LEL	zebrafish	Du et al. 2016				
			>300	mg/kg/d		Mouse	Liver weight, Chen et al., 2015b				
Teratogenicity	Developmental cardiotoxicity	Heart rate	0.38	µM	LEL	Medaka	Sun et al., 2016b	317, 358	150, 21	**Activation AhR**	Increased Embryolethality, Mortality
			1.5	µM	LEL	Zebrafish	Du et al., 2015				
		in vitro				3	POD Cardiotoxicity 30min, Sirenko et al., 2017				
						4	POD Cardiotoxicity 24h, Sirenko et al., 2017				
						2	TOXPi (cardio and cyto toxicity), Sirenko et al.,				
		Pericardial edema	6.25-12.5	µM	LEL	Zebrafish	Isales et al., 2015				
			1.5	µM	LEL	Zebrafish	Kim et al., 2015				
			1	µM	LEL	Zebrafish	McGee et al., 2013				
			2	µM	EC50	Zebrafish	Du et al., 2015				
		LV thickness in male	1	mg/kg/d	LOAEL	rat	PM550! US-EPA, 2015				
			690	mg/kg/d	>	?	US-EPA, 2015	1001, 339	43, **29**	Inh VEGFR2	
			2	µM	POD	zebrafish	Behl et al., 2015				
			1.9	µM	LEL	Medaka	Sun et al., 2016b				
			10000	mg/kg/d	>	Rat	Welsh et al., 1987				
other toxicities		Reduction in body weight	161	mg/kg/d	LOAEL	Rat	US-EPA, 2015	864	6	Antagonist PPARα	
			345	mg/kg/d	LOAEL	Rat	US-EPA, 2015				
			300	mg/kg	LOAEL	Mouse	Chen et al., 2015b				
			1	%	LEL	Rat	Hinton et al., 1987				
		oxydative stress	100	mg/kg	LOAEL	Mouse	antioxydant enzymes, Chen et al., 2015b	209, 1088, 1392, 210, 211	27, 108, **144**, 149, 171, 138, 177, 186, **200, 220**, 17, 31	Inh. Bile Salt Export Pump (27) ; Inh. PDK (108) ; Disruption Lysosome (144) ; **Act. Cyp2E1 (220)** ...	Cholestasis (27) ; **heptaocellular adenomas (108)** ; Mortality (138, 177, 186) ; **Breast Cancer (200) ; Liver Cancer (220)** ...
			50	µM	LEL	A549	An et al., 2017				
			1	µM	LEL	B35	Hendriks et al., 2014				
			75	µM	LEL	E. Coli	Krivoshiev et al., 2015				
			10	µM	LEL	MA-10	Schang et al., 2016				
			46	µM	LEL	TM3 Leydig cell	Chen et al., 2015a				

4.4 Linking Polyaromatic Hydrocarbons (PAHs) to AOPs

4.4.1 Objectives

The present document supports WP13, Task 13.1 of HBM4EU project by summarising existing information on toxicity of PAHs (polyaromatic hydrocarbons) with the aim to define corresponding Adverse Outcome Pathway(s) (AOP(s)). The information collected is organised in a way, which allows establishment of links between reported biological effects with individual formalised "Key Events" currently present in AOPWiki.

4.4.2 Toxicity of PAHs (EFSA report from 2008 and update with recent publications)

There is already a large amount of information on PAHs toxicity including several *in vivo* papers, and a respective number of papers in mammals). We therefore decided to summarize the conclusions from the ATSDR (1995) and the EFSA report (2008) and update those conclusions with more recent relevant information.

Overall conclusions:

Based on the previous studies, PAHs are potentially genotoxic and carcinogenic to humans. Concerning the other toxicological endpoints, the reports concludes on either:

- indications of endocrine disruption
- indications of immunotoxicity
- limited information on the reproductive toxicity of individual PAHs

Additional evidence is expected to be provided by recent studies.

