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Incorporating Lines of Evidence from New Approach Methodologies (NAMs) to Reduce Uncertainties in a Category Based Read-Across: A Case Study for Repeated Dose Toxicity

Incorporating Lines of Evidence from New Approach Methodologies (NAMs) to Reduce Uncertainties in a Category Based Read-Across: A Case Study for Repeated Dose Toxicity

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Abstract

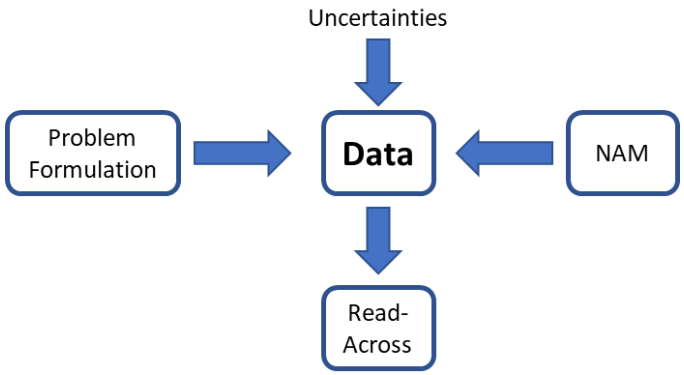
A group of triazole compounds was selected to investigate the confidence that may be associated with read-across of a complex data gap: repeated dose toxicity. The read-across was evaluated using Assessment Elements (AEs) from the European Chemicals Agency's (ECHA's) Read-Across Assessment Framework (RAAF), alongside appraisal of associated uncertainties. Following an initial read-across based on chemical structure and properties, uncertainties were reduced by the integration of data streams such as those from New Approach Methodologies (NAM) and other existing data. In addition, addressing the findings of the ECHA RAAF framework, complemented with specific questions concerning uncertainties, increased the confidence that can be placed in read-across. Although a data rich group of compounds with a strong mechanistic basis was analysed, it was clearly demonstrated that NAM data available from publicly available resources could be applied to support read-across. It is acknowledged that most read-across studies will not be so data rich or mechanistically robust, therefore some targeted experimentation may be required to fill the data gaps. In this sense, NAMs should constitute new experimental tests performed with the specific goal of reducing the uncertainties and demonstrating the read-across hypothesis.

Key Words: Read-Across; Uncertainty; Read-Across Assessment Framework; New Approach Methodology; Triazole Fungicides

Highlights

- Uncertainties in read-across for repeated dose toxicity are identifiable
- A variety of *in silico* and *in vitro* NAMs can be obtained easily
- Strategic use of NAM data reduces uncertainty in read-across
- Resources to support read-across are illustrated

Graphical Abstract



Abbreviations

1,2,4-T, 1,2,4-triazole; AEs, Assessment Elements; AHR, aryl hydrocarbon receptor; AOP, Adverse Outcome Pathway; AR, androgen receptor; BBB, blood-brain barrier; bit, bitertanol; CA, Chromosomal Aberration; CAG, Cumulative Assessment Group; CAR, constitutive androstane receptor; CERAPP, Collaborative Estrogen Receptor Activity Prediction Project; CompTox, US EPA Computational Toxicology; CYP, cytochrome P450; cypr, cyproconazole; dif, difenoconazole; DART, Developmental and Reproductive Toxicity; ECHA, European Chemicals Agency; EFSA, European Food Safety Authority; ER, oestrogen receptor; epo, epoxiconazole; EU, European Union; FAO, Food and Agriculture Organization of the United Nations; fen, fenbuconazole; FXR, farnesoid X receptor; GI, gastrointestinal; GRAP, Good Read-Across Practice; HB, halogenated benzenes alerts; hex, hexaconazole; HTS, high-throughput screening; IPCS INCHEM, International Programme on Chemical Safety; ISS, Istituto Superiore di Sanità; iTTC, internal Threshold of Toxicological Concern; JMPR, Joint FAO/WHO Meeting on Pesticide Residues; KE, Key Event; log P, logarithm of the octanol-water partition coefficient; LXRα, liver X receptor alpha; MIE, Molecular Initiating Event; MNT, micronucleus test; myc, myclobutanil; NA, No (*in silico* profiling) alerts identified; NAM, New Approach Methodology; NOAEL, No Observed Adverse Effect Level; NR, ruclear receptor; NRMEA, Nuclear Receptor-Mediated Endocrine Activity Model; OCM, Organotypic Culture Models; OECD, Organisation for Economic Co-operation and Development; pac, paclobutrazol; pen, penconazole; P-gp, permeability glycoprotein; prop, propiconazole; prot, prothioconazole; PXR, pregnane X receptor; (Q)SAR, (Quantitative) Structure-Activity Relationship; RAAF, Read-Across Assessment Framework; RARα, retinoic acid receptor alpha; REACH, Registration, Evaluation, Authorisation and Restriction of Chemicals; SAA, n-alkylcarboxylic acid alert; STOT-RE, Specific Target Organ Toxicity - Repeated Exposure; TA, triazole alanine; TAA, triazole acetic acid; Tc, Tanimoto coefficient; teb, tebuconazole; TK, toxicokinetic(s); ToxCast, US EPA's Toxicity Forecaster; tril, triadimenol; trin, triadimefon; TRα, TRβ, thyroid receptors alpha and beta; TTC, Threshold of Toxicological Concern; US EPA, United States

67 Environmental Protection Agency; UGT1A1, uridine diphosphate glucuronosyltransferase 1A1; WHO,

68 World Health Organization; WoE, Weight of Evidence.

69

1. Introduction

Read-across is the process of interpolating similar biological effects for related chemicals. It is based around the identification of analogues with suitable data to make a prediction for a compound with no, or insufficient data (Cronin, 2013; Kovarich et al., 2019). As such, read-across is an increasingly widely used as a method of filling data gaps for toxicological and other endpoints (Myatt et al., 2018). Due to the robustness, simplicity and transparency of the approach, it has seen widespread use for the prediction of complex toxicities and adverse effects, e.g. repeated dose toxicity. Whilst there are many potential applications of read-across (Cronin and Yoon, 2019; Mahony et al., 2020), it is for regulatory applications where it has come to prominence - notably in the well-documented uptake within the European Union (EU) Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) legislation (ECHA, 2020).

There is copious guidance on methods and means of forming a similarity argument to support a read-across to make a prediction of toxicity (Schultz et al., 2015). There are also established frameworks to develop these read-across arguments, which have been summarised and rationalised by Patlewicz et al. (2018) into a harmonised hybrid development and assessment framework. The harmonised framework leads the user through a process of problem formulation, determination of the overarching similarity rationale through to analogue identification and evaluation resulting in data gap filling. A final step is foreseen as being the assessment of uncertainties. Within this framework, a number of steps are relatively well established and have been supported by case studies, international guidance as well as recommendation such as Good Read-Across Practice (GRAP) (Ball et al., 2016).

A number of case studies of read-across for repeated dose toxicity (Schultz et al., 2017a,b; Przybylak et al., 2017; Mellor et al., 2017; Firman et al., 2018) have provided the catalyst for the understanding of weaknesses in read-across. Most specifically, these weaknesses are seen as being potentially restrictive towards regulatory acceptance (Chesnut et al., 2018; Escher et al., 2019). Schultz and Cronin (2017) reported a number of key areas in read-across where there was significant uncertainty, typically

95 focussing on definition and justification of similarity between molecules and the quantity and quality
96 of data associated with the source chemicals. Later, the same authors (Schultz et al., 2019) provided
97 a framework to identify, and more significantly (semi-)quantify, these uncertainties.

