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CASE STUDY ON THE USE OF INTEGRATED APPROACHES TO TESTING AND ASSESSMENT FOR PREDICTION OF A 90 DAY REPEATED DOSE TOXICITY STUDY (OECD 408) FOR 2-ETHYLBUTYRIC ACID USING A READ-ACROSS APPROACH FROM OTHER BRANCHED CARBOXYLIC ACIDS

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Paris 2020

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Environment, Health and Safety Division
2, rue André-Pascal
75775 Paris cedex 16
France
Fax : (33-1) 44 30 61 80
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Forward

OECD member countries have been making efforts to expand the use of alternative methods in assessing chemicals. The OECD has been developing guidance documents and tools for the use of alternative methods such as (Q)SAR, chemical categories and Adverse Outcome Pathways (AOPs) as a part of Integrated Approaches for Testing and Assessment (IATA). There is a need for the investigation of the practical applicability of these methods/tools for different aspects of regulatory decision-making, and to build upon case studies and assessment experience across jurisdictions.

The objective of the IATA Case Studies Project is to increase experience with the use of IATA by developing case studies, which constitute examples of predictions that are fit for regulatory use. The aim is to create common understanding of using novel methodologies and the generation of considerations/guidance stemming from these case studies.

This case study was developed by EU ToxRisk project (BIAC) for illustrating practical use of IATA and submitted to the 2019 review cycle of the IATA Case Studies Project. This case study was reviewed by the project team. The document was endorsed at the 4th meeting of the Working Party on Hazard Assessment in June 2020.

The following case study was also reviewed in the project in 2019:

1. CASE STUDY ON USE OF AN INTEGRATED APPROACH TO TESTING AND ASSESSMENT (IATA) AND NEW APPROACH METHODS TO INFORM A THEORETICAL READ-ACROSS FOR DERMAL EXPOSURE TO PROPYLPARABEN FROM COSMETICS, ENV/JM/MONO(2020)16.
2. CASE STUDY ON THE USE OF INTEGRATED APPROACHES FOR TESTING AND ASSESSMENT FOR SYSTEMIC TOXICITY ARISING FROM COSMETIC EXPOSURE TO CAFFEINE, ENV/JM/MONO(2020)17.
3. CASE STUDY ON THE USE OF INTEGRATED APPROACHES FOR TESTING AND ASSESSMENT FOR 90-DAY RAT ORAL REPEATED-DOSE TOXICITY OF CHLOROBENZENE-RELATED CHEMICALS, ENV/JM/MONO(2020)18.
4. CASE STUDY ON THE USE OF INTEGRATED APPROACHES FOR TESTING AND ASSESSMENT TO INFORM READ-ACROSS OF P-ALKYLPHENOLS: REPEATED-DOSE TOXICITY, ENV/JM/MONO(2020)19.
5. CASE STUDY ON THE USE OF INTEGRATED APPROACHES TO TESTING AND ASSESSMENT FORREAD-ACROSS BASED FILLING OF DEVELOPMENTAL TOXICITY DATA GAP FOR METHYL HEXANOIC ACID, ENV/JM/MONO(2020)21.
6. CASE STUDY ON THE USE OF INTEGRATED APPROACHES TO TESTING AND ASSESSMENT FOR IDENTIFICATION AND CHARACTERISATION OF PARKINSONIAN HAZARD LIABILITY OF DEGUELIN BY AN AOP-BASED TESTING AND READ ACROSS APPROACH, ENV/JM/MONO(2020)22.
7. CASE STUDY ON THE USE OF INTEGRATED APPROACHES TO TESTING AND ASSESSMENT FOR MITOCHONDRIAL COMPLEX-III-MEDIATED

NEUROTOXICITY OF AZOXYSTROBIN - READ-ACROSS TO OTHER STROBILURINS, ENV/JM/MONO(2020)23.

These case studies are illustrative examples, and their publication as OECD monographs does not translate into direct acceptance of the methodologies for regulatory purposes across OECD countries. In addition, these cases studies should not be interpreted as official regulatory decisions made by the authoring member countries.

In addition, a considerations document summarising the learnings and lessons of the review experience of the case studies is published with the case studies:

REPORT ON CONSIDERATIONS FROM CASE STUDIES ON INTEGRATED APPROACHES FOR TESTING AND ASSESSMENT (IATA) -Fifth Review Cycle (2019) -, ENV/JM/MONO(2020)24.

This document is published under the responsibility of the Joint Meeting of the Chemicals Committee and Working Party on Chemicals, Pesticides and Biotechnology.

Abstract

Regulatory framework: In this read-across we assume, that 2-Ethylbutyric acid (2-EBA) has to be registered under REACH and is produced in Europe at tonnages of more than 100 t/a. The standard REACH information requirements ask for a 90 days study with oral exposure. We use a category approach to predict the outcome of a subchronic toxicity study. The category comprises nine branched carboxylic acids.

Synopsis: The structure of the target compound 2-EBA comprises a short chain, branched aliphatic carboxylic acid in position 2. Nine aliphatic carboxylic acids with different branched aliphatic side chains are regarded as most similar to the target compound. Beside high structural similarity the grouped compounds show a consistent trend for physico-chemical (pc) parameters, e.g. logPow and MW increases slightly with side chain length, whereas water solubility and vapour pressure decreased. The pc-parameters do however not alert for a potential bioaccumulation *in vivo*. Two compounds have *in vivo* animal studies with repeated oral exposure. 2-Ethylhexanoic acid (2-EHA) has subchronic guideline studies, in which liver hypertrophy was observed together with an increase of the relative liver weight. Valproic acid (VPA) induced liver steatosis in shorter-term subacute studies. The read-across hypothesis is therefore, that 2-EBA is a liver toxicant with special concern for steatosis. In addition to the nine structural analogues, Pivalic acid (PVA) is tested as negative control compound. PVA has a third substituent in position 2 and did not induce any liver toxicity in a subacute study up to the highest tested dose. A negative compound is needed to judge on the accuracy of NAM data.

The selection of NAMS and testing scope is dependent on the read-across hypothesis as recently published (Escher &Kamp *et al.* 2019). In example, the grouped compounds might have in common i) an AOP (adverse outcome pathway), ii) a specific toxicological adverse effect or iii) an unspecific toxicological effect/ a generally low toxicity. In this read-across assessment, an AOP network for liver steatosis is known for the primary toxicological effect. From this AOP network, molecular initiation events (MIEs) and one key event (KE) are tested to see, in how far the grouped compounds might induce this adverse outcome. In addition, the perturbation of general biological pathways and cellular functions are tested together with cytotoxicity to discover potential major differences between the grouped compounds (section 4.).

NAM data showed a consistent trend with regard to toxikokinetics and toxikodynamics within the grouped compounds. The results are briefly summarised in the following:

Toxikokinetics: A rat physiology-based pharmacokinetic (PBPK) model was established, based on *in vivo* data, and used to calculate plasma and target organ concentrations, which guided the selection of a relevant concentration range for *in vitro* testing. Human PBPK models were established for all read-across compounds based on physiochemical properties and *in vitro* clearance data (e.g. plasma protein binding (ppb) and intrinsic hepatic clearance ($CL_{int, Hep}$). Human *in vivo* pharmacokinetic data for VPA was identified and verified good predictive performance based on observed plasma concentration data in humans. Based on this proof of concept IVIVE-PBPK models were used for *in vitro* to *in vivo* extrapolations for all analogues.

Toxikodynamics: Several adverse outcome pathways are available describing the development of liver steatosis. About 50 published signalling pathways leading to steatosis

were compiled from literature and summarised in an adverse outcome pathway (AOP) network. The AOP network guided the selection of *in vitro* assays, to determine MIE (molecular initiation event) and KE (key event) activation. Two high throughput models, the CALUX and GFP reporter assays, measured six MIEs being present in the AOP network. With increasing chain length, the number of activated MIEs related to steatosis increased. The target compound 2-EBA activated one MIE, PPAR-α. It can therefore not be excluded that a pathway towards lipid accumulation is activated by 2-EBA. In addition, three liver models measured intracellular triglyceride accumulation, a key event regarded as direct surrogate for liver steatosis. After single and/or repeated exposure, lipid accumulation was mainly observed for long chain analogues, whereas short-chain analogues remained inactive. The two compounds with *in vivo* data on liver steatosis induced lipid accumulation, whereas the *in vivo* negative compound was inactive up to the highest *in vitro* tested dose. 2-EBA was inactive in HepG2 and HepaRG cells (primary human hepatocytes were not measured) up to the highest *in vitro* tested dose. No difference was observed within the grouped analogues with regard to cytotoxicity (in liver and kidney cells), MIEs not present in the AOP network or other endpoints pointing towards general biological perturbations (e.g. glutathione depletion, mitochondrial membrane potential, mitochondrial superoxide formation etc.).

Data integration: The decision theory Dempster Shafer (DS) indicated that the absence of lipid accumulation for 2-EBA can be predicted with 100% certainty from the *in vitro* assays used in this case study. DS further showed that the results of lipid accumulation and cytotoxicity from HepG2 cells and the Calux reporter gene panel give already enough information for this conclusion.

Conclusion:

We have shown in this dossier that the NAMs predict all three compounds with *in vivo* data correctly, either those that induce or do not induce liver steatosis. 2-EBA was in all assays less toxic then the two liver toxic analogues with *in vivo* animal data, 2-VPA and 2-EHA.

The NAM data investigated in this case study indicate that 2-EBA will not induce liver steatosis up to the highest tested *in vitro* dose. Kidney models did not show any difference in cytotoxicity, with all compounds being of general low cytotoxicity. Also, endpoints measuring the perturbation of general biological processes like mitochondrial membrane potential or cytotoxicity support the trend of higher activity with longer side chain length.

We used the 10th percentile of the most sensitive *in vitro* endpoint of 2-EBA to derive an oral equivalent dose. QIVIVE results in an oral equivalent dose of 730 to 948.6 mg/kg bw/d for rats, which might be used to fill the data gap of a subchronic toxicity study. Furthermore, QIVIVE was used to determine directly a corresponding human oral equivalent dose, which is estimated to be 243 to 245.7 mg/kg bw/d.

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1. Introduction and purpose

This case study characterises the hazard of 2-Ethylbutyric acid using a read-across approach to other branched carboxylic acids with suitable subchronic *in vivo* studies.

The regulatory context chosen for this mock-submission is REACH. We assume that 2-Ethylbutyric acid (2-EBA, CAS 88-09-5) has to be registered under REACH, as it is produced and marketed in Europe at tonnages of more than 100 t/a. The standard REACH information requirements, as outlined in the REACH Annexes VII to VIII, state that the hazard of compounds of this annual volume have to be assessed by using a subchronic 90 days study with oral exposure e.g. according to OECD guideline 408.

2-Ethylbutyric acid does not have a 90 days study with oral exposure. Therefore, we use a read-across approach to extrapolate the toxicity of 2-Ethylbutyric acid from suitable 90 days studies of relevant analogues. This approach corresponds to scenario 4 as outlined in the Read-Across Assessment Framework (RAAF, ECHA 2017). Ten source compounds with and without subchronic *vivo* data have been identified. NAM data substantiate the read-across hypothesis by showing a consistent trend with regard to toxikokinetic and toxikodynamic properties within the grouped compounds.

1.1. Target chemical definition

Target chemical of this read-across is 2-Ethylbutyric acid. Experimental (source: http://esc.syrres.com/fatepointer/search.asp) and estimated physico-chemical properties (est; Episuite, ACD) are listed below:

Name: 2-Ethylbuytric acid

CAS: 88-09-5

MW: 116.08 Da

Melting point: -31.8°C; 15.24 (est; Episuite)

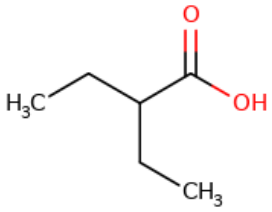
Boiling point: 194 °C; 195.6 (est; Episuite)

Water solubility: 18.000 mg/l at 20°C ([1]);

VP: 0.25 hPa ([2])

logPow: 1.68 ([3]); 1.98 (est; Episuite)

pka: 4.71 ([4]); 4.8 (est, ACD)



Smiles:(C(O)=O)(CC)CC

2-Ethylbutyric acid is an aliphatic carboxylic acid, which comprises a branched, aliphatic side chain at position 2 (Table 1, Annex I).

The physico-chemical data indicate that 2-EBA is water soluble and not volatile. Its dissociation constant (pka = 4.71) indicates that the 2-EBA is a weak acid. Main routes of exposure to humans will therefore be dermal or oral exposure. The low logPow value of

[1] Riemenschneider, W. (1986)

[2] Daubert, T.E. and Danner R.P. (1989)

[3] Sangster (1993)

[4] Kortum G. *et al.* (1961)

1.68 does at a first glance not alert for a potential bioaccumulation of the compound in the human organism. Profiling of several QSAR models did not alert for any other potential toxicological hazard like e.g. mutagenicity, skin or eye irritation/sensitisation (Annex III). The properties of the target compound guides the selection of the source compounds. In this read-across approach, we selected branched carboxylic acids with similar physico-chemical and properties for the read-across evaluation (section 3.).

1.2. Endpoint(s) for which the read-across is performed

The read-across is performed for the endpoint repeated dose toxicity, 90days exposure period (subchronic), oral application.

1.3. Exposure information

Not applicable for the read-across case described here.

2. Hypothesis for the category approach

In this read-across we will predict the outcome of a 90 days repeated dose toxicity study with oral exposure for the target compound 2-Ethylbutyric acid.

We selected nine structurally similar analogues, which are branched aliphatic carboxylic acids, with different aliphatic side chain length. Structural similarity scores ranges from 73% (VPA) to 92% (2-Methyl-butyric acid). The available *in vivo* studies with repeated oral exposure indicate that the primary effect is liver toxicity with specific concern about microvesicular steatosis. 2-EHA induce liver hypertrophy in high quality subchronic studies with oral exposure at the lowest observed effect levels (LOELs). VPA shows liver steatosis in supporting oral *in vivo* studies with shorter timeframes. It can therefore not be excluded that the hypertrophy of 2-EHA is caused by lipid accumulation.

The read-across hypothesis is that the nine source and one target compounds form a category. The toxicological properties in this category follow a consistent trend and biotransformation to a critical metabolite is regarded to be not critical. This hypothesis corresponds to a scenario 4 under REACH.

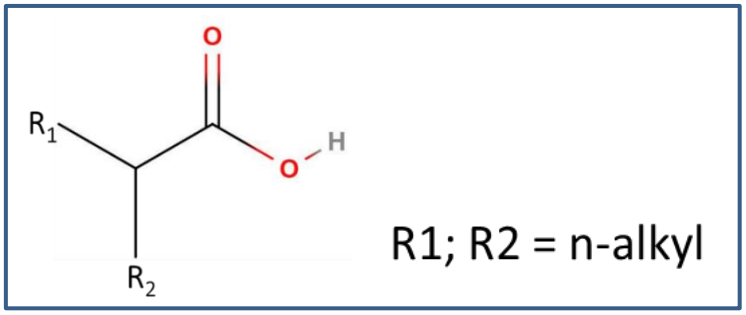
3. Identification and selection of source chemicals/category members

Target chemical of this read-across is 2-Ethylbutyric acid. The structure 2-Ethylbutyric acid is best characterised as a carboxylic acid, which comprises a short, branched, alkyl side chain at position 2. Each side chain contains two linear alkyl chains, termed 2/2/0 in the following. Aliphatic carboxylic acids with short side chains, all branched in position 2 were regarded as most similar to 2-EBA.

We did not include short chain carboxylic acids with i) one linear side chain, ii) branched side chains at other positions relative to the carboxylic acid or iii) unsaturated side chains, iv) more than one carboxylic acid, v) branched aliphatic carboxylic acids comprising additional functional groups like halogens, alcohols, aldehydes, thiols, amines, amides or aromatic moieties.

9 structurally similar aliphatic carboxylic acids, which have a branched alkyl side chains in position 2 in common, were identified (Table 1, Annex I). Structural differences were allowed only with regard to side chain length, which ranges in principle from C1 to C5. The common chemical structure of all category members can therefore be expressed as shown in Figure 1.

Figure 1. Generalised chemical structure of the category members.



Structural similarity scores of all analogues relative to the target compound were calculated using the MACCS fingerprint from RDKIT and DICE algorithm (Annex III, Table 1). Scores range from 73% (Valproic acid) to 92% (2-Methyl-butyric acid).

Closest similar to the target 2-EBA (side chain 2/2/0) are those that differ with regard to one carbon atom in one of the side chains, e.g. i) minus one carbon moiety: 2-Methylbutyric acid (2-MBA; 2/1/0, score 92%) and ii) plus one carbon moiety: 2-Ethylpentanoic acid (2-EPA, 2/3/0, score 80%). Both compounds, 2-EPA and 2-MBA do not have any *in vivo* animal study with repeated exposure. The group of analogues was therefore expanded to analogues with *in vivo* endpoint data, in this case 2-EHA (2/4/0, score 77%) and VPA (3/3/0, score 73%). The final selection of analogues include homologues compounds (plus/minus side chain length) that connect the compounds with *in vivo* data to those without *in vivo* data in a systematic way (see Table below):

Table 1. Compounds ordered according to side chain length relative to target compound

Red target compound 2-EBA, green analogues with *in vivo* endpoint data

		2-MPA (1/3/0)		
2-MBA (2/1/0)	2-EBA (2/2/0)	2-EPA (2/3/0)	2-EHA (2/4/0)	2-EHP (2/5/0)
2-MPA (3/1/0)		VPA (3/3/0)		
2-MHA (4/1/0)		2-PHA (4/3/0)		
		2-PHP (5/3/0)		

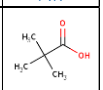
In all follow up analyses, compounds are ordered according to molecular weight and side chain length (Table 2).

Pivalic acid, with a third methyl substituent in position 2 (PVA, 1/1/1, score 66%), is included as negative control compound, as it does not induce any adverse liver effect in available *in vivo* animal studies (see chapter 4.2.2 *In vivo* endpoint data). The use of a negative compound is needed to prove predictivity and specificity of the NAM models.

All compounds tested in NAMs in this case study have been ordered in analytical grade (purity >98%, Annex I). For a few, the purity was not specified. Because of this high purity levels, we do not expect an impact of impurities on the *in vitro* results obtained in this case study. Chiral compounds with asymmetric side chain length are tested in the NAM assays as racemates like 2-EHA. The corresponding *in vivo* studies were also performed with the racemate of this chemical. In this case, study a difference in toxicity of the enantiomers is not investigated. In the following, the analogues will always be given in order of decreasing molecular weight and side chain length (Table 2).

Table 2. Overview on selected source and target compounds

Compounds are given in order of decreasing molecular weight and side chain length relative to the target compound 2-EBA.

	Source and Target Compounds										
	Source1	**Source 2**	**Source 3**	**Source 4**	**Source 5**	**Source 6**	**Source 7**	**Source 8**	**Target**	**Source 9**	**negative control**
CAS	31080-39-4	3274-29-1	3274-28-0	149-57-5	99-66-1	20225-24-5	4536-23-6	97-61-0	**88-09-5**	1730-91-2	75-98-9
Name	2-Propylheptanoic acid	2-Ethylheptanoic acid	2-PHA	-Ethylhexanoic ac	Valproic acid	2-Ethylpentanoic acid	2-Methyl-hexanoic acid	2-Methyl-pentanoic acid	**2-Ethylbutyric acid**	2-Methylbutyric acid	Pivalic acid
Abbreviation	2-PHP	2-EHP	2-PHA	2-EHA	VPA	2-EPA	2-MHA	2-MPA	**2-EBA**	2-MBA	PVA
Structure											
Structure (smiles)	CCCCCC(CCC)C(=O)O	CCCCCC(CC)C(=O)O	CCCCC(CCC)C(=O)O	CCCCC(CC)C(=O)O	CCCC(CCC)C(=O)O	CCCC(CC)C(=O)O	CCCCC(C)C(=O)O	CCCC(C)C(=O)O	**CCC(CC)C(=O)O**	CC[C@H](C)C(=O)O	CC(C)(C)C(=O)O
Similarity score	77%	77%	77%	77%	73%	80%	83%	86%	**100%**	92%	66%
Chain length from position 2	5/3/0	5/2/0	4/3/0	4/2/0	3/3/0	3/2/0	4/1/0	3/1/0	**2/2/0**	2/1/0	1/1/1

4. Data gap filling and justification

4.1. Testing and assessment strategy

The assessment and testing strategy in this case study follows the recently published EuToxRisk concept on integration of NAMs into read-across assessments (Escher & Kamp *et al.* 2019). Figure 1 illustrates the traditional six assessment steps that usually contribute to a read-across assessment (blue boxes), enriched by hypothesis driven testing of NAM models (green boxes; starting from after step 4).

In this case study, an AOP is known for the critical primary toxicological effect, which is liver steatosis (see section 4.3.1). A primary effect in this report is defined as an adverse effect as opposed to secondary effects occurring as a consequence of primary effects, e.g. extramedullary haematopoiesis e.g. in spleen or liver and all the related haematology effects secondary to the primary effect aplastic anaemia. The primary effect is likely to determine the point of departure (PoD) for risk assessment (e.g. NOAEL/LOAEL) in the *in vivo* study.

The AOP on liver steatosis illustrates potential MIEs and KEs that can be tested with different *in vitro* models to verify that the adverse outcome will be reached. The selection rational of the MIEs/KEs is described in section 4.3.1.

Figure 2. Overview of the six traditional assessment steps within the read-across assessment (blue boxes).

Generation of NAM data will be done based on the overarching read-across hypothesis. All steps to which NAM data might contribute are indicated in green. In this assessment, we assume based on the *in vivo* data of the analogues that 2-EBA might be a liver toxicant with special concern for liver steatosis. For liver steatosis an AOP is known, and the subsequent testing steps are given in Figure 3 (Figure adopted from Escher & Kamp *et al.* 2019).