Thyroid hormone homeostasis

Polycyclic aromatic hydrocarbons are regarded as endocrine disruptors, which can lead to thyroid hormone homeostasis perturbations. Since endocrine disruptors can mimic or antagonise thyroid hormones, they could potentially produce permanent neurodevelopmental disorders (Mastorakos et al. 2007). Several studies have indicate that exposure to PAHs is positively correlated with the following six thyroid variables: TT3, FT3, TT4, FT4, TSH, and TGN (Kelishadi et al. 2017; Mastorakos et al. 2007). The findings of a most recent study indicate that there are conflicting effects of PAHs on TPO activities. Although there is no known mechanism to explain why such effects occur, the authors concluded that certain PAHs, notably pyrene, benzo(k)fluoranthene, and benzo(e)pyrene, inhibited TPO activity, which is a key enzyme, upregulated in expression by thyroid stimulating hormone (TSH) and responsible for iodination of tyrosine to form mono, di-, triiodothyronine (T3), and tetraiodothyronine (T4). The *in vivo* significance of these changes has not been determined (Song et al. 2012).

Adipogenicity and obesity

Polycyclic aromatic hydrocarbons (PAHs) have been, also, associated with childhood obesity in epidemiological studies (Scinicariello and Buser 2014; Yan et al. 2014). However, the underlying mechanisms are unclear. According to Yan et. Al, offspring of dams exposed to greater PAH during gestation had increased weight, fat mass, as well as higher gene expression of PPAR γ, C/EBP α, Cox2, FAS and adiponectin and lower DNA methylation of PPARγ. Similar differences in phenotype and DNA methylation extended through the grand-offspring mice (Yan et al. 2014).

Moreover, developmental exposure in rats to PAHs in diesel exhaust have been shown to lead to increased obesity, insulin resistance and inflammation; these effects were observed only in males fed a high fat diet, indicating a sexually dimorphic effect (Bolton et al. 2014; Strakovsky et al. 2015). Specific exposure to benzo (a) pyrene during development also resulted in increased visceral adipose tissue weight in female offspring (Ortiz et al. 2013). There are limited human data on the

association of childhood obesity with maternal exposure to ambient PAHs, however a study by Rundle and colleagues (Rundle et al. 2012) shows children born to mothers with the highest PAH exposures during pregnancy had higher body weights both at 5 and 7 years of age. The extensive exposure of populations to air pollution necessitates a further examination of its overall effects and its specific contribution to increased risk of obesity.

Mutagenicity

In a recent study, human alveolar epithelial type II cells (A549) have been treated with nonmutagenic PAH pyrene and with the mutagenic PAHs benzo-[a]-pyrene, 1-nitropyrene, or 1,8-dinitropyrene, in order, to evaluate the mutagenicity of PAHs and constituents of environmental pollutants in lung tissue, including metabolic activation. Comparison of genome-wide microarray expression profiles between a nonmutagenic and a mutagenic PAH-treated group revealed that xenobiotic response genes such as CYP1B1 were commonly upregulated in two groups and that DNA damage induced genes, especially p53-downstream genes such as p21 (CDKN1A). Pretreatment with cytochrome P450 inhibitor α-naphthoflavone or p53 inhibitor pifithrin-α inhibited the benzo-[a]-pyrene-induced p21 expression. These data suggest that when PAHs enter the cells, lung epithelium induces PAH metabolic activating enzymes, and then the DNA damages-recognition signal is converged with p53 downstream genes (Hirano et al. 2013).

Carcinogenicity

The best known PAH compound is benzo[a]pyrene (B[a]P), which in 2012 was classified among the highly genotoxic compounds, since there are several animal studies that confirm carcinogenic properties of B[a]P. According to the International Agency for Research on Cancer (IARC) it belongs to group 1—carcinogenic to humans (IARC 2012). Moreover, the products containing B[a]P and other PAHs (tobacco smoke, indoor emissions from household combustion of coal, diesel exhaust fumes, outdoor air pollution, and particulate matter) are also classified to group 1 (IARC 2016). Also, the EPA has classified the following seven PAH compounds as being probable human carcinogens: benz(a)anthracene, BaP, benzo(b)fluoranthene, benzo(k)fluoranthene, chrysene, dibenz(ah)anthracene, and indeno(1,2,3-cd)pyrene. Recently studies suggest that low molecular weight (LMW) polycyclic aromatic hydrocarbons (PAH) in combination with B[a]P can elicit increased carcinogenic potential. Future studies will further address the mechanisms of co-carcinogenesis driving these responses (Schwerdtle, et al., 2010).

