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NAM-supported read-across: from case studies to regulatory guidance in safety assessment

Case Studies for Breakout discussion groups

1 Prediction of a 90-day repeated dose toxicity study (OECD 408) for 2-Ethylbutyric acid using a read-across approach to other branched carboxylic acids.

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1.1 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, according to a scenario 4. A category of branched carboxylic acids is evaluated, for which we see a consistent trend between category members with regard to the primary toxic effect, identified in the in vivo studies of analogues. New approach methodologies (NAM) like in vitro and in silico models are used in addition to in vivo data to confirm the consistent trend and for hazard characterization.

1.2 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 analogue, 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. NAM data showed a consistent trend with regard to toxicokinetics and toxicodynamics within the grouped compounds.

1.3 Toxicokinetics

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.

1.4 Toxicodynamics

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 summarized 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 of the AOP related MIEs. With increasing chain length, the number of activated MIEs related to steatosis increased. The target compound 2-EBA activated the MIE, PPAR-α. It can therefore not be excluded that a pathway toward 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 pertubations (e.g. glutathion depletion, mitochondrial membrane potential, mitochondrial superoxide formation etc.).

1.5 Data integration

The decision theory Dempster Shafer (DS) indicated that the absence of lipid accumulation for 2-EBA can be predicted with 100% certainty up to the highest tested dose tested in the present in vitro assays. 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.

1.6 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. Therefore, we used the most sensitive in vitro endpoint, 10^{th} percentile of all assays measuring cytotoxicity in liver and kidney cells, 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 can be used to fill the data gap of a subchronic toxicity study. Furthermore, QIVIVE was used to determine the human oral equivalent dose, which is 138 mg/kg bw/d. Below this threshold, a risk for humans to develop liver steatosis or general liver/kidney toxicity is not expected.

2 Read-across based filling of developmental and reproductive toxicity data gap for methyl hexanoic acid

2.1 Abstract

2-Methylhexanoic acid (MHA) is a compound for which developmental and reproductive toxicity test (DART) data is taken to be lacking. We have searched for structural analogues that have this data in order to explore the possibility to read across information of these source chemicals to MHA. The following structural related aliphatic carboxylic acids were selected: 2-ethylhexanoic acid (EHA), 2-propylpentanoic acid (VPA), 2-propylheptanoic acid (PHA), 2-methylpentanoic acid (MPA), 2-ethylbutanoic acid (EBA), 4-pentenoic acid (PA), 2-propyl-4-pentenoic acid (4-ene-VPA), and 2-dimethylpentanoic acid (DMPA). Some of these analogues proved to be clear reproductive toxicants, i.e. VPA, PHA, EHA, and 4-ene-VPA, while others were identified as not being toxic to reproduction, i.e. EBA, PA, and DMPA; i.e. they did or did not induce neural tube defects upon *in vivo* exposure. Thus, structural similarity alone doesn't allow a conclusion on the reproductive properties of MHA. Therefore, we have also tested MHA and all the selected source chemicals in a battery of *in vitro* tests with clear relevance to DART, i.e. the Zebrafish Embryo Test (ZET), mouse Embryonic Stem cell Test (mEST), iPSC-based neurodevelopmental model (UKN1), and a series of CALUX Reporter assays, that we combined with toxicokinetic models to calculate effective cellular concentrations and associated *in vivo* exposure doses. With these new approach methodologies (NAM) we wanted to explore whether they could correctly predict the *in vivo* reproductive properties of these aliphatic carboxylic acids, and thus could be used to predict the *in vivo* reproductive properties of MHA itself. This data would also allow to further explore the relationship between structure and reproductive toxicity within this series of aliphatic carboxylic acids. For that reason, we have also tested MPA in these NAM, despite the absence of *in vivo* data. We have also investigated the potential to inhibit histone deacetylase activity in ZET, mEST, and UKN1 models, as this enzyme is postulated to be the molecular initiating target leading to neural tube defects observed with the reprotoxic analogues.

The NAM results show that VPA, PHA, EHA, and 4-ene-VPA were correctly predicted as *in vivo* reproductive toxicants, and EBA, PA, and DMPA as non-reproductive toxicants. The NAM results also predict MHA, and DMPA as not reprotoxic, and shed some more light on the structural requirements for reproductive properties of these aliphatic carboxylic acids. On the basis of this data it is concluded that MHA is not expected to be an *in vivo* reproductive toxicant.

3 Waiving of repeat-dose neurotoxicity study (TG 424) for azoxystrobin based on Read-Across to other strobilurins

3.1 Abstract

The synthetic strobilurin fungicides are derived from the naturally occurring strobilurin A and B. The strobilurins bind to the quinol oxidation site of cytochrome b of complex III (CIII) of the mitochondria which is also their fungicidal mode of action. There are some signals of potential neurotoxicity from in vitro studies by a CIII-mediated mechanism.