98 As read-across techniques are better developed, with the expectation of improvements in regulatory
99 acceptance, a number of enhancements have been made. For instance, the last decade has seen a
100 shift in read-across from simply being a consideration of structurally similar analogues, to being a more
101 robust compilation of various lines of evidence to support a similarity hypothesis. In particular, greater
102 consideration is now given to compiling information on biological (toxicological), metabolic and
103 pharmacokinetic data (Gadaleta et al., 2020). Whereas read-across was originally foreseen as simply
104 being the extrapolation of information from one homologous analogue to another, which could, for
105 example, be as straightforward as an increase in carbon chain length, it is now expanded to
106 compounds with similar modes/mechanisms of toxic action and or metabolic profiles. Therefore,
107 there is much greater emphasis on identifying information relating to the target and source chemicals,
108 much of which is now referred to as being New Approach Methodology (NAM) data, to support the
109 read-across hypothesis (Rovida et al., 2020). From the outset in this study it is acknowledged that
110 there is currently no harmonised definition of a NAM. Whilst it had been used before, the term "NAM"
111 was first brought to broader public attention in 2016 as part of a Workshop report (ECHA, 2016), with
112 its first use in a peer-reviewed publication appearing in 2017 (Schultz and Cronin, 2017). Amongst
113 others, a definition of NAM is provided by the US EPA as being "*any technology, methodology,*
114 *approach, or combination thereof that can be used to provide information on chemical hazard and risk*
115 *assessment that avoids the use of intact animals.*" (US EPA, 2018). These are usually defined as being
116 *in vitro*, *in chemico* and *in silico* techniques. Several studies have shown the utility of NAM data to
117 support read-across arguments, especially when based on a mechanistic (Escher et al., 2019) or
118 metabolic (Yordanova et al., 2019) hypothesis. It is also possible to combine together these
119 information streams using techniques such as Dempster-Shafer theory (Rathman et al., 2018). In this
120 study we have also included existing data as part of the package of information that can be used to

121 support a read-across. It is acknowledged that, in the strictest sense, existing data are not NAMs, but
122 it is our assumption that they provide useful and useable lines of evidence that will support an overall
123 weight of evidence for a read-across. They were used by Schultz et al., (2017a,b), Przybylak et al.,
124 (2017), Mellor et al., (2017) and Firman et al., (2018) for this purpose, without being termed NAMs.
125 The types of information used as NAMs, and the sources of existing data that may be useful to support
126 read-across, are summarised in Table 1.

127

128 Table 1. Lines of evidence from NAMs and existing data that can be brought together to support a
129 weight of evidence in a read-across justification (adapted from Mahony et al (2020)).

Data source	Use as a line of evidence in read-across
Conventional NAMs[a] – *in vitro*	
Functional *in vitro* assays at the cellular, tissue, etc level	Supporting mechanistic hypotheses, toxicokinetic and exposure determination
High throughput screening	
Organotypic Culture Models (OCMs) i.e. organoid, microphysiological systems, organ-on-a-chip	
Omics technologies e.g. metabolomics	
Conventional NAMs[a] – *in chemico*	
Reactivity assays e.g. peptide reactivity	Supporting mechanistic hypotheses for reactive toxicity
Conventional NAMs[a] – *in silico*	
Structural alerts / profilers	Supporting hypotheses of similarity and dissimilarity based on calculated properties and effects, molecular fragments and descriptors etc
Structural similarity	
Read-across techniques e.g. metrics of chemical similarity	

Calculated physico-chemical properties / molecular descriptors	
QSARs	
3-D docking	
Virtual tissue modelling	
Conventional NAMs[a] – Toxicokinetics (TK)/ Exposure	
In vitro and in silico estimates of TK properties	Supporting similarity in terms of bioavailability
Exposure e.g. use, internal concentrations, biomonitoring	
Internal Threshold of Toxicological Concern (iTTC) and TTC	Not formally used in read-across although may be applied in risk assessment
Other Lines of Evidence from Existing Data	
In vitro data for hazard and TK	Supporting evidence of presence or absence of adversity and / or potency
In vivo data for the endpoint of interest, these may be non-standard data, for hazard and TK	
In vitro / *in vivo* data for related endpoints e.g. Ames test to support skin sensitisation assessment.	
Human data e.g. clinical or epidemiological for hazard and exposure.	

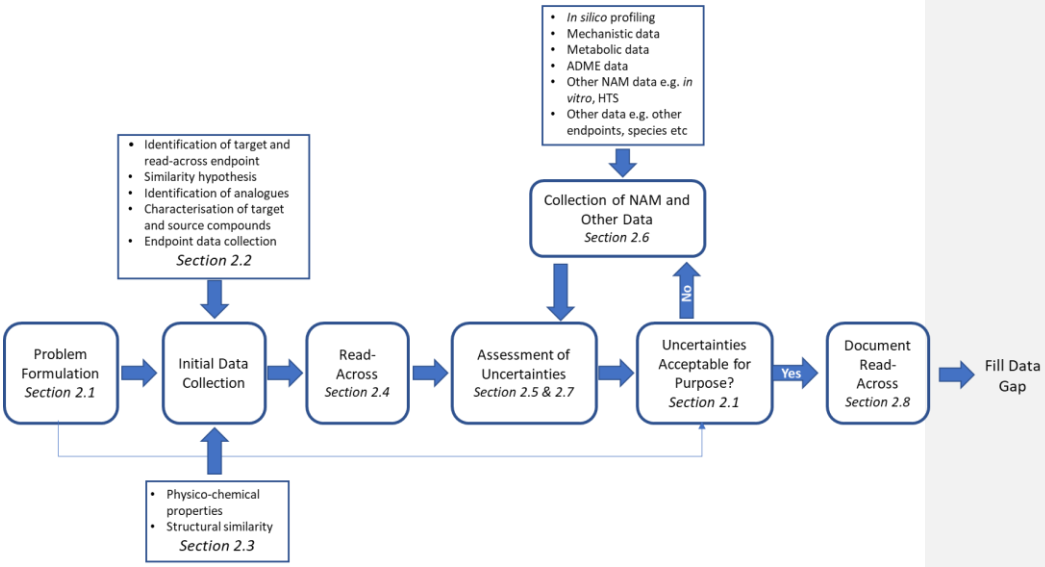
[a]The term "Conventional NAMs" is used advisedly in this context. It refers to NAMs as defined by ECHA (2016) and US EPA (2018). Please note this is not an accepted term and is used to differentiate what may be considered to be "non-NAM" lines of evidence.

Currently, NAM-derived data have started to be used as complementary information to support the read-across hypothesis by providing data to confirm if a group of substances share the same biological mechanism. The strength of NAM in read-across is that all members of the group can be tested simultaneously with the same test method and the results assessed as a category, demonstrating

similarities and dissimilarities or providing clues to link the chemical structure to the biological activity (Rovida et al., 2020). With many hundreds of data resources available (Pawar et al., 2019) and a range of tools for *in silico* profiling that can be applied, it is essential to establish the means by which the data can be considered. At this time a variety of methods have been applied, however, there is no comprehensive overview of how *in silico* NAM data can provide an overall view of the uncertainty of a read-across and hence potentially drive, in a rational manner, experimental work using NAM.

In the present analysis, we selected the triazole fungicides, a data-rich group of compounds, to demonstrate the confidence that may be associated with read-across of a complex data gap: repeated dose toxicity. Triazoles are widely used in agriculture as antifungal agents in plant protection products as well as in pharmaceuticals. These compounds share a similar mechanism of action based on the inhibition of the enzyme sterol 14α demethylase, which belongs to the cytochrome P450 (CYP) family. Inhibition of this enzyme leads to ergosterol depletion, fungal cell membrane disruption and prevention of infection (JMPR, 2008a). As triazoles are produced at high volumes and frequently occur as residues in foods, the European Food Safety Authority (EFSA) proposed a Cumulative Assessment Group (CAG) to assist in the assessment of their toxicity (EFSA, 2009).