The carcinogenicity of PAHs administered by dermal, subcutaneous, inhalation or oral routes has been assessed in a large number of studies. In most studies, the site of tumour development was related to the route of administration, e.g. gastric tumours after oral administration, skin tumours after dermal application. However, tumours at sites other than the site of application were also observed. For the current opinion data on the carcinogenicity of PAHs following oral administration are most relevant. Several studies on individual PAHs have been evaluated earlier by SCF (EC, 2002) and the JECFA (FAO/WHO, 2006). Benzo[a]pyrene, when administered by the oral route, has been reported to produce tumours of the gastrointestinal tract, liver, lungs and mammary glands of mice and rats. None of the other priority PAHs have been tested for carcinogenicity after oral administration.

Exposure to B[a]P and/or its mixture causes immunotoxic, teratogenic effects, and induces apoptosis and cell proliferation, as well as increased DNA methylation. In humans, occupational exposure to mixtures containing benzo[a]pyrene was associated with different kinds of cancer: 1. lung cancer (coke production, soot, paving and roofing), 2. lung and bladder (coal gasification and aluminum smelting), 3. skin (coal-tar distillation), and 4. lung, lip, oral cavity, pharynx, oesophagus, larynx, and bladder (tobacco smoking) (IARC 2010, 2012). However, it is not clear from these studies whether exposure to PAHs was the main cause, as these workers had been simultaneously exposed to other cancer-causing agents (*e.g.*, aromatic amines) (Ewa and Danuta 2017).

Although unmetabolized PAHs can have toxic effects, a major concern is the ability of their reactive metabolites, such as epoxides and dihydrodiols, to bind to cellular proteins and DNA. Studies examining the carcinogenicity of B[a]P have identified the 7,8-oxide B[a]P and 7,8-dihydrodiol B[a]P as proximate carcinogens, and the 7,8-diol-9,10-epoxide B[a]P (BPDE) as a strong mutagen and ultimate carcinogen (Ewa and Danuta 2017). The most common mechanisms of metabolic activation of PAHs, such as B[a]P, are involving and generating a large number of metabolites due to the activity of phase I (activation) and phase II (detoxification) enzymes. In the phase I oxidation, reactions catalysed by cytochrome P450 enzymes (CYPs: 1A1, 1A2, 1B1, 3A4) and hydroxylation by epoxide hydrolase occur. CYP1A1 or CYP1B1 are highly inducible by the exposure to PAHs via the aryl hydrocarbon receptor (AhR). The AhR is present in the cytoplasm as a complex with other proteins such as heat shock protein 90 (Hsp90), p23, and AhR-interacting protein. Having formed a complex with PAHs, the Hsp90 is released and an AhR–PAH complex is translocated to the nucleus. There, the AhR–PAH complex creates a heterodimer with an ARNT (AhR nuclear translocator), and afterwards binds to DNA via the xenobiotic response element (XRE) situated in the promoter region of CYP1A and CYP1B genes. Therefore, the AhR plays an important role in the tumorigenesis mediated by PAHs, which has been illustrated previously (Arenas-Huertero F 2011; Shimada 2006).

BaP is the most common PAH to cause cancer in animals, and this compound is notable for being the first chemical carcinogen to have been discovered. Liamin et. al (Liamin et al. 2017) characterised the genotoxic response of primary activated T lymphocytes to B[a]P. They demonstrated that, following T lymphocyte activation, B[a]P treatment triggers a marked increase in CYP1 expression and activity generating, upon metabolic activation, DNA adducts and double-strand breaks (DSBs) after a 48-h treatment. At this time point, B[a]P also induces a DNA damage response with ataxia telangiectasia mutated kinase activation, thus producing a p53-dependent response and T lymphocyte survival. B[a]P activates DSB repair by mobilising homologous recombination machinery but also induces gene mutations in activated human T lymphocytes which could consequently drive a cancer process.