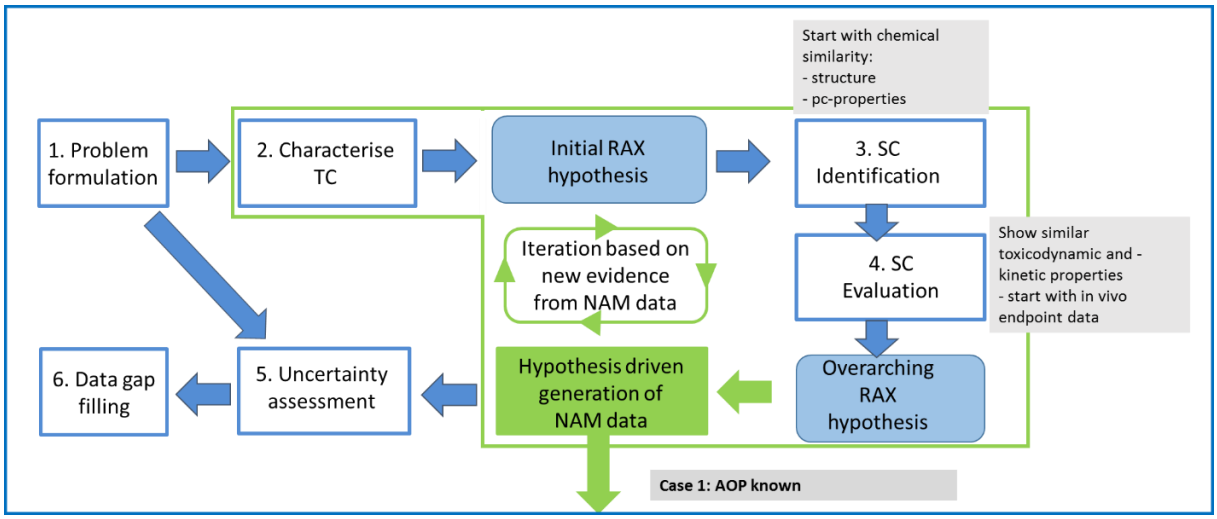
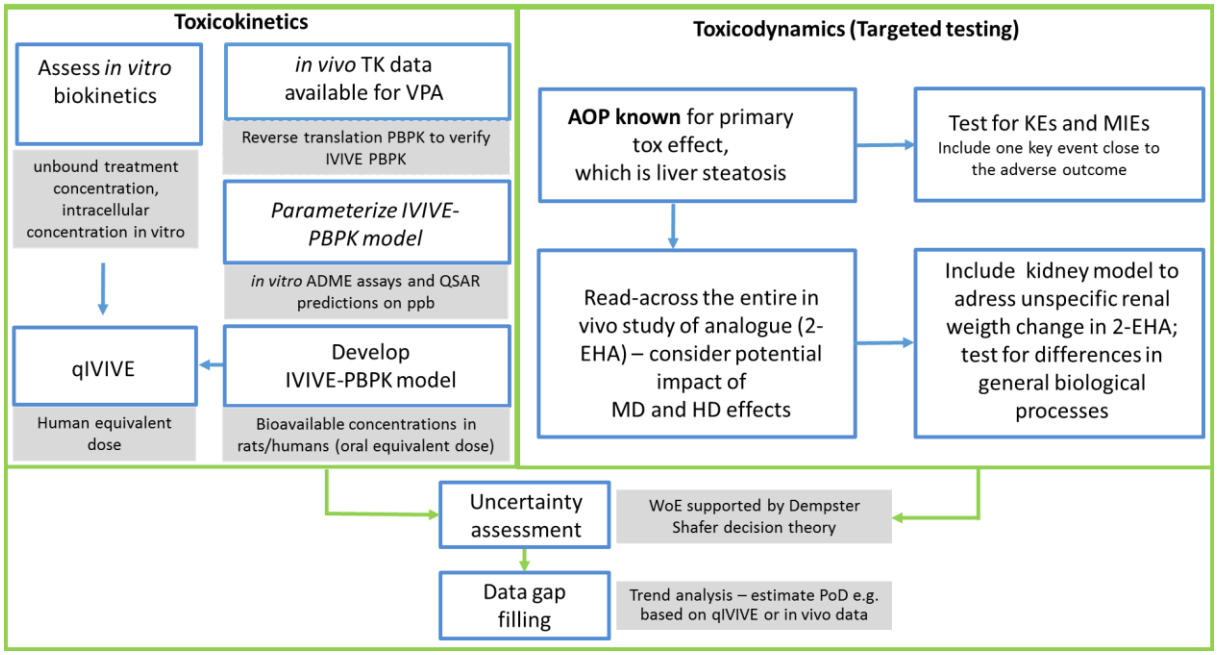


Figure 3. Schematic presentation illustrating the generation of NAM data for toxicokinetic and dynamic properties for the assessment of 2-EBA.

In this read-across an AOP for the primary toxicological *in vivo* observed liver effect (steatosis) is known and the testing of toxicodynamic properties is guided by the AOP (Figure adopted from Escher & Kamp *et al.* 2019).



Section 4. gives a comprehensive summary on the different data and assays used in the read-across evaluation. Up to step 4 (Figure 2), all existing (mainly experimental) data are used to characterise the grouped compounds, with the aim to derive an overarching read-across hypothesis (section 4.2). The read-across hypothesis guides the targeted generation of NAM data, including hazard (section 4.3) and toxicokinetic data (section 4.4).

Section 4.2: Hazard evaluation using existing data

- Physico-chemical properties and other molecular descriptors
- *In vivo* endpoint data
- Information obtained from other endpoints

Section 4.3: Hazard characterisation by targeted testing of NAMs based on read-across hypothesis

- AOP network on steatosis - Selection of MIEs and KEs for testing
- *In vitro* testing battery
- Reverse dosimetry to select *in vitro* relevant concentrations
- Evaluation of MIEs and KEs data
- Additional testing for perturbation of general cellular processes

Section 4.4: Toxikokinetic data – Characterisation of ADME properties by NAMs

- Proof of concept- Development of a human IVIVE-PBPK model based on *in vivo* and *in vitro* data on one analogue, VPA.
- Human IVIVE-PBPK model for all remaining analogues: Generation of *in vitro*/*in silico* data to parameterise the IVIVE-PBPK models for all analogues
- Analysis of differences in metabolism within the grouped compounds

The three sections give a high level summary of the data. Full documentation of the applied *in silico* or *in vitro* models is provided in the Annexes:

Annex I Data matrix, including all measured, predicted and calculated (experimental) data

Annex II Description of *in vivo* data and *in vitro* experiments

Annex III Description of *in silico* models

4.2. Hazard evaluation using existing data

For the evaluation of the grouped compounds, first all relevant existing experimental and *in silico* data have to be assessed. In this case study we consider pc-properties (section 4.2.1), *in vivo* endpoint data (section 4.2.2) and *in silico* predictions (section 4.2.3).

4.2.1. Physical-chemical properties and other molecular descriptors

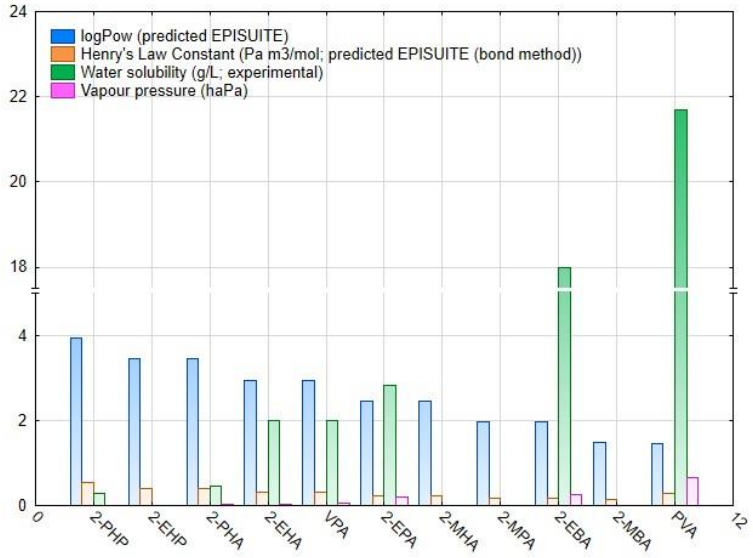
We gathered experimental and predicted physico-chemical descriptors for all 11 compounds of the read-across group (Annex I). Experimental and predicted logPow data show a high correlation (Annex I). 2-Ethylbutyric-acid shows a high solubility in water (18 g/l at 20°C), an octanol water partition coefficient of 1.68, both indicating that it is not likely that this compound will bioaccumulate in fatty tissues *in vivo*. The boiling point, melting point, vapour pressure and henry constant indicate that this compound is not volatile. The dissociation constant (4.71) indicates that the target compound is a weak acid.

The 9 selected analogues show a consistent trend dependent on side chain length with regard to vapour pressure, water solubility and logPow values. The logPow values increase with increasing chain length, ranging from 1.48 (Pivalic acid) to 3.2 (2-Propylheptanoic acid), whereas water solubility and vapour pressure decrease. Water solubility range from 21.7 g/l (Pivalic acid) to 0.465 g/l (2-Propylhexanoic acid, Figure 4). The vapour pressure is far below 1000 hPa (normal pressure), indicating that all compounds are not volatile. The pka values are in the same range as for 2-Ethylbutyric acid.

It has to be noted that experimental physico-chemical data were not available for all compounds of the dataset. The available data cover compounds with higher and smaller chain length, which allows to compare the properties of the analogues to the target compound and to identify trends. Predicted values, available for all analogues, confirm the above described observations (Annex I).

This analysis clearly indicates that all analogues are water soluble and not volatile. Taking the logPow values as first rough estimate on bioaccumulation, it is not likely that any of the grouped compound will bioaccumulate *in vivo*.

Figure 4. Physico-chemical profile of the target and source compounds including logPow, pka, vapour pressure and water solubility



4.2.2. In vivo endpoint data (preclinical in vivo data in rodents)

Three out of the 10 source compounds have repeated dose toxicity studies with oral or intraperitoneal exposure (Table 3). Only the source compound 2-Ethylhexanoic acid (2-EHA, CAS 149-57-5) has two high quality subchronic studies with oral exposure, which can directly be used for read-across. The other source compounds have shorter-term repeated dose studies, which we use as supporting information. Annex II gives a detailed overview on the observed effects per compound and study, whereas Annex I lists the LOAELs and most sensitive effects per compound.

Table 3. Overview on available repeated dose studies with oral and intraperitoneal exposure.

Name	No of studies			
	up to 14 days[2]	28 days[1]	90days[1]	Chronic[1]
Pivalic Acid		1		
2-EHA	4		2	
VPA	14	2		

1: oral 2: intraperitoneal

The critical adverse effects per compound are summarised per compound in the following:

2-Ethylhexanoic acid (2-EHA, CAS 149-57-5)

2-Ethylhexanoic acid was tested in two high quality subchronic repeated dose toxicity studies up to 1000 mg/kg bw/d in rats and about 3000 mg/kg bw/d in mice. Liver hypertrophy was observed in the mice study together with an increase of the relative liver weight, at 2 mmol/kg bw/d in rats and 6 mmol/kg bw/d in mice.

This change is accompanied by a relative weight changes in kidney (relative to body weight) and testes and by body weight and relative brain weight decrease at the next higher dose group in rats. Histopathological changes are not reported.

In mice further effects include weight changes of the kidney (at 7 mmol/kg bw/d) and an increase of the enzyme alanine aminotransferase (ALAT) and total protein (albumin) suggested a slight change in liver function at this dose level, however, there was no associated histopathological changes.

Overall, these findings indicate that 2-EHA is hepatotoxic, showing unspecific liver toxicity.

Valproic acid (VPA, CAS 99-66-1)

Several preclinical studies up to 28 days exposure are publically available. In addition, we got one oral GLP rat study from the etox project, which tested VPA with 10 days exposure in males and females with doses ranging from 100 mg/kg/bw/d to 1000 mg/kg. Only high dose effects were reported. At 1000 mg/kg bw/d weight changes in thymus and adrenals were observed as well as clinical signs like ataxia and reduced muscle tone, together with decreased haemoglobin, leucocytes and lymphocyte values. Liver effects were not observed (Annex II).

However, many other studies show that VPA predominantly provokes liver steatosis, probably by an impairment of β-oxidation of fatty acids (Annex I and Annex II). The LOAEL for liver steatosis ranges from 0.008 mmol/kg bw/d to 0.43 mmol/kg bw/d.

Overall, these findings indicate that VPA is a hepatotoxicant, which induces microvesicular liver steatosis.

Pivalic acid (CAS 75-98-9)

Pivalic acid has one subacute GLP study (28 days) with gavage exposure in Fischer 344 rats conducted according to OECD guideline 307 (details in Annex II).

There was no treatment related effect on survival. The only clinical sign seen in this study was a transient effect. Immediately after dosing the 100 or 300 mg/kg/day test formulations, rats were observed to sneeze and produce a dark nasal discharge. This transient behaviour probably resulted from a mild irritant effect of the volatile acid test substance on nasal mucosa.

A slightly lower body weight in the 300 mg/kg/day dose group females was considered to be of uncertain relation to treatment. There was no effect on male body weight, food intake, haematological parameters, or histopathological observations at any dose level.

Changes in alkaline phosphatase, cholesterol, creatinine and bilirubin in females suggested a slight change in liver function at the 100 and 300 mg/kg/day dose level, however, there was no associated histopathological changes.

Minor differences in hepatic macroscopic appearance, liver weight and kidney weight were not accompanied by any histopathological change. These differences were considered to be related to adaptive or functional changes and not a toxic effect.

There was no evidence of a cumulative toxic effect at any dose level. However, transient irritancy or adaptive changes were seen at 30, 100 and 300 mg/kg/day. Minor differences in liver and kidney weight were seen at 10 mg/kg/day, these were of uncertain relationship with treatment.

The no adverse effect level for treatment with Pivalic acid was 30 mg/kg/day for systemic and for local irritation.

Overall, these findings indicate that Pivalic acid is not toxic up to the highest tested dose. Adverse liver effects and/or steatosis were not observed.

4.2.3. Information obtained from other endpoints

QSAR profiling for DNA and protein binding

We use the profiler of the OECD QSAR toolbox to gather information on DNA (two models) and protein binding (seven models). The applied profilers flag the "presence of alerts" and the absence of known alerts should not be interpreted as a lack of toxicity. Therefore, the documentation embedded within the OECD QSAR Toolbox describing the profilers does not report their specificity since "negative predictions" should be taken cautiously. We use the predictions in sense of a similarity assessment within the grouped compounds. None of the grouped compounds has an alert for DNA binding, the DPRA profiles indicate that only a minimal or no reactivity" is likely for all compounds (Figure XY, data in Annex I). Overall, the profiler does not highlight any structural alert associated with enhanced chemical reactivity.

Profiling for skin and eye irritation

We use the profiler of the OECD QSAR toolbox to gather information on skin and eye irritation. The applied profilers flag the "presence of alerts" and the absence of known alerts should not be interpreted as a lack of toxicity. Therefore, the documentation embedded within the OECD QSAR Toolbox describing the profilers does not report their specificity since "negative predictions" should be taken cautiously. We use the predictions in sense of a similarity assessment within the grouped compounds (Figure 5; data in Annex I).

Only "2-Propylheptanoic acid" flags an alert for the exclusion rules for eye/skin irritation (i.e. this molecule should not be an irritant). It has to be noted that such an alert is based on an estimated melting point and it is known that QSPR methods are characterised by predictions errors well in excess of the error of experimental measurements. Therefore, the uncertainty attached to the pertinence of such an alert is rather high (Dearden, J. C., *et al.*, 2013).

All compounds are flagged as irritating to skin, because they contain an aliphatic acid. This corresponds well to the pka values of about 4.5 for all compounds. Both alerts are not indicating any dissimilarities between the target and the source compounds.

Figure 5. The QSAR profiler for DNA and protein binding as well as for skin and eye irritation do not indicate any dissimilarities within the grouped compounds.

Chemical name(s)	DNA binding		Eye irritation/corrosion		Protein binding									Skin irritation/corrosion	
	DNA binding by OASIS	DNA binding by OECD	Exclusion rules by BfR	Inclusion rules by BfR	Potency h-CLAT	alerts for Chromosomal aberration by OASIS	alerts for skin sensitization according to GHS	alerts for skin sensitization by OASIS	by OASIS	by OECD	potency Cys (DPRA 13%)	potency GSH	potency Lys (DPRA 13%)	Exclusion rules by BfR	Inclusion rules by BfR
2-Propylheptanoic	-	-	Group C: MP > 55 C	ND	-	-	-	-	-	-	xx	ND	xx	Group C: MP > 55 C	ND
2-Ethylheptanoic ac	-	-	x	ND	-	-	-	-	-	-	xx	ND	xx	x	Aliphatic acids
2-Propylhexanoic a	-	-	x	ND	-	-	-	-	-	-	xx	ND	xx	x	Aliphatic acids
Valproic acid	-	-	x	ND	-	-	-	-	-	-	xx	ND	xx	x	Aliphatic acids
2-Ethylhexanoic aci	-	-	x	ND	-	-	-	-	-	-	xx	ND	xx	x	Aliphatic acids
2-Ethylpentanoic ac	-	-	x	ND	-	-	-	-	-	-	xx	ND	xx	x	Aliphatic acids
2-Ethylbutyric acid	-	-	x	ND	-	-	-	-	-	-	xx	ND	xx	x	Aliphatic acids
2-Methylbutyric aci	-	-	x	ND	-	-	-	-	-	-	xx	ND	xx	x	Aliphatic acids
2-Methylpentanoic	-	-	x	ND	-	-	-	-	-	-	xx	ND	xx	x	Aliphatic acids
2-Methylhexanoic a	-	-	x	ND	-	-	-	-	-	-	xx	ND	xx	x	Aliphatic acids
2,2-Dimethylpropa	-	-	x	ND	-	-	-	-	-	-	xx	ND	xx	x	Aliphatic acids

- =no alert found; x= undefined; xx = not reactive; ND- not in applicability domain; red = flagged alert

4.3. Hazard characterisation by targeted testing of NAMs based on read-across hypothesis

4.3.1. AOP network on steatosis

Several adverse outcome pathways lead to the development of liver steatosis. Adverse outcome pathways (AOPs) are a recent toxicological construct that connects, in a formalised, transparent and quality-controlled way, mechanistic information to apical endpoints for regulatory purposes. The AOP links a molecular initiating event (MIE) to the adverse outcome (AO) via key events (KE), in a way specified by key event relationships (KER) (Leist *et al.* 2017).

The steatosis AOP network builds primarily on information accessible through AOPWiki (https://aopwiki.org) (Figure 6). The following AOPs were used:

- LXR activation leading to hepatic steatosis (https://aopwiki.org/aops/34)
- Peroxisomal Fatty Acid Beta-Oxidation Inhibition Leading to Steatosis (https://aopwiki.org/aops/36)
- AhR activation leading to hepatic steatosis (https://aopwiki.org/aops/57)
- NR1I3 (CAR) suppression leading to hepatic steatosis (https://aopwiki.org/aops/58)
- NR1I2 (Pregnane X Receptor, PXR) activation leading to hepatic steatosis (https://aopwiki.org/aops/60)
- NFE2L2/FXR activation leading to hepatic steatosis (https://aopwiki.org/aops/61)

These AOP were accessed on February 2016, are still under development and are therefore not endorsed by OECD. This gives a certain uncertainty to this AOP network, which is taken into account within the uncertainty assessment (section 6.). The detailed descriptions of the molecular initiation (MIEs) and key-events (KEs) and their relationships can be found by following the above links to the individual AOP web pages.

This AOP network is congruent with the one proposed by Mellor *et al.* (2016) and van Breda *et al.* (2018). The involvement of THRSP (thyroid hormone responsive) was added on the basis of an analysis of the toxicogenomic database TG-GATES (Igarashi Y *et al.* 2015). This gene has been shown to be expressed in liver and adipocytes, particularly in lipomatous modules. It is also expressed in lipogenic breast cancers, which suggests a role in controlling tumour lipid metabolism. Genes CPT1A, HMGCS2, CD36, HSD17B10, NRF2, SHP, LXR, PPARγ, SREBF1, and SRXN1 expressions were also found to be significantly altered following VPA exposures in TG-GATES.

An AOP is chemical agnostic and describes the development of the adverse outcome starting from the molecular initiation event, followed by key events on the cellular and organ level. Nevertheless some of the MIEs and KE were detected by testing VPA, which is one of the analogues in this case study (Figure 6, red and yellow colour).

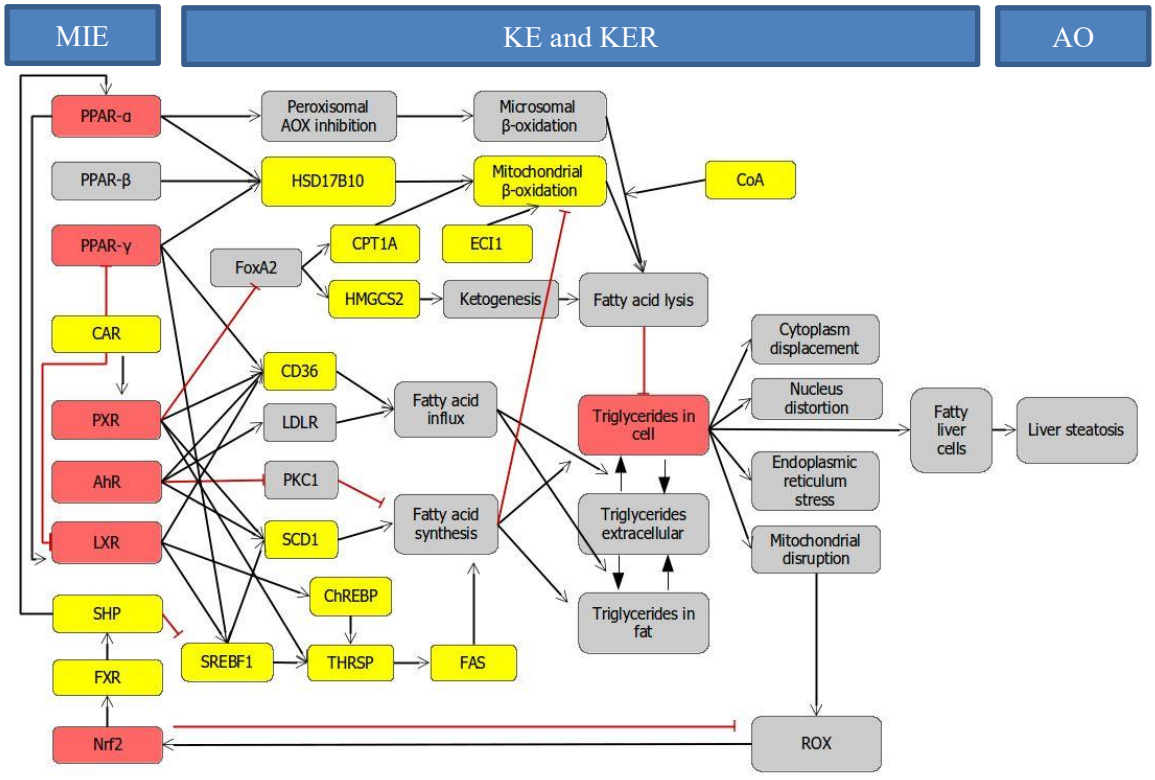
ECI1 (enoyl-CoA delta isomerase 1) was added based on TG-GATES data for VPA. This gene encodes a member of the hydratase/isomerase superfamily. The protein encoded is a key mitochondrial enzyme involved in beta-oxidation of unsaturated fatty

acids (according to NCBI). ECI1 knockdown in mice suffer from steatosis and the database CTD also indicates that ECI1 is affected by VPA. The sequestration of co-enzyme A e.g. by VPA can impair mitochondrial β-oxidation (Aires CCP *et al.* 2010, Schumacher JD & Guo GL 2015). The action of VPA on PPAR-γ, CD36, SCD1, ChREBP, SREBF1, and FAS was documented by van Breda *et al.* (2018), who also confirmed its activity on PPAR-α, LXR, PXR and AhR testing primary human hepatocytes.