Triazole compounds have extensive animal toxicity data. Also, a significant amount of *in vitro* data have been generated for these chemicals as a proof-of-concept for the potential health effects when compared to the animal toxicity results (Seeger et al., 2019). We analysed read-across using Assessment Elements (AE) from the European Chemicals Agency (ECHA) Read-Across Assessment Framework (RAAF) (ECHA, 2017) alongside uncertainties, as defined by Schultz et al. (2019), to identify weaknesses. Where possible, substances were compared based on the chemical and biological points of view through several tools including NAMs and existing data – the general workflow for this study is shown in Figure 1. The issue of how NAM data could reduce uncertainty in the prediction, and therefore lead to its future use for similarity definition, category formation and data gap filling in the case of lack of *in vivo* data, is discussed.



164

165 Figure 1. Generic workflow for the development of a read-across and the inclusion of NAM data
166 including reference to the relevant section in the methods that details how to perform each step.

167

168 **2. Methods**

169 *2.1 Problem Formulation*

170 The purpose of this study was to investigate the possibility of reading across repeated dose toxicity.
171 90-day oral sub-chronic toxicity in rats was selected, specifically aligning withthe Organisation for
172 Economic Co-operation and Development (OECD) Test Guideline 408. The triazole fungicides were
173 chosen as a data rich group and tetraconazole as the target molecule. For the purposes of this read-
174 across exercise and case study, existing *in vivo* data for tetraconazole were omitted from the analysis
175 until the conclusion, although the authors acknowledge such data are easily available. The data for
176 tetraconazole were considered only to assess the validity of the read-across argument.

The starting point for the read-across was as follows, which is analogous to a read-across that might be undertaken within the OECD QSAR Toolbox:

- The initial similarity hypothesis was based around structural similarity – in this case all compounds were triazoles with known fungicidal activity i.e. all molecules contained a triazole functional group.
- The triazole structure was considered to be responsible for similar toxicity, as mediated through effects at the liver, with all compounds having similar mode of action.
- This was considered to be a category approach to read-across, which is used to group a number of structurally similar substances: i.e. one-to-many.

Furthermore, this read-across exercise aimed to demontrate how and where NAM and other existing data could strengthen read-across arguments following assessment using the ECHA RAAF and analysis of uncertainties. The study was intended to verify if structural and mechanistic similiarity were sufficient for this well-characterised group of compounds that the Point of Departure, here the No Observed Adverse Effect Level (NOAEL), could be read-across with definable uncertainties.

The intended purpose of the read-across was to provide a NOAEL value that could be used for risk assessment, i.e. would be associated predominately with low uncertainty with few understandable and acceptable instances of moderate uncertainty. Any instances of high uncertainty would be unacceptable for this purpose.

2.2 Substance Characterisation and Endpoint Data Collection

Initial grouping was performed on a structural analogue basis. The following substances were chosen as members of the category of triazoles initially analysed since they each share a common defining chemical unit, namely the triazole moiety: bitertanol, cyproconazole, difenoconazole, epoxiconazole, fenbuconazole, hexaconazole, myclobutanil, paclobutrazol, penconazole, propiconazole, prothioconazole, tebuconazole, tetraconazole, triadimenol and triadimefon. Structurally, the triazoles

are a class of five-membered aromatic heterocycles composed of three nitrogen and two carbon atoms as part of the ring. Table 2 shows the chemical structures as well as characterisation of the group members, including name and CAS number. Toxicity data for the category were compiled from publicly available sources, notable the EFSA database and OECD QSAR Toolbox – the details of these data sources are given in Table 3.

2.3 Assessment of Structural Similarities and Calculation of Physico-Chemical Properties

In addition to the structural information and properties described in Table 2, the Tanimoto coefficient (Tc) based on PubChem fingerprints was calculated in the OECD QSAR Toolbox (ver 4.4.1) to assess the similarities between Tetraconazole and all source molecules in the group. There is no absolute cut-off for similarity on the basis of such indices, indeed it is recognised that the overall similarity value is dependent on the method applied i.e. the metric (here the Tanimoto coefficient) and the basis of similiarty (here the PubChem fingerprints) (Mellor et al., 2019). Physicochemical properties including molecular weight, water solubility, logarithm of the octanol-water partition coefficient (log P), vapour pressure and boiling point were retrieved from the US EPA CompTox Chemicals Dashboard and are also report in Table 2.

2.4 Read-Across

The read-across of the target substance (tetraconazole) was performed from the lowest NOAEL value of the data collection, in order to provide the most conservative value.

2.5 Application of the RAAF and Identification of Uncertainties

The similarity hypothesis in the initial read-across, and hence the uncertainty assosciated, was analysed according to the systematic approaches described in ECHA's RAAF (ECHA, 2017). In this context, different read-across approaches are described in the form of "scenarios" which comprise different AE and address varying scientific considerations deemed crucial to evaluate reliability.

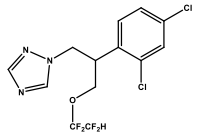
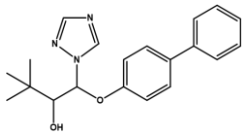
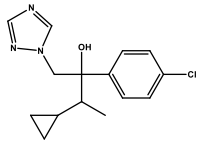
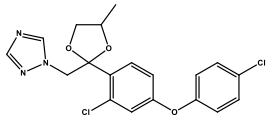
Selection of the applicable RAAF scenario must identify the type of approach applied (analogue or category approach) and whether quantitative variations in the properties are observed among the category members. The structured framework of Schultz et al. (2019) aided in the analysis of the uncertainties by a semi-qualitative ranking of low, moderate or high through twelve sources of uncertainty.

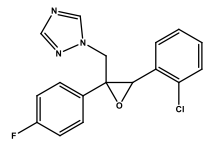
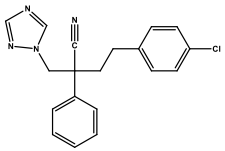
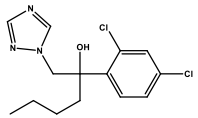
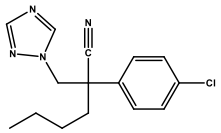
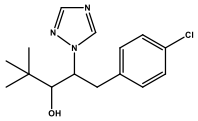
In the current analysis relevant uncertainty criteria and AEs were classified according to their relative uncertainty according to the following scheme:

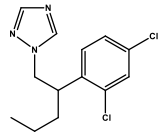
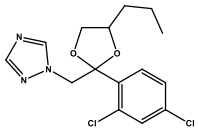
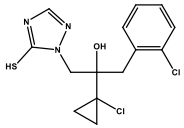
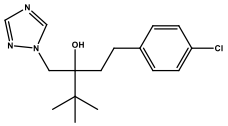
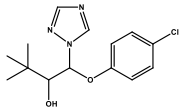
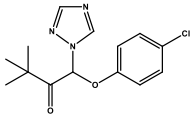
- Low Uncertainty: Strong or compelling evidence that the molecules are similar with regard to the criterion being assessed as related to the defined toxicity or adverse effect e.g. demonstrable similarity from relevant or pertinent experimental (preferably) or *in silico* predictions.
- Moderate Uncertainly: Partial evidence that molecules are similar with regard to the criterion being assessed as related to the defined toxicity or adverse effect e.g. some demonstrable similarity from experimental or *in silico* data. Some experimental data may be missing or from non-standard or only related tests.
- High Uncertainly: No or very little evidence that molecules are similar with regard to the criterion being assessed as related to the defined toxicity or adverse effect e.g. No or very limited experimental data and / or no consideration of *in silico* predictions.

An overall assessment of uncertainty was made on the basis of the highest levels of uncertainty. This was compared with that stated for acceptability in the Problem Formulation in Section 2.1. For criteria or AEs in the initial read-across which had moderate or high uncertainty, a strategy was proposed, usually based around the collection of NAM and other exiting data, to reduce the uncertainty.