Reproductive toxicity

There is limited information on the reproductive toxicity of individual PAHs. For benzo[a]pyrene no effect on reproductive capacity was found in a one-generation study in mice receiving diets containing doses of up to 133 mg/kg b.w. per day. However, impaired fertility was seen in the offspring of female mice given benzo[a]pyrene by gavage at doses >10mg/kg b.w. per day. Developmental toxicity of benzo[a]pyrene was observed in mice of a susceptible genotype following administration of 120 mg benzo[a]pyrene/kg b.w. per day via the diet. A NOAEL for reproductive and developmental effects via the oral route has not been established (FAO/WHO, 2005).

Reproduction toxicity has been noted by the dermal and parenteral exposure routes. Inhalation exposure of rats to B[*a*]P during pregnancy decreased plasma estrogen, progesterone and prolactin levels in dams in association with decreased pup survival and development, thus partly explaining the effects (Archibong et al. 2002). B[*a*]P exposure before conception caused Ah-receptor-dependent fetal intrauterine growth restriction in mice, which was associated with altered vasculature in the placenta and a decreased placental cell death rate (Detmar et al. 2008). Maternally toxic doses of B[*a*]P and 7,12-dimethylbenz[*a*]anthracene caused necrosis in the placenta and haemorrhages in fetuses in rats (Sanyal and Li 2007), suggesting that the vascular system in general is one target of PAHs. In male rats, B[*a*]P reduced testis weight decreased the testosterone level in the blood and sperm motility (Inyang et al. 2003; Ramesh et al. 2008), probably contributing to reproduction toxicity.

Genotoxicity

Genotoxicity plays an important role in the carcinogenicity process and maybe in some forms of developmental toxicity as well (Schwerdtle et al. 2010). Based on the available information the JECFA concluded that 15 individual PAHs are clearly genotoxic, both in vitro and in vivo (FAO/WHO, 2006). These genotoxic PAHs are benz[a]anthracene, benzo[a]pyrene, benzo[b]fluoranthene, benzo[ghi]perylene, benzo[j]fluoranthene, benzo[k]fluoranthene, chrysene, cyclopenta[cd]pyrene, dibenz[a,h]anthracene, dibenzo[a,e]pyrene, dibenzo[a,h]pyrene, dibenzo[a,i]-pyrene, dibenzo[a,l]pyrene, indeno[1,2,3-cd]pyrene and 5-methylchrysene.

B[a]P and other PAHs have been investigated for genotoxic effects in numerous systems, for example in both rodents and *in vitro* tests using in cultured human cells (A549, VH10hTert). They produced a large variety of different types of mutations (e.g. base substitutions, frameshifts, selective DNA amplifications as well as structural and numerical chromosomal aberrations) and other genotoxic effects (e.g. UDS, SCE). They were also found to be active in diverse target cells ranging from bacteria and cultured eukaryotic cells to somatic and germinal cells in laboratory animals.

A high number of different PAH metabolites demonstrated genotoxic effects in one or another system. An important observation was the binding of the active metabolites of PAHs to DNA, predominantly to amino groups of guanine and adenine. The major stable adduct is formed at the N2 position of desoxyguanosine. DNA adduct formation is generally regarded as one of the first steps in carcinogenicity of the mutagenic PAHs. However, there is a poor quantitative relationship between levels of tissue adduct and tumour formation. Bay- and fjord-region diol-epoxides are electrophilic PAH metabolites that are highly genotoxic in numerous test systems, as they readily permeate cell membranes and are relatively resistant towards detoxifying enzymes. They appear to play an important role in the in vivo genotoxicity of various PAHs, including the prototypic compound B[a]P. Some benzylic alcohol derivatives show strong genotoxicity in SULT-expressing cells in culture and can form exceptionally high levels of DNA adducts in animal models under certain conditions. (Luch 2005).