The MIEs and KEs illustrated in this AOP network summarise all to date known mechanisms which might lead to liver steatosis. This does, however, not mean that branched caboxylic acids will activate all these MIEs/KEKS to induce liver steatosis. For VPA a complex mode of action has been described which includes decrease in mitochondrial β-oxidation of lipids via PPARs antagonism and potentially Nrf2 activation (reported here by the SRNX1 GFP assay), increase in fatty acids influx in hepatocytes (via PXR, AhR and LXR activation), potential increase in lipids synthesis via the same nuclear receptors and THRSP. MIEs and KEs belonging to these mechanism are coloured in red and yellow (Figure 6).

Figure 6. AOP network on liver steatosis.

MIEs and KEs which are reported for VPA (one of the source compounds) are coloured-coded: red – these six MIEs and KEs were measured in this case study; yellow - literature documented, or data-mining inferred. In addition the key event "endoplasmic reticulum stress" was measured in this case study. Black arrows indicate activation of events, whereas red lines indicate inhibitory processes.



In this case study we followed the strategy to measure a set of early events (red colour, Figure 6) together with lipid accumulation in the cell (reported as "triglycerides in cell"

in Figure 6), which is a late key event relatively close to the adverse *in vivo* outcome (lipid accumulation in the liver/or steatosis). In addition the key event "endoplasmic reticulum stress" was measured in this case study. The concordance/trend within the resultant biological fingerprint is used to conclude on a shared MoA within the grouped compounds. The selected *in vitro* assays are described in more detail in the next section.

4.3.2. In vitro testing battery

The read-across hypothesis, based on available subchronic *in vivo* data of analogues, is, that 2-EBA is potentially toxic to liver with special concern about liver steatosis.

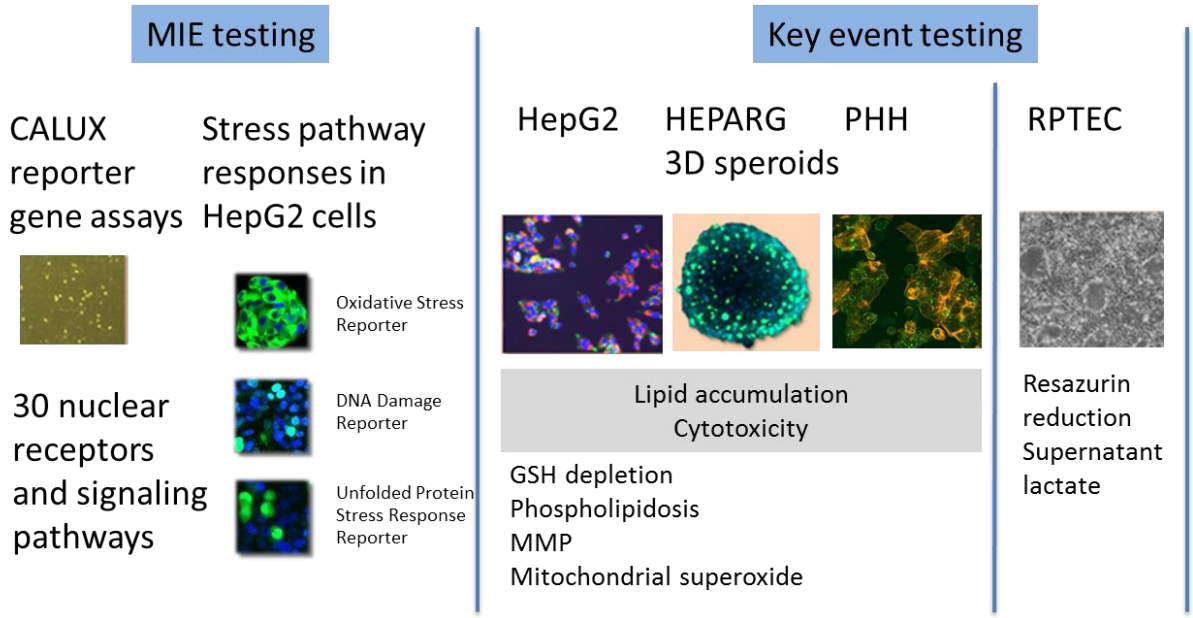
We applied several *in vitro* tests to characterise the liver toxicity of 2-EBA, compared to its 9 source and one negative compounds. Assays were selected from the EU-ToxRisk *in vitro* toolbox. *In vitro* assays specifically testing for molecular initiation and key events described in AOP network on steatosis (Figure 6) were combined with assays, that inform about the perturbation of main biological cellular functions (Figure 7).

Two reporter gene assays, Calux and stress pathway responses in HepG2 cells, were used to test the activation PPAR-a, PPAR-y, PXR, AhR, LXR and Nrf2, all belonging to the AOP network on liver steatosis (Figure 6). We further selected three liver models which differ with regard to their metabolic capacity (2D HepG2 cells < 3D HepaRG cells < primary human hepatocytes) and tested cytotoxicity and accumulation of trigylcerides.

To reveal any other major difference between the grouped compounds we further addressed perturbation of basic biological processes in HepG2 cells like glutathione (GSH) depletion, disruption of mitochondrial membrane potential, superoxide formation and phospholipidosis. In addition, a number of other receptor gene assays were tested to cover a broader mechanistic range.

The analogue 2-EHA showed a renal weight increase at higher dosing compared to the lead liver effect. Therefore, in addition to liver models viability was evaluated in the human kidney cell line RPTEC by measuring resazurin reduction and lactate in the supernatant.

Figure 7. Overview on test systems used for hazard characterisation



4.3.3. Reverse dosimetry to select in vitro relevant concentrations

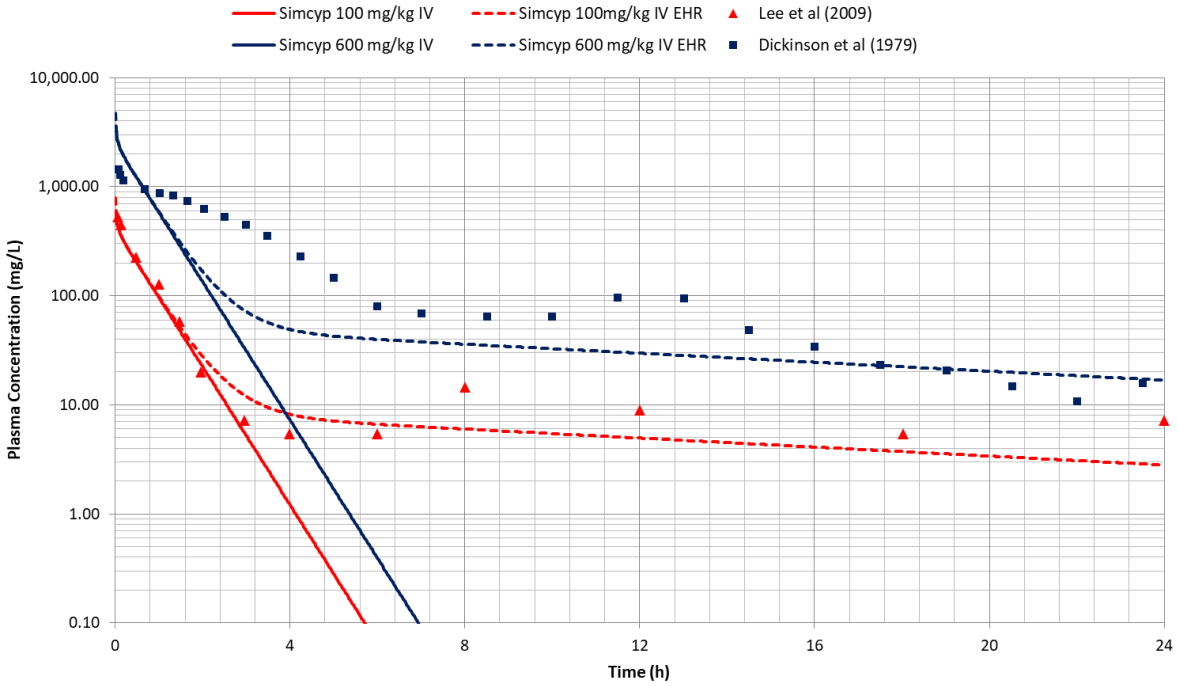
In vitro test might lead to unspecific responses in case of unrealistic high dosing regimens. Therefore, the a rat PBPK model was developed to guide the selection of a concentration range for *in vitro* testing by translating nominal LOEL doses from *in vivo* rat studies to unbound plasma concentrations. This rat PBPK model was established based on published data for VPA.

Kinetic studies in rats show that enterohepatic recirculation (EHR) is a critical mechanism in the *in vivo* exposure of rats (Dickinson, Harland *et al.* 1979, Lee, Lee *et al.* 2010). In contrast to the human VPA model developed based on *in vitro* data, the rat VPA model was developed using a reverse translation approach, back-calculating hepatic intrinsic clearance based on systemic *in vivo* clearance. The rat model was also constructed incorporating EHR through back conversion of biliary cleared glucoronidated metabolites in the gut to parent compound. Simulations with this model recovered the observed data across a doing range from two independent studies; model inputs and simulation outputs are shown below (Figure 8).

Figure 8. PBPK modelling of rat plasma concentration of VPA, for two doses applied intra venous (red- 100 mg/kg bw; blue 600 mg/kg bw).

The models with integrated enterohepatic recirculation (HER, dashed lines) simulates the plasma concentrations fairly well, compared to the experimentally reported values (squares and triangles). Models without EHR (straight lines) fail to model the plasma concentration 4h after application.

Parameter	Value	Method / Comment	Source/Reference
MW [g/mol]	144.21		EPI-Suite (v4.1, US-EPA)
logP	2.75	experimental	EPI-Suite (v4.1, US-EPA)
Compound Type	Monoprotic acid		
pKa	4.8		ACD/Percepta (2012 release, build 2254, ACD Labs)
TPSA (Å^2)	37.3		https://pubchem.ncbi.nlm.nih.gov/compound/3121#section=Chemical-and-Physical-Properties
Hydrogen bond donors	1		https://pubchem.ncbi.nlm.nih.gov/compound/3121#section=Chemical-and-Physical-Properties
fu	0.35	experimental	(Loscher 1978)
B/P ratio	0.74	assumed	(Loscher 1978)
fa	0.997	predicted using HBD and PSA (Simcyp v17r1), ADAM	(Winiwarter, Bonham *et al.* 1998, Winiwarter, Ax *et al.* 2003)
ka (h^{-1})	5.955	predicted using HBD and PSA (Simcyp v17r1), ADAM	
fu_{Gut}	1	assumed	
CLint (µl/min/mg protein)	10.71	Obtained through reverse translation from observed data (Kameya, Hokama *et al.* 2009)	See Annex III



In vivo to in vitro translation simulations in rat

The verified rat PBPK VPA model incorporating EHR was used to guide the selection of a concentration range for *in vitro* testing by translating established LOEL doses to unbound plasma concentrations. Rat *in vivo* studies, using intraperitoneal and intravenous dosing, have determined a LOEL of 500 mg/kg (Loscher, Fisher *et al.* 1989,

Abdel-Dayem, Elmarakby *et al.* 2014). An oral repeat dosing study (500mg/kg, 4 days, τ=24h) was simulated. Based on this a maximum unbound plasma concentration of 2.5mM was identified as corresponding to the LOEL established *in vivo*. *In vitro* studies, using the Huh7 human hepatocyte cell line, have previously shown accumulation of lipid droplets after 24hr treatment with 0.5 mM VPA (Elphick, Pawolleck *et al.* 2012).

The *in vitro* concentrations chosen for all assays include the *in vivo* observed relevant dose of about 2 mM and tests above and below this concentration. Typically, the assays used five to eight concentrations e.g. 8mM - 4mM - 2 mM – 1 mM – 0.5 mM – 0.25 mM – 0.125 mM – 0.0625 mM.

4.3.4. Evaluation of MIEs

High through put assays were used to measure the activation of several MIEs as described in the AOP (Figure 9), the other nuclear receptors and signalling pathways are seen as indicators for general biological perturbation.

Experimental data on MIEs

The CALUX test battery measures receptor agonism, antagonism or cell signalling pathways by assessing luminescence in human osteosarcoma cell line U2_OS or the rat hepatoma H-4-II-E cell line (AhR CALUX only, see Figure XY, all details in Annex II). The U2_OS cell line does not endogenously express most nuclear receptors and/or metabolic enzymes. Therefore, this cell line is ideally suited to study the interaction of a compound with a specific receptor or pathway, without interference of metabolism or receptor cross-talk. This makes interpretation of the results relatively straightforward, and allows comparison of compounds on the level of MIEs. Reported values are lowest effect concentrations (LECs) given in Log(M) units (details in Annex II). Some of the reporter gene assays directly measure MIEs as described in the AOP network (see Figure 9 above). These are: Nrf2 (oxidative stress), PXR (pregnane X receptor agonist); PPAR-α and γ (peroxisome proliferator activated receptor agonist), AhR (Aryl hydrocarbon receptor agonist) and LXR (liver X receptor agonist).

The HepG2 BAC GFP reporter platform was used to measure the activation of cellular stress pathways accurately and over time on a single cell level. The GFP reporter platform includes HepG2 reporters for oxidative stress (e.g. SRXN1) and DNA damage (e.g. p21). HepG2 reporters can be imaged after test compound exposure in a high throughput format (384 well plates) at chosen time points or throughout a 72h live imaging period.

A general finding is, that none of the compounds were cytotoxic according to the CALUX assay up to the highest tested dose of 100 µM. The HTS panel results show a trend towards an increase of potency with increasing chain length as indicated by the lowest effect concentrations (LEC, Figure 10). Also the number of activated reporters increase with increasing chain length (Figure 11. Lipid accumulation in HepG2 cells, HepaRG cells and PHH.

Test results, which did not show any lipid accumulation up to the highest tested dose are depicted as “zero” in this plot. The value of PVA is considered as an artefact of the test analysis - it was flagged as not significant. The MEC or IC20 values, summarized as lowest effect concentrations (LEC) represent -logM units.). The longer chain compounds (2-EHA and below) are consistently active on the p21, ESRE, anti-PR, p53, TRβ, TCF

and PXR, while the short chain compounds are (2-Methylhexanic acid and above) do only active PPAR-α and 2-MBA in addition Nrf2.

The target compound 2-EBA activates PPAR-α, but does not show any other response up to the highest tested dose of 100 µM. PPAR-α is activated by all compounds except of Pivalic acid and 2-Methylbutyric acid. PPAR- α is however a MIE reported in the AOP network (Figure 9), indicating that 2-EBA might activate this AOP. The closest similar analogues 2-MBA activates stress response reporters (Nrf2, SRXN1) and p21, 2-MPA activates only PPAR-α comparable to the target compound.

VPA, which induces steatosis *in vivo*, activates 10 reporter genes, namely PR-anti; TRb; PXR; PPAR-α; TCF; ESRE; p21 and p53; SRXN1 and p21 from GFP panel. PPAR-α, PXR and SRXN1 are MIEs in the AOP network for liver steatosis. 2-EHA, which shows liver hypertrophy and potentially lipid accumulation *in vivo*, activates the same reporters as VPA and in addition, Nrf2 and AR-anti, but not TRβ. PPAR-α, PXR and Nrf2 and anti-AhR are MIEs in the AOP network for liver steatosis.

Pivalic acid does not show any activity up to the highest tested *in vitro* dose and was also not liver toxic in the available *in vivo* studies.

None of the category members activated the "unfolded protein stress response" reporter, which indicates endoplasmic reticulum stress, one key event reported in the AOP network.

Overall, these results indicate a trend towards a greater number of activated receptors/pathways with increasing chain length (Figure 9, "No. of activated endpoints).

Figure 9. Scatterplot of LEC/EC_{20} values for MIEs being part of the AOP network (Figure 8, minus log molar units are shown).

Red - Calux results; blue - HepG2 GFP results

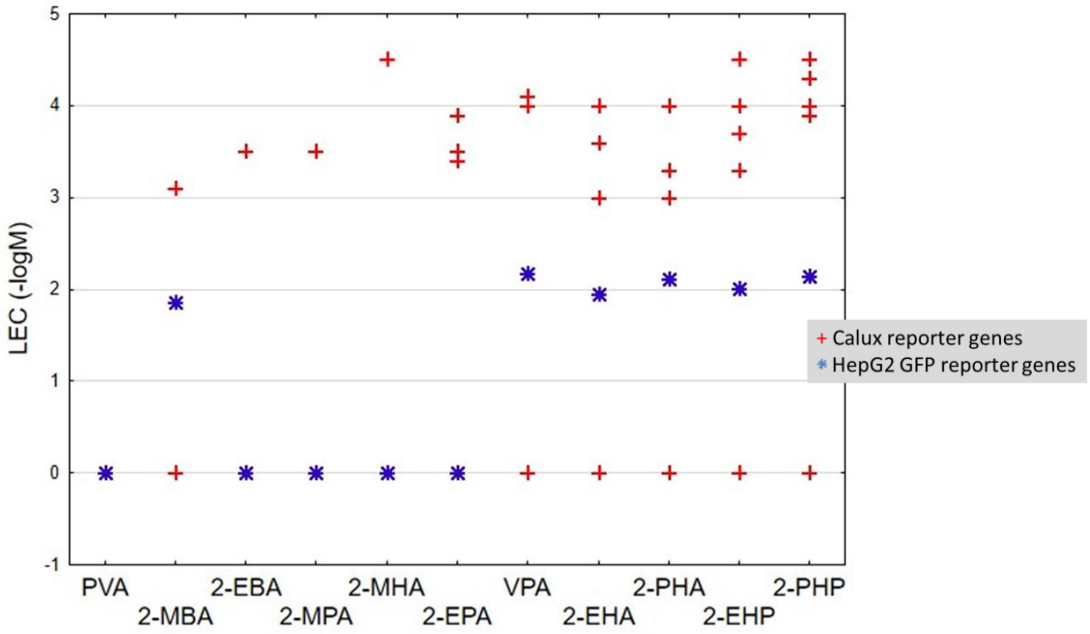


Figure 10. Outcome of the high throughput assay CALUX and GFP.

Results are shown in log molar units. Compounds inactive up to the highest tested dose are indicated in green, all others in red. Compounds are shown in order of increasing chain length from top to bottom. The number of activated endpoints/MIEs is counted based on activity per individual endpoint, e.g. p21 is counted only once if activated by CALUX or GFP panel. Nrf2 and SRXN1 are also counted only once, as they both measure different events in the same oxidative stress pathway.

		Calux assay																										GFP assay		No. of active endpoints	No. of MIEs in AOP
CAS	Name	Cytotox	Era	ERa-anti	AR	AR-anti	PR	PR-anti	GR	GR-anti	TRb	TRb-anti	RAR	LXR	PXR	PPARa	PPARd	PPARg	AhR	Hif1a	TCF	AP1	ESRE	NFkB	Nrf2	p21	p53	p53 S9	SRXN1	P21		
75989	Pivalic acid	>3	>3	>3	>3	>3	>3	>3	>3	>3	>3	>3	>3	>3	>3	>3	>3	>3	>3	>3	>3	>3	>3	>3	>3	>3	>3	>3	>-1,79	>-1,79	0	0
1730912	2-Methylbutyric acid, (S)-	>3	>3	>3	>3	>3	>3	>3	>3	>3	>3	>3	>3	>3	>3	>3	>3	>3	>3	>3	>3	>3	>3	>3	-3,1	>3	>3	>3	-1,85	-1,92	2	1
88095	2-Ethylbutyric acid	>3	>3	>3	>3	>3	>3	>3	>3	>3	>3	>3	>3	>3	>3	-3,5	>3	>3	>3	>3	>3	>3	>3	>3	>3	>3	>3	>3	>-1,79	>-1,79	1	1
97610	2-Methylpentanoic acid	>3	>3	>3	>3	>3	>3	>3	>3	>3	>3	>3	>3	>3	>3	-3,5	>3	>3	>3	>3	>3	>3	>3	>3	>3	>3	>3	>3	>-1,79	>-1,79	1	1
4536236	2-Methylhexanoic acid	>3	>3	>3	>3	>3	>3	>3	>3	>3	>3	>3	>3	>3	>3	-4,5	>3	>3	>3	>3	>3	>3	>3	>3	>3	>3	>3	>3	>-1,79	-1,84	2	1
20225245	2-Ethylpentanoic acid	>3	>3	>3	>3	>3	>3	>3	>3	>3	>3	>3	>3	>3	-3,5	-3,9	>3	>3	>3	>3	-3	>3	>3	>3	-3,4	>3	-3	>3	>-1,79	>-1,79	5	3
149575	2-Ethylhexanoic acid	>3	>3	>3	>3	-3,4	>3	-3	>3	>3	>3	>3	>3	>3	-3,6	-4	>3	>3	>3	>3	-3,5	>3	-3	>3	-3	-3	-3,1	>3	-1,95	>-1,79	9	3
99661	Valproic acid	>3	>3	>3	>3	>3	>3	-3,1	>3	>3	-3	>3	>3	>3	-4,1	-4	>3	>3	>3	>3	-4	>3	-4	>3	>3	-3,2	-3,6	>3	-2,18	-1,98	8	3
3274280	2-Propylhexanoic acid	>3	>3	>3	>3	-3,5	>3	-3,9	>3	>3	-3,1	>3	>3	>3	-4	-4	>3	-3,3	>3	>3	-3,5	-3	-3	>3	-3	-3,2	-3,7	>3	-2,11	-2,07	12	3
3274291	2-Ethylheptanoic acid	>3	>3	>3	>3	-3,5	>3	-3,5	>3	>3	-3,4	>3	>3	>3	-4	-4,5	-3,1	-3,3	>3	>3	-3,6	>3	-3,5	>3	-3,7	-3,4	-3,5	>3	-2,01	>-1,79	12	3
31080394	2-Propylheptanoic acid	>3	>3	>3	>3	-3,5	>3	-3,6	>3	>3	-3,5	>3	>3	>3	-4,3	-4,5	-3	-3,9	-3	>3	-4,3	-4	-4	>3	-4	-3,7	-4	>3	-2,15	-2,34	14	4

MIE predictions by QSAR modelling

We applied four *in silico* QSAR models for predicting molecular initiating events (MIEs) for the 11 compounds of this read-across group. All four models were developed and validated using data from ToxCast (https://www.epa.gov/chemical-research/toxicity-forecaster-toxcasttm-data).