247 Table 2. The category of triazole compounds considered, incorporating structures and identifiers in addition to key physico-chemical properties
248

Name	Structure	CAS No	Molecular Weight	Log P	Water Solubility (mol/L)	Vapour Pressure (mmHg)	Boiling Point (˚C)
Tetraconazole (Target)		112281-77-3	372	3.53	0.000928	1.75×10^{-6}	339
Bitertanol		55179-31-2	337	4.11	1.72×10^{-5}	8.89×10^{-9}	340
Cyproconazole		94361-06-5	292	3.01	0.000284	4.00×10^{-7}	321
Difenoconazole		119446-68-3	406	4.08	2.02×10^{-5}	6.17×10^{-10}	336

Epoxiconazole		133855-98-8	330	3.45	1.53×10^{-5}	2.38×10^{-7}	336
Fenbuconazole		114369-43-6	337	3.14	1.30×10^{-6}	4.42×10^{-8}	336
Hexaconazole		79983-71-4	314	3.83	5.39×10^{-5}	2.02×10^{-7}	321
Myclobutanil		88671-89-0	289	2.99	0.000736	1.012×10^{-6}	338
Paclobutrazol		76738-62-0	294	3.34	0.000113	3.82×10^{-8}	322

Penconazole		66246-88-6	284	4.26	0.000351	1.88×10^{-6}	325
Propiconazole		60207-90-1	342	3.55	0.000223	4.09×10^{-7}	350
Prothioconazole		178928-70-6	344	2.75	0.000286	2.21×10^{-8}	338
Tebuconazole		107534-96-3	308	3.68	0.000106	2.20×10^{-8}	321
Triadimenol		55219-65-3	296	2.99	0.000261	8.87×10^{-10}	322
Triadimefon		43121-43-3	294	2.81	0.000394	2.84×10^{-8}	337

249

250

251 *2.6 Retrieval of NAMs and Existing Data*

252 *2.6.1 Data Sources*

253 NAMs and other existing data to support the read-across assessment were retrieved from a variety of
254 publicly-accessible sources, as summarised in Table 3. All chemical structures were entered with the
255 appropriate identifiers e.g. SMILES strings, CAS Numbers etc.

256 Table 3. Information resources utilised to obtain NAMs and other existing data to support the read-
257 across assessment (adapted from Madden et al., 2020).
258

Source	Data retrieved	Information on Resource	Reference and / or URL
OECD QSAR Toolbox (ver 4.4.1)	Chemical structure identifiers i.e. name, CAS, SMILES, *In silico* profiling, chemical similarity assessment.	A freely available computational tool designed to support hazard assessment of chemicals as well as to increase mechanistic and other knowledge on chemical substances in a cost-efficient way.	https://qsartoolbox.org/
EFSA OpenFoodTox database	*In vivo* toxicity data for read-across	Provides information about the toxicity of chemicals found in the food and feed chain, as well as toxicological information for chemical risk assessment of pesticides, food and feed additives, and contaminants.	https://www.efsa.europa.eu/en/data/chemical-hazards-data
PubChem	NAM data, especially *in vitro* and mechanistically based activities.	A database of molecules and their activities against biological assays, which contains bioactivity results from 1.25 million high-throughput screening (HTS) programs with several million values.	https://pubchem.ncbi.nlm.nih.gov/
United States Environmental Protection Agency (US EPA) Computational Toxicology (CompTox) Chemicals Dashboard	Physicochemical properties, HTS data.	An online tool that integrates available information on physicochemical properties, environmental fate and transport, exposure, usage, *in vivo* toxicity and assays associated with HTS data allowing an efficient evaluation for over 875,000 chemicals.	https://comptox.epa.gov/dashboard

Toxtree (ver 3.1).	Identification of the carcinogenic alerts.	An open source application to estimate toxic hazard by applying a decision tree approach, which allows several types of prediction, such as skin and eye irritation, biodegradation and persistence, *in vitro* mutagenicity, as well as identification of structure alerts for mutagenicity, carcinogenicity and skin sensitisation.	http://toxtree.sourceforge.net/
VEGA (ver 1.1.3)	Properties relating to endocrine disruption.	QSAR predictions from a variety of models are available within the VEGA software, which covers multiple endpoints and provides an estimate of whether a molecule is in the applicability domain of the model. These, and other, models are available as part of the VEGA HUB.	https://www.vegahub.eu/
SwissADME	ADME properties.	This website estimates physicochemical descriptors as well as predicting ADME parameters, pharmacokinetic properties, druglike nature and medicinal chemistry.	http://www.swissadme.ch/
International Programme on Chemical Safety (IPCS INCHEM)	Pesticide toxicological Information (*in vivo* data on toxicity and ADME properties).	This publicly-accessible website consolidates international chemical safety-related publications and database records and offers easy electronic access to thousands of searchable full-text documents on chemical risks and management of chemicals.	www.inchem.org
Joint WHO/ FAO Meeting on Pesticide Residues	Pesticide toxicological Information (*in vivo* data on toxicity and ADME properties).	International expert scientific group reviews on pesticide residues, estimate the maximum residue levels, toxicological data and estimate acceptable daily intakes for humans.	https://www.who.int/foodsafety/areas_work/chemical-risks/jmpr

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260 2.6.2 *In Silico* Toxicological Profilers and QSARs

261 In order to provide evidence of the similiarities and differences among the compounds, the possibility
262 of other effects, aside from those addressed by the hypothesis, were investigated through the use of
263 computational profilers and QSAR models. These included several *in silico* profilers for the target and

source substances, including alerts related to genotoxic, endocrine disruption and developmental effect as well as carcinogenticity and ADME properties. The following profilers were applied to the compounds in the OECD QSAR Toolbox (version 4.4.1): DNA binding alerts for point mutation, micronucleus formation or chromosome aberration, protein binding alerts (Protein binding by OASIS), protein binding alerts for chromosome aberration and the Developmental and Reproductive Toxicity (DART) scheme. In addition, the Istituto Superiore di Sanità (ISS) rulebase for carcinogenicity and mutagenicity in Toxtree (version 3.1) was used to profile the compounds for genotoxic and non-genotoxic carcinogenicity.

QSAR predictions were made using the Estrogen Receptor Relative Binding Affinity Model as part of the Collaborative Estrogen Receptor Activity Prediction Project (CERAPP) and Thyroid Receptors alpha (TRα) and beta (TRβ) binding using the Nuclear Receptor-Mediated Endocrine Activity Model (NRMEA) available through the VEGA software (version 1.1.5) from the VEGA HUB.

To provide an estimation of the passive human gastrointestinal absorption (GI), blood-brain barrier (BBB) permeation, permeability glycoprotein (P-gp) and inhibition of isoenzymes CYP1A2, CYP2C19 and CYP2C9, ADME and pharmacokinetic parameters were obtained from the SwissADME website (Daina et al., 2017).

2.6.3 *In Vitro* Data

Toxicogenomic assays, among them, the nuclear receptor binding and activity could be assessed by monitoring expression of suites of genes that are the transcriptional targets for specific nuclear receptors of interest. The appropriate target genes can be identified by a complementary suite of positive internal control ligands utilised in ToxCast cellular assays. Receptor activities could then be assessed based on the expression of receptor-modulated genes and utilised as an efficient toxicogenomics *in vitro* assay for the characterisation of chemicals.

