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Working Party on Hazard Assessment

CHEMICAL SAFETY ASSESSMENT WORKFLOW BASED ON EXPOSURE CONSIDERATIONS AND NON-ANIMAL METHODS

1st Meeting of the Working Party on Hazard Assessment
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Mr.Yuki SAKURATANI, Administrator, QSARs and Hazard Assessment
Tel: +331 45 24 98 19 - Email: Yuki.SAKURATANI@oecd.org

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This case study was developed by JRC and BIAC for illustrating practical use of IATA and submitted to the 2016 review cycle of the IATA Case Studies project. This case study was reviewed by the project team and revised to consider the comments from reviewers. The final version of the case study has been accepted by the project team to be published.

The learnings and lessons obtained from the review experience of the case study were summarized with other case studies in a considerations document [ENV/JM/HA(2017)1].

ACTION REQUIRED: ***The Working Party on Hazard Assessment is invited to discuss and endorse the document.***

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ABSTRACT

Based on the SEURAT-1 conceptual framework for safety assessment (White, Knight 2014; Daston et al 2015), a general workflow was developed in an attempt to structuring knowledge and data in a logical sequence for an integrated chemical safety assessment relying specifically on alternative methods and based on exposure considerations.

Considerations included the possible application of the Threshold of Toxicological Concern (TTC) approach and read-across assessment. Physiologically-based kinetic (PBK) modelling was applied to identify target organs and internal concentrations. *In silico* structural alerts and (quantitative) structure-activity relationship ((Q)SAR) profilers as well as *in vitro* information including high throughput assays were used to build a weight of evidence, based on an Adverse Outcome Pathway (AOP)-anchored mode-of-action hypothesis and supported by *in vitro* to *in vivo* extrapolation (IVIVE) modelling and refinement, with the aim of concluding on the safety of use, regarding repeated-dose toxicity.

Piperonyl butoxide (PBO) was selected to illustrate the case study in a hypothetical exposure scenario as a new ingredient introduced in a daily applied body lotion. PBO was chosen as it was considered relevant in the context of cosmetics and known to have hepatotoxic effects, which both were the focus of the SEURAT-1 cluster and the methods developed. The supportive alternative data (*in vitro* and *in silico*) were generated in the SEURAT-1 projects besides being obtained from ToxCast.

The case study focusing on alternative approaches highlights the challenge in integrating multiple data streams for safety assessment decisions, points out knowledge gaps and proposes a way forward.

UP FRONT – PURPOSE

The intention of this case study is not to be an assessment of a specific chemical (class) in view of regulatory acceptance, but demonstration of an exposure-based chemical safety assessment workflow without relying on animal testing. The aim of the case study was to construct a decision logic on how to bring together different elements of a risk assessment in a harmonised and logical way, with a sequential look at different levels of information, building on each other, with special emphasis on external and internal exposure and kinetics considerations.

The example of PBO is used for illustration purposes in a hypothetical scenario and is not intended to be a complete safety assessment evaluation of the chemical.

INTRODUCTION

The presented chemical safety assessment workflow was developed in and beyond the SEURAT-1 ("Safety Evaluation Ultimately Replacing Animal Testing") Research Initiative[1] which aimed at finding alternative safety assessment approaches for repeated dose toxicity (Gocht et al 2015). Within the SEURAT-1 framework of proof-of-concept case studies for applied safety assessment, the *ab initio* case study set out to identify and subsequently quantify relevant biological pathway concentration effect levels of a cosmetic ingredient for specific exposure scenarios.

Based on the SEURAT-1 conceptual framework (White, Knight 2014; Daston et al 2015), this workflow assists in structuring information ─ *in silico* knowledge and predictions alongside *in vitro* data ─ in a logical decision workflow and in providing guidance on which additional alternative data is needed to establish and then test a mode-of-action hypothesis. Considerations include the possible application of the Threshold of Toxicological Concern (TTC) approach and read-across evaluation. Physiologically-based kinetic (PBK) modelling was applied to identify target organs and internal concentrations. *In silico* structural alerts and (quantitative) structure-activity relationship ((Q)SAR) profilers as well as *in vitro* information including high throughput assays were used to build a weight of evidence, based on an Adverse Outcome Pathway (AOP)-anchored mode-of-action hypothesis and supported by *in vitro* to *in vivo* extrapolation (IVIVE) modelling and refinement, with the aim of concluding on the safety of use, regarding repeated dose toxicity.

The aim of the presented case study (Berggren et al 2017) is to bring together and transparently describe the whole process of considering different pieces of information in the sense of the Integrated Approach to Testing and Assessment (IATA) concept, i.e.

> *"an approach based on multiple information sources used for the hazard identification, hazard characterisation and/or safety assessment of chemicals, integrating and weighting all relevant existing evidence and guiding the targeted generation of new data, where required, to inform regulatory decision-making regarding potential hazard and/or risk. Within an IATA, data from various information sources (i.e. physico-chemical properties, in silico models, grouping and read-across approaches, in vitro methods, in vivo tests and human data) are evaluated and integrated to draw conclusions on the hazard and/or risk of chemicals." (OECD 2015)*

The emphasis of the chemical assessment workflow is on

- providing safety assessment for a specific use scenario
- therefore considering in particular exposure ─ external and internal concentrations, kinetics of distribution in the organism
- relying completely on alternative methods.

It goes beyond the integration of different data and results in providing a decision logic to guide through the different consecutive steps building on each other.

The safety assessment and workflow starts with Tier 0, where the exposure scenario and chemical identity are defined and existing data is collected. Exit points were identified, i.e. application of the TTC approach or a read-across assessment taking into account similar substances. When neither is considered to be applicable and sufficient to allow a decision, the assessment continues to Tiers 1 and 2, which further exploit these concepts and define the *ab initio* assessment. For the purpose of this case study, emphasis is put on the *ab inito* approach. Under Tier 1, data from alternative methods are collected to support evidence for possible modes-of-action. Tier 2 includes targeted (*in vitro*) testing based on the hypothesis set up under Tier 1. Exposure considerations and PBK modelling are

[1] http://www.seurat-1.eu

important parts of the workflow, to define the target organs and internal concentrations applicable as well as to set the appropriate concentrations for the targeted testing and to interpret results relative to exposure conditions.

The workflow and chemical safety assessment process is illustrated with the example of a hypothetical use scenario of piperonyl butoxide (PBO) as a new cosmetic ingredient in a daily applied body lotion. It aims at showcasing the evaluation procedure which can be applied to the assessment of other chemicals in the future and potentially for risk assessment based on alternative methods for regulatory use.

Definition of the purpose and regulatory relevance

The aim of this case study is not an assessment of a specific chemical (class) in view of regulatory acceptance, but demonstration of an exposure-based chemical safety assessment workflow without relying on animal testing.

The case study shows how the different elements of a risk assessment can be brought together in a harmonised and logical way, with a sequential look at different levels of information, building on each other, and thus to integrate information from different sources and methods to be able to make a conclusion of the safety of a chemical. It goes beyond a weight of evidence of considering and bringing all the information together by going step-wise through the information in a sequential way and constructing a decision logic, considering the exposure scenario from the beginning in order to make conclusions on safety for a specific use. The workflow includes exclusively alternative methods (apart from possible existing *in vivo* study data).