The activity of all compounds did not differ for all predicted MIEs. As many results were not in the applicability of the models, these data are not used in the assessment of this read-across case study in the following. The QSAR results are in full detail described in Annex III.

4.3.5. Testing of KE - Lipid accumulation

One KE in the AOP is accumulation of lipids (Figure 6). This KE is very close to the actual adverse outcome in vivo, which is accumulation of lipids in the liver and is therefore considered to be of crucial importance to confirm the read-across hypothesis.

Lipid accumulation was measured after 24h and 72h in HepG2 cells (single exposure) and after repeated exposure in HepaRG (10 days exposure; 5 treatments) and single and repeated exposure in freshly isolated primary human hepatocytes (PHH) from three different donors. All assays tested 5 to 8 concentrations from 0.062 mM to 8 mM, with up to 30mM in PHH.

HepG2: We used a robust and reliable *in vitro* test system that is able to detect microvesicular steatosis in response to VPA treatment. Having tested the different protocols, timepoints, and run the interlab comparison using two compounds a testing strategy and protocol for the remaining compounds was decided upon. The original concentration range (0.0625 - 8 mM) was found to be appropriate in that it captured the point of departure from the baseline for the endpoint of interest whilst also capturing the cytotoxic range at higher concentrations. The ideal time points in HepG2 cells were found to be 24 hours, 72 hours single dose (details in Annex II).

HepaRG: We tested 3D HepaRG speroids for 10 days with 5 treatments. As above the concentrations of the compounds ranged from 0.0625 to 8mM. Lipid accumulation and cytotoxicity were measured.

PHH: Lipid accumulation was tested for three different compounds, Valproic acid, Pivalic acid, 2- Propylhexenoic acid, in primary human hepatocytes from three different donors using sandwich culture conditions. The lipid accumulation was tested after 1 day and 5 days of treatment with the study compound in medium containing 62µM fatty acids.

Description of results

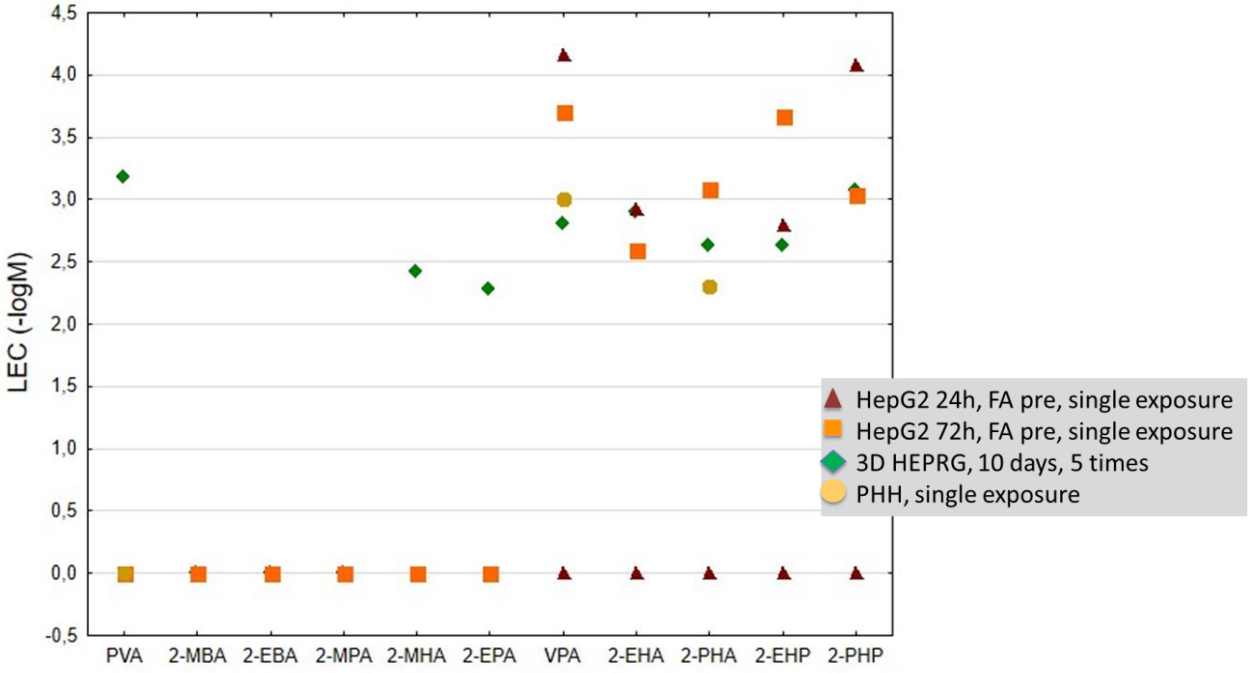
2-EBA is inactive in HepG2 and HepaRG assays. VPA (CAS 99-66-1) showed an increase of lipids in all assays. 2-EHA (CAS 149-57-5) also induced lipid accumulation in HepG2 and HepaRG cells, with slight lower potency compared to VPA. Pivalic acid was inactive in the majority of test systems, namely in both HepG2 assays and PHH from two out of three donors. A slight increase of lipids was observed, however, in 3D HepaRG cells after 10 day exposure and 5 treatments. This finding has a slight downward trend and was probably an artefact of the data analysis rather than a real response. Therefore the validated analysis software flagged it as "NS" (not significant). In conclusion, we classify Pivalic acid as negative in all liver assays up to the highest tested dose.

It can therefore be concluded, that all three compounds with *in vivo* data were well predicted by the three different *in vitro* liver assays.

The other compounds show a chain length dependent trend. Compounds with longer side chains compared to 2-EBA (from 2-MHA on) induced lipid accumulation, whereas the two most similar analogues 2-MPA and 2-MBA do not induce lipid accumulation. 2-EBA was inactive up to the highest tested dose in all three liver models. Pivalic acid was also inactive. The result for 2-MHA is equivocal; lipid accumulation is observed at a minimal effect concentration of 3.82 mM in HepaRG cells, but not in both assays with HepG2 cells (Figure 11, Annex I). The closest similar compounds to 2-EBA (2-MBA and 2-MPA) do not show lipid accumulation in any of the *in vitro* tests, comparable to the target compound.

Figure 11. Lipid accumulation in HepG2 cells, HepaRG cells and PHH.

Test results, which did not show any lipid accumulation up to the highest tested dose are depicted as "zero" in this plot. The value of PVA is considered as an artefact of the test analysis - it was flagged as not significant. The MEC or IC_{20} values, summarized as lowest effect concentrations (LEC) represent -logM units.



4.3.6. General perturbation of biological processes in vitro

Cytotoxicity

All assays measure the viability of the cells induced by the tested compounds (Annex I,).

In primary human hepatocytes with and without fatty acid pretreatment the mean IC_{20} from three individual donors is above 8 mM (>3.9 logM). Only Pivalic acid shows a logIC_{20} value of 3.6 mM.

In 3D HepaRG cells cytoxicity decreases with side chain length. The target compound 2-EBA has a minimal effect concentration (MEC) comparable to VPA and 2-EHA. In HepG2 cells, after 24h, there is also a trend of decreasing toxicity with side chain length, which is less evident after 72h exposure.

In the kidney cell line RPTEC, all compounds were practically non toxic up to the highest tested dose.

Overall, it can be concluded that there is a trend to a slightly lower cytotoxicity with decreasing side chain length. VPA and 2-EHA were always more toxic compared to PVA. 2-EBA was in all assays practically non-toxic up to the highest tested dose, with the exception of the ATP measurement in HepaRG cells, where the AC_{50} value was 4-6 fold higher than VPA and 2-EHA, the MEC however in the range of the MEC of both compounds.

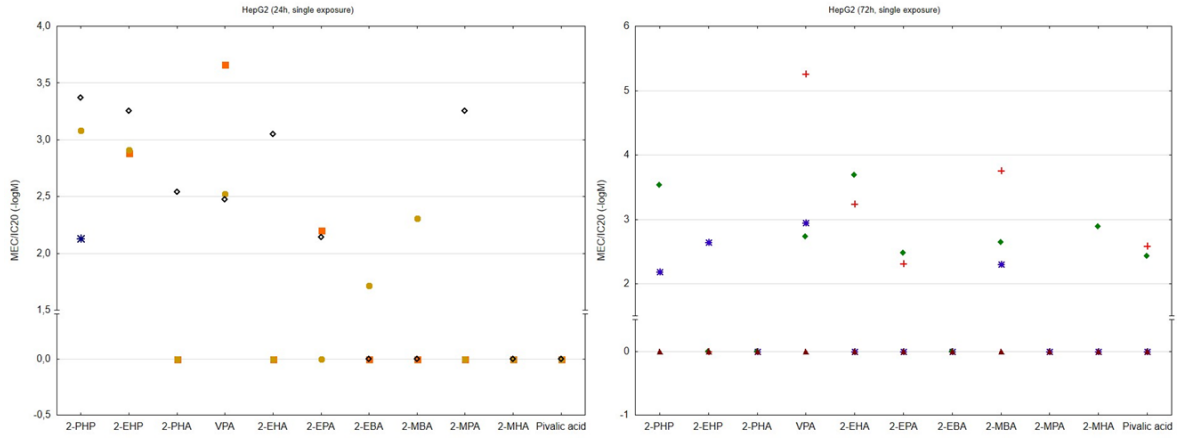
In vitro testing of perturbation of general biological processes

HepG2: In HepG2 four important cellular mechanisms were tested, such as glutathione depletion, phospholipidosis, mitochondrial superoxide production or mitochondrial membrane potential (Figure 12; details in Annex II).

None of the tested compounds induced phospholipidosis. 2-EBA activates only one of these cellular mechanisms which is a change in mitochondrial potential (MMP) after 24h. The TC is less potent than all other tested compounds, except of PVA, which remains inactive. VPA induced GSH depletion, MMP and mitochondrial superoxide formation, whereas 2-EHA also only showed induction of mitochondrial superoxide formation. After 24h there is a trend towards higher activity and potency with increasing chain length. This trend is less evident after 72h exposure.

Figure 12. Other functional endpoints tested in HepG2 cells

Left 24h; right 72h exposure - GSH depletion (orange (left), red cross (right)), MMP (yellow, blue star), Mitochondrial superoxide formation (black, green) and phospholipidosis (red triangle), (-logM) units are shown.



4.4. Characterisation of ADME properties by NAMs

In vivo ADME data are not available for the majority of the source and target compounds. Therefore, we use PBPK modelling informed by *in vitro* assays, termed IVIVE-PBPK modelling, to calculate the plasma and tissue concentrations of the individual compounds.

Physiochemical data, *in vitro* data (e.g. hepatic intrinsic clearance, $CL_{int, Hep}$; µl/min/10^6 cells) and, in the absence of in vitro data, QSAR based predictions (e.g. fraction unbound in plasma, fu) were used to parametrise human PBPK models in the Simcyp simulator (Simcyp v17r1, Certara Ltd. Simcyp Division, Sheffield, UK). Clinical pharmacokinetic data in humans was only available for VPA. This data to were used to verify the performance of the IVIVE-PBPK modelling approach and demonstrate a proof of concept for the RAX compound series of branches carboxylic acids. Simulations with the human VPA PBPK model showed good recovery of the observed data. Subsequently, bioavailable concentrations were predicted and *in vitro* to *in vivo* extrapolation performed using PBPK based reverse-dosimetry.

4.4.1. Development of a human IVIVE-PBPK model

In constructing the PBPK models for the read-across study, the following aspects were considered as suggested in the WHO PBPK guidance (WHO publication Harmonisation Project Document No. 9. CHARACTERIZATION AND APPLICATION OF PHYSIOLOGICALLY BASED PHARMACOKINETIC MODELS IN RISK ASSESSMENT):

1) Parent compound was assumed to be the toxic moiety (i.e. plasma and tissue levels of formed metabolites were not routinely considered in the PBPK models).
2) Metabolism is thought to be the major clearance pathway of the compounds in this read-across case study and the clearance in human subjects was predicted using an *in vitro – in vivo* extrapolation approach using intrinsic clearance data generated in human hepatocytes.
3) The physiology (i.e. tissue weights and blood flow rates) of the species of interest were the default values in the Simcyp rat and human simulator (V17r1, Certara Ltd. Simcyp Division, Sheffield, UK).

The following assumptions were made in the development of PBPK models for the read-across study:

1) The metabolism of the compounds is assumed to be linear (i.e. not to saturate) over the range of doses simulated.
2) Plasma protein binding is assumed to be linear (i.e. not to saturate) over the range of doses simulated (this is explained in detail in the following)
3) There is no intestinal metabolism or metabolism in any of the tissues in the PBPK model apart from in the liver.
4) Potential cleavage of the compounds in the blood was not considered in the models.

A summary of the human VPA model parameters are given in the table below:

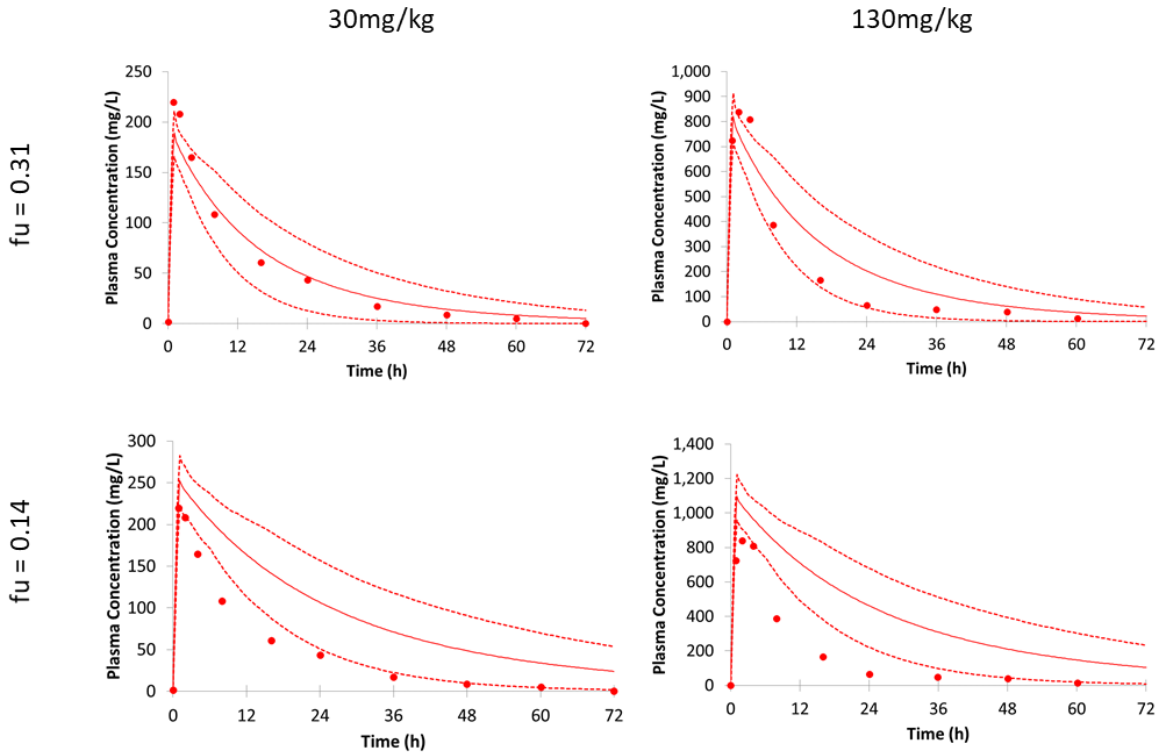
Parameter	Value	Method	Source/Reference
MW [g/mol]	144.21		EPI-Suite (v4.1, US-EPA)
logP	2.75	exp.	EPI-Suite (v4.1, US-EPA)
Compound Type	Mono-protic acid		
pKa	4.8		ACD/Percepta (2012 release, build 2254, ACD Labs)
TPSA (Å^2)	37.3		https://pubchem.ncbi.nlm.nih.gov/compound/3121#section=Chemical-and-Physical-Properties
Hydrogen bond donors	1		https://pubchem.ncbi.nlm.nih.gov/compound/3121#section=Chemical-and-Physical-Properties
fu	0.138 0.310	Pred. (min) Pred. (max)	Lhasa CORAL model
B/P ratio	0.55	assumed	
fa	0.996	Pred. (Simcyp v17r1)	(Winiwarter, Bonham *et al.* 1998, Winiwarter, Ax *et al.* 2003)
ka(h^{-1})	2.546	Pred.(Simcyp V17r1)	
fu_{Gut}	1	assumed	
CLint (µl/min/10^6 cells)	0.219	Exp.	HREL co-culture system; Cyprotex data (CYP1440)
Hepatocyte binding (fu heps)	0.954	Pred.	(Kilford, Gertz *et al.* 2008)

Legend: fu=faction unbound; Clint = intrinsic clearance rate in primary hepatocytes; ka=absorption rat; fa= fraction absorbed; B/P ratio= blood plasma ratio; TPSA topological polar surface area.

Experimental values for B/P and fraction unbound (fu) were unavailable for the development of this model and so predicted/assumed values were used; the justification of these decisions is discussed below. Using the data available to verify VPA model performance, simulations using the minimum and maximum QSAR predicted fu showed good recovery of observed plasma concentration-time profiles. Simulations across an iv dosing range from 15-150 mg/kg predicted observed plasma C_{max} (mg/L) and AUC_{inf} (mg/L.h) within -1-2 and 1-6 fold, respectively.

Figure 13. Verification of IVIVE-PBPK modelling for human plasma concentration of VPA.

100 virtual individuals (proportion of females = 0.1) were simulated, mean of mean of simulated population (red line), percentile (5th, 95th, red dashed lines), observed experimental data for VPA (red points; Georgoff, Nikolian *et al.* 2018).



Sensitivity analyses, parameter uncertainty and model assumptions

For some model parameters there was uncertainty as to what is the correct input value, or modelling assumptions to use. For these parameters sensitivity analysis and the discussed prototype modelling was conducted, using Valproic acid as an exemplar compound, and the impact on the simulation results (namely systemic plasma exposure as judged by the area under the plasma concentration time profile) was evaluated.

Blood to plasma ratio

Acidic compounds, significantly ionised at physiological pH, do not extensively distribute into erythrocytes. While experimental data was generated for the read-across compounds, this was confounded by analytical issues with remaining data gaps for many of the source, and the target, compound of the read-across. Sensitivity analysis with the available prototype models showed only low sensitivity of the model to variability in this input parameter (see table below). Simulations with the prototype model using the assumption that the B/P is equal to one minus the human reference haematocrit value (0.45), showed good recovery of the observed data (above). Based on this and the comparable ionisation of these compounds at the pH of human plasma, this assumption was applied to the modelling of all read-across compounds.

Plasma protein binding (fu)

An assessment of the most reliable model to use is limited to a review of the predictions generated for Valproic acid (CAS 99-66-1), since this is the only compound for which experimental human values are available. Reported values for the plasma protein binding of Valproic acid vary as a result of concentration dependent (saturable) binding (Ogungbenro, Aarons *et al.* 2014). Sensitivity analysis covering the range of plasma protein binding values predicted for VPA (fu = 0.1-0.4), shows that predicted plasma exposure (AUC) decreases with increasing fu parameter values.

While the non-linearity in plasma protein binding for VPA is described in the literature, and simulations incorporating this mechanism showed good recovery of the observed data, there is insufficient data to inform this modelling decision across all the other read-across compounds. The assumption of saturable, non-linear protein binding, whilst also assuming linear metabolism, shows a tendency to under-prediction of exposure with increasing dose; AUC was predicted within 2 to -2 fold of observed data with increasing dose (iv, 30-130 mg/kg). However, predictions of plasma C_{max} across the same dosing range were comparable simulations using the minimum and maximum predicted fu values, 1-2 fold of observed data.

Including the concentration-dependent plasma protein binding is therefore not consistent with a conservative modelling approach. Experimental plasma protein binding data were not available for the grouped compounds. Therefore, based on data availability, and the adoption of a conservative predictive modelling strategy, we used four different QSAR models in the course of the read-across. Each compound was modelled at the lowest and highest predicted fu value assuming linear, non-saturable plasma protein binding.

Other parameters tested that the model was not sensitive to included cardiac output, hepatic and renal blood flow. Sensitivity analysis results are presented in the table below as high (absolute value greater than or equal to 0.5), medium (absolute value greater than or equal to 0.2 but less than 0.5) or low (absolute value greater than or equal to 0.1 but less than 0.2); parameters with sensitivities less than 0.1 are not tabulated. A sensitivity ratio of 1 implies that a 1% change in input of a parameter value leads to a 1% change in dose metric prediction. Uncertainty is a subjective assessment of how reliable the input parameters are. A formal uncertainty analysis as suggested in the WHO PBPK guidance is difficult to perform with a bottom up PBPK model as the ratio of median to 95th percentile reflects a measure of variability rather than true uncertainty as could be obtained if the PBPK model parameters were fitted to an observed dataset. The *in vitro* intrinsic hepatic clearance (CL_{int}) value is considered medium uncertainty based on analytical challenges of measuring this parameter *in vitro* for these compounds.

		UNCERTAINTY		
		High	**Medium**	**Low**
SENSITIVITY	**High**	plasma fu		
	Medium			
	Low		*in vitro* CLint	B/P

4.4.2. Human IVIVE-PBPK model for all remaining analogues

Based on the assumptions approved by using the VPA exemplar model, all remaining read-across source and target compounds were modelled using an IVIVE-PBPK approach. In the IVIVE –PBPK model hepatic intrinsic clearance data from *in vitro* assays are used as well as the lowest and highest predicted value for fu (see above).

Intrinsic hepatic clearance

Intrinsic hepatic clearance was measured using a low clearance assay as described in Annex II. Briefly this method utilised the Hμrel low clearance assay which is a system that contains plated primary hepatocytes able to remain viable in culture for 72 hours. The relative compound concentration was measured at specific time points using mass spectrometry during the incubation and a Clint value obtained for each compound.

The table below contains the data obtained using the low clearance assay for 2-EBA and analogues. It was not possible to detect Pivalic acids and 2-MBA using mass spectrometry.