US EPA Toxicity Forecaster (ToxCast) data was retrieved for each compound through use of the CompTox Chemicals Dashboard resource (https://comptox.epa.gov/dashboard). Assays corresponding to the nuclear receptor gene symbols NR1H3 (liver X receptor alpha – LXRα), NR1H4 (farnesoid X receptor – FXR), RARA (retinoic acid receptor alpha – RARα), AHR (aryl hydrocarbon receptor – AHR), NR1I3 (constitutive androstane receptor – CAR) and NR1I2 (pregnane X receptor – PXR) were examined, as were those of the glucuronyl transferase UGT1A1 and cytochrome P450 (CYP) enzymes CYP1A1, CYP1A2, CYP2B6, CYP2C9, CYP2C19, CYP3A4. Endpoints covered included, depending upon availability, reporter gene activation and direct binding site agonism/antagonism. Binary activity calls, assigned within ToxCast sources, formed the basis of final judgments: compounds registering a positive score "1" within an assay were deemed active at the related gene symbol (please refer to Supplementary Information Table S1 for additional detail). Such information was complemented with data from a recent review by Marx-Stoelting et al. (2020).

2.7. Reanalysis of Uncertainties

Once key uncertainties were established for the group of triazoles, inclusion of further NAM data was considered and the uncertainties defined according to the ECHA RAAF and Schultz et al. (2019) were reassessed according to the procedure outlined in Section 2.5 to determine if they were acceptable according to the pre-defined limit assigned in the Problem Formulation (Section 2.1).

2.8 Documentation of Read-Across

Once the uncertainties in the read-across were found to be at a level that met the criteria identified in the Problem Formulation (Section 2.1) the read-across was complete and could be documented. For this case study the read-across and associated data for reported below, it is acknowledged that there are a number of formal templates for reporting a read-across, see for instance Schultz et al., (2015) amongst many others.

3. Results

3.1 Initial Analysis and Mechanistic Hypothesis

Tetraconazole (the target for read-across) and an initial set of 14 triazole compounds (the source molecules for read-across) were selected on the basis of a common triazole functionality, and grouped as a category to read-across repeated dose toxicity. Whilst a simplistic concept, structural similarity is at the heart of read-across and is highly useful to cluster similar compounds to initiate analyses (Date et al., 2020). The read-across was analysed utilising both the AE from the ECHA RAAF and the scheme proposed by Schultz et al. (2019) to identify uncertainties. To start the analysis, the 90-day oral toxicity study in rats was selected as the endpoint for read-across and hence for initial data collection. The data for these studies for all selected substances are reported in Table 4 in terms of the reported NOAEL established for liver toxicity. Whilst data are available for tetraconazole, they were intentionally omitted from the read-across assessment.

Based on structural similarity of the triazole structure, a common mechanism of action was assumed, although not initially proven. The *in vivo* 90-day toxicity data provide evidence of a common effect elicited by the triazoles, namely liver dysfunction. Hepatotoxicity is supported in the toxicological read-across through a mode of action based on the inhibition of the enzyme sterol 14α demethylase, an enzyme from the CYP P450 family (Martinez-Matias et al., 2018). However, as described below, for complex endpoints such as repeated dose toxicity, the read-across prediction based on structural similarity alone is likely to have a high level of uncertainty. Thus, the possibility of using NAM data to confirm the mechanism and hence reduce uncertainty was investigated.

3.2 Case Study: Initial Read-Across for Tetraconazole

In order to probe the read-across hypothesis for the triazoles and, more specifically, illustrate the possibility of improving the read-across and similarity justification as assessed through RAAF and the quantification of uncertainties, tetraconazole was chosen as a target substance and an initial

assessment undertaken. As stated previously, the target and source substances were grouped initially based on chemical structure since all substances share a triazole moiety and, it is assumed, a similar mode of toxic action. All substances have clear chemical identities and characterisation, as presented in Table 2.

3.2.1 *In Vivo* Toxicity Data for the Source Molecules

Table 4 lists the available *in vivo* toxicity data for the source substances, There was good availability of experimental test results for the purpose of grouping the compounds and investigating the mechanistic hypothesis. An examination of these studies revealed that substances could be separated into two groups relating to the Specific Target Organ Toxicity - Repeated Exposure (STOT-RE) classification: Class 1 (< 10 mg/kg bw/d) and Class 2 (between 10 and 100 mg/kg bw/d). Lower NOAELs (< 10 mg/kg bw/d) were reported for 7 out of 14 source compounds. The remaining substances had NOAEL values between 10 and 25 mg/kg bw/d, except for myclobutanil and prothioconazole (51.5 mg/kg bw/d and 100 mg/kg bw/d, respectively). All values reported were based on liver toxicity, providing confidence in the initial assumption of a common hepatotoxic effect on source substances in this group and the probability of it being read across to the target.

Table 4: NOAEL of triazoles obtained from oral 90-day studies.

Substances	Target organ	Oral NOAEL (mg/kg bw/d)	Source	STOT-RE Class
Bitertanol	Liver	8	JMPR, 1998	1
Cyproconazole	Liver	6.4	EFSA, 2010a	1
Difenoconazole	Liver	20	EFSA, 2011	2
Epoxiconazole	Liver	7	EFSA, 2008a	1
Fenbuconazole	Liver	5.7	EFSA, 2010b	1
Hexaconazole	Liver	2.5	EPA, 1999	1
Myclobutanil	Liver	51.5	JMPR, 2014	2
Paclobutrazol	Liver	20	EFSA, 2010c	2
Penconazole	Liver	25	EFSA, 2008b	2
Propiconazole	Liver	15.9	ECHA, 2016	2
Prothioconazole	Liver	100	JMPR, 2008	2
Tebuconazole	Liver	9	EFSA, 2014a	1

Triadimenol	Liver	9	EFSA, 2008d	1
Triadimefon	Liver	13.6	FAO, 2011	2

352

353 3.2.2 Physico-Chemical Properties

354 To initiate the assessment of similarity, target and source substances were compared with regard to
355 their physico-chemical properties (molecular weight, water solubility, log P, vapour pressure and
356 boiling point) using the data presented in Table 2. No significant differences in the properties of
357 tetraconazole compared to the source compounds were observed. All substances were relatively
358 lipophilic (log P between 2.8 and 4), poorly water soluble (< 1 ppm) and possessed low vapour
359 pressures (beneath $2x10^{-6}$ mmHg). Molecular mass was approximately 300 Da for target and source
360 compounds, ranging from 289 Da to 406 Da. Boiling points in the vicinity of 400°C were observed for
361 all substances.

362

363 3.2.3 Structural Similarity

364 Target and source compounds were compared using Tanimoto coefficients (Tc) calculated from
365 Pubchem fingerprints (Table 5). Tetraconazole was found to be similar to 9 out of 14 source substances
366 in terms of Tc (> 0.75). The other six substances showed Tc between 0.6 and 0.7 (bitertanol,
367 difenoconazole, prothioconazole, triadimenol and triadimefon). It is important to mention that as no
368 scientific or regulatory guidance defines similarity calculations, indices could vary within a large range
369 depending on the specific features employed within (Mellor et al., 2019). As such, whilst similarity
370 metrics such as Tc may form a useful line of evidence in the overall WoE they but cannot be used
371 definitively.

372 Table 5. Similarity defined as the Tanimoto coefficients calculated from PubChem fingerprints
373 between target (in bold) and source substances within the triazoles considered in this study. Paris of
374 compounds with high similarity are identified with Tc > 0.75 as an arbitrary cutoff in red and
375 underlined.