This exposure-based chemical safety assessment workflow was developed within the SEURAT-1 research initiative which was focused on cosmetics-related substances, but is also applicable to any other chemicals.

The workflow is illustrated with a hypothetic scenario example in this case study, but is generally intended to be possibly used as guidance for an IATA type risk assessment in a regulatory context.

Endpoint addressed

The endpoint addressed is repeated dose toxicity for human health safety assessment.

The aim of the case study was to determine the safe use of a cosmetic ingredient, assuming a specific exposure scenario.

The workflow can be used/adapted for other endpoints or effects.

Considered chemical and specific exposure scenario

The substance used for illustration of the workflow steps in this case study is the safrole derivate piperonyl butoxide (PBO). It is not a cosmetic ingredient but fits within a chemical space relevant to cosmetics (which was the focus of the SEURAT-1 Initiative) and has prior use in medicated shampoos.

The hypothetical exposure scenario created is: a new ingredient, with unknown properties and no *in vivo* study data available, introduced in a body lotion applied twice daily on the skin (overall body surface).

The hypothetical question posed is "Can we safely use 12.5% PBO in a daily body lotion?"

CHEMICAL SAFETY ASSESSMENT WORKFLOW PROPOSED

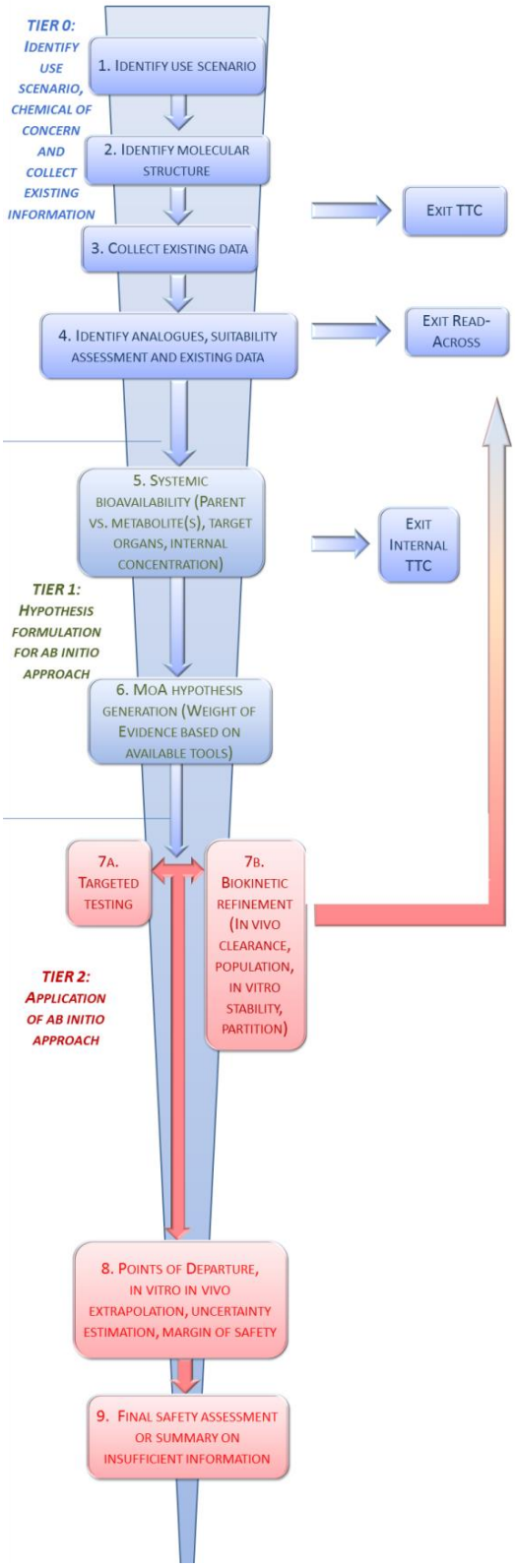


Figure 1. Schema of the chemical safety assessment workflow

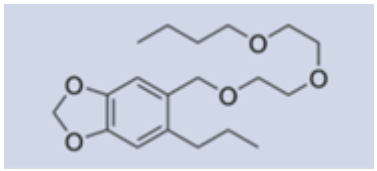
The proposed workflow integrating the different steps in the chemical safety assessment in three tiers is summarised in Figure 1.

TIER 0: Identification of the use scenario, chemical of interest and collection of existing information

1. Identification of the chemical of interest

The assessed chemical and its molecular structure should be identified at the beginning of the assessment, with other chemical identifiers available.

The chemical considered in this case study is piperonyl butoxide (PBO; 5-[2-(2-butoxyethoxy)ethoxymethyl]-6-propyl-1,3-benzodioxole, CAS 51-03-6) in a hypothetical scenario based on the assumption that this is a new ingredient with no data available.



The safrole derivate PBO fits within a chemical space relevant to cosmetics and has prior use in medicated shampoos.

2. Identification of the use scenario

For risk assessment, actual exposure to the substance has to be known and thus the use scenario and route of exposure have to be defined. The conclusion on the risk depends on the exposure and can be different for different use scenarios.

In this case study, the use scenario is twice daily application of a body lotion on skin (overall body surface). The question posed in the hypothetical scenario is: Can 12.5% piperonyl butoxide be safely used as ingredient in a twice daily applied body lotion?

An average exposure of a body lotion applied twice a day on the whole body (female, 60 kg, 1.6 m^2) is estimated to 145 mg lotion/kg/day (95^{th} percentile of distribution for European consumers in Hall et al 2007), assuming 100% skin penetration corresponding to 18.1 mg/kg/day of PBO.

First decision and possible exit point: TTC

In the absence of compound-specific data, the Threshold of Toxicological Concern (TTC) approach can be applied (EFSA 2012, SCCS/SCHER/SCENIHR 2012), a risk assessment approach that establishes a human exposure threshold value below which there is a low probability of an appreciable risk to human health. Applying the most protective TTC value, if the chemical structure is not well defined and genotoxicity cannot be excluded, an exposure below 0.15 µg/person/day would be considered a safe use in most cases. For a known structure, the Cramer class (Cramer et al 1978) can be derived based on data on exposure, chemical structure, metabolism, and including QSAR for genotoxicity prediction to rule out the concern for genotoxicity. Safe use of a substance is assumed if the exposure is considered to be below the assigned TTC threshold.

However, the TTC values are derived from oral exposure data. The extrapolation to the dermal route of exposure has been evaluated in the COSMOS project[2] within the SEURAT-1 cluster and a tiered decision tree was developed assessing the chemical's bioavailability, taking into account the absorption/permeability via dermal or oral routes as well as metabolism differences between skin and liver (Williams et al 2016). The dermal absorption can be calculated using an established predictive algorithm (Potts and Guy 1992) to derive the maximum skin flux adjusted to the actual 'dose' applied and the systemic availability predicted (assuming no local metabolism), which can then be compared with the oral TTC for the respective structural class.