Clearance in hepatocytes decrease with longer side chain length. Clearance rates of the long chain analogues 2-PHP, 2-EHP and 2-PHA are below the limit of detection of the low clearance assay (approximately CL_{int} = 0.137 µl/min/million cells), and therefore are regarded to be not reliable. Oral equivalent doses for 2-PHP, 2-EHP and 2-PHA have been calculated based on the assumption that these compounds have an intrinsic hepatic clearance half that of VPA (CL_{int} = 0.11 ul/min/million cells), which has the lowest experimentally determined intrinsic clearance of the compound series (see chapter 5. Read-across justification).

Table 4. Intrinsic hepatic clearance and calculated half-lives of all grouped compounds.

Compounds indicated in yellow show clearance rates at or below limit of detection and are therefore considered to be outside the applicability domain of this assay.

Compound ID	CAS number	CL_{int} (µL/min/10^6 cells)	SE CL_{int}	$t_{1/2}$ (min)	n
2-PHP*	31080-39-4	0.0902	0.0374	20500	6
2-EHP*	3274-29-1	0.0361	0.0423	51200	6
2-PHA*	3274-28-0	-0.0464	0.0537	-39800	6
2-EHA	149-57-5	0.551	0.115	3360	6
VPA	99-66-1	0.219	0.200	8420	6
2-EPA	20225-24-5	0.779	0.370	2370	4
2-MHA	4536-23-6	3.95		468	2
2-MPA	97-61-0	10.2	1.97	182	3
2-EBA	88-09-5	9.62	2.00	192	3
2-MBA	Not detectable in mass spectrometry				
PVA	Not detectable in mass spectrometry				

Legend: CL_{int}: intrinsic clearance in hepatocytes; SE: standard error; $t_{1/2}$: half-life ;*outside applicability domain of assay – for these compounds a low clearance rate of 0.11 µl/min/106 cells was assumed).

Plasma Protein binding

We applied four different *in silico* QSAR models for predicting plasma protein binding for all the 11 compounds of the read-across group (Annex I and Annex III).

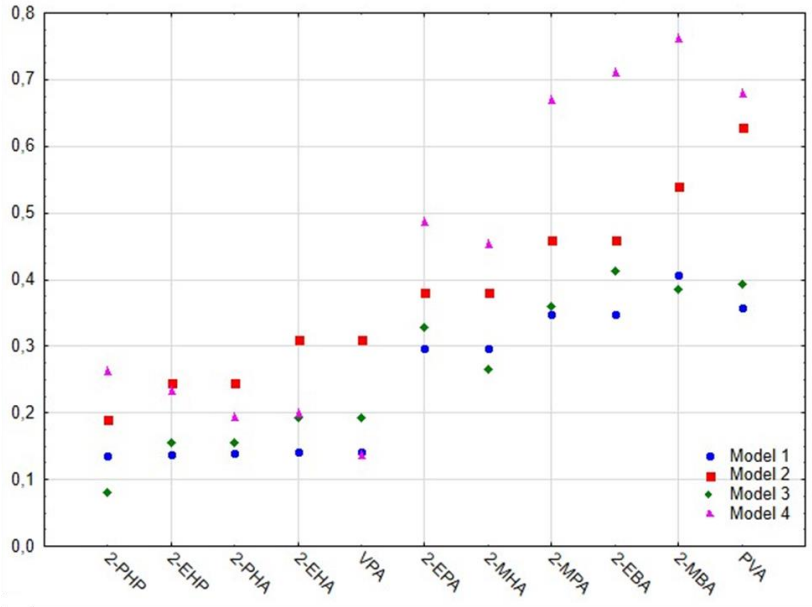
1. Model 1 (blue, Figure 14): is a Random Forest model based on chemical descriptors calculated with Dragon software (v7.0.3, Kode s.r.l.).
2. Model 2 (red): is derived by mean the freely available CORAL software (http://www.insilico.eu/coral/).
3. Model 3 (green): is a local one for the prediction of acidic chemicals.

4. Model 4 (pink): is a commercial model developed by Lhasa and is based on a Random Forest algorithm. The model was developed by 10 times cross-validation using a dataset of PPB collected from the literature and the ChEMBL dataset (3600 chemicals in total, where 20% was used as test sets). Using an in-house pKa model all chemicals were converted to their ionisation state at the pH= 7.4.

In all the cases, models returned predictions in form of fraction unbound (fu) that are reported in Annex I. All derived predictions are in the applicability domain of the relative models.

As shown in Figure 14, the predicted fraction unbound increased with decreasing side chain length. The lower freely dissolved fraction for bulkier derivatives is justified by the fact that binding of chemicals with the main blood circulating proteins (e.g. albumin) mainly involve hydrophobic interactions (Gleeson, 2007, JMC 50, 101-112). Nevertheless, the individual values per compound differ by factor of 2 to 3 comparing the outcomes of all models. We can not decide which of the predicted values for the fraction unbound represents the true value. It is more likely that the depicted range is realistic per compound or at least the true values is covered. Therefore, we used the lowest and the highest values from all four models for the *in vitro* to *in vivo* extrapolation (see paragraph "Reverse dosimetry to establish oral equivalent dose in human").

Figure 14. Results of ppb prediction from four different models (Annex I; model description is in Annex III).



Reverse dosimetry to establish oral equivalent dose in human

Biokinetic modelling (see Annex III) was used to predict the intracellular concentrations corresponding to effective concentrations determined experimentally based on nominal treatment concentrations used in *in vitro* assays. For assay systems confirming to steady-state assumptions, a biokinetic model accounting for cellular composition and physicochemical properties was used to predict intracellular concentration *in vitro*.

Alternatively, a simplified approach was used to predict free-concentrations in treatment medium (Annex III).

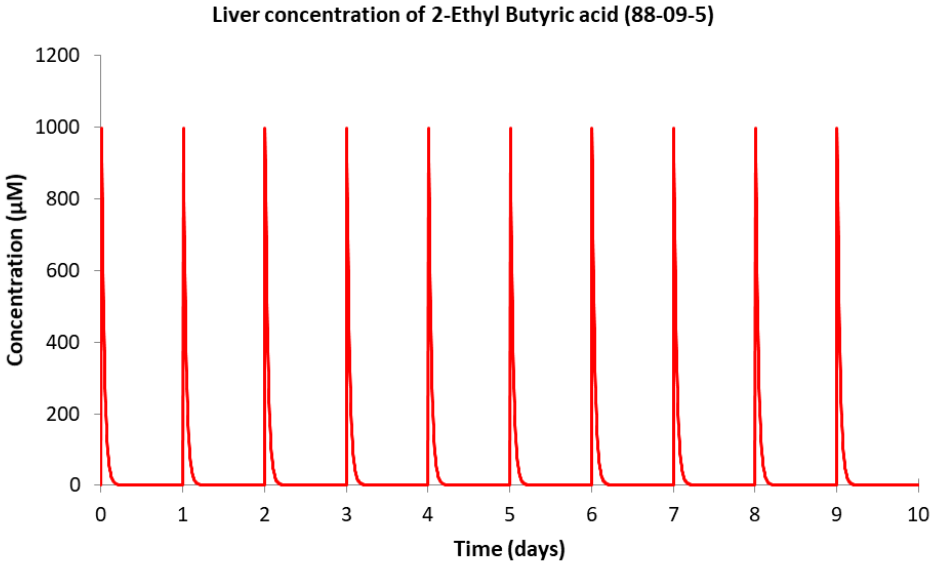
These data were assigned to one of six categories based on endpoint relation to the steatosis AOP (chapter 5.). Oral equivalent doses (OED) were then determined for the 10th and 90th percentile in each category using the above described human IVIVE-PBPK model. As described for VPA, the minimum and maximum predicted value of fu was used, resulting in a range of human equivalent doses.

Annex I lists the peak concentration in liver equal to the 10th and 90th percentile for each compound in each category identified *in vitro* experiments. Furthermore, Annex I lists the calculated oral equivalent dose- which has to be given to a human to achieve this liver concentration. As min and max fu are for both used as model, simulation results for each the 10th and 90th percentile are reported.

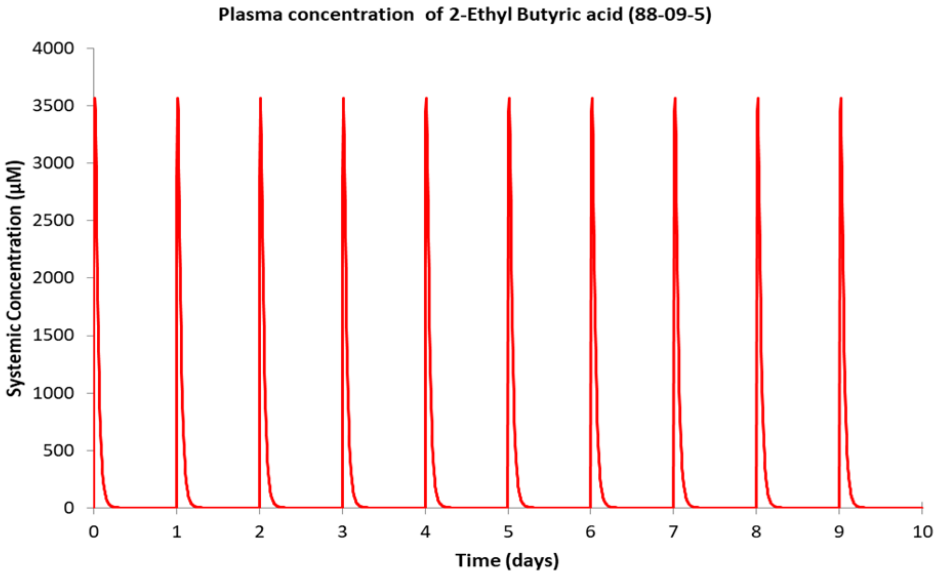
Modelling of bioaccumulation in vivo

We also modelled whether or not 2-EBA will have a potential to bioaccumulate *in vivo* after repeated exposure. If so the single and repeated exposure regimens used in our *in vitro* assays would not be able to mimic the repeated exposure scenario as being present in *in vivo* studies with 90 days exposure.

As explained in chapter 5. and 7. , we used the finally selected dose for read-across in this modelling, which is at maximum 242.8 mg/kg. The modelling approach assumed repeated single oral dose (bolus application) every 24 hours for a period of 10 days. Simulation results show no accumulation of 2-EBA in the liver or the plasma following this repeated oral exposure (Figure below). This is unsurprising given the complete and relatively rapid clearance of 2-EBA within 24hrs. The PBPK model used assumes that intrinsic hepatic metabolic clearance is not saturable at this high dosing level. While this model assumption must be considered in interpreting these simulation results, at this dose 2-EBA is cleared from the plasma within 6hrs. Saturation of metabolism resulting in a 10-fold reduction in intrinsic clearance, would still see complete clearance of compound from the plasma within 24hrs. We therefore conclude that bioaccumulation is not a critical topic in our read-across approach.



Modelling the bioavailable concentration in liver after repeated exposure to 2-EBA. Bioaccumulation in the target organ is not seen.



Modelling the bioavailable concentration in plasma after repeated exposure to 2-EBA. Bioaccumulation is not seen.

4.4.3. Metabolism

A search of the literature has unearthed variable amounts of information on the metabolism of four out of the ten chosen analogue compounds. Analogue compounds with some known metabolism are: Valproic acid, 2-Ethylhexanoic acid, 2-Methylhexanoic acid and Pivalic acid (Annex III). In addition, we used Meteor Nexus, a knowledge based system, to predict the biotransformations for the remaining six analogues (Annex III).

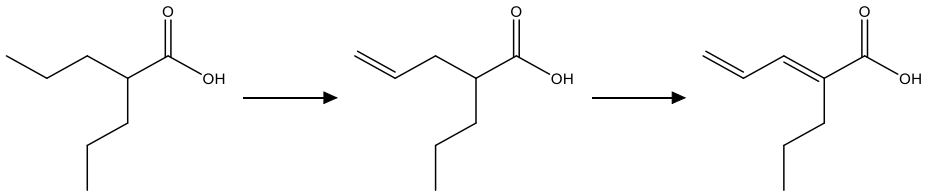
The resulting biotransformations scores (as e.g. outlines in Figure 6) give us a level of confidence in the occurrence of a particular biotransformation. They are predicated on a set of nearest neighbour parent compounds drawn from the underlying Meteor Nexus dataset. All 10 compounds of this read-across evaluation are in the applicability domain of the applied model (Annex III).

Comparison of predicted biotransformations compared to published literature

The most extensively studied and metabolically characterised analogue in this study is Valproic acid itself and this has an extensive and complex metabolic profile. Meteor Nexus is able to predict all Phase II biotransformations with the exception of the adenylate (adenosine monophosphate) conjugate reported in a couple of studies. This is generally not a significant or detectable conjugate in the study of the vast majority of compounds with a carboxylic acid function.

Whilst beta-oxidation is predicted for most analogues, the score assigned to the biotransformations is always zero. The reason for this is that the Meteor choice of nearest neighbour algorithm requires the metabolite structure in the data set, in the case of beta-oxidation (and in respect of the meteor definition of this biotransformation) the final product that results after the final thiolytic cleavage and CoA deconjugation. This final thiolytic cleavage step has been hinted at in the literature but never observed for Valproic acid or any of its analogues. The theoretical structures and intermediates are available for inspection in the Meteor Nexus tree.

For substrates with a branched propyl chain, Meteor Nexus predicts conjugation with glutathione (biotransformation 506). Within this pathway, there are intermediates which arise as a result of sequential Cyp-oxidation (terminal dehydrogenation) and beta-oxidation (internal dehydrogenation):



which is followed by 1,6-conjugate addition of glutathione to the 2-propyl-penta-2,4-dienoic acid. This reaction has been observed only for Valproic, 4-ene- and 2,4-diene Valproic acids but would in any case seem to be peculiar to propyl groups as a terminal dehydrogenation is involved.

All of the major Cyp oxidations seen in the literature are predicted by Meteor Nexus including some of the non-obvious metabolites such as those resulting from lactone formation. However, it is necessary to examine the larger, multi-generational trees in order to observe the predictions of these downstream metabolites. Understanding the

limitation of the zero score for beta-oxidations, Meteor predicts well for this class in respect of the major and most often observed metabolites.

The general (and expected) trend in prediction (and limited observation) is that the relative amount of carboaliphatic oxidation increases as the length of the alkyl chain and the side branch increases. Some degree of conjugation (by a combination of glucuronic acid, glycine, glutamine and taurine) is always predicted and these are usually the most significant contributors to clearance of these compounds *in vivo*. This is illustrated by comparing a long chain and short chain analogue to the target compound 2-EBA (Figure 16; all data in Annex III).

The biotransformations of the target compound 2-EBA include mainly phase II metabolism like glucuronidation and of the carboxylic acids and its conjugation to glutamine; glucuronidation of the alkyl carboxylic acids with glycine. Phase I metabolism includes hydroxylation of penultimate alkyl methylene. The short chain analogue 2-methylbutyric acid shares most of these biotransformations and shows in addition hydroxylation of terminal methyl groups; whereas the longer chain analogue 2-propylheptanoic acid share the four biotransformations predicted for 2-EBA, but show seven additional phase I biotransformations.

This analysis shows that, 2-EBA is most similar to the short chain analogue 2-Methylbutyric acid with regard to potential biotransformations *in vivo*. This observation is illustrated in form of a similarity score. The occurrence/absence of biotransformation was translated into a binary fingerprint for all compounds in this category and similarity scores were calculated using the standard algorithm Tanimoto (Annex III). Structural as well as biotransformation similarity are given in Figure 15. From a biotransformation point of view 2-EBA is most similar to 2-Methylbutyric acid, but is also rather similar to 2-Ethylhexanoic acid and 2-Ethylheptanoic acid. The prediction of potential biotransformations did not reveal a clear difference between the grouped compounds or a trend with regard to chain length. Same biotransformations will result in metabolites which differ with regard to side chain length as the parent compounds do. Therefore, we did not calculate and compare the similarity of the predicted metabolites in the read-across approach.

Figure 15. Comparison of structural and biotransformation fingerprint similarities for 2-EBA and analogues.

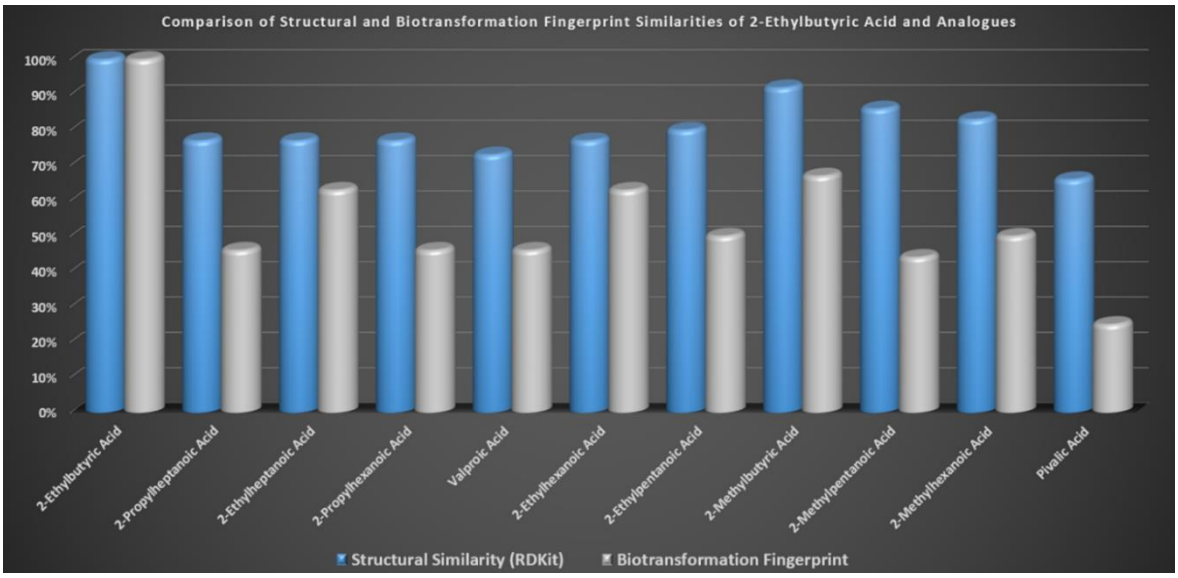
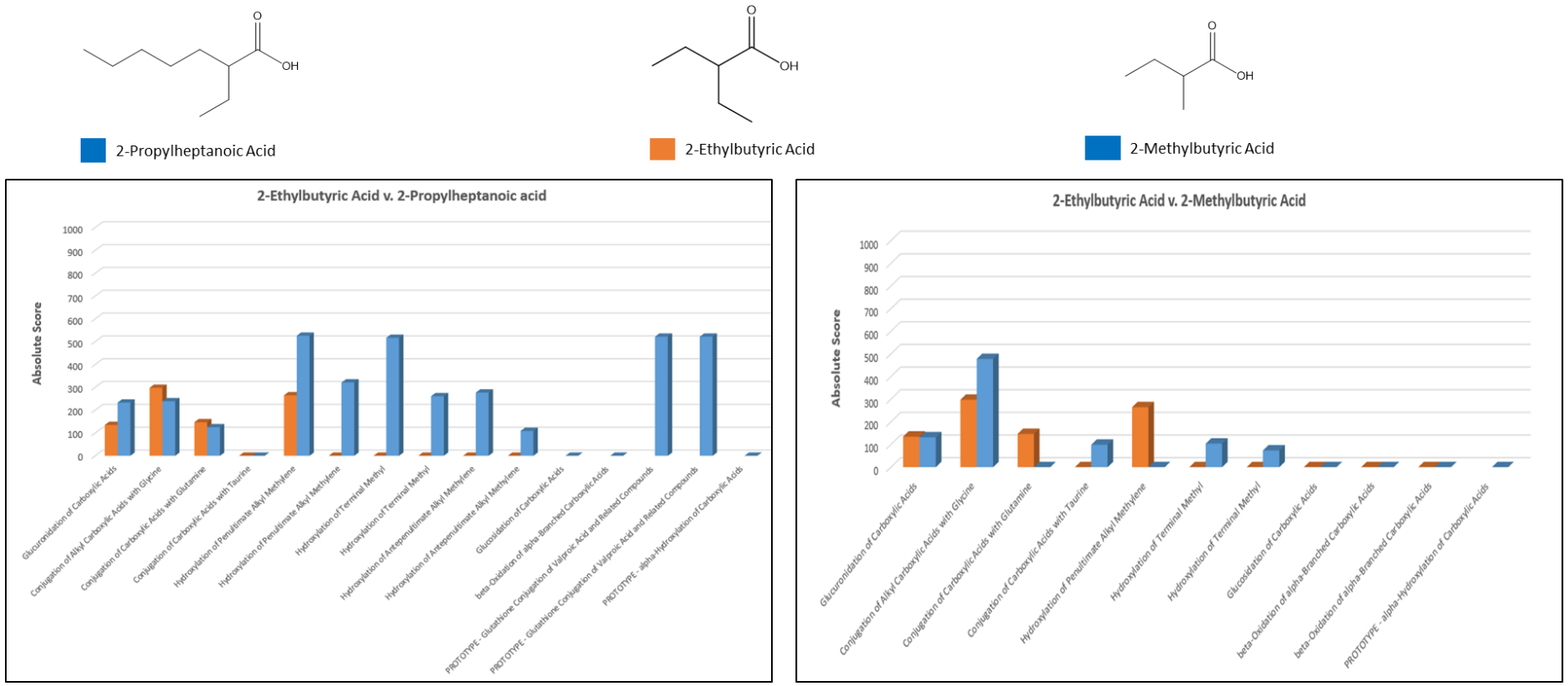


Figure 16. Comparison of predicted biotransformation types at first generation, exemplarily shown for the long chain analogue 2-Propylheptanoic acid to 2-EBA and one short chain analogue 2-Methylbyutyric acid to 2-EBA.

The biotransformation scores describe the likelihood of occurrence but do not give a quantitative information; the depicted scores are not normalised.



4.5 Information on fate in the environment (hydrolysis, biodegradation)

Not applicable for the read-across case described here.

4.6 The route and duration of expected exposure.

Not applicable for the read-across case described here.

5. Read-across justification

The read-across hypothesis is that 2-EBA might induce liver toxicity with special concern about liver steatosis after 90 days treatment with oral application. 2-EHA is the only analogues with suitable subchronic *in vivo* data, which can be read-across to the TC 2-EBA.

In this dossier, we support this read-across hypothesis using *in vitro* and *in silico* data to evaluate the toxicodynamic and toxikokinetic properties of a series of homologues analogues.

The experimental *in vitro* data show a clear trend of decreasing activity with decreasing chain length with regard to MIE activation and lipid accumulation. Although 2-EBA activated a single MIE related to steatosis (PPAR-α), lipid accumulation was not observed in two different liver models up to the highest *in vitro* tested dose (HepG2 and HepaRG cells), after single and/or repeated exposure. The activation of PPAR-α is therefore not used for the derivation of oral equivalent doses in the following.