Teratogenicity

Yuan et al. (Yuan et al. 2013) reported the association between the low levels of placental PAH-DNA adducts with an increased risk of neural tube defects, especially when a low adduct level was coupled with a high placental PAH concentration. During PAH metabolism, enzymatic activity can result in the formation of reactive intermediates that can form covalent bonds with DNA (Rice and Baker 2007). DNA adducts have been demonstrated to result in a spectrum of cellular mutations that may be teratogenic (Wells et al. 2010). Embryotoxic effects of PAHs have been described in experimental animals exposed to PAHs such as benzo(a)anthracene, BaP, and naphthalene. Laboratory studies conducted on mice have demonstrated that ingestion of high levels of BaP during pregnancy results in birth defects and a decreased body weight in the offspring (Ng et al. 2009). It is not known whether these effects can occur in humans. However, the Center for Children's Environmental Health reports studies demonstrate that exposure to PAH pollution during pregnancy is related to adverse birth outcomes including low birth weight, premature delivery, and heart malformations. High prenatal exposure to PAH is also associated with a lower IQ at age three, increased behavioral problems at ages six and eight, and childhood asthma (Perera et al. 2012).

Lupo et al. (Lupo et al. 2012) indicated an association between occupational exposure to PAHs among mothers who are over 20 years and occurrence of gastroschisis. Langlois et al. (2013) found a statistically significant relationship between maternal occupational exposure to PAHs for cleftlip with or without cleft palate (P_{trend}= 0.02). According to experimental model systems, exposure to PAHs was expected to result in congenital heart defects (CHDs). However, in a case–control study, in significant associations were observed between estimated maternal occupational exposure to

PAHs and CHDs in offspring (Lupo et al. 2012). Ren et al. (Ren et al. 2011) investigated placental PAH levels in 80 fetuses or newborns with neural tube defects in China. The results of their study showed that the risk of a defect was 4 to 5 times greater, when the levels of PAHs were above the average of 597 ng g^{-1} of lipids.

Immunotoxicity

PAHs have been reported to suppress immune reactions in rodents. The precise mechanisms of PAH-induced immunotoxicity are still not clear; however, it appears that immunosuppression may be involved in the mechanisms by which PAHs induce cancer (Gao 2014).

There is some debate in the literature concerning whether AhR-triggered PAH metabolic pathway is the predominant pathway for PAH-induced immunotoxicity. In the experience of many investigators, DMBA is a weak AhR ligand (Bigelow and Nebert 1982). DMBA induces only low level expression of CYP1A1 and other AhR-dependent enzymes in the presence of AhR in the liver. Therefore, there is little or no first pass clearance occurring after the oral administration. CYP1A1 has low constitutive expression levels in the liver and spleen (Choudhary et al. 2003). In vitro studies of DMBA-induced murine splenic immunosuppression have shown that DMBA acts on lymphocytes by mechanisms largely independent of the AhR. Thus, DMBAinduced immunotoxicity is not AhR dependent in vivo.

In previous studies, has been observed that BaP and DMBA produced suppression of the PFC response in C57BL/6N WT mice, and mEH null mice were protected against BaP and DMBA-induced splenic immunotoxicity (Gao et al. 2005). These results demonstrate that mEH (EPHX1 gene) is a crucial enzyme for metabolic activation of PAH in vivo leading to immunosuppression effects. PAHs require mEH for the formation of the toxic diol-epoxide metabolite after mono-oxygenation by P450s. mEH is one of the most important pivotal enzymes for biotransformation of epoxide substrates, such as DMBA-3,4-epoxide, produced by CYP1B1 to form DMBA-3,4-dihydrodiol, resulting in carcinogenesis (Miyata et al. 1999) and immunosuppression (Miyata et al. 2001). The other example is DB[a,l]P (also known as DBC), which needs mEH to 252 12 Genotoxic Mechanisms of PAH-Induced Immunotoxicity form DB[a,l]-11,12-diol, and finally convert to DB[a,l]-11,12-diol-13,14-epoxide by P450 to produce immunotoxic effects (Shimada and Fujii-Kuriyama 2004).