For the use scenario of PBO considered, assuming the worst case of 100% dermal absorption, the oral TTC threshold (Cramer Class III, oral TTC threshold 1.5 µg/kg/day) is exceeded by far. Even taking into account less systemic bioavailability through dermal exposure, according to predicted values of skin permeability (for the calculations according to the decision tree see Williams et al 2016), exposure waiving through TTC was not applicable due to the too high exposure.

After identifying the structure of the target chemical and defining the exposure scenario, the applicability of the TTC approach to provide a safety decision based on exposure is evaluated. In the considered example, the exposure is too high for a safe exit at this point.

3. Collection of existing data

Available information on physicochemical properties of the substance should be documented, some properties can be derived by prediction from the chemical structure.

These properties can be useful in the subsequent assessment procedure, e.g. the molecular weight and partition coefficients may inform about possible penetration through skin and other tissues, volatility would determine possible intake through inhalation.

Examples of physicochemical data for PBO, as used for calculations in the Virtual Cell Based Assay (VCBA) model in Tier 2, are shown in Table 1.

Table 1. PBO physicochemical properties.

Chemical name	CAS	Molecular weight [1]	Boiling point [2]	$LogK_{ow}$ [1]	Henry constant [1]	Air degradation[1]	Water degradation[1]	Atomic diffusion [3]	Molar Volume [2]
Piperonyl butoxide	51-03-6	338.44 g/mol	180°C	4.75 (exp)	$3.69 \cdot 10^{-1}$ Pa m^3/mol	$8.02 \cdot 10^{-5}$ 1/s	$2.14 \cdot 10^{-7}$ 1/s	359.9	282.94 cm^3/mol

[1] data from from EPI suite v4.0 (experimental values preferred over predictions);
[2] calculated according to method from Schotte (1992); [3] calculated according to Fuller et al (1966)

[2] http://www.cosmostox.eu

Furthermore, it should be considered whether the formation of metabolites is likely and whether they should be taken into consideration in the further assessment. For the prediction of metabolites a range of simulation programmes is available, it is however not always easy to make a judgement on how likely the possible formation of the metabolites will actually occur and whether the metabolites will be biologically active.

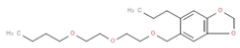
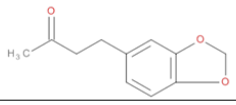
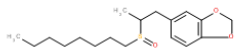
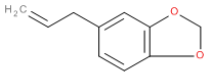
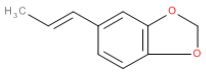
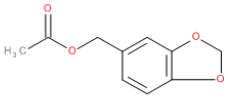
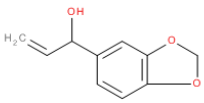
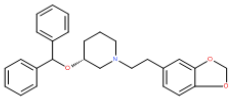
For the chemical considered and possible metabolites data from existing *in vivo* or *in vitro* studies is collected if available. Even only sparse data may support the hypothesis building, further following the workflow.

4. Identification of analogues, suitability assessment and existing data

Second decision and possible exit point: Read-across

Suitable potential analogues should be identified to be able to infer properties and toxicity from similar substances with known properties (existing data) by reading across to the unknown target substance. For the chemical similarity assessment it is important, in a first step, to identify the relevant structural features of the molecule. A systematic strategy for the formation of a category of similar substances, which is considering chemical and toxicological (toxicodynamic and toxicokinetic) similarity, as well as for the assessment of the uncertainty in the category and read-across justification, has been described for example in Schultz et al (2015) within the SEURAT-1 Initiative.

Table 2. Molecules structurally similar to PBO

NAME	STRUCTURE	SIMILARITY MEASURE
Piperonyl butoxide		-
Piperonyl acetone		>65
Piperonyl sulfoxide		>65
Safrole		>65
Isosafrole		>65
Piperonyl acetate		>65
1'-Hydroxysafrole		>60
Zamifenacin		>60

For the case of PBO, examples of molecules (alkenylbenzenes) with a similar core structure to PBO are listed in Table 2.

Based on structural similarity a read-across approach was considered, however, it is not applicable in this case for PBO, as there were no suitable analogues with sufficiently high similarity to read across confidently from or lack of repeated dose toxicity data.

For example, one metabolic pathway predicted for PBO, i.e. O-dealkylation at the aromatic system (see Table 3), leading to ring opening and formation of catechol and guaiacol derivatives, is similar to safrole. Hepatotoxicity via the potential to generate reactive oxygen species inducing oxidative stress responsible for hepatocarcinogenicity is suggested (Vitcheva et al 2015). Safrole and its metabolite 1'-hydroxysafrole are known rodent hepatocarcinogens (IARC 1972, Miller et al 1983). However the hepatotoxicity mechanisms of safrole include genotoxicity of its metabolites. This is not the case of PBO, which for example lacks the allyl group in the side chain important for the metabolic activation via oxidation causing genotoxicity. The side chain will give additional differences in potential metabolic pathways, e.g. the prosteatogenic mode-of-action via PPARγ receptor agonism of the glycol side chain of PBO (Vitcheva et al 2015). Thus there are differences in the toxicity pathways of PBO compared to safrole and its metabolite 1'-hydroxysafrole.

Therefore the chemical safety assessment further progresses to the next tier. Information from the analogues on possible toxic effects and related mechanisms can be used in the next tier to build the mode-of-action (MoA) hypothesis, where applicable. If in the weight-of-evidence more evidence substantiating the similarity and relevance of analogues is gathered, a read-across can still be considered at this stage, if the necessary data are available for the analogues (see Figure 1).

After collection of available (e.g. physicochemical) data for the target substance, consideration of possible metabolites, and identification of possible similar analogues with associated toxicity data, it is assessed whether reading across the target endpoint from suitable analogues is possible in order to come to a conclusion.
For the example considered no suitable analogues with relevant data were available.
However, the collected information may contribute to building the MoA hypothesis and read-across can be re-evaluated at a later stage when more data becomes available.

TIER 1: Hypothesis formulation for *ab initio* approach

5. Systemic bioavailability (target organs, internal concentration)

In this step, systemically available concentrations are predicted in different body compartments and relevant target organs are identified for further assessment according to these concentrations, and contribute to formulating the MoA hypothesis together with results from the *in silico* and (existing) *in vitro* profiles (step 6). The concentration range simulated for a target organ can further be used to establish doses to be tested *in vitro* in Tier 2.

Further to the primary route of exposure, in this case dermal application, it should be considered if other routes of exposure have to be taken into account, e.g. inhalation.

Physiologically-Based Kinetic (PBK[3]) models mathematically describe interconnected compartments representing the human body, considering absorption, distribution, metabolism and excretion (ADME) properties of a chemical within the organism. These models facilitate extrapolations, i.e. predict concentrations in different compartments, across studies, species, routes and dose levels. They are therefore fundamental to the development of biologically based dose-

[3] PBK is synonymous of PBPK, PBBK, and PBTK, respectively Physiologically-Based Pharma-, Bio- and Toxicokinetic.

response models which address uncertainty and variability related to the chemical's kinetics and dynamics.

A six compartment PBK model was built for PBO, based on the human safrole model by Martati et al (2012, 2014), including a skin compartment to simulate dermal exposure (see Figure 2). The skin compartment was divided into viable skin and stratum corneum. Predicted values for the physiological and physicochemical parameters for the human PBO PBK model were used according to the quantitative property-property relationship (QPPR) approach described in Dejongh et al (1997). They were compared to experimental safrole values from Martati et al 2012. A general description of the validation of human PBK models based on literature data can be found for example in Gajewska et al (2014) and Punt et al (2009).