The objective of this read-across case is to justify the waiving of an OECD TG 424 study for azoxystrobin by means of NAM data. The source compounds are other strobilurin fungicides. The formation of the category is based on the hypothesis that the compounds share similar chemical structure, similar pesticidal mode of action, similar toxicophore, similar neurotoxic potential and similar toxicokinetics to azoxystrobin. The source compounds chosen were pyraclostrobin, picoxystrobin, trifloxystrobin, and kresoxim-methyl. Furthermore, in vitro testing was conducted on Antimycin A, a well-established CIII inhibitor with neurotoxic effects, which serves as a reference compound for this mode of action. The degree of *in vivo* inhibition of the mitochondrial respiratory system depends on the respiratory activity and thus the tissues like brain can be more susceptible if exposed.

Existing regulatory *in vivo* data was collected for the source and target compounds with a focus on ADME, neurotoxicity as well as target organ toxicity data. The source compounds do neither show signs of neurotoxicity in neurotoxicity studies nor in other repeat dose toxicity studies.

The scientific hypothesis is: Can the absence of a neurotoxic potential (as detected with a TG424 study) mediated by inhibition of Complex III of the mitochondria be predicted by toxicodynamic and toxicokinetic NAM data?

The hypothesis is supported by mechanistic data, anchored to a putative AOP (based on the recently OECD adopted AOP on CI inhibition leading to parkinsonian disorder), and kinetic PBTK data. Thus, the following data was obtained: phys/chem, structural similarity, effects on oxygen consumption (mitochondrial complexes and whole cells), effects on mitochondrial membrane potential, cellular damage measured by effects on glycolysis and viability in three different cell types including neuronal cells, neuronal degeneration and neurite outgrowth.

The overall structural similarity of the compounds, although having the same pesticidal mode of action and toxophore is less, however, they have the same pesticidal mode of action and toxophore.

Inhibition of CIII complexes measured by oxygen consumption, by the target compound azoxystrobin seemed to be slightly less strong than by the source compounds pyraclostrobin and picoxystrobin, while antimycin A resulted in a much stronger inhibition. This was confirmed with whole cells as well. Effects on membrane potential were marked by Antimycin A and orders of magnitude less with the target and source compounds. Effects on glycolysis and cell viability were similar between the compounds. The target compound was negative in the neurite outgrowth assay in SH-SY5Y cells, while

some of the source compounds did show weak effects, and neither the target nor the source compounds were regarded as neurotoxic in the neuritetox assay in LUHMES cells.

The kinetic data and simulations confirm comparable kinetics and that the exposure of the brain to the strobilurines is limited being approx. twice the plasma concentration.

Overall, based on the generated data on kinetics and effect data, there is no evidence for a stronger neurotoxic potential of azoxystrobin mediated by a complex III inhibitory mode of action as compared to the source compounds. Since the source compounds do not show neurotoxicity in vivo, it is concluded that also the target compound azoxystrobin is not a neurotoxicant and therewith no TG 424 is warranted.

4 CASE STUDY ON THE USE OF INTEGRATED APPROACHES FOR TESTING AND ASSESSMENT (IATA) FOR ESTROGENICITY OF THE SUBSTITUTED PHENOLS

Case study submitted by Health Canada and US EPA

4.1 Introduction

This case study is intended to offer practical insights to inform the development of guidance for deriving and applying Integrated Approaches for Testing and Assessment' (IATA) in a regulatory context. The case study is the result of a collaborative effort by staff at both the Existing Substances Risk Assessment Bureau (ESRAB) at Health Canada and the National Center for Computational Toxicology (NCCT) at the U.S. Environmental Protection Agency (EPA). The case study focuses on a set of substituted phenols (hindered and non-hindered) and uses IATA to examine their estrogenic potential. The selected phenols used in this case study were of particular interest because they are listed on the Canadian Domestic Substances List (DSL)1 and will be addressed under the third phase of Canada's Chemicals Management Plan (CMP)2. The final approach applied for the CMP screening risk assessment report currently under development by Health Canada is considered separate from this document and the approaches taken are subject to change. This document is not intended to provide complete characterisation of health effects for the CMP phenolic substances (target chemicals), nor does it provide information regarding their exposure for the general population of Canada. The goal of this case study is to demonstrate that in silico and in vitro data can be used to screen for estrogenic potential of chemical substances, and that these data sources provide a good proxy for estimating the in vivo point of departure dose. The estrogenic potential of the three target chemicals was determined using an IATA that combines (Q)SAR approaches and data from in vitro and in vivo studies. (Q)SAR predictions were generated using selected publicly available and commercial models. The in vitro high throughput screening (HTS) data from multiple assays were combined into a consensus prediction of estrogenic potential. Extrapolation of HTS bioactivity to an estimated applied dose equivalent (ADE) was performed through the application of reverse dosimetry. For the target substance that showed estrogenic potential, the ADE was compared to effect levels from traditional in vivo animal studies to demonstrate the utility of these HTS data for use during prioritisation and assessment. The methods and application of the IATA illustrated here may broadly support priority setting for further evaluation as well as hazard characterisation for risk assessment.