The two compounds (VPA and 2-EHA), which showed steatosis or hepatocellular vacuolisation *in vivo*, induced lipid accumulation in all tested liver models and activated three or more MIEs related to steatosis. Pivalic acid, used as negative control, was inactive with regard to MIE activation or induction of lipid accumulation *in vitro*, which is in good agreement to the available *in vivo* data.

Cytotoxicity results showed that all compounds in this category are low toxic. A slight trend with regard to side chain length could be derived from the other functional assays, indicating that perturbation of basic cellular mechanisms increase with increasing side chain length. The TC was however, always less potent than 2-EHA or VPA.

Quantitative *in vitro* to *in vivo* extrapolation (QIVIVE) is applied to derive oral equivalent doses, which can be used as point of departure for risk assessment.

NAM outcomes are grouped according to the following categories (cat):

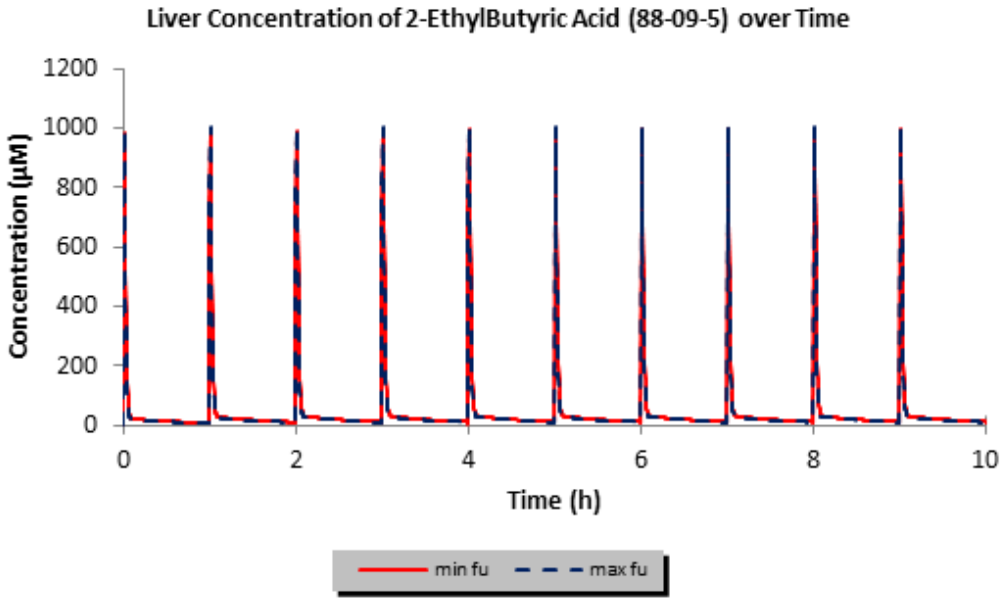
> **Cat 1:** High throughput assays measuring MIEs, which are part of the AOP network "liver steatosis" ("MIE in AOP")
>
> **Cat 2:** Key events of the AOP network, in this case lipid accumulation in different cellular systems ("lipid accumulation")
>
> **Cat 3:** Cytotoxicity values from all assays tested, including liver and kidney cells lines (" cytotoxicity")
>
> **Cat 4:** High throughput assays with not direct link to AOP ("HTS")
>
> **Cat 5:** Other functional endpoints indicating general cellular mechanisms ("general cellular mechanisms")

The purpose of the read-across is to fil a data gap for a missing subchronic *in vivo* animal study. Therefore a rat PBPK model was used for QIVIVE.

No experimental *in vivo* data in rat were available to parameterise a PBPK model for 2-EBA in rat and no species specific *in vitro* CL_{int} data could be generated. To establish a PBPK for prediction of OED in the rat, the PBPK simulated human *in vivo* clearance was allometrically scaled (using an exponent = 0.75) and then applied in the rat. Based

on biotransformation predictions, 2-EBA will primarily be metabolised through phase II metabolism (e.g. glucoronidation), comparable to VPA. As such, 2-EBA may also undergo enterohepatic recirculation which would increase exposure and could result in bioaccumulation with repeat dosing. No experimental data was available on the EHR of 2-EBA, so 2-EBA was assumed to undergo the same extent of EHR as VPA, and so the same EHR parameters were used in PBPK simulations of 2-EBA in rat. The OED in rat for EBA is calculated to range from 730 to 948.6 mg/kg bw/d.

Simulations of repeat dosing (τ=24h) in rat at 730mg/kg and 948mg/kg (cat 3; min and max fu, respectively) show no accumulation of 2-EBA in the liver, despite the inclusion of VPA equivalent EHR.



The human PBPK model is applied to derive a human oral equivalent dose (hOED) based on the *in vitro* outcomes for all compounds in this category (Table 5).

hOED values are predicted based on both the minimal and maximum predicted fraction unbound (fu, Table 5). The hOED for the longest chain analogues, 2-PHP, 2-EHP, 2-PHA, inherit a higher uncertainty as intrinsic hepatic clearance was below limit of detection in the low clearance assay and for the purpose of QIVIVE, we assumed a clearance two times lower than the lowest measured clearance in the category.

The calculated human equivalent doses are again based on the 10th percentiles per category. For 2-EBA, the lowest values is derived from the category 3 “cytotoxicity” as the activation of PPAR-α is regarded to be not critical for the development of liver steatosis. The hOED for 2-EBA is 243– 245.7 mg/kg bw/d (corresponding to 2.1-2.4 mmol/kg bw/d), which is more than a factor of 100 higher than the predicted values for VPA (1.13 to 1.22 mg/kg bw/d // 7.84 x10^{-3} to 8.5 x 10^{-3} mmol/kg bw/d, category 1 MIE related to AOP) and 2-EHA (1.4 to 1.5 mg/kg bw/d// 9.7 x 10^{-3} – 10,4 * 10^{-3}; category 1 MIE related to AOP, Table 5, Table 8).

Based on these data we conclude that 2-EBA will not induce liver steatosis up to the highest *in vitro* tested dose/or the derived oral equivalent dose and is overall less toxic than 2-EHA and VPA.

We propose a threshold of 243 mg/kg bw/d for 2-EBA for human exposure (2.1 mmol/kg bw/d), as worst case estimate from all tested *in vitro* assays. As outlined above the available *in vitro* and *in vivo* data clearly indicate that 2-EBA will not induce steatosis *in vivo* (more detailed description in chapter 7.).

Table 5. Human oral equivalent dose (mg/kg bw/d) calculated using PBPK based reverse dosimetry from categorised *in vitro* endpoints.

Rows show OED for the 10th percentile in each category using the PBPK models parameterised with the min and max predicted fraction unbound in plasma; light orange indicate inactivity up to highest tested *in vitro* dose, grey fields absence of data e.g. for PVA, 2-MBA, which could not be detected by mass spectrometry.

Cmpds	Cat_1 MIE in AOP		Cat_2 Lipid accum		Cat_3 Cytotox		Cat_4 HTS		Cat_5 General cellular mechanisms	
	min	max	min	max	min	max	min	max	min	max
2-PHP	0.66	0.59	6.07	5.46	7.05	6.32	1.65	1.48	8.53	7.67
2-EHP	0.74	0.64	9.30	8.06	63.60	55.00	4.00	3.50	14.60	12.70
2-PHA	1.86	1.80	22.00	21.27	68.70	66.60	3.40	3.30	77.90	75.50
2-EHA	1.50	1.40	12.70	12.20	20.50	19.60	3.58	3.50	2.45	2.38
VPA	1.22	1.13	2.60	2.39	11.50	10.70	1.50	1.40	2.74	2.52
2-EPA	2.60	2.30	654.70	578.30	57.00	50.40	15.60	13.80	93.50	82.60
2-MHA	0.75	0.74	680.70	619.50	149.30	135.90	545.90	496.80	46.00	41.80
2-MPA	10.40	10.40			410.00	417.80			28.10	28.60
2-EBA	10.00	10.10			243.00	245.70				
2-MBA										
PVA										

6. Uncertainty Assessment

Chapter 4. describes the combination of results from several *in vitro* and *in silico* approaches and in chapter 5. we conclude on a final human equivalent dose, which is based on an *in vitro* to *in vivo* extrapolation. We used a weight-of-evidence (WoE) approach to conclude that the activation of the single MIE PPAR-α does most likely not lead to the following key event "lipid accumulation" in the AOP, as triglyceride activation is not observed up to the highest tested *in vitro* dose. Therefore, the dose level at which PPAR-α activation is observed is not considered for threshold derivation. The uncertainty of such a combination is described in a semi-quantitative manner based on a weight of evidence approach closely related to the approach recently described by Blackburn *et al.* (2014, Table 6). The uncertainty is graded as low/moderate or high and a short explanation is given for justification.

Table 6. Uncertainty assessment

Assessment step	Uncertainty (low/medium/high)	Explanation
Read across hypothesis	low	Available *in vivo* data of two source compounds alert for liver toxicity indicating that the potential hazard of 2-Ethylbutyric acid is liver steatosis.
Structural similarity	low	This read-across approach investigates the toxicity of a series of branched carboxylic acids that differ by 1 to several carbon entities with regard to side chain length of the target compound. A potential bias can therefore be excluded.
Trend of physico-chemical descriptors	low	A consistent trend with regard to side chain length is observed. None of the selected compounds show a remarkable deviation from this trend. Experimental and predicted data are used, the data matrix is conclusive.
Trend of toxikokinetic data	low	A trend between fraction unbound and side chain length is observed. Only predicted data for plasma protein binding are available (from four different QSAR models). All compounds are within the models' applicability domain. The highest and lowest predict ppb value was used for the *in vitro* to *in vivo* calculation, By this approach a range of the human equivalent dose is predicted, which gives in our view a more realistic picture than taking only one single value.
	low with regard to 2-EBA	Intrinsic hepatic clearance was measured for all compounds of the category in 3D-HepaRG cells. It turned out that the longer chain analogues 2-PHP, 2-EHP and 2-PHA our outside the applicability domain of this assay, whereas 2-EBA and the analogues with *in vivo* data like VPA and 2-EHA can be measured. PVA was not detectable by mass spectrometry.
	low	A functional PBPK model could be built based on *in vivo* data for one analogue in rat, VPA. This model predicts bioavailable concentrations of VPA in plasma well. A good prediction was obtained for human by parameterizing the PBPK model with *in vitro* values on ppb and intrinsic hepatic clearance. This proof of concept gives confidence in the IVIVE approach used for all analogues in this case study.
Relevance of AOP	low	The AOP refers to the concern that 2-Ethylbutyric acid might induce liver steatosis as seen in one source compound. MIEs and KE derived from investigations with the analogue VPA are summarised into an AOP network, as well as other MIEs and KE being also reported to induce liver steatosis. This AOP network is not endorsed by OECD, but based on several peer-reviewed publications and therefore the overall uncertainty of the AOP is considered to be low.
Trend of MIE data associated to the AOP	low	Long chain analogues activate three or more MIEs, which are involved in the development of steatosis. 2-EBA, however, activates only one MIE, PPAR-. Similar to 2-EBA all short-chain analogues activated one MIE, whereas PVA was inactive.
Trend of KE associated to the AOP	low	Lipid accumulation in different liver assays showed a good concordance between *in vivo* data (VPA and 2-EHA active; PVA inactive). A consistent trend was shown for all analogues as the ability to induce lipid accumulation decrease with chain length. 2-EBa was inactive up to highest tested dose. Assays were conducted at realistic dose range,

		as confirmed by reverse dosimetry. Cytotoxicity did not occur at dose level, where lipid accumulation was seen.
Other supporting *in vitro* data	low	Cytotoxicity and other functional endpoints or MIEs did not provide any further information on a consistent trend within the analogues. The whole group is low toxic, and 2-EBA is one of the less toxic compounds.
Number of analogues	Low	Overall uncertainty
… with suitable *in vivo* endpoint data	high	Only one analogue - 2-EHA has guideline 90 days *in vivo* studies with oral exposure. These data on its own would result in an analogue approach. Therefore, the uncertainty associated with a one to one prediction is judged to be high.
… with supporting *in vivo* data	moderate to high	Two compounds - VPA and PVA have shorter term *in vivo* studies. The toxicological data of these analogues are not concordant, with VPA being a liver toxicant, PVA being practically non-toxic up to the highest tested dose group. Therefore, the uncertainty associated with a read-across prediction based on 2-EHA together with VPA and PVA is still judged to be moderate to high.
…with supporting *in vitro* and *in silico* data	low	Nine analogues - including the above mentioned compounds with *in vivo* data. Analogues differ with regard to side chain length. All potential analogues were included into testing. Therefore, the remaining uncertainty based on the number of analogues is judged to be low.
Quality of endpoint data used for read-across	low	Relevance and accuracy of *in vitro* and *in silico* data with regard to the investigated endpoint
Similarity of endpoint data (among source chemicals)	low	
Concordance of *in vitro* and *in vivo* data	low	High concordance between those compounds with *in vivo* and *in vitro* data
Concordance and weight of evidence of all data used for justifying the hypothesis	low	High concordance between several assays - same trend observed
Overall uncertainty of the read-across	low	

In addition, we applied a statistical based decision theory, called Dempster-Shafer (DST) (Shafer G., 1976; Dempster AP, 1967). DST is an extension of generalised Bayesian statistical inference in which evidence can be associated with multiple sources. DST represents is a rigorous decision-theory approach that provides a framework to generate predictions, estimate the uncertainty associated with each prediction, and combine multiple sources of evidence resulting in a weight-of-evidence (WoE) prediction by quantitatively accounting for the reliability of each of the individual sources (details in Annex III). In general, the outcome of such a decision theory will support the decision making process done by the toxicologist.

In this submission DST was used to combine the evidence from different *in vitro* assays for the source compounds in order to provide a WoE estimate for the target compound with respect to steatotic *in vivo* outcome.

First the subset of assays were identified, which gives the best validation results from a leave-one-out (LOO) cross validation. This LOO validation enables to detect the reliability, positive prediction accuracy (PPV) and negative prediction accuracy (NPV). The LOO procedure is a standard approach for selecting a set of sources (in this case assays) based only on the training set (in this case the source compounds).

As result, the read-across was performed using three sets of assays (BDS HTS assays, HULAFE *in vitro* data 24h and HULAFE *in vitro* data 72h). Binary data were used, indicating active/inactive per assay result. A leave-out-out cross-validation was performed in order to calculate the positive (PPV) and negative prediction (NPV) value, respectively, for the assays based on the three source compounds (Equations are provided in Annex III).

Using the correlations between *in vivo* and *in vitro* outcomes from three sets of assays (BDS HTS assays, HULAFE *in vitro* data 24h and HULAFE *in vitro* data 72h), for the 3 source compounds the *in vivo* predicted outcome for the target compound in all 3 cases (one from each set of assays) is that it is not steatotic (Table 7; Annex III).

The DST analysis shows that by using only the three best HULAFE assays (ID88095 - lipid accumulation after 72h (EC_{50} or EC_{20} values) combined with viability (IC_{50} values); Table 7) according to PPV, NPV and reliability provides the same outcome as using all of the assays. While deliberately using the three worst assays (ID 88095: HepG2 viability after 72h; HepG2 induction of mitochondrial superoxide formation (EC_{50} or EC_{20})) would lead to an inconclusive results due to the fact that the belief is still 0 (<0.5, non steatotic) but with a very high uncertainty (plausibility) of 0.833 (> 0.5).

This result also turns out, that the minimal scope to conclude on the absence of liver steatosis comprises the results of lipid accumulation and viability results from the HULAFE assays (72h; ID 88095) or the BDS assays (ID 88095, Table 7).

Table 7. Results from DST on target compound 2-EBA

id	belief	plausibility	DST outcome	classification	true_class	assay set			comment
88095	0	0	low	non-steatotic	non-steatotic	cs1_dst_BDS_selected_assays_binary_excluded_nodata			BDS set
88095	0	0	low	non-steatotic	non-steatotic	cs1_dst_HULAFE_24h_binary_excluded_nodata			HULAFE_24h set
88095	0	0	low	non-steatotic	non-steatotic	cs1_dst_HULAFE_72h_binary_excluded_nodata			HULAFE_72h set
88095	0	0	low	non-steatotic	non-steatotic	HULAFE_HepG2_72h_Lipids_IC50	HULAFE_HepG2_72h_Lipids_MEC/EC20	HULAFE_HepG2_72h_Viability_IC50	HULAFE_72h set, 3 best assays
88095	0	0.833	moderate	?	non-steatotic	HULAFE_HepG2_72h_Viability_MEC/IC20	HULAFE_HepG2_72h_MitoSOX_EC50	HULAFE_HepG2_72h_MitoSOX_MEC/EC20	HULAFE_72h set, 3 worst assays

7. Integrated conclusion

The integrated conclusion of this read-across is, that 2-EBA will not induce liver steatosis and liver/kidney toxicity *in vivo* up to the derived oral equivalent dose level. Despite the fact that 2-EBA showed activation of the nuclear receptor PPAR-α, no evidence for lipid accumulation was seen in two different cellular systems (HepG2 and HepaRG) after single and/or repeated exposure. This is well in line with the observed trend in this group of analogues, for which MIE activation and the potential to induce lipid accumulation decrease with decreasing side chain length.

Based on the data provided from the tested liver cells, we can conclude that the potency to induce liver steatosis differ, which sets the basis of a safe human threshold, the hOED. Transcriptomic data will be used to further substantiate the hypothesis, that all compounds follow the same mode of action *in vivo*, with the already proven difference in toxicological potency.

A renal weight change was seen *in vivo* for one analogue. We tested viability of a human kidney cell line to see differences in renal toxicity within the grouped compounds. Based on cytotoxicity measurements, no clear difference or trend was observed. VPA was, however, slightly more toxic than all other compounds in the group (Annex I). So far, a specific concern for kidney toxicity is not seen for the TC. Further data, like transcriptome data, might help to better define a shared mode of action with regard to kidney toxicity.

We applied a worst case approach to extrapolate the *in vitro* assay results to human oral equivalent doses, using the highest and lowest predicted fraction unbound in human plasma.

2-EBA derived the lowest oral equivalent doses from the 10 percentile of *in vitro* values measuring cytotoxicity, which is 2.1 to 2.4 mmol/kg bw/d for humans (243-245.7 mg/kg bw/d; Table 8).

2-VPA and 2-EHA derived the lowest human values from the 10^{th} percentile of the category 1 "MIE in AOP", 7.8×10^{-3} – 8.5×10^{-3} mmol/kg bw/d for VPA and 9.7×10^{-3} to 10.4×10^{-3} mmol/kg bw/d for 2-EHA.

The lowest observed effect level *in vivo* animal studies for VPA is 8×10^{-3} to 0.43 mmol/kg bw/d. Application of a standard risk extrapolation factor of 100, accounting for inter and intraspecies differences, leads to threshold values in the range of 8×10^{-5} to 4.3×10^{-2} mmol/kg bw/d. A similar approach lead to 5×10^{-3} to 9×10^{-3} mmol/kg bw/d for 2-EHA.The extrapolated values from the *in vitro* studies are in the same range as the corresponding *in vivo* animal studies.

Table 8. Integrated conclusion on a human threshold value for 2-EBA.