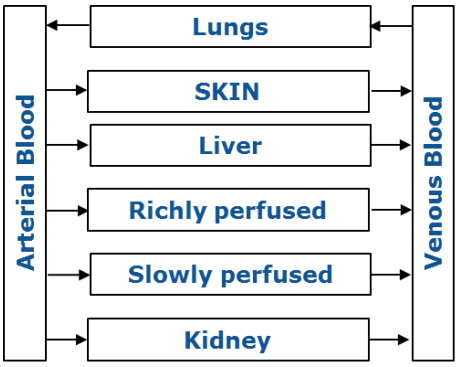


Figure 2. Six compartment PBK model for the simulation of PBO distribution within the human body.

Systemic concentrations of PBO were generated for repeated dose exposures based on the expected consumer use for body lotions. Monte Carlo simulations (Bois et al 2010) were run to predict blood and liver concentrations in a general group of 10000 persons exposed daily. The predicted concentrations within the consumer population and 95% confidence intervals are shown in Figure 3.

High concentrations of PBO accumulate in viable epidermis (data not shown). Significantly lower levels are systemically available with highest concentrations in adipose tissue followed by kidney (data not shown). Lower levels are predicted in venous blood and liver with rapid cycling between. This would highlight fatty tissue and kidney as a priority for concern. Further conclusions will be drawn taking into account the results from the *in silico* / *in vitro* hazard evaluation in step 6.

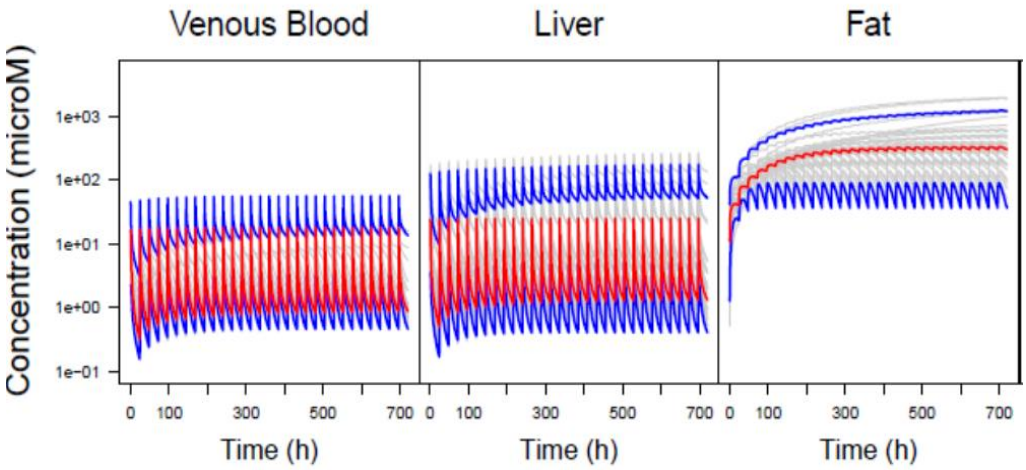


Figure 3. Results of the PBK modelling of PBO concentrations vs time in venous blood, liver, and fat, in 10000 random human subjects generated with Monte Carlo simulations. Red: mean values, blue: 95% confidence intervals.

6. Mode-of-Action hypothesis generation (Weight-of-Evidence based on available methods)

In order to further identify (or exclude some) target organs and the modes-of-action for human adversity, for the specific route of exposure, all data are considered in a weight-of-evidence (WoE) approach: existing *in vivo* or *in vitro* studies, if available, for the target substance or similar compounds, supplemented by a broad scan of possible effects for hazard characterisation with *in silico* methods. If not sufficient for definitive conclusions, the WoE helps identifying data gaps and building a targeted testing strategy.

In particular the increasing availability of *in vitro* data libraries, with data from high throughput and high content screening (HTS, HCS) and -omics (transcriptomics, metabolomics, proteomics) techniques, such as ToxCast (EPA 2014) or TG Gates (Igarashi et al 2015) increase the information available from alternative methods and help to build a toxicological profile of a substance.

Similarly, information from screening with *in silico* profilers or QSAR models for specific endpoints, or applying docking simulators to predict which groups would bind to specific proteins, adds to the understanding of possible biological activity and formulating a MoA hypothesis. The collection of data for the weight-of-evidence and *in silico* screening also applies to possible metabolites to evaluate whether they might cause adverse effects and the MoA is based on biotransformation.

In the present case study *in silico* profilers from the COSMOS project in the SEURAT-1 Initiative and others have been used (see Table 3). They do not represent an exhaustive list of adverse effects to screen for, but are used as examples in this case study as profilers focusing on liver toxicity (inherent to the SEURAT-1 context). When applying the general workflow, a larger battery of profilers should be selected to enable the prediction of possible molecular initiating events. Here the profilers applied were screening for: potential hepatotoxicity (Hewitt et al 2013), protein binding and DNA binding (Enoch et al 2010, 2011), mitochondrial toxicity (Nelms et al 2015a, 2015b) as well as for phospholipidosis (Przybylak et al 2014), also associated with liver toxicity. Potential LXR (liver X receptor) binding was predicted using a combination of different *in silico* approaches, including ensemble docking, pharmacophore matching, fingerprint-based similarity and a QSAR classification model (Fioravanzo et al 2013). Furthermore, the potential for full PPARγ (peroxisome proliferator-activated receptor) agonism was predicted by a virtual screening procedure, including docking with filtering by four PPARγ pharmacophores (Tsakovska et al 2014, Al Sharif et al 2016). In addition, a set of profilers for nuclear receptor binding was used to identify potential binding to the following

nuclear receptors: PPAR, androgen receptor (AR), aryl hydrocarbon receptor (AHR), estrogen receptor (ER), farnesoid X receptor (FXR), glucocorticoid receptor (GR), LXR, pregnane X receptor (PXR), progesterone receptor (PR), retinoic acid receptor (RXR), thyroid hormone receptor (THR), and vitamin D receptor (VDR) (Mellor et al 2016, Steinmetz et al 2015). The receptors AHR, ER, GR, FXR, LXR, PPAR, PXR, RXR are associated with the development of hepatosteatosis.

In addition to these *in silico* profilers compiling structural alerts for specific endpoints/effects, publicly available -omics data and results from ToxCast were collected. 92 out of the 700 examined ToxCast assays were active, with an activity across a broad concentration range from 0.1 µmol/l to 100 µmol/l in cell free assay of different cell types.

The results of the *in silico* predictions (Table 3) as well as ToxCast results (Table 4) highlight nuclear receptor binding including PXR and PPARγ and suggest metabolism may occur. Metabolism prediction with the OECD QSAR Toolbox v.3.0 also pointed at the formation of metabolites with a potential to bind to proteins and DNA, as well as possible reactive oxygen species (ROS) formation and non-genotoxic carcinogenicity.