5 Case Study on the use of Integrated Approaches for Testing and Assessment for Testicular Toxicity of Ethylene Glycol Methyl Ether (EGME)-Related Chemicals

Case study submitted to OECD by Japan

Highlights: Integrating in vivo, in vitro and in silico metabolism information for the category assessment and read-across with use of toxicity data of EGME-related source chemicals. NO(A)EL values are derived for the hazard classification under the CSCL in Japan.

5.1 Executive Summary

This specific case study was developed to address how read-across can be applied to fill data gaps in reproductive toxicity endpoint for the screening assessment under the Japanese Chemical Substances of Control Law (CSCL). A category approach was applied to assessing the testicular toxicity of ethylene glycol methyl ether (EGME)-related chemicals. Based on toxicity information for EGME and related chemicals and accompanied by possible adverse outcome pathway information on the testicular toxicity of EGME, this category members were defined as chemicals that are metabolized to methoxy- or ethoxyacetic acid, a substance responsible for testicular toxicity.

A Japanese chemical inventory was screened using metabolism simulator of Hazard Evaluation Support System (HESS). Quantitative metabolism information on the related chemicals was then considered, and fifteen chemicals were finally obtained from the inventory as a shortlist for the category. Available data in the literature shows that chemicals for which information is available on the metabolic formation of methoxy- or ethoxyacetic acid possess testicular toxicity. The results suggest that testicular toxicity is a concern for the untested chemicals which are predicted to produce the toxic metabolites.

Overall uncertainty of the case study is low. However, some of the original compounds are structurally diverse. Metabolic hydrolysis or dealkylation might produce additional toxic compounds, which needed to be explicitly mentioned. Database search for toxicity and metabolism information was useful to suggest that possible metabolites do not affect the toxicity levels through different mechanism of action.

5.2 Introduction

Reproductive and developmental toxicity is one of the key regulatory endpoints in the hazard assessment of chemicals. For risk assessment under the Japanese Chemical Substances of Control Law (CSCL), a screening assessment is conducted to select Priority Assessment Chemical Substances. Human health endpoints for the assessment include, i) repeated dose toxicity, ii) reproductive and developmental toxicity, iii) genotoxicity, and iv) carcinogenicity. A hazard class for the reproductive and developmental toxicity is currently assignable when

both reproductive and developmental toxicity data are obtained by animal testing. Due to lack of animal study data, some of the chemicals cannot be classified for the reproductive and developmental toxicity. In such cases, the hazard on the reproductive and developmental toxicity is not further considered for prioritization. When reproductive or developmental toxicity potential to a target chemical is suggested by analogue substances, the read-across approach can be useful for more valid prioritization under the CSCL.

Ethylene glycol methyl ether (EGME) is manufactured and imported in amounts of about 5 kt/year in Japan (METI, 2018) although it has gradually been substituted by other chemicals. Due to concerns about exposure to this chemical, numerous toxicological studies have been conducted (NIOSH, 1991). One of the most studied organ toxicities of EGME is testicular toxicity, which is characterized by atrophy, degeneration and necrosis of the pachytene spermatocytes, and a decrease in sperm count in rats, mice and rabbits (Foster *et al*., 1983; Miller *et al*., 1983; Nagano *et al*., 1984; NTP, 1993). Substantial new information concerning the human health consequences of exposure to this class of chemicals was summarized in the report by ECETOC (ECETOC, 2005). Moreover, read-across approach was applied to proposal for harmonized classification and labelling of tetraglyme, which contains EGME moiety in the molecule (Environmental Agency Austria, 2017)). This case study focuses on testicular toxicity endpoints for category assessment of EGME-related chemicals, some of which are used as solvent, plasticizer or synthetic intermediate.

Category assessment is not currently utilized in the screening assessment, but it is recommended for chemical assessment under the CSCL (METI *et al*., 2012). This specific case study was developed to address how read-across can be applied to fill data gaps in the screening assessment under the CSCL. This case study is mostly based on the work published in a previous paper by Yamada *et al.* (2014) but updated with related literature.