The 10th percentile of the lowest calculated effect concentrations was used to extrapolate the human equivalent dose (Annex I, Table 5)

Summary of Data Gap Filling											
CAS	31080-39-4	3274-29-1	3274-28-0	149-57-5	99-66-1	20225-24-5	4536-23-6	97-61-0	88-09-5	1730-91-2	75-98-9
Name	2-PHP	2-EHP	2-PHA	2-EHA	VPA	2-EPA	2-MHA	2-MPA	**2-EBA**	2-MBA	**PVA**
Repeated dose studies with oral exposure.	No data	No data	No data	2 x 90days studies	Several short term studies (up to 14 days exposure); 2x 28 days studies	No data	No data	No data	No data	No data	1 x 28 day study
Main effects reported and LOAEL(mmol/kg bw/d).				Main effect- liver hypertrophy. LOAEL liver: 0.5 -0.9	10days rat gavage: No liver effect observed; 14 days mouse study LOAEL: 0.03. Ip exposure.						No adverse effects observed
					LOAEL liver steatosis: 8 x 10^{-3} to 0.43						
hOED (mg/kg bw/d)	0.59 to 0.66	0.64 to 0.74	1.8 to 1.86	1.4 to 1.5	1.13 to 1.22	2.3-2.6	0.74-0.75	410-417.80	243-245.7	out of domain	out of domain
hOED (mmol/kg bw/d)	3.43 x 10^{-3} to 3.83 x10^{-3}	4.05x10^{-3} to 6.7 x 10^{-3}	0.01	9.7x10^{-3} to 10.4x10^{-3}	7.84x10^{-3} to 8.5x10^{-3}	17.7x10^{-3} to 20 x 10^{-3}	5.6x10^{-3} to 6.4 x 10^{-3}	3.53-3.6	2.1-2.4	out of domain	out of domain

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1 Fraunhofer Institute for Toxicology and Experimental Medicine, Chemical Safety and Toxicology, Germany

2 Vrije Universiteit Amsterdam, Amsterdam, The Netherlands

3 BioDetection Systems, Amsterdam, The Netherlands

4 INERIS, Verneuil-en-Hallate, France

5 Novo Nordisk Foundation Center for Protein Research, University of Copenhagen, Denmark

6 Université de Paris, France

7 University of Vienna, Vienna, Austria

8 MTM Research Centre, Örebro University, Sweden

9 Cyprotex, Cheshire, United Kingdom

10 Istituto di Ricerche Farmacologiche Mario Negri IRCCS, Milano, Italy

11 Edelweiss Connect GmbH, Basel, Switzerland

12 Lhasa Limited, Leeds, United Kingdom

13 Certara UK Ltd, Simcyp Division, Sheffield, United Kingdom

14 Unilever Safety and Environmental Assurance Centre, Sharnbrook, Bedfordshire, United Kingdom

15 Leiden Academic Centre for Drug Research (LACDR), Leiden University, Leiden, The Netherlands

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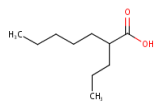
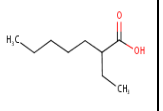
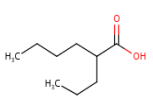
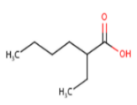
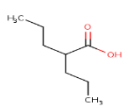
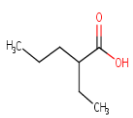
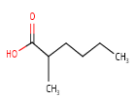
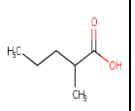
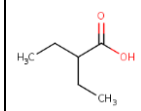
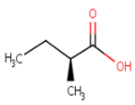
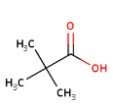
Annex I. Data matrix

Results

Source and Target Cmpds: Description of analogues and target compound; name, structure information

	Source and Target Compounds										
	Source1	**Source 2**	**Source 3**	**Source 4**	**Source 5**	**Source 6**	**Source 7**	**Source 8**	**Target**	**Source 9**	**negative control**
CAS	31080-39-4	3274-29-1	3274-28-0	149-57-5	99-66-1	20225-24-5	4536-23-6	97-61-0	**88-09-5**	1730-91-2	75-98-9
Name	2-Propylheptanoic acid	2-Ethylheptanoic acid	2-PHA	2-Ethylhexanoic acid	Valproic acid	2-Ethylpentanoic acid	2-Methyl-hexanoic acid	2-Methyl-pentanoic acid	**2-Ethylbutyric acid**	2-Methylbutyric acid	Pivalic acid
Abbreviation	2-PHP	2-EHP	2-PHA	2-EHA	VPA	2-EPA	2-MHA	2-MPA	**2-EBA**	2-MBA	PVA
Structure											
Structure (smiles)	CCCCCC(CCC)C(=O)O	CCCCCC(CC)C(=O)O	CCCCC(CCC)C(=O)O	CCCCC(CC)C(=O)O	CCCC(CCC)C(=O)O	CCCC(CC)C(=O)O	CCCCC(C)C(=O)O	CCCC(C)C(=O)O	**CCC(CC)C(=O)O**	CC[C@H](C)C(=O)O	CC(C)(C)C(=O)O
Similarity score	77%	77%	77%	77%	73%	80%	83%	86%	**100%**	92%	66%
Chain length from position 2	5/3/0	2/5/0	4/3/0	2/4/0	3/3/0	2/3/0	4/1/0	3/1/0	**2/2/0**	2/1/0	1/1/1
Purity	not given	98%	not given	≥ 99%	not given	not given	>99%	≥ 98%	99%	98%	99%

Reverse Dosimetry (target; rat): QIVIVE: Calculation of the 90 days oral equivalent dose for rats using 6 categories of in vitro values

		target
CAS		88-09-5
Name		2-Ethylbutyric acid
category 1 MIE (µM) - peak concentration in liver	10th Percentile	41
	90th Percentile	41
oral dose equivalent (min fu model) (mg/kg)	10th Percentile	30.3
	90th Percentile	30.3
oral dose equivalent (max fu model) (mg/kg)	10th Percentile	39.04
	90th Percentile	39.04
category 2 lipid accumulation (µM) - peak concentration in liver	10th Percentile	
	90th Percentile	
oral dose equivalent (min fu model) (mg/kg)	10th Percentile	
	90th Percentile	
oral dose equivalent (max fu model) (mg/kg)	10th Percentile	
	90th Percentile	
category 3 cytotox (µM) - peak concentration in liver	10th Percentile	997
	90th Percentile	3248
oral dose equivalent (min fu model) (mg/kg)	10th Percentile	730
	90th Percentile	2379.7
oral dose equivalent (max fu model) (mg/kg)	10th Percentile	948.6
	90th Percentile	3093.4
category 4 functional HTS (µM) - peak concentration in liver	10th Percentile	
	90th Percentile	
oral dose equivalent (min fu model) (mg/kg)	10th Percentile	
	90th Percentile	
oral dose equivalent (max fu model) (mg/kg)	10th Percentile	
	90th Percentile	
category 5 other functional (µM) - peak concentration in liver	10th Percentile	
	90th Percentile	
oral dose equivalent (min fu model) (mg/kg)	10th Percentile	
	90th Percentile	
oral dose equivalent (max fu model) (mg/kg)	10th Percentile	
	90th Percentile	
category 6 AC_{50} (µM) - peak concentration in liver	10th Percentile	
	90th Percentile	
oral dose equivalent (min fu model) (mg/kg)	10th Percentile	
	90th Percentile	
oral dose equivalent (max fu model) (mg/kg)	10th Percentile	
	90th Percentile	

	active
	inactive
	no data

Reverse Dosimetry (human): QIVIVE: Calculation of human equivalent doses for all a analogues using 6 categories of in vitro values

		Supporting Data Related to the Target Endpoint(s)										
		Source1	Source 2	Source 3	Source 4	Source 5	Source 6	Source 7	Source 8	target	Source 9	negative
CAS		31080-39-4	3274-29-1	3274-28-0	149-57-5	99-66-1	20225-24-5	4536-23-6	97-61-0	88-09-5	1730-91-2	75-98-9
Name		2-PHP	2-Ethylheptanoic acid	2-PHA	2-Ethylhexanoic acid	Valproic acid	2-Ethylpentanoic acid	2-Methyl-hexanoic acid	2-Methyl-pentanoic acid	2-Ethylbutyric acid	2-Methylbutyric acid	Pivalic acid
category 1 MIE (µM) - peak concentration in liver	10th Percentile	4	5	11	10	9	20	4	41	41	402	
	90th Percentile	620	833	836	958	1011	46	4	41	41	2795	
oral dose equivalent (min fu model) (mg/kg)	10th Percentile	0.66	0.74	1.86	1.5	1.22	2.6	0.75	10.4	10.0		
	90th Percentile	101.7	123.2	141.2	138.3	136.6	5.98	0.75	10.4	10.0		
oral dose equivalent (max fu model) (mg/kg)	10th Percentile	0.59	0.64	1.8	1.4	1.13	2.3	0.74	10.4	10.1		
	90th Percentile	91.1	106.6	136.8	132.4	127	5.29	0.74	10.4	10.1		
category 2 lipid accumulation (µM) - peak concentration in liver	10th Percentile	37	63	130	88	19	5026	3629				
	90th Percentile	234	1179	1243	884	977	5026	3629				
oral dose equivalent (min fu model) (mg/kg)	10th Percentile	6.07	9.3	22	12.7	2.6	654.7	680.7				
	90th Percentile	38.39	174.4	210	127.6	132	654.7	680.7				
oral dose equivalent (max fu model) (mg/kg)	10th Percentile	5.46	8.06	21.27	12.2	2.39	578.3	619.5				
	90th Percentile	34.4	150.9	203.4	122.2	122.7	578.3	619.5				
category 3 cytotox (µM) - peak concentration in liver	10th Percentile	43	430	407	142	85	438	796	1634	997	861	1020
	90th Percentile	3244	3033	3617	1597	4581	4937	2038	2371	3248	2801	4686
oral dose equivalent (min fu model) (mg/kg)	10th Percentile	7.05	63.6	68.7	20.5	11.5	57	149.3	410	243		
	90th Percentile	532	448.7	610.9	230.6	619	643.1	382.3	595	791.6		
oral dose equivalent (max fu model) (mg/kg)	10th Percentile	6.32	55	66.6	19.6	10.7	50.4	135.9	417.8	245.7		
	90th Percentile	476.7	388.1	592	220.8	575.3	568.1	347.9	606.2	801.2		
category 4 functional HTS (µM) -peak concentration in liver	10th Percentile	10	27	20	25	11	120	2910			2668	
	90th Percentile	163	47	380	70	853	120	2910			2668	

oral dose equivalent (min fu model) (mg/kg)	**10th Percentile**	1.65	4	3.4	3.58	1.5	15.6	545.9				
	90th Percentile	26.7	7	64.2	10.1	115.3	15.6	545.9				
oral dose equivalent (max fu model) (mg/kg)	**10th Percentile**	1.48	3.5	3.3	3.5	1.4	13.8	496.8				
	90th Percentile	24	6	62.2	9.8	107.1	13.8	496.8				
category 5 other functional (µM) - peak concentration in liver	**10th Percentile**	52	99	461	17	20	718	245	112		161	566
	90th Percentile	1053	256	461	50	572	1317	245	112		987	749
oral dose equivalent (min fu model) (mg/kg)	**10th Percentile**	8.53	14.6	77.9	2.45	2.74	93.5	46	28.1			
	90th Percentile	172.7	37.9	77.9	7.2	77.3	171.6	46	28.1			
oral dose equivalent (max fu model) (mg/kg)	**10th Percentile**	7.67	12.7	75.5	2.38	2.52	82.6	41.8	28.6			
	90th Percentile	154.7	32.8	75.5	6.9	71.82	151.5	41.8	28.6			
category 1 MIE (µM) - peak concentration in liver	**10th Percentile**	4	5	11	10	9	20	4	41	41	402	
	90th Percentile	620	833	836	958	1011	46	4	41	41	2795	
oral dose equivalent (min fu model) (mg/kg)	**10th Percentile**	0.66	0.74	1.86	1.5	1.22	2.6	0.75	10.4	10.0		
	90th Percentile	101.7	123.2	141.2	138.3	136.6	5.98	0.75	10.4	10.0		
oral dose equivalent (max fu model) (mg/kg)	**10th Percentile**	0.59	0.64	1.8	1.4	1.13	2.3	0.74	10.4	10.1		
	90th Percentile	91.1	106.6	136.8	132.4	127	5.29	0.74	10.4	10.1		

active
inactive
no data

Summary data gap filling: Integrated conclusion on data gap filling; human equivalent doses are provided using PBPK modelling

	Summary of Data Gap Filling										
	Source1	**Source 2**	**Source 3**	**Source 4**	**Source 5**	**Source 6**	**Source 7**	**Source 8**	**target**	**Source 9**	**negative compound**
CAS	31080-39-4	3274-29-1	3274-28-0	149-57-5	99-66-1	20225-24-5	4536-23-6	97-61-0	88-09-5	1730-91-2	75-98-9
Name	2-PHP	2-Ethylheptanoic acid	2-PHA	2-Ethylhexanoic acid	Valproic acid	2-Ethylpentanoic acid	2-Methyl-hexanoic acid	2-Methyl-pentanoic acid	2-Ethylbutyric acid	2-Methylbutyric acid	Pivalic acid
Repeated dose studies with oral exposure.	No studies available	No studies available	No studies available	2 x 90days studies	Several short term studies (up to 14 days exposure); 2x 28 days studies, supporting information	No studies available	No studies available	No studies available	No studies available	No studies available	1 x 28 day study
Main effects reported and LOAEL(mmol/kg bw/d).				Main effect- liver hypertrophy.LOAEL liver: 0.5 -0.9	10days rat gavage: No liver effect observed; 14 days mouse study LOAEL: 0.03. Interperitoneal exposure. LOAEL liver steatosis: 8 x 10-3 to 0.43						No adverse effects observed
Integrated conclusion on human equivalent dose, lowest calculated effect concentration was used (mg/kg bw/d)	0.59 to 0.66	0.64 to 0.74	1.8 to 1.86	1.4 to 1.5	1.13 to 1.22	2.3 to 2.6	0.74-0.75	410-417.8	243 to 245.7	out of domain	out of domain
Integrated conclusion on human equivalent dose, lowest calculated effect concentration was used (mmol/kg bw/d)	3.43 x 10-3 to 3.83 x10-3	4.05x10-3 to 6.7 * 10-3	0.01	9.7x10-3 to 10.4x10-3	7.84x10-3 to 8.5x10-3	17.7x10-3 to 20 x 10-3	5.6 x 10-3 - 6.4 x 10-3	3.53-3.61	2.1 - 2.4	out of domain	out of domain

	active
	inactive
	no data

Input data

Molecular profiling: Data on metabolites

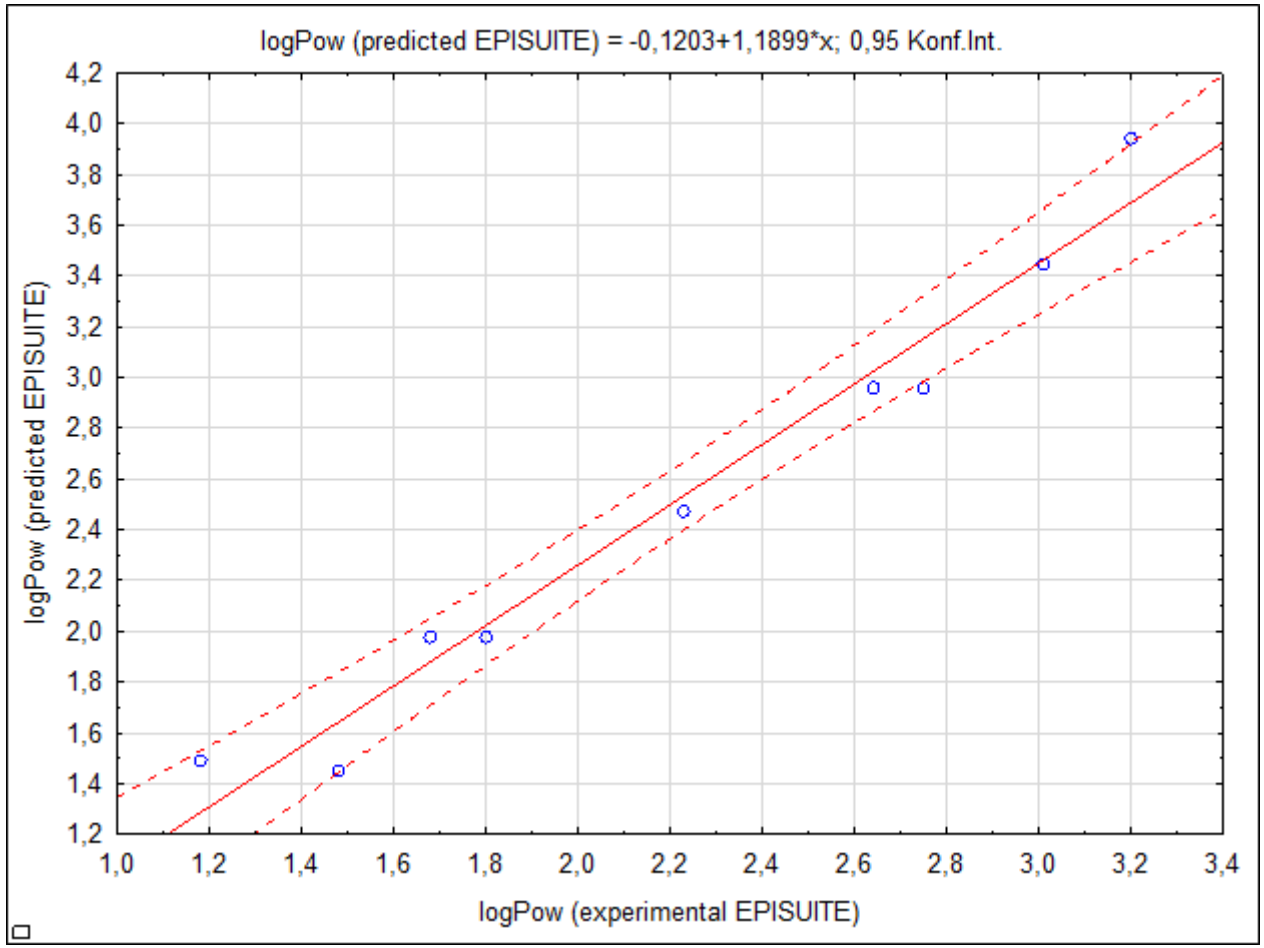
		Molecular Profiling Related to the Analogue Approach Hypothesis										
		Source1	**Source 2**	**Source 3**	**Source 4**	**Source 5**	**Source 6**	**Source 7**	**Source 8**	**target**	**Source 9**	**negative**
CAS		31080-39-4	3274-29-1	3274-28-0	149-57-5	99-66-1	20225-24-5	4536-23-6	97-61-0	**88-09-5**	1730-91-2	75-98-9
Name		2-Propylheptanoic acid	2-Ethylheptanoic acid	2-PHA	2-Ethylhexanoic acid	Valproic acid	2-Ethylpentanoic acid	2-Methyl-hexanoic acid	2-Methyl-pentanoic acid	**2-Ethylbutyric acid**	2-Methylbutyric acid	Pivalic acid
Abb		2-PHP	2-EHP	2-PHA	2-EHA	VPA	2-EPA	2-MHA	2-MPA	**2-EBA**	2-MBA	PVA
Parent chemical	Profiler 1 (name, version)											
	Expert system 1 (name, version)											
Metabolites*	Profiler 1 (name, version)											
	Expert system 1 (name, version)											

Assumption in this read-across: metabolism will result in metabolites that differ with regard to side chin length as the parents do.

Physchem: Predicted physchem properties

			predicted values							experimental values				
Name	**Abb**	**Molecular weight**	**Melting point (deg C; predicted EPISUITE)**	**Boiling point (deg C; predicted EPISUITE)**	**logPow (experimental EPISUITE)**	**logPow (predicted EPISUITE)**	**Henry's Law Constant (Pa m^3/mol; predicted EPISUITE (bond method))**	**Henry's Law Constant (Pa m^3/mol; experimental EPISUITE)**	**pKa (predicted ACD/Percepta)**	**Water solubility (mg/L; experimental)**	**pKa (experimental)**	**Vapour pressure (mm Hg; experimental)**	**Vapour pressure (haPa)**	**logPow (experimental)**
2-PHP	2-PHP	172.15	59.15	268.98	3.2	3.94	0.535		4.8	275.6		0.0048	0.01	3.2
2-Ethylheptanoic acid	2-EHP	158.13	48.57	252.04		3.45	0.403		4.8					
2-PHA	2-PHA	158.13	48.57	252.04	3.01	3.45	0.403		4.8	465.3		0.0146	0.0195	3.01
2-Ethylhexanoic acid	2-EHA	144.12	37.72	234.2	2.64	2.96	0.304	0.289	4.8	2000	4.7	0.03	0.04	2.64
Valproic acid	VPA	144.12	37.72	234.2	2.75	2.96	0.304		4.8	2000	4.6	0.0458	0.061	2.75
2-Ethylpentanoic acid	2-EPA	130.1	26.62	215.45	2.23	2.47	0.229		4.8	2840	4.71	0.148	0.1973	2.23
2-Methyl-hexanoic acid	2-MHA	130.1	26.62	215.45		2.47	0.229		4.8					
2-Methyl-pentanoic acid	2-MPA	116.08	15.24	195.8	1.8	1.98	0.172		4.8					1.8
2-Ethylbutyric acid	**2-EBA**	116.08	15.24	195.8	1.68	1.98	0.162		4.8	18000	4.71	0.188	0.25	1.68
2-Methylbutyric acid	2-MBA	102.07	3.61	175.25	1.18	1.49	0.130	0.149	4.8					
Pivalic acid	PVA	102.07	9.87	166.85	1.48	1.45	0.282		4.9	21700	5.031	0.5	0.67	

	active
	inactive
	no data


logPow (predicted EPISUITE) = -0,1203+1,1899*x; 0,95 Konf.Int.
logPow (predicted EPISUITE)
logPow (experimental EPISUITE)
4,2
4,0
3,8
3,6
3,4
3,2
3,0
2,8
2,6
2,4
2,2
2,0
1,8
1,6
1,4
1,2
1,0
1,2
1,4
1,6
1,8
2,0
2,2
2,4
2,6
2,8
3,0
3,2
3,4

ADME-Toxicokinetics: ppb and (intrinsic) clearance measured or predicted

	ADME-Toxicokinetics										
	Source1	**Source 2**	**Source 3**	**Source 4**	**Source 5**	**Source 6**	**Source 7**	**Source 8**	**target**	**Source 9**	**negative**
CAS	31080-39-4	3274-29-1	3274-28-0	149-57-5	99-66-1	20225-24-5	4536-23-6	97-61-0	**88-09-5**	1730-91-2	75-98-9
Name	2-Propylheptanoic acid	2-Ethylheptanoic acid	2-PHA	2-Ethylhexanoic acid	Valproic acid	2-Ethylpentanoic acid	2-Methyl-hexanoic acid	2-Methyl-pentanoic acid	**2-Ethylbutyric acid**	2-Methylbutyric acid	Pivalic acid
id											
Abbreviation	2-PHP	2-EHP	2-PHA	2-EHA	VPA	2-EPA	2-MHA	2-MPA	**2-EBA**	2-MBA	PVA
Fraction unbound in plasma (human; predicted – Model 1 Mirko-all in domain)	0,135	0,138	0,139	0,142	0,141	0,296	0,296	0,348	**0,348**	0,407	0,358
Fraction unbound in plasma (human; predicted – Model 2 Mirko-all in domain)	0,191	0,245	0,245	0,310	0,310	0,382	0,382	0,460	**0,460**	0,541	0,629
Fraction unbound in plasma (human; predicted – Model 3 Mirko-all in domain)	0,081	0,155	0,155	0,193	0,193	0,329	0,265	0,359	**0,413**	0,386	0,393
Fraction unbound in plasma (human; predicted) - Model 4 (Lhasa-all in domain)	0.26	0.23	0.20	0.20	0.14	0.49	0.45	0.67	**0.71**	0.76	0.68
Intrinsic Hepatic Clearance ($CL_{int,H}$; µl/min/10^6 Hepatocytes) (human; experimental Cyprotex)	No available data*	No available data*	No available data*	0.551	0.219	0.779	3.95	10.2	**9.62**	No available data	No available data
***In vivo* Clearance (CL; L/h) (human; predicted Simcyp, min fu)**	No available data	No available data	No available data	0.85	0.33	2.38	9.10	21.09	**20.42**	No available data	No available data
***In vivo* Clearance (CL; L/h) (human; predicted Simcyp, max fu)**	No available data	No available data	No available data	1.80	0.73	3.80	13.68	29.07	**29.09**	No available data	No available data
Steady-state volume of distribution, human (Vss; L/kg; predicted)	0.12	0.14	0.13	0.12	0.14	0.15	0.15	0.16	**0.16**	0.18	0.2
Steady-state volume of distribution, human (Vu,ss; L/kg; predicted)	0.63	0.57	0.53	0.39	0.45	0.38	0.39	0.35	**0.35**	0.33	0.33
Intrinsic clearance – Insphero											

* The heaptic lcearnce was at limit of detection in this *in vitro* assay. QIVIVE modelling is therefore based on the assumption that these compounds have an intrinsic hepatic clearance half that of VPA (CLint = 0.11 ul/min/million cells). This is in line with the data we have, but the minimum CLint that can be accurately measured, according to the manufacturers, using the *in vitro* system we used is approx 0.137ul/min/million cells.