Table 3. Results of screening with *in silico* profilers

***In silico* Model**	**Prediction**	**Details/Reference**
Hepatotoxicity (COSMOS *in silico* profiler)	0 structural alerts hit	16 structural alerts Hewitt et al 2013 DB-ALM[1] Method Summary no. 179
Phospholipidosis (COSMOS *in silico* profiler)	0 structural alerts hit	45 structural alerts Przybylak et al 2014
Mitochondrial toxicity (COSMOS *in silico* profiler)	1 structural alert hit	21 mechanistic structural alerts, 21 chemotypes Nelms et al 2015a, 2015b DB-ALM[1] Method Summary no. 180
Nuclear receptor binding (COSMOS *in silico* profiler)	7 hits (AHR, AR, ER, GR, PR, THR, PXR)	alerts for 12 nuclear receptors (AHR, AR, ER, FXR, GR, LXR, PR, PPAR, PXR, RXR, THR, VDR) Mellor et al 2016, Steinmetz et al 2015 DB-ALM[1] Method Summary no. 177
PPARγ (COSMOS virtual screening procedure)	PPARγ full agonist	including docking with filtering 4 featured PPARγ pharmacophores Tsakovska et al 2014, Al Sharif et al 2016 DB-ALM[1] Method Summary no. 168
LXR binding prediction (COSMOS)	LXR likely not a target	combining different *in silico* approaches Fioravanzo et al 2013 DB-ALM[1] Method Summary no. 169
Protein binding (COSMOS *in silico* profiler)	0 structural alerts hit	108 structural alerts Enoch et al 2011 DB-ALM[1] Method Summary no. 181
DNA binding (COSMOS *in silico* profiler)	0 structural alerts hit	111 structural alerts Enoch et al 2010 DB-ALM[1] Method Summary no. 178
Metabolic classification (Molecular Networks public set of metabolic classes)	7 metabolic classes activated, including O-dealkylation, aliphatic hydroxylation, carboxylation	25 generic metabolic classes
Carcinogenicity (CAESAR *in silico* model)	carcinogen	available through http://www.vega-qsar.eu
Mutagenicity (CAESAR *in silico* model)	non mutagen	available through http://www.vega-qsar.eu
Mutagenicity (SarPy model)	non mutagen	

[1] https://ecvam-dbalm.jrc.ec.europa.eu

Table 4. Results from ToxCast assays hit by PBO

PBO active assay hits	Intended target family	AC_{50}
ATG_PXRE_CIS_up*	Nuclear receptor	2.69
ATG_SREBP_CIS_up*	Sterol regulatory element TF	5.55
ATG_NRF2_ARE_CIS_up*	Stress response	7.11
ATG_PBREM_CIS_up*	Nuclear receptor	7.23
ATG_PPRE_CIS_up*	Nuclear receptor	10.23
ATG_ERa_TRANS_up*	Nuclear receptor	11.11
ATG_PXR_TRANS_up*	Nuclear receptor	12.07
ATG_VDRE_CIS_up*	Nuclear receptor	12.29
Cytotoxicity threshold		11.40
ATG_PPARg_TRANS_up*	Nuclear receptor	14.17

AC_{50}: half maximal activity concentration (µmol/l)
*in common with Zamifenacin

Analysis of ToxCast and gene expression data for defined Adverse Outcome Pathway (AOP) markers highlights liver toxicity, CYP450 metabolism and weak/moderate potential for steatosis (data not shown). Zamefenacin, a chemical with similar structural elements (see Table 2) and a ToxCast profile that was not limited to but included PBO hits (see Table 4), is known to cause hepatotoxicity in species able to metabolise it into an O-methylated derivative (Amacher et al 1998). Enrichment analysis of ToxCast assays highlighted metabolism of xenobiotics by cytochrome P450 and steroid hydroxylase activity.

There is weight of evidence across all approaches for metabolism effects and steroid perturbation through binding to nuclear receptors. Some conflicting evidence for impact on liver toxicity between *in silico* and *in vitro* and between different *in vitro* systems may be due to a parent vs metabolite issue. It highlights the importance of the use of metabolically relevant systems.

The results of the *in vitro* and *in silico* screening should be considered together with the predicted concentrations and likely target organs from step 5.

PXR is mainly expressed in liver, colon and small intestine, while PPARγ is prevalent in adipose tissue. Effects in adipose tissue would need to be further addressed. Effects on kidney cannot be ruled out at this stage, given the predicted accumulation of PBO in kidney.

The results of the PBK concentration predictions of systemic bioavailability to identify likely target organs (step 5) and of the broad *in vitro* and *in silico* screening for hazard characterisation (step 6) are considered together in a weight-of-evidence in order to build a mode-of–action hypothesis. Data gaps are identified.
In the example of PBO considered, there is weight-of-evidence across all approaches for metabolism effects and steroid perturbation through binding to nuclear receptors.

TIER 2 Application of *ab initio* approach

7. Targeted testing and biokinetic refinement

Refinement on Adverse Outcome Pathway (AOP) altering dose and exposure, based on in vitro repeated dosing exposure

Following up on the indications on target organs/tissues obtained in Tier 1, if a well-known AOP is concerned, the respective key events can be investigated to confirm the hypothesis. Furthermore, quantitative (dose-response) estimates of biological effects should be derived under mimicked realistic conditions. For this targeted testing different types of assays can be used, e.g. 2D based, organotypic models or organ-on-a-chip models; pathways with specific biomarkers if specific biological effects such as binding to a nuclear receptor are involved (Madureira at al 2014).

Figures 4 and 5 show the AOPs for liver steatosis and liver fibrosis, as general examples of AOP schemes leading from a molecular initiating event (MIE) to adverse effects via several steps and key events. In the case of these two AOPs, the MIEs include PPARγ activation and covalent protein binding, respectively. In the case of PBO, the broad screening results from *in silico* or *in vitro* HTS assays from Tier 1 pointed to MIEs and effects within the steatosis AOP, i.e. binding of PPARγ, PXR, activation of a subset of cytochrome P450s and biotransformation pathways, leading to potential liver toxicity (see Figure 4). The fibrosis AOP (see Figure 5) was also considered as covering potential liver toxicity effects, supported for example by alerts for protein binding for PBO metabolites.

Based on the alerts for PXR and PPARγ and their tissue localisation (expression in liver and adipose tissue), targeted testing is warranted for both liver and adipose tissue. In the frame of the SEURAT-1 Initiative, assays have been carried out related to liver toxicity only. It must be stressed again that the scope of the assays and the outlined AOPs does not cover all biological functions and toxicological endpoints, but are illustrations of the application of the safety assessment workflow.

The targeted testing was thus based on both the steatosis and liver fibrosis AOPs[4] (Landesmann et al 2012, Horvat 2017) and assays developed in SEURAT-1.

In order to determine if the considered events could lead to steatosis after repeated dose exposure, lipid accumulation was measured in metabolically competent SEURAT-1 HepaRG test systems (Joossens et al 2015). Lipid droplet accumulations indicated that potential steatosis could not be ruled out.

An assessment of fibrotic potential was performed using the SEURAT-1 multicellular assay for hepatic stellate cell activation (Leite et al 2016). Comparison to the positive control suggested upregulation of stellate cell activation and collagen deposition at both doses tested (180 and 540 µmol/l) with increasing activation following repeated exposure.