Substances	**Tet**	Bit	Cypro	Dif	Epo	Fen	Hex	Myc	Pac	Pen	Prot	Prop	Teb	Tril	Trin
Tetraconazole	**1.00**														
Bitertanol	**0.62**	1.00													
Cyproconazole	**0.80**	0.69	1.00												
Difenoconazole	**0.66**	0.79	0.68	1.00											
Epoxiconazole	**0.81**	0.72	0.81	0.76	1.00										
Fenbuconazole	**0.75**	0.61	0.76	0.61	0.72	1.00									
Hexaconazole	**0.83**	0.68	0.94	0.70	0.81	0.75	1.00								
Myclobutanil	**0.77**	0.59	0.78	0.60	0.69	0.96	0.77	1.00							
Paclobutrazol	**0.79**	0.69	0.86	0.62	0.76	0.79	0.85	0.82	1.00						
Penconazole	**0.85**	0.57	0.80	0.67	0.72	0.85	0.84	0.89	0.79	1.00					
Prothioconazole	**0.63**	0.50	0.69	0.52	0.62	0.55	0.69	0.56	0.64	0.60	1.00				
Propiconazole	**0.80**	0.69	0.79	0.83	0.82	0.67	0.84	0.70	0.78	0.75	0.59	1.00			
Tebuconazole	**0.79**	0.69	0.90	0.65	0.77	0.83	0.89	0.84	0.93	0.82	0.68	0.75	1.00		
Triadimenol	**0.63**	0.84	0.71	0.79	0.70	0.60	0.70	0.62	0.73	0.60	0.52	0.73	0.70	1.00	
Triadimefon	**0.64**	0.74	0.66	0.74	0.67	0.60	0.66	0.62	0.66	0.60	0.48	0.69	0.65	0.88	1.00

bitertanol (bit), cyproconazole (cypr), difenoconazole (dif), epoxiconazole (epo), fenbuconazole (fen), hexaconazole (hex), myclobutanil (myc), paclobutrazol (pac), penconazole (pen), propiconazole (prop), prothioconazole (prot), tebuconazole (teb), triadimenol (tril) and triadimefon (trin).

3.2.4 Initial Read-Across for the 90 day Repeated Dose Toxicity of Tetraconazole

The toxicity data in Table 4 show remarkable consistency, varying by just over one order of magnitude. The initial read-across for tetraconazole could therefore be conducted from the lowest, and thus most conservative, NOAEL, i.e. the value of 2.5 mg/kg bw/d for hexaconazole on the basis of a one-to-many read-across with similarity based on chemical structure and putative mechanism of action. In order to determine the robustness of this relatively simplistic read-across, it was assessed according to the ECHA RAAF and uncertainties defined by Schultz et al. (2019).

3.2.5 Identification of Uncertainties

The similarity hypothesis of target and source substances was analysed according to each RAAF AE, and then associated to specific uncertainties as described by Schultz et al. (2019). The (semi)-

quantitative ranking of uncertainties as low, moderate or high was applied according to the evidence in the initial data collection (i.e. Sections 2.2 and 2.3). A strategy to reduce each uncertainty classified as moderate or high was defined. The steps taken to confirm structural similarity, identify structural differences, analyse the quality and consistency of data and determine metabolic and toxicokinetic similarity in order to make a conclusion on the mechanism of action are described in the following paragraphs. Overall, the uncertainty that would be associated with the read-across value of 2.5 mg/kg bw/d for the 90 day Repeated Dose Toxicity of tetraconazole would be moderate-high on the basis structural similarity and lack of mechanistic confirmation. This level of uncertainty makes it highly unlikely that this read-across would be acceptable for many regulatory purposes and did not meet the criteria stipulated in the Problem Formulation (Section 2.1).

3.3 Inclusion of NAMs and Other Existing Data to Reduce Uncertainty in the Read-Across

In order to reduce uncertainty in the read-across of the 90 day Repeated Dose Toxicity of tetraconazole, a number of NAM and other existing data were compiled (as described in Section 2.6). It is important to note that purpose here was not to change the value read across but to identify and provide further information to increase confidence in the read-across hypothesis.

3.3.1 *In Silico* Profiling

Results from other possible effects aside from those addressed by the hypothesis investigated through computational profilers are summarised in Table 6. No alerts related to genotoxic effects (DNA binding, alerts for point mutation, micronucleus formation or chromosome aberration, protein binding alerts and protein binding alerts for chromosome aberration) were detected using the OECD QSAR Toolbox profilers, except for tetraconazole and epoxiconazole. A genotoxic alert related to Schiff base formation was identified for epoxiconazole and confirmed as a genotoxic carcinogenicity alert by the Toxtree plataform. The genotoxic alert related to S_N2 reactivity detected for the target compound

(tetraconazole) was not confirmed in Toxtree. In addition, structural non-genotoxic carcinogenicity alerts were detected for 10 out of 15 compounds: one compound presented the substituted n-alkylcarboxylic acid (SAA) alert and 9 presented halogenated benzenes (HB) alerts (three of them also containg the SAA alert) (Table 6). The DART scheme from the OECD QSAR Toolbox identified all compounds to have known precedent reproductive and developmental toxic potential, since they all are triazole derivatives. Oestrogen receptor (ER), TRα and TRβ binding investigated by VEGA platform predicted no binding properties for tetraconazole and source substances. Generally speaking, the investigated *in silico* profiles showed consistent results among target and source substances, except for non-genotoxic carcinogenicity, which will be further discussed.

ADME properties estimated using the SwissADME web resource showed consistent *in silico* profiles for tetraconazole, as well as all source substances, i.e. there were no differences in the predicted passive human GI absorption and BBB permeation (except for prothioconazole) – both of which govern important pharmacokinetic behaviours. In addition, tetraconazole as well as the source substances (except for prothioconazole and epoxiconazole) were not predicted to be substrates of P-gp. *In silico* investigation of the inhibition of four important CYP enzymes (CYP1A2, CYP2C19, CYP2C9 and CYP3A4) showed a heterogeneous pattern.

434 Table 6. Results of the *in silico* profiling for relevant toxicological and ADME alerts.

Compound	*In silico* profilers and QSARs															
	OECD QSAR Toolbox					Vega		Toxtree		SwissADME						
	DNA binding	Protein binding	Protein binding for CA	DNA alerts for AMES, CA and MNT	DART scheme	ER binding	TRα and TRβ binding	Non-genotoxic carcinogenicity	Genotoxic carcinogenicity	GI	BBB	Pgp	CYP1A2[1]	CYP2C19[1]	CYP2C9[1]	CYP3A4[1]
Tetraconazole	Schiff base formation	NA	NA	NA	+	NA	NA	NA	NA	High	Yes	No	Yes	Yes	Yes	No
Bitertanol	NA[2]	NA	NA	NA	+	NA	NA	SAA	NA	High	Yes	No	No	Yes	Yes	Yes
Cyproconazole	NA	NA	NA	NA	+	NA	NA	HB	NA	High	Yes	No	No	Yes	No	No
Difenoconazole	NA	NA	NA	NA	+	NA	NA	NA	NA	High	Yes	No	Yes	Yes	Yes	No
Epoxiconazole	S_N2[3]	S_N2[3]	S_N2[3]	NA	+	NA	NA	HB	Epoxides and aziridines	High	Yes	Yes	No	Yes	Yes	No
Fenbuconazole	NA	NA	NA	NA	+	NA	NA	HB	NA	High	Yes	No	No	Yes	Yes	Yes
Hexaconazole	NA	NA	NA	NA	+	NA	NA	NA	NA	High	Yes	No	Yes	Yes	Yes	No
Myclobutanil	NA	NA	NA	NA	+	NA	NA	HB	NA	High	Yes	No	Yes	Yes	Yes	No
Paclobutrazol	NA	NA	NA	NA	+	NA	NA	HB and SAA	NA	High	Yes	No	No	Yes	No	No
Penconazole	NA	NA	NA	NA	+	NA	NA	NA	NA	High	Yes	No	Yes	Yes	Yes	No
Propiconazole	NA	NA	NA	NA	+	NA	NA	NA	NA	High	Yes	No	No	Yes	Yes	No
Prothioconazole	Non-specific[4]	NA	NA	Non-specific[4]	+	NA	NA	HB; thicarbonyl	NA	High	No	Yes	Yes	Yes	Yes	No
Tebuconazole	NA	NA	NA	NA	+	NA	NA	HB and SAA	NA	High	Yes	No	Yes	Yes	No	No
Triadimenol	NA	NA	NA	NA	+	NA	NA	HB and SAA	NA	High	Yes	No	No	Yes	No	No
Triadimefon	NA	NA	NA	NA	+	NA	NA	HB	NA	High	Yes	No	No	Yes	No	No

[1]CYP inhibition; [2] No alert identified; SN2 reaction: [3]Epoxides, Aziridines and Sulfuranes; [4]Specific Imine and Thione Derivatives; MNT: micronucleus test
CA: chromosomal aberration; + Known reproductive and developmental toxic potential.