It is not completely known how these kinases respond to PAH-induced DNA damage. Jun Gao and Scott W. Burchiel have, for the first time, reported involvement of ATM and ATR in PAH-induced immunotoxicity. They observed that ATM and ATR are phosphorylated following exposure of WT mice to DMBA, but not in CYP1B1 or mEH knockout mice, suggesting that metabolic activation of DMBA is important in ATM/ATR activation (Gao et al. 2008). In addition, they showed that p53 null mice are protected from DMBA-induced immunotoxicity, demonstrating that genotoxicity is a major mechanism of immunotoxicity. In response to PAH-induced DNA damages, ATM and ATR may signal differently, but we believe that they may crosstalk with each other and function through the same effector: p53. Thus, a putative mechanism of immunosuppression by DMBA involves the binding of DMBA metabolites to DNA, leading to sensing by ATM/ATR, and upregulation and activation of p53, resulting in blockage of cell cycle at checkpoints or activation of apoptotic machinery leading to cell death.

4.4.3 Search for AOPs underlying PAHs toxicity

Approach

Following the first step of literature search, we collected information from both *in vivo* and *in vitro* original research papers and EFSA reports, and we sorted it according to the biological effect (eg, “hepatotoxicity”). For each biological effect, we indicated the supporting evidence that it is affected by selected PAHs.

We then searched within the AOP wiki (https://aopwiki.org/) to which key events (KE) those biological effects correspond and then to which AOPs those KEs belong. We also indicated the corresponding molecular initiating event (MIE) that were not already described in the literature, with the aim of identifying predictive adverse outcomes or potential molecular targets.

All this information and links between reported effects and corresponding AOP-Wiki KEs is collected and organised in the “PAHs-AOPs.xlsx” file (See Table1).

We could identify 48 AOPs for which there is evidence that PAHs affects at least one KE and there are 5 AOPs for which PAHs have been linked to 3 or more KEs.

Results and comments

1. **Considering carcinogenicity**

The following AOPs concern selected PAHs or simple mixtures of the 16 EPA priority PAHs that regulate pathways of carcinogenicity.

AOP 37: PPARalpha-dependent liver cancer

KEs affected:

KE 1170: Modulation, Genes/proteins that regulate hepatocyte fate

KE 716: Increase, Mitogenic cell proliferation (hepatocytes)

KE 1171: Increase, clonal expansion / cell proliferation to form pre-neoplastic altered hepatic foci

AOP 107: Constitutive androstane receptor activation leading to hepatocellular adenomas an carcinomas in the mouse and the rat

KEs affected:

KE 716: Increase, Mitogenic cell proliferation (hepatocytes)

KE 774: Increase, Preneoplastic foci (hepatocytes)

AOP 117: Androgen receptor activation leading to hepatocellular adenomas and carcinomas (in mouse and rat)

KEs affected:

KE 716: Increase, Mitogenic cell proliferation (hepatocytes)

KE 774: Increase, Preneoplastic foci (hepatocytes)

AOP 118: Chronic cytotoxicity leading to hepatocellular adenomas and carcinomas (in mouse and rat)

KEs affected:

KE 787: Increase, Regenerative cell proliferation (hepatocytes)

KE 774: Increase, Preneoplastic foci (hepatocytes)

2. **Considering hepatotoxicity**,

AOP 220 : Chronic Cyp2E1 Activation Leading to Liver Cancer

KEs affected :

KE 1392 : Oxidative stress

KE 1393 : Hepatocytotoxicity

KE 1395 : Liver Cancer

Conclusion and perspective

1. **AOPs/mechanisms for PAHs carcinogenicity**

- AOP 37 is plausible mode of action for PAH-induced liver cancer in rodents, involving peroxisome proliferator-activated receptor alpha (PPARα) activation that results in liver cancer.
- AOP 41 provides a mechanism for PAH-induced hepatocellular carcinomas, involving Sustained AhR Activation.
- AOP 107 provides a mechanism for PAH-induced hepatocellular carcinomas, involving constitutive androstane receptor activation leading to hepatocellular adenomas and carcinomas in the mouse and the rat.
- AOP 220 provides a mechanism for PAH-induced liver cancer, involving constitutive androstane receptor activation leading to hepatocellular adenomas and carcinomas in the mouse and the rat.