Following up on the indication from Tier 1, pointing at specific AOPs, targeted testing can be applied in Tier 2 to investigate the respective key events in order to confirm the MoA hypothesis.
For the example of PBO, the AOPs of liver steatosis and liver fibrosis were considered and selected targeted assays were performed. Evidence for liver droplet accumulation as well as upregulation of stellate cell activation and collagen deposition were found, respectively.

[4] https://aopwiki.org/aops/34, https://aopwiki.org/aops/38

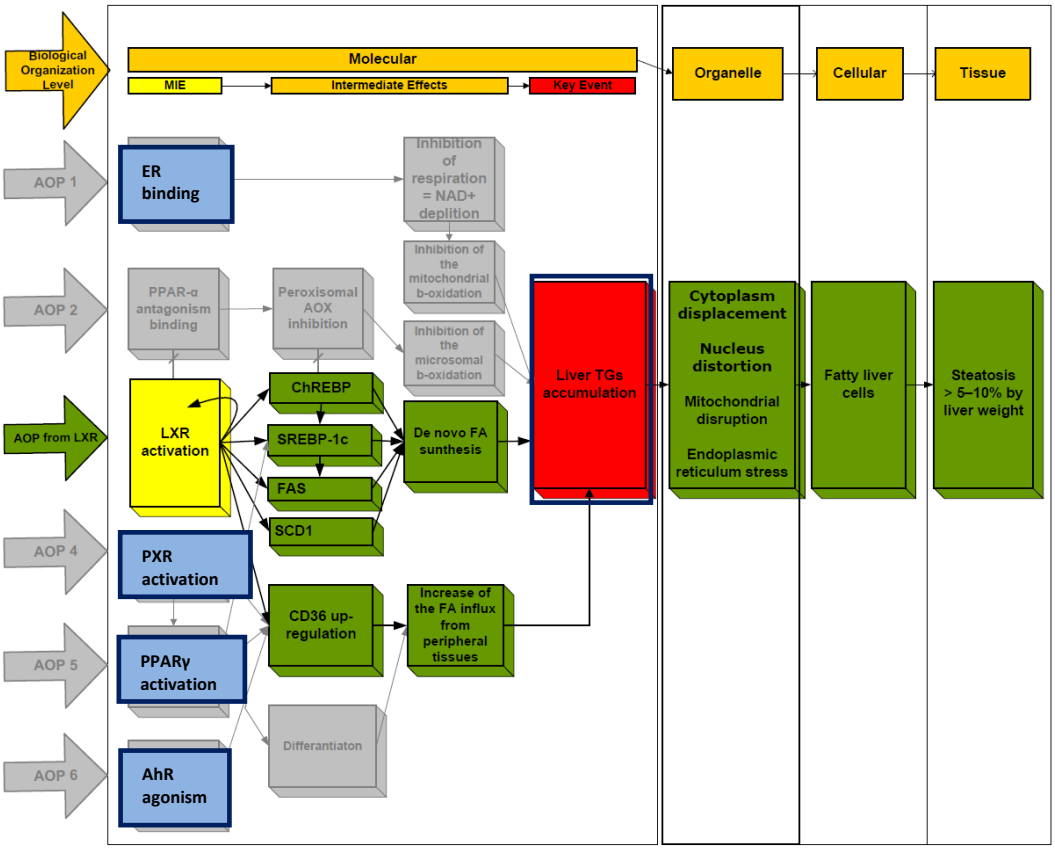


Figure 4. Flow diagram of the AOPs leading from different MIEs related to nuclear receptors to liver steatosis (Landesmann et al 2012). Triglyceride (TG) accumulation is a key event which can be tested in Tier 2.

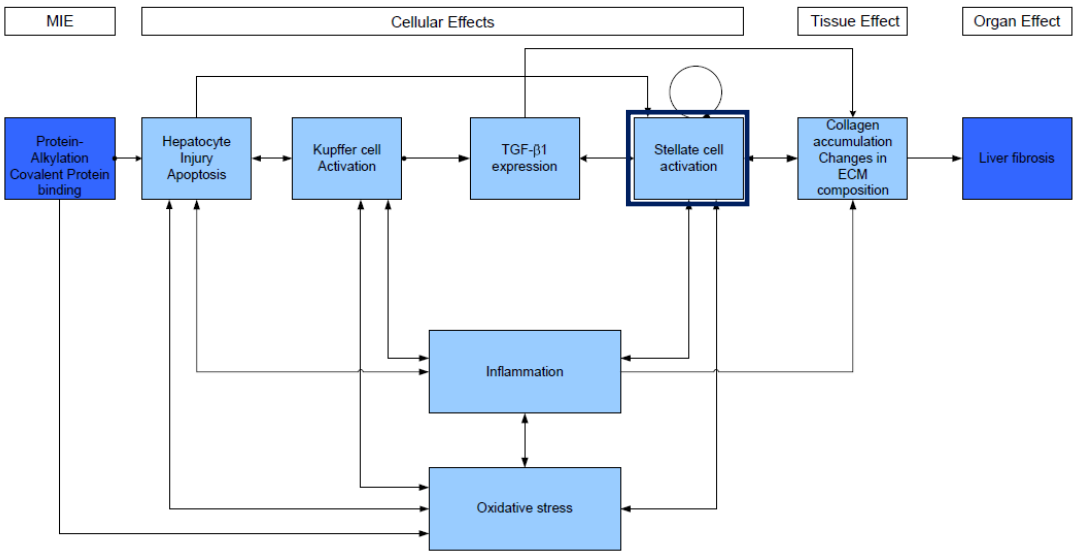


Figure 5. Adverse Outcome Pathway for liver fibrosis (Landesmann et al 2012). Stellate cell activation is a key event which can be tested in Tier 2.

Biokinetic and in vitro refinement

Exposure should be taken into consideration, both as an estimation of the internal dose at the possible target organs, as well as the concentrations in the test systems. It is possible to calculate internal dose/concentration from external exposure and predict a realistic corresponding dose/concentration in the *in vitro* experiments with PBK models, i.e. the calculated internal concentrations will guide the decision on the concentration range to test in the selected targeted assays.

The Virtual Cell Based Assay (VCBA) is one mathematical tool to perform this extrapolation to account for *in vitro* dosimetry effects and to calculate the intracellular concentration. Thus, the simulated concentration of the chemical in the target organs (or in the blood) can be related to the intracellular concentration available in the cells in the *in vitro* test system. In addition, internal exposure is translated into a realistic external exposure through an *in vitro* to *in vivo* extrapolation (IVIVE) calculation, to predict the exposure needed to initiate an effect. This should make certain that the worst case dose is considered in framing the test concentrations. It refines the consideration of the chemical's solubility limit as the highest dose tested, which might differ from the IVIVE extrapolation by orders of magnitude. The PBK and VCBA models are applied for extrapolation from *in vitro* to *in vivo* but also from high to low doses/concentrations and from route to route extrapolation. PBK models help to fill in the data/knowledge gap related to low dose exposure often relevant to human exposure scenarios, which in general is less well covered by traditional animal data.