3.3.2 Mechanistic Studies

Although classified as "conventional NAMs", existing data from the *in vivo* rodent studies were available and analysed to identify the metabolic pathways affected by triazoles for target and seven source compounds (cyproconazole, epoxiconazole, myclobutanil, propiconazole, tebuconazole, triadimenol and triadimefon). Tetraconazole, as well as the other source substances tested were shown to be active for CAR in rodents. Activity was also demonstrated for PXR and AHR in source compounds (cyproconazole, epoxiconazole, myclobutanil, propiconazole, tebuconazole and triadimefon) when tested in rodents (Table 7), although epoxiconazole and propiconazole are suggested to be weak activators and cyproconazole is at most only a very weak activator of AHR-dependent signal transduction (Marx-Stoelting et al., 2020). There is strong evidence that activation of the nuclear receptors including CAR, PXR and AHR, is associated with liver toxicity (Tully et al., 2006; Martin et al., 2007; Goetz & Dix 2009; Mellor et al., 2016), and again there is consistency from *in vivo* data. Although this in isolation does not prove mechanistic similarity, it is an important line of evidence to support the overall WoE.

Commented [C1]: Abbreviations in the method section.

Table 7. *In vivo* activation of nuclear receptors related to liver toxicity for tetraconazole and other triazoles with available data.

In vivo activation	CAR	PXR	AHR
Tetraconazole	Active	Not determined	Not determined
Cyproconazole	Active	Active	Low Activity
Epoxiconazole	Active	Active	Low Activity
Myclobutanil	Active	Active	ND
Propiconazole	Active	Active	Low Activity
Tebuconazole	Active	Active	Active
Triadimenol	Not determined	Not determined	Active
Triadimefon	Active	Active	Not determined

Results from Marx-Stoelting et al. (2020), except for Tetraconazole retrieved from: http://pmep.cce.cornell.edu/profiles/fung-nemat/tcmtb-ziram/tetraconazole/tetraconazole_reg_0913.pdf.

3.3.3 *In Vivo* ADME Studies

There was sufficient ADME information obtained from rat *in vivo* studies to establish toxicokinetic similarity for the target and source substances used in the read-across (summarised in Table 8). Tetraconazole was reported to be extensively absorbed and metabolised, and excreted in 48 hours mostly by urine and less from faeces, resulting in an *in vivo* half life of 15 hours. Distribution was reported to be in the liver, kidneys, gonads, brain and bones. Patterns of metabolism for source substances were similar (rapid and extensive), including similar *in vivo* half life values. The main organ of concentration of substances was confirmed in each instance to be the liver. While dissimilarities in metabolism considerably complicate the read-across approach, the pattern observed for tetraconazole and source substances is an important line of evidence to support similarity. These *in vivo* ADME studies also indicated no significant differences in the toxicokinetics of the two STOT-RE groups.

Table 8. ADME properties from rat *in vivo* data for the triazole group.

Compound	Absorption	Distribution	Metabolism	Excretion (h)	Route of excretion	In vivo half life (h)
Tetraconazole	extensive	liver, kidneys, gonads, brain and bones	extensive	48	urine (51-76%); faeces (9-36%)	15[13]
Bitertanol	rapid	liver and kidneys	extensive	72	faeces (>90%); urine (7%).	~ 26[1]
Cyproconazole	rapid and extensive	liver, kidneys and pancreas	extensive	144	urine and faeces	24 to 96[2]
Difenoconazole	rapid and extensive	liver, kidneys, fat and pancreas	extensive	24	predominantly via faeces	33 to 48[3]
Epoxiconazole	rapid	liver, blood, lung kidneys, spleen and adrenals	extensive	168	~17% via urine; ~78% via faeces	5 to 30[4]
Fenbuconazole	complete	higher in the liver, kidneys and adrenals.	extensive	48	10% urine; ≥80% bile	Biphasic: rapid 7; slow 50[5]
Hexaconazole	readily	kidney, liver and pâncreas	extensive	76	43% urine/53% faeces (m) 66% urine/29% faeces (f)	10 to 27[6]
Myclobutanil	rapid and extensive	widely distributed	rapid and extensive	96	even distribution via urine and faeces	Biphasic: rapid 5; slow 26[7]
Paclobutrazol	rapid	liver	extensive	48	bile and urine	72[8]

Penconazole	rapid	liver, kidneys and adrenal glands	rapid and extensive	<72	urine and faeces (47-44%, m); urine and faeces (69%-21%, f)	3 to 13[9]
Propiconazole	rapid[10]	widely; mainly in the liver and kidneys[10]	extensive[10]	24[10]	even distribution via urine and faeces[10]	24 to 31[10]
Prothioconazole	rapid	liver and kidneys	rapid and extensive	48	Urine, faeces and bile	47.7[11]
Tebuconazole	complete	higher in the liver	rapid and extensive	48	61 to 82% faeces; urine (18 to 39%)	32 to 52.5[12]
Triadimenol	rapid	widely distributed	extensive	96	14–21% (m); 48% (f) urine	6 to 15[14]
Triadimefon	complete	widely distributed; mainly in the liver and kidneys	rapid and extensive	96	67% in males; 28% in females via urine	~ 4[15]

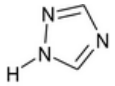
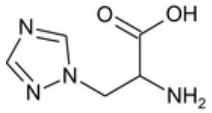
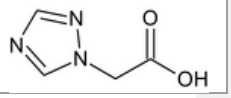
References from all ADME follow the sources listed on Table 3, except for in vivo half life (h) which are:
1: JMPR, 1998;
2:https://echa.europa.eu/documents/10162/13626/02_cyproconazole_syngenta_public_comments_toxicokinetics_en.pdf
3: Pubchem (https://pubchem.ncbi.nlm.nih.gov); 4: USEPA - Pesticides - Fact Sheet for Epoxiconazole;
5: Inchem http://www.inchem.org/documents/jmpr/jmpmono/v097pr07.htm;
6: Inchem http://pmep.cce.cornell.edu/profiles/fung-nemat/febuconazole-sulfur/hexaconazole/Hexaconazole_tol_699.html
7: Inchem http://www.inchem.org/documents/jmpr/jmpmono/v92pr13.htm
8: Inchem http://www.inchem.org/documents/jmpr/jmpmono/v88pr08.htm
9: ECHA https://echa.europa.eu/documents/10162/3c32ece7-6b51-e32d-1d66-6cd893096713
10: Pubchem (all information);
11: Pubchem;
12:http://www.fao.org/fileadmin/templates/agphome/documents/Pests_Pesticides/JMPR/Report10/Tebuconazole.pdf
13: Pubchem; 14: FAO 191 Pesticide Residues in Food 2007 (http://www.fao.org/3/a-a1556e.pdf);
15: http://www.inchem.org/documents/jmpr/jmpmono/v83pr39.htm

3.3.4 Metabolites

1,2,4-Triazole (1,2,4-T), triazole alanine (TA) and triazole acetic acid (TAA) are the most important common metabolites derived from triazole-containing fungicides that act by inhibiting sterol synthesis (JMPR, 2009), as illustrated in Figure 2. According to the JMPR report (2009), in a 90-day oral study in rats with 1,2,4-T, clinical effects and histopathological findings in the liver were observed. The reported NOAEL values were 37.8 (males) and 54.2 mg/kg bw/d (females). The same report mentions a dietary study of toxicity in rats with TA, where small toxicologically significant changes were observed leading to a NOAEL equal to 370 mg/kg bw/d. For TAA, no target organ or any treatment-related toxicity was observed in a short-term study in rats. Radiolabelled 1,2,4-T in orally treated rats

was rapidly and completely absorbed and excreted unchanged, mainly in the urine, within 24h. TA and TAA have similar toxicokinetic profiles in that they are rapidly eliminated, primarily in the urine and mostly as the parent compound. Toxicological data extracted from the JMPR (2009) report for the three metabolites indicate they follow the same pattern of toxicity and metabolism of the parent compounds and add support to the similarity hypothesis.