Target Endpoints (exp.): Measured values from in vitro experiments for hazard characterisation

			Supporting Data Related to the Target Endpoint(s)										
			Source 1	**Source 2**	**Source 3**	**Source 4**	**Source 5**	**Source 6**	**Source 7**	**Source 8**	**target**	**Source 9**	**negative**
CAS			31080-39-4	3274-29-1	3274-28-0	149-57-5	99-66-1	20225-24-5	4536-23-6	97-61-0	**88-09-5**	1730-91-2	75-98-9
Name			2-PHP	2-Ethylheptanoic acid	2-PHA	2-Ethylhexanoic acid	Valproic acid	2-Ethylpentanoic acid	2-Methyl-hexanoic acid	2-Methyl-pentanoic acid	**2-Ethylbutyric acid**	2-Methylbutyric acid	Pivalic acid
In Vitro	**RPTEC/TERT1 cells**	**Resazurin reduction POD (nominal; µM)**	4000	4000	8000	8000	>8000	4000	8000	>8000	>8000	8000	>8000
		Supernatant lactate POD (nominal; µM)	8000	8000	>8000	>8000	>8000	>8000	>8000	>8000	8000	>8000	>8000
	CALUX reporter gene assays, LEC in Log(M), CALUX (DB-ALM 197)	**Cytotox CALUX (nominal conc.)**	>-3	>-3	>-3	>-3	>-3	>-3	>-3	>-3	**>-3**	>-3	>-3
		ERa CALUX (nominal conc.)	>-3	>-3	>-3	>-3	>-3	>-3	>-3	>-3	**>-3**	>-3	>-3
		Anti-ERa CALUX (nominal conc.)	>-3	>-3	>-3	>-3	>-3	>-3	>-3	>-3	**>-3**	>-3	>-3
		AR CALUX (nominal conc.)	>-3	>-3	>-3	>-3	>-3	>-3	>-3	>-3	**>-3**	>-3	>-3
		Anti-AR CALUX (nominal conc.)	-3.5	-3.5	-3.5	-3.4	>-3	>-3	>-3	>-3	**>-3**	>-3	>-3
		PR CALUX (nominal conc.)	>-3	>-3	>-3	>-3	>-3	>-3	>-3	>-3	**>-3**	>-3	>-3
		Anti-PR CALUX (nominal conc.)	-3.6	-3.5	-3.9	-3	-3.1	>-3	>-3	>-3	**>-3**	>-3	>-3
		GR CALUX (nominal conc.)	>-3	>-3	>-3	>-3	>-3	>-3	>-3	>-3	**>-3**	>-3	>-3
		Anti-GR CALUX (nominal conc.)	>-3	>-3	>-3	>-3	>-3	>-3	>-3	>-3	**>-3**	>-3	>-3
		TRb CALUX (nominal conc.)	-3.5	-3.4	-3.1	>-3	-3	>-3	>-3	>-3	**>-3**	>-3	>-3
		Anti-TRb CALUX (nominal conc.)	>-3	>-3	>-3	>-3	>-3	>-3	>-3	>-3	**>-3**	>-3	>-3
		RAR CALUX (nominal conc.)	>-3	>-3	>-3	>-3	>-3	>-3	>-3	>-3	**>-3**	>-3	>-3
		LXR CALUX (nominal conc.)	>-3	>-3	>-3	>-3	>-3	>-3	>-3	>-3	**>-3**	>-3	>-3
		PXR CALUX (nominal conc.)	-4.3	-4	-4	-3.6	-4.1	-3.5	>-3	>-3	**>-3**	>-3	>-3
		PPARa CALUX (nominal conc.)	-4.5	-4.5	-4	-4	-4	-3.9	-4.5	-3.5	**-3.5**	>-3	>-3
		PPARd CALUX (nominal conc.)	-3	-3.1	>-3	>-3	>-3	>-3	>-3	>-3	**>-3**	>-3	>-3

		PPARg CALUX (nominal conc.)	-3.9	-3.3	-3.3	>-3	>-3	>-3	>-3	>-3	**>-3**	>-3	>-3
		AhR CALUX (nominal conc.)	-3	>-3	>-3	>-3	>-3	>-3	>-3	>-3	**>-3**	>-3	>-3
		Hif1a CALUX (nominal conc.)	>-3	>-3	>-3	>-3	>-3	>-3	>-3	>-3	**>-3**	>-3	>-3
		TCF CALUX (nominal conc.)	-4.3	-3.6	-3.5	-3.5	-4	-3	>-3	>-3	**>-3**	>-3	>-3
		AP1 CALUX (nominal conc.)	-4	>-3	-3	>-3	>-3	>-3	>-3	>-3	**>-3**	>-3	>-3
		ESRE CALUX (nominal conc.)	-4	-3.5	-3	-3	-4	>-3	>-3	>-3	**>-3**	>-3	>-3
		NFkB CALUX (nominal conc.)	>-3	>-3	>-3	>-3	>-3	>-3	>-3	>-3	**>-3**	>-3	>-3
		Nrf2 CALUX (nominal conc.)	-4	-3.7	-3	-3	>-3	-3.4	>-3	>-3	**>-3**	-3.1	>-3
		p21 CALUX (nominal conc.)	-3.7	-3.4	-3.2	-3	-3.2	>-3	>-3	>-3	**>-3**	>-3	>-3
		p53 GENTOX CALUX (nominal conc.)	-4	-3.5	-3.7	-3.1	-3.6	-3	>-3	>-3	**>-3**	>-3	>-3
		p53 S9 GENTOX CALUX (nominal conc.)	>-3	>-3	>-3	>-3	>-3	>-3	>-3	>-3	**>-3**	>-3	>-3
	GFP-reporter gene assay, 24h exposure; single application, LEC in Log(M)	**SRXN1 (FPC_2m) (nominal conc.)**	-2.1	-2.0	-2.1	-2.0	-2.2	>-1.79	>-1.79	>-1.79	**>-1,79**	-1.9	>-1.79
		P21 (FPC_2m) (nominal conc.)	-2.3	>-1.79	-2.1	>-1.79	-2.0	>-1.79	-1.8	>-1.79	**>-1.79**	-1.9	>-1.79
	HepaRG, 10day treatment, 5 exposures (Cyprotex)	**Steatosis 3D HepaRG (MEC, µM)**	834	2330	2350	1270	1570	5290	3820	>8000	**>8000**	>8000	>8000 (665 (NS))
		ATP 3D HepaRG (MEC, µM)	109	601	320	409	209	558	568	1600	**579**	3000	4720
	HepG2, 24h exposure, single application (HULAFE) (µM)	**24h Viability MEC/IC20**	323	>8000	4536	125	>8000	1822	>8000	>8000	**>8000**	>8000	>8000
		24h GSH IC50	No data available	4328	>8000	>8000	1144	>8000	>8000	>8000	**>8000**	>8000	>8000
		GSH MEC/IC20	7383	1311	>8000	>8000	218	6235	>8000	>8000	**>8000**	>8000	>8000
		MMP IC50	150	803	>8000	>8000	No data available	>8000	>8000	>8000	**>8000**	>8000	>8000

		MMP MEC/IC20	826	1223	>8000	>8000	2999	>8000	>8000	>8000	**19224 (NS)**	4937	>8000
		Lipid accumulation IC50	831	2905	>8000	1222	1898	>8000	>8000	>8000	**>8000**	>8000	>8000
		Lipid accumuation MEC/EC20	83	1598	810	1203	69	>8000	>8000	>8000	**>8000**	>8000	>8000
		MitoSOX EC50	690	838	>8000	1758	3383	>8000	>8000	837	**>8000**	>8000	>8000
		MitoSOX MEC/EC20	426	560	2883	895	3355	7231	>8000	560	**>8000**	>8000	>8000
		Phospholipids EC50	>8000	>8000	>8000	>8000	>8000	>8000	>8000	>8000	**>8000**	>8000	>8000
		Phospholipids MEC/EC20	>8000	>8000	>8000	>8000	>8000	>8000	>8000	>8000	**>8000**	>8000	>8000
	HepG2, 72h exposure, single application (µM) (HULAFE)	**Viability IC50**	400	>8000	>8000	>8000	6763	2299	>8000	>8000	**>8000**	1256	>8000
		Viability MEC/IC20	670	3789	>8000	>8000	258	4106	>8000	>8000	**>8000**	510	7853
		GSH IC50	No data available	>8000	>8000	3505	1121	>8000	No data available	No data available	**>8000**	915	>8000
		GSH MEC/IC20	No data available	>8000	>8000	578	5.5	4897	No data available	No data available	**>8000**	177	2587
		MMP IC50	>8000	>8000	>8000	>8000	>8000	>8000	>8000	>8000	**>8000**	7156	>8000
		MMP MEC/IC20	6481	2249	>8000	>8000	1128	>8000	>8000	>8000	**>8000**	4937	>8000
		Lipids IC50	1688	527	>8000	>8000	1912	>8000	>8000	>8000	**>8000**	>8000	>8000
		Lipids MEC/EC20	913	211	810	2537	197	>8000	>8000	>8000	**>8000**	>8000	>8000
		MitoSOX EC50	340	>8000	>8000	>8000	>8000	>8000	>8000	No data available	**>8000**	>8000	>8000
		MitoSOX MEC/EC20	293	>8000	>8000	203	1847	3297	1289	No data available	**>8000**	2272	3678
		Plipids EC50	>8000	>8000	>8000	>8000	>8000	>8000	>8000	>8000	**>8000**	>8000	>8000
		Plipids MEC/EC20	>8000	>8000	>8000	>8000	>8000	>8000	>8000	>8000	**>8000**	>8000	>8000
	PHH, 24h exposure, single treatment (mM) (IFADO)	**Viability 62uM pre treat IC20 median**	27	23	27	27	25	26	11	12	**17**	13	22
		Viability 48h without FA IC20 median	18	23	16	26	26	26	7	9	**15**	11	4
		Lipid accumulation without FA	No data available	No data available	5	No data available	1	No data available	No data available	No data available	**No data available**	No data available	NS

		pretreatment MEC											
		value, unit, test method (eg. test guide line)											

	active
	inactive
	no data

Target Endpoints (pred.): in vitro available doses predicted by using an in vitro distribution model (Annex III)

			Supporting Data Related to the Target Endpoint(s)										
			Source1	**Source 2**	**Source 3**	**Source 4**	**Source 5**	**Source 6**	**Source 7**	**Source 8**	**target**	**Source 9**	**negative**
CAS			31080-39-4	3274-29-1	3274-28-0	149-57-5	99-66-1	20225-24-5	4536-23-6	97-61-0	88-09-5	1730-91-2	75-98-9
Name			2-PHP	2-Ethylheptanoic acid	2-PHA	2-Ethylhexanoic acid	Valproic acid	2-Ethylpentanoic acid	2-Methyl-hexanoic acid	2-Methyl-pentanoic acid	2-Ethylbutyric acid	2-Methylbutyric acid	Pivalic acid
In Vivo	**Toxicogenomics**												
	…												
In Vitro	**RPTEC/TERT1 cells**	**corr. factor**	0.27	0.29	0.26	0.08	0.26	0.25	0.25	0.25	0.25	0.25	0.27
		Resazurin reduction POD (intracellular; µM)	1080	1160	2080	640	NA	1000	2000	NA	NA	2000	NA
		Supernatant lactate POD (intracellular; µM)	2160	2320	NA	NA	NA	NA	NA	NA	2000	NA	NA
	Zebrafish												
		Yolk malabsorption in zebrafish embryo; morphological observation (intracellular EC20; µM)	is this supposed to be here?										
	CALUX reporter gene assays, LEC (µM), CALUX (DB-ALM 197)	**corr. factor**	0.10	0.09	0.11	0.07	0.11	0.12	0.12	0.13	0.13	0.13	0.13
		Anti-AR CALUX (intracellular conc.)	31.6	28.5	35	28	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
		Anti-PR CALUX (intracellular conc.)	25.1	28.5	13.8	70.0	87.4	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
		TRb CALUX (intracellular conc.)	31.6	35.8	87.4	#VALUE!	110.0	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
		PXR CALUX (intracellular conc.)	5.0	9.0	11.0	17.6	8.7	37.9	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!

		PPARa CALUX (intracellular conc.)	3.2	2.8	11.0	7.0	11.0	15.1	3.8	41.1	41.1	#VALUE!	#VALUE!
		PPARd CALUX (intracellular conc.)	100.0	71.5	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
		PPARg CALUX (intracellular conc.)	12.6	45.1	55.1	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
		AhR CALUX (intracellular conc.)	100.0	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
		TCF CALUX (intracellular conc.)	5.0	22.6	34.8	22.1	11.0	120.0	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
		AP1 CALUX (intracellular conc.)	10.0	#VALUE!	110.0	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
		ESRE CALUX (intracellular conc.)	10.0	28.5	110.0	70.0	11.0	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
		Nrf2 CALUX (intracellular conc.)	10.0	18.0	110.0	70.0	#VALUE!	47.8	#VALUE!	#VALUE!	#VALUE!	103.3	#VALUE!
		p21 CALUX (intracellular conc.)	20.0	35.8	69.4	70.0	69.4	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
		p53 GENTOX CALUX (intracellular conc.)	10.0	28.5	21.9	55.6	27.6	120.0	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
	GFP-reporter gene assay, 24h exposure; single application, LEC (µM) (intracellular conc.)	**corr. factor**	0.16	0.14	0.17	0.12	0.19	0.21	0.20	0.22	0.22	0.22	0.22
		SRXN1 (FPC_2m)	1139.1	1358.4	1320.1	1338.7	1260.7	#VALUE!	#VALUE!	#VALUE!	#VALUE!	3093.7	#VALUE!
		P21 (FPC_2m)	727.6	#VALUE!	1458.6	#VALUE!	1967.4	#VALUE!	2909.7	#VALUE!	#VALUE!	2668.3	#VALUE!
		corr. factor	0.31	0.61	0.61	0.84	0.84	0.95	0.95	0.98	0.98	1.00	1.00

	HepaRG, 10day treatment, 5 exposures (Cyprotex)	Steatosis 3D HepaRG (MEC, µM; free media)	258.5	1421.3	1433.5	1066.8	1318.8	5025.5	3629.0	#VALUE!	#VALUE!	#VALUE!	#VALUE!
		ATP 3D HepaRG (MEC, µM; free media)	33.8	366.6	195.2	343.6	175.6	530.1	539.6	1568.0	567.4	3000.0	4720.0
	HepG2, 24h exposure, single application (HULAFE) (µM) (intracellular conc.)	corr. factor	0.15	0.13	0.16	0.06	0.18	0.19	0.19	0.20	0.20	0.20	0.21
		24h Viability MEC/IC20	48.5	#VALUE!	725.8	7.5	#VALUE!	346.2	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
		24h GSH IC50	#VALUE!	562.6	#VALUE!	#VALUE!	205.9	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
		GSH MEC/IC20	1107.5	170.4	#VALUE!	#VALUE!	39.2	1184.7	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
		MMP IC50	22.5	104.4	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
		MMP MEC/IC20	123.9	159.0	#VALUE!	#VALUE!	539.8	#VALUE!	#VALUE!	#VALUE!	#VALUE!	987.4	#VALUE!
		Lipid accumulation IC50	124.6	377.7	#VALUE!	73.3	341.6	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
		Lipid accumuation MEC/EC20	12.4	207.7	129.6	72.2	12.5	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
		MitoSOX EC50	103.6	108.9	#VALUE!	105.5	608.9	#VALUE!	#VALUE!	167.4	#VALUE!	#VALUE!	#VALUE!
		MitoSOX MEC/EC20	63.9	72.8	461.3	53.7	603.9	1373.9	#VALUE!	112.0	#VALUE!	#VALUE!	#VALUE!
	HepG2, 72h exposure, single application (µM) (HULAFE) (intracellular conc.)	Viability IC50	60.0	#VALUE!	#VALUE!	#VALUE!	1217.3	436.8	#VALUE!	#VALUE!	#VALUE!	251.2	#VALUE!
		Viability MEC/IC20	100.5	492.6	#VALUE!	#VALUE!	46.4	780.1	#VALUE!	#VALUE!	#VALUE!	102.0	1649.1
		GSH IC50	#VALUE!	#VALUE!	#VALUE!	210.3	201.8	#VALUE!	#VALUE!	#VALUE!	#VALUE!	183.0	#VALUE!
		GSH MEC/IC20	#VALUE!	#VALUE!	#VALUE!	34.7	1.0	930.4	#VALUE!	#VALUE!	#VALUE!	35.4	543.3
		MMP IC50	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	1431.2	#VALUE!
		MMP MEC/IC20	972.2	292.4	#VALUE!	#VALUE!	203.0	#VALUE!	#VALUE!	#VALUE!	#VALUE!	987.4	#VALUE!

		Lipids IC50	253.2	68.5	#VALUE!	#VALUE!	344.2	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
		Lipids MEC/EC20	137.0	27.4	129.6	152.2	35.5	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
		MitoSOX EC50	51.0	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
		MitoSOX MEC/EC20	44.0	#VALUE!	#VALUE!	12.2	332.5	626.4	244.9	#VALUE!	#VALUE!	454.4	772.4
	PHH, 24h exposure, single treatment (µM) (IFADO) (intracellular conc.)	**corr. factor**	0.15	0.13	0.16	0.06	0.18	0.19	0.19	0.20	0.20	0.20	0.21
		Viability 62uM pre treat IC20 median	4064.3	3026.1	4270.2	1638.4	4477.7	4956.1	2054.2	2488.5	3313.2	2503.3	4606.4
		Viability 48h without FA IC20 median	2697.1	3040.4	2637.5	1534.6	4625.6	4918.9	1394.9	1898.7	3096.3	2256.0	750.1
		Lipid accumulation without FA pretreatment MEC	#VALUE!	#VALUE!	800	#VALUE!	180	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
		value, unit, test method (eg. test guide line)											

	active
	inactive
	no data

In chemico: QSAR profiler of the OECD QSAR toolbox

		In chemico										
		Source1	**Source 2**	**Source 3**	**Source 4**	**Source 5**	**Source 6**	**Source 7**	**Source 8**	**target**	**Source 9**	**negative**
CAS		31080-39-4	3274-29-1	3274-28-0	149-57-5	99-66-1	20225-24-5	4536-23-6	97-61-0	88-09-5	1730-91-2	75-98-9
Name		2-PHP	2-Ethylheptanoic acid	2-PHA	2-Ethylhexanoic acid	Valproic acid	2-Ethylpentanoic acid	2-Methyl-hexanoic acid	2-Methyl-pentanoic acid	2-Ethylbutyric acid	2-Methylbutyric acid	Pivalic acid
***In silico* models of the OECD QSAR toolbox**	**DNA binding by OASIS**	No alert found	No alert found	No alert found	No alert found	No alert found	No alert found	No alert found	No alert found	No alert found	No alert found	No alert found
	DNA binding by OECD	No alert found	No alert found	No alert found	No alert found	No alert found	No alert found	No alert found	No alert found	No alert found	No alert found	No alert found
	Eye irritation/corrosion Exclusion rules by BfR	Group C Melting Point > 55 C|Undefined	Undefined	Undefined	Undefined	Undefined	Undefined	Undefined	Undefined	Undefined	Undefined	Undefined
	Eye irritation/corrosion Inclusion rules by BfR	Inclusion rules not met	Inclusion rules not met	Inclusion rules not met	Inclusion rules not met	Inclusion rules not met	Inclusion rules not met	Inclusion rules not met	Inclusion rules not met	Inclusion rules not met	Inclusion rules not met	Inclusion rules not met
	Protein Binding Potency h-CLAT	No alert found	No alert found	No alert found	No alert found	No alert found	No alert found	No alert found	No alert found	No alert found	No alert found	No alert found
	Protein binding alerts for Chromosomal aberration by OASIS	No alert found	No alert found	No alert found	No alert found	No alert found	No alert found	No alert found	No alert found	No alert found	No alert found	No alert found
	Protein binding alerts for skin sensitisation according to GHS	No alert found	No alert found	No alert found	No alert found	No alert found	No alert found	No alert found	No alert found	No alert found	No alert found	No alert found
	Protein binding alerts for skin sensitisation by OASIS	No alert found	No alert found	No alert found	No alert found	No alert found	No alert found	No alert found	No alert found	No alert found	No alert found	No alert found
	Protein binding by OASIS	No alert found	No alert found	No alert found	No alert found	No alert found	No alert found	No alert found	No alert found	No alert found	No alert found	No alert found
	Protein binding by OECD	No alert found	No alert found	No alert found	No alert found	No alert found	No alert found	No alert found	No alert found	No alert found	No alert found	No alert found
	Protein binding potency GSH	Not possible to classify	Not possible to classify	Not possible to classify	Not possible to classify	Not possible to classify	Not possible to classify	Not possible to classify	Not possible to classify	Not possible to classify	Not possible to classify	Not possible to classify

	Protein binding potency Cys (DPRA 13%)	Non-Conjugated carboxylic acids and esters (non reactive)	Non-Conjugated carboxylic acids and esters (non reactive)	Non-Conjugated carboxylic acids and esters (non reactive)	Non-Conjugated carboxylic acids and esters (non reactive)	Non-Conjugated carboxylic acids and esters (non reactive)	Non-Conjugated carboxylic acids and esters (non reactive)	Non-Conjugated carboxylic acids and esters (non reactive)	Non-Conjugated carboxylic acids and esters (non reactive)	Non-Conjugated carboxylic acids and esters (non reactive)	Non-Conjugated carboxylic acids and esters (non reactive)	Non-Conjugated carboxylic acids and esters (non reactive)
	Protein binding potency Lys (DPRA 13%)	Non-Conjugated carboxylic acids and esters (non reactive)	Non-Conjugated carboxylic acids and esters (non reactive)	Non-Conjugated carboxylic acids and esters (non reactive)	Non-Conjugated carboxylic acids and esters (non reactive)	Non-Conjugated carboxylic acids and esters (non reactive)	Non-Conjugated carboxylic acids and esters (non reactive)	Non-Conjugated carboxylic acids and esters (non reactive)	Non-Conjugated carboxylic acids and esters (non reactive)	Non-Conjugated carboxylic acids and esters (non reactive)	Non-Conjugated carboxylic acids and esters (non reactive)	Non-Conjugated carboxylic acids and esters (non reactive)
	Skin irritation/corrosion Exclusion rules by BfR	Group C Melting Point > 55 C|Undefined	Undefined	Undefined	Undefined	Undefined	Undefined	Undefined	Undefined	Undefined	Undefined	Undefined
	Skin irritation/corrosion Inclusion rules by BfR	Inclusion rules not met	Aliphatic acids	Aliphatic acids	Aliphatic acids	Aliphatic acids	Aliphatic acids	Aliphatic acids	Aliphatic acids	Aliphatic acids	Aliphatic acids	Aliphatic acids

Annex II. Detailed description of *in vivo* and *in vitro* experiments

Please refer to the separate publication for full Annex II

ENV/JM/WRPR(2020)31/ANN2

Annex III. Detailed description of *in silico* models

Please refer to the separate publication for full Annex III

ENV/JM/WRPR(2020)31/ANN3