The VCBA (Zaldivar Comenges et al 2016) has been developed within the COSMOS project to simulate a chemical's fate in an *in vitro* assay and was applied for obtaining the dissolved concentration that could enter the cell. Briefly, it comprises five interconnected models: fate and transport, chemical partition inside the cell, cell growth, toxicity and effects, as well as the experimental set up. It is equivalent to the Armitage model (Armitage et al 2014) and to Kramer (2010). The relevant parameters for the VCBA can be predicted *in silico*. An *in vitro* concentration response curve for model optimisation can be based on experimental cytotoxicity testing. Parameters needed to perform the VCBA simulation for PBO (based on Zaldivar et al 2010, 2011, 2012, 2016) are listed in Table 1.

Furthermore, it is necessary to evaluate which exposure scenario is relevant for the experimental testing, i.e. single exposure or repeated exposure. Since the chemical might accumulate in the target organ cells, elimination rates should be estimated. The scenario needs also to be understood in terms of the toxicity being caused by a maximum concentration (Cmax), either occurring after one dose or after accumulation of repeated doses reaching the same dose, or by a repeated disturbance of the system with many non-toxic doses that lead to the adverse outcome, even without accumulation in the cell (AUC; area under the concentration-time curve). The toxicodynamic model can accordingly be based on Cmax, AUC, or on a combination of both parameters. It should also be noted regarding the translation of the *in vivo* system to the *in vitro* model, that a significant divergence of the time scale is possible due to the *in vitro* system's limitations in metabolic activity. Thus the repeated exposure mimicked in a short time interval might be more relevant.

For the PBO case study scenario, refinement of the systemic availability after skin absorption (which can be calculated according to the decision tree in Williams et al 2016; here the literature value of human skin penetration (COSMOS skin permeability database, Wester et al 1994) was used) reduced skin penetration for systemic exposure to 2.1% of the applied dose. Application of this estimation resulted in a reduced predicted concentration range of 0.01-1 µmol/l and 0.01-3 µmol/l for plasma and liver repeated dose respectively (Figure 6). Higher concentrations are still seen with accumulation in fatty tissues: 1-12 µmol/l at 95% confidence interval for the Monte Carlo simulation.

Characterisation of PBO stability in the culture medium indicated a significant reduction in available parent compound compared to the nominal applied *in vitro* dose, suggesting that dose-response curves may be shifted to a lower concentration.

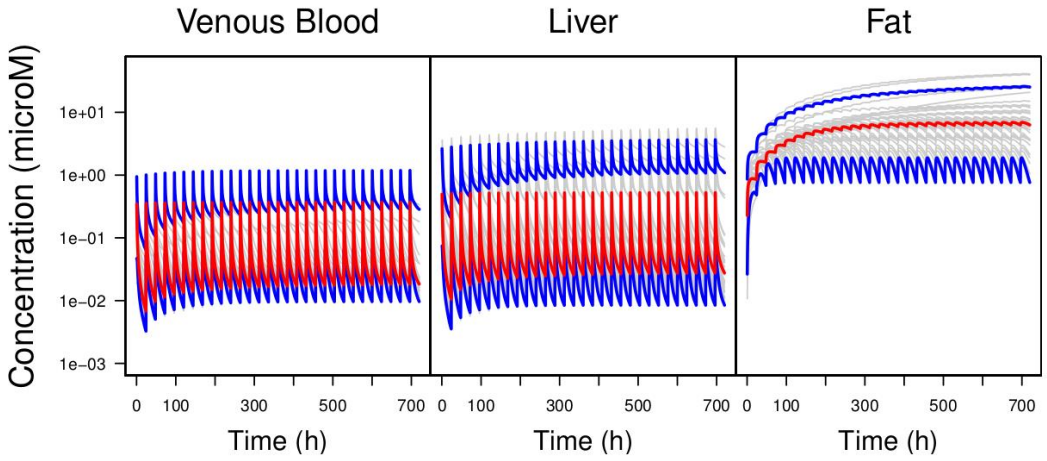


Figure 6. Refined results of the PBK model simulating PBO concentrations in venous blood, liver, and fat.

Generally, to prepare targeted testing, it is important to understand uptake, metabolism and clearance to define the substance actually present in the cell. Similarly, for example the binding to proteins in the plasma or to the plastic in the test system should be evaluated and considered for tests aimed at deriving points of departure for risk assessment. Another important consideration is to include positive and negative controls to benchmark the experimental system, at different concentrations tested, and to carry out assays in replicates.

Biokinetic refinement is considered at this stage, both to refine the estimation of the internal dose at the possible target organs, as well as the concentrations to be used for the assays performed, and to correlate the internal exposure in the assays with the external exposure initiating an adverse effect.

8. Points of Departure, in vitro in vivo extrapolation, uncertainty estimation

The final steps in the chemical safety assessment workflow would be:

- Prediction of a point of departure (PoD) for safety assessment based on the relevant AOP incorporating kinetics and biomarker data from repeated dose assays
- Definition of the margin of safety based on variability and uncertainty estimates
- Description of the safety decision and any open issues that could assist in gaining higher confidence.

The data collected in the previous steps can confirm the hypothesis of mode(s)-of-action and elucidate points for departure for a quantitative risk assessment. Considering the results from the *in vitro* assays, it could be concluded that there is no concern for toxic adverse effect regarding the specific endpoint, if the internal exposure is estimated to be far below any biologically active dose in the assays. However, depending on the confidence in the outcome and its relevance for a specific adverse effect, additional proof might be considered to be required to confirm the effect level.

The benchmark dosing approach could be used to obtain a PoD for risk assessment considering the overall concentration response curve (Benchmark Concentration - BMC), processing the *in vitro* assay results. The data from assays and *in silico* predictions have to be extrapolated to the relevant human situation, by using approaches as *in vitro* to *in vivo* extrapolations (IVIVE) and reverse PBK modelling (see for example Strikwold et al 2013).

Before making a final conclusion on the safety of the chemical considered, or conclude on a data gap and requirement of further testing (step 9), all data analyses should be completed and the uncertainty of all results and of all steps should be evaluated and characterised, as well as the overall uncertainty in applying the safety assessment approach.

The results from *in vitro* assays available for the case study substance PBO are summarised in Figure 7. (It is appreciated that in the context and time available in the SEURAT-1 Initiative the obtained information could not be provided as completely as required for example for regulatory submission, however the development of the procedure and workflow were the main aim of this case study.) The *in vitro* points of departure are plotted against the concentration range simulated by PBK modelling, represented by a red line, which describes a population range set up with 10000 people, to show the predicted relevant human concentrations of PBO in liver and blood, both for 100% skin absorption assumed (predicted concentration range in liver about 1-100 µmol/l at 95% confidence interval) and the refinement based on 2.1% absorption (predicted concentration range in liver about 0.01-3 µmol/l at 95% confidence interval). Thus, all the results from *in vitro* testing in this concentration range are related to the 12.5% of PBO in the body lotion.

The performed assays summarised in Figure 7 include: Microassay Pathway NOTELr, lipid accumulation repeat dose 4/24h, loss of Cyp3A activity (in metabolically active HepRG cells), repeated dose activation of stellate cell and collagen mRNA production, NOAEL neutral lipid staining repeated dose 1/24h, NOAEL lipid accumulation and size dose 1/24h (all in metabolically active test systems); Attagene nuclear receptor activation (ToxCast), Microassay Pathway NOTELr (in metabolically inactive test systems); NovaScreen ADMS Cyp3A4 (ToxCast) (cell free system)[5].