2. **Indication for molecular targets**

There are 3 molecular targets for which direct evidence is supported by AOP search:

- PPARα
  PPARα may also be a potential molecular target as its activation is a MIE for hepatocellular carcinoma (AOP 37) and eventually increased proliferation (AOP 51).
- Aryl hydrocarbon receptor
  Several of the effects of PAH, such as enzyme induction, immunosuppression, teratogenicity, and tumour promotion are believed to be mediated by the sustained activation of the arylhydrocarbon receptor (AhR) and the subsequent disturbance of cellularhomeostasis. Aryl hydrocarbon receptor is a MIE that has been associated with liver cancer and hepatotoxicity (AOP 41).
- Constitutive androstane receptor

  Constitutive androstane receptor in a molecular target that has been also associated with hepatocellular and bile duct tumors
- Gap-junctional intercellular communication

A strong correlation has been found between the tumour promoting activity of a compound in the two-stage skin carcinogenesis model and its ability to inhibit gap-junctional intercellular communication (GJIC). Using an in vitro test system, it was found that different PAH inhibit GJIC. Several PAH considered to possess low carcinogenic activity belong to the most potent inhibitors of GJIC, e.g. fluoranthene, 5-methylchrysene and picenes.

3. Indications for predictive adverse outcome

In addition to the observed adverse outcomes of PAHs that have been described above, additional AO can be predicted as possible consequences of PAHs effects according to AOPs,

- Impaired fertility is an AO expected from activation of PPARα and increased proliferation (AOP 51).

Abbreviations

AO, Adverse Outcome
AOP, Adverse Outcome Pathway
PAHs, Polyaromatic Hydrocarbons
KE, Key event
MIE, Molecular Initiating Event

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Table 1:

	Biological effect			Key Event	AOPs	Corresponding MIE (when absent from list of biological effects
		species	Ref.			
Reproductive toxicity/Genotoxicit	Alteration of pregnacy related hormones	Rats	Archibong et al. 2008	1230, 1232,	37, 41, 46, 107, 117, 200, 202, 220	Androgen receptor activation leading to hepatocellular adenomas and carcinomas (in mouse and rat) (117)
	Ah-receptor-dependent fetal intrauterine growth restriction	Mice	Detmar et al. 2008	1170, 1171, 716		Activation, PPARa (227)
	Alteration of fertility endpoints	Rats	Ramesh et al. 2008	716, 774		Activation, PPARa (227)
	Influence of PAHs in cardiotoxicity	Fish	Incardona et al. 2016	1171		
CarcinogenicityCarcinogenicity	Alteration of pregnancy related hormones and fetal survival in F-	Rats	Archibong et al. 2002	853, 854	1, 32, 37, 41, 105, 107, 108, 109, 110, 111, 116, 117, 118, 119, 120, 121, 162, 163, 167, 168, 169, 170, 220	Sustained AhR Activation leading to Rodent Liver Tumours (41)
	Biological Response to Aromatic Hydrocarbons from Urban Air in Humans	Human lung tissue	Arenas-Huertero et al. 2	774, 1230, 1232		
	Benzo[a]pyrene-induced DNA damage associated with mutagenesis in primary human activated T lymphocytes	Human T-cells	Liamin et al. 2017	853, 854, 1171		
	Cancer initiation by polycyclic aromatic hydrocarbons results from formation of stable DNA adducts rather than apurinic sites.	Mice	Melendez-Colon et al. 1	716, 774, 853, 854		Sustained AhR Activation leading to Rodent Liver Tumours (41)