Differences in dose response can be seen between the different test systems, e.g. an approximatively 100 fold difference in concentration between the cell free and the metabolically active HepaRG cell assay for Cyp3A4. This needs to be taken into account when translating the data into *in vivo* relevance.

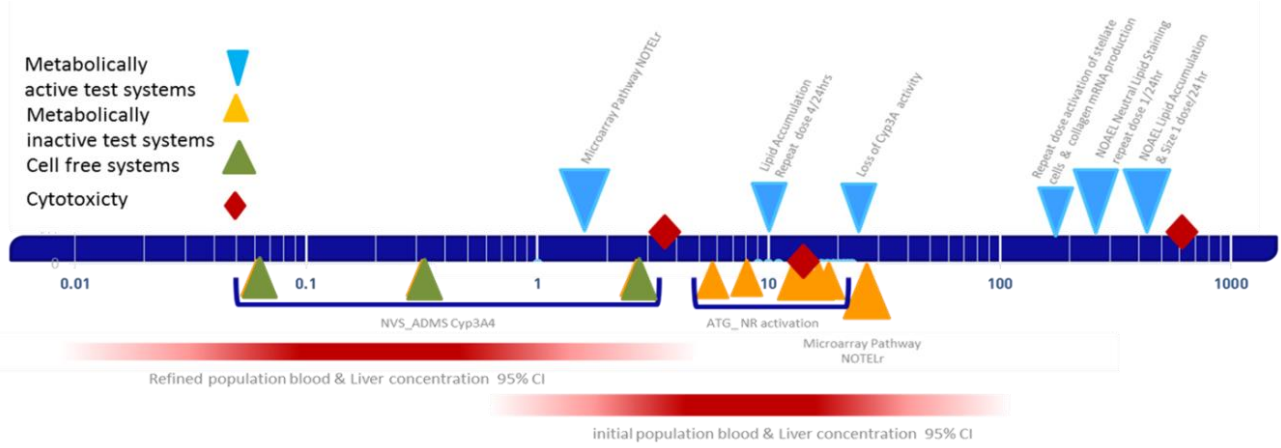


Figure 7. Illustration of predicted liver and blood concentrations of PBO simulated by PBK modelling and the *in vitro* assay results, which can be considered as possible points of departure for PBO. The concentration ranges (red lines) correspond to a population range of

[5]See ToxCast Assay Annotation Data User Guide: https://www.epa.gov/chemical-research/toxcast-assay-annotation-data-user-guide

10000 people (Monte Carlo simulation), the first concentration range based on 100% absorption through the skin, the second corresponding to the refinement based on 2.1% absorption.

9. Final safety assessment or summary on insufficient information

Considering all evidence and uncertainties, a final conclusion on the safety assessment is made. The decision on the safety of the chemical in the considered exposure scenario is described or the remaining data gaps which may prevent a final conclusion are identified.

In sum, even with the remaining variability and uncertainty it appears there is not an adequate margin of safety for a use scenario of 12.5% PBO in a daily body lotion using the new approach data.

The predicted concentration in liver, also after refinement (concentration range about 0.01-3 µmol/l at 95% confidence interval predicted for the simulated population), is in the order of magnitude of the points of departure identified from the applied methods as illustrated in Figure 7. Therefore the systemic concentration reached after application of the considered dose of PBO cannot be considered to be below any concern for an adverse effect.

Given the PPARy response, its high expression in adipose tissue and the prediction of fat accumulation, this would be a key area to be addressed to understand potential effects such as on adipocyte differentiation and hormone levels. Further work would also be needed to refine the human systemic exposure estimates, including addressing uncertainties in plasma protein binding, liver metabolism, clearance and renal excretion. Similarly, the *in vitro* exposure scenario needs to be better characterised to enable comparison with the human systemic exposure, and ultimately to be able to compare or convert the *in vitro* concentration to an amount applied to the skin.

All data and evidence is collected for the overall evaluation, the uncertainty of all results and of the safety assessment conclusion needs to be considered. The points of departure of the *in vitro* assays can be translated into the dose relevant for human risk assessment by reverse PBK modelling, *in vitro* to *in vivo* extrapolations (IVIVE). A margin of safety should be defined. The safety decision on the chemical considered is described or the remaining data gaps which might prevent a final conclusion are identified.
For the example of PBO, the predicted concentration in liver is in the order of magnitude of the points of departure identified from the *in vitro* methods. Overall, the use scenario of 12.5% PBO in a daily body lotion cannot be concluded as safe.

CONCLUDING REMARKS

This general workflow was developed in an attempt to structuring knowledge and data in a logical sequence for an integrated safety assessment relying specifically on alternative methods and specifically taking into account exposure considerations and kinetics.

The safety assessment and workflow starts with Tier 0, where the exposure scenario and chemical identity are defined and existing data is collected. Exit points were identified, i.e. application of the TTC approach or a read-across assessment taking into account similar substances. When neither is considered to be applicable and sufficient to allow a decision, the assessment continues to Tiers 1 and 2, which further exploit these concepts and define the *ab initio* assessment. In this case study

emphasis was put on the latter, whereby, under Tier 1, data from alternative methods are collected to support evidence for possible modes-of-action. Tier 2 includes targeted (*in vitro*) testing based on the hypothesis set up under Tier 1. Exposure considerations and PBK modelling are important parts of the workflow, to define the target organs and internal concentrations applicable as well as to set the appropriate concentrations for the targeted testing.

Overall, the case study highlights the challenge in integration and visualisation of multiple data streams for safety assessment decisions. It shows how using a combination of *in silico*, high throughput and high content data streams can be used to infer a mode-of-action. Biological and chemical sub-structure similarity screening provides anchoring to build confidence and points at adverse outcomes on the organism level.

To provide confidence in the assessment, uncertainty should be identified and evaluated for the different steps of the workflow, the methods used and the data considered. Schemes and strategies for data quality and uncertainty assessment have been discussed for example in Klimisch et al (1997), Schultz et al (2015) and EFSA (2015). A final decision on safe use cannot be taken if the uncertainty is too high at the end of the assessment. However, the evaluation then also helps identifying the remaining information gaps, so that recommendations for further specific targeted *in vitro* testing can be made. The assessment may also identify general needs for new, reliable and relevant, methods.

The presented workflow could be the basis for a full risk assessment. It was conceived as a tool to guide a risk assessment evaluation through the different steps that needs to be considered and thus to support decision making. The workflow is a general framework that can cover different types of chemicals, endpoints and exposure scenarios.

It was not considered realistic to complete such a risk assessment for a chosen substance within the timeframe of the SEURAT-1 initiative. However, the case study is the basis for a first integrated assessment relying only on alternative methods, showcasing the feasibility but also indicating weaknesses and knowledge gaps, which will assist in shaping a more focused strategy to advance alternative assessment approaches. In particular, the systematic analysis can help to point out where new methods and techniques would be needed to answer remaining questions.

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