Figure 2. Chemical structures of the three main metabolites for the triazole compounds.

1,2,4-triazole (1,2,4-T)	1*H*-1,2,4-triazole	
Triazole alanine (TA)	3-(1*H*-1,2,4-triazol-1-yl)-D,L-alanine	
Triazole acetic acid (TAA)	1*H*-1,2,4-triazol-1-ylacetic acid	

3.3.5 *In vitro* and High Throughput Screening Data

The ToxCast database was searched for the activity of biomarkers related directly or indirectly to liver functions, such as the following nuclear receptors: LXRα, FXR, RARα, CAR, PXR, AHR, CYP enzymes and UGT1A1. Information on CAR, PXR, AHR and CYP enzymes was complemented with data from Marx-Stoelting et al. (2020). Overall, tetraconazole was active towards CAR, PXR and AHR. Combining results from ToxCast with those of Marx-Stoelting et al. (2020), all triazoles showed activity towards PXR (except penconazole) as well as for CAR (except bitertanol and epoxiconazole). Activity for AHR was seen in 11 of 14 source compounds. Tetraconazole was also active against all other nuclear receptors. However, activity for the source substances was less frequent, about 50% for LXR and RARα. FXR and UGT1A1 showed more active compounds (11 of 15 and 11 of 11, respectively), including

514 tetraconazole. Considering the 15 substances (target and sources) and the six nuclear receptors
515 investigated, nuclear receptor activity was confirmed as a common hepatotoxicity marker (Table 9).

516 Additionally, CYP enzymes responsible for metabolism of many exogenous compounds were
517 investigated, including CYP1A2, CYP2B6, CYP2C9, CYP2C19 and CYP3A4. The ToxCast database
518 combined with data from Marx-Stoelting et al. (2020) indicated that tetraconazole, as well as all source
519 substances, were active against all tested CYPs (Table 10). The concordance of activity of all researched
520 CYP enzymes for both the target and source substances is a clear line of evidence to support the overall
521 mechanistic hypothesis of hepatotoxicity, as well as the robustness of the category.

522 Table 9. Data relating to the activity against the human nuclear receptors (NR) and UGT1A1 for the 15
523 triazoles.

Compound	LXR	FXR	RARα	AHR*	AHR#	CAR*	CAR#	PXR*	PXR#	UGT1A1
Tetraconazole	1	1	1	1	ND	1	ND	1	ND	1
Bitertanol	1	1	1	0	ND	0	ND	1	ND	ND
Cyproconazole	1	0	0	0	1	1	1	1	1	1
Difenoconazole	0	1	0	0	1	1	ND	1	ND	1
Epoxiconazole	0	1	1	1	1	0	ND	1	ND	ND
Fenbuconazole	0	0	0	0	1	1	1	1	1	1
Hexaconazole	1	1	1	0	1	1	1	1	1	1
Myclobutanil	1	0	1	0	1	1	1	1	1	1
Paclobutrazol	0	1	0	0	ND	1	ND	1	ND	1
Penconazole	ND	1	0	0	ND	1	ND	0	ND	ND
Propiconazole	0	1	1	1	1	1	1	1	1	1
Prothioconazole	1	0	0	0	ND	1	ND	1	ND	ND
Tebuconazole	1	1	1	1	1	1	1	1	1	1
Triadimenol	0	1	0	1	1	1	ND	1	ND	1
Triadimefon	0	1	1	1	1	1	ND	1	ND	1
No. Active	7	11	8	6	10	13	6	14	6	11
Total No. Tested	14	15	15	15	10	15	6	15	6	11

524
525 * Results from ToxCast; # Results from Marx-Stoelting et al. (2020). Where 1 indicates activity, 0 is
526 inactive and ND is not determined
527

528 Table 10. Data relating to the binding to the human CYP enzymes for the 15 triazoles.

Compound	CYP1A1*	CYP1A1#	CYP1A2*	CYP1A2#	CYP2B6*	CYP2B6#	CYP2C9*	CYP2C19*	CYP3A4*	CYP3A4#
Tetraconazole	1	ND	1	ND	1	ND	1	1	1	ND
Bitertanol	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Cyproconazole	1	1	1	1	1	1	1	1	1	1
Difenoconazole	1	1	1	ND	1	ND	1	1	1	ND
Epoxiconazole	0	1	1	ND	ND	ND	1	1	1	ND
Fenbuconazole	1	ND	1	1	1	1	1	1	1	1
Hexaconazole	1	ND	1	1	1	1	1	1	1	1
Myclobutanil	1	1	1	1	1	1	1	1	1	1
Paclobutrazol	1	ND	1	ND	1	ND	1	1	1	ND
Penconazole	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Propiconazole	1	1	1	1	1	1	1	1	1	1
Prothioconazole	ND	ND	ND	ND	ND	ND	ND	1	ND	ND
Tebuconazole	1	1	1	1	1	1	1	1	1	1
Triadimenol	1	ND	1	ND	1	ND	1	1	1	ND
Triadimefon	1	ND	1	ND	1	ND	1	1	1	ND
No. Active	11	6	12	6	11	6	12	13	12	6
Total No. Tested	12	6	12	6	11	6	12	13	12	6

* Results from ToxCast; # Results from Marx-Stoelting et al. (2020). Where 1 indicates activity, 0 is inactive and ND is not determined

3.3.6 Impact of NAMs on Read-Across Uncertainties

Table 10 illustrates the outcome of the evaluation of the similarity hypothesis of target and source substances using the RAAF AEs and uncertainties as described by Schultz et al. (2019). When analysing each item separately, uncertainties could be reduced after including NAM data as illustrated above, although some items could not have the initial uncertainty completely lowered. Briefly, structural similarity, category hypothesis, quality of the data, consistency of in the *in vivo* effects and robustness of the supporting data sets were initially judged to have moderate uncertainties and were reduced to low uncertainties at the end of the exercise. The most important achievement was to reduce the high uncertainty of the mechanistic plausibility to low uncertainty, obtaining an overall moderate to low uncertainty for the process. Nevertheless, it should be stressed that while the use of novel data has

been encouraged in many publications, the reporting and the understanding of the assessment of this data is not implicit in the RAAF framework. It can be concluded that the overall uncertainty associated with this category-based read-across has been reduced from “moderate-high” on the basis of a common structural feature alone to “low-moderate” following the inclusion of systematically derived NAM data. Thus, the inclusion of NAMs data has increased the possibility of acceptance of the read-across value.

548 Table 11. Outcome of the strategies to reduce uncertainties in the read-across of triazoles following the inclusion of NAM data and other lines of evidence as
549 explained in the text.

AE	RAAF AE	Major uncertainties	Initial level of uncertainty	Strategy to reduce uncertainty	Level of uncertainty following inclusion of NAMs and other data
	Type of approach	None	Low	No strategy required	Low
AE C.1	Substance characterisation	None	Low	No strategy required	Low
AE C.2	Structural similarity and category hypothesis	Category hypothesis	High	Tanimoto similarity, *in silico* profiling	Moderate
AE C.3	Structural similarities and diferences	Structural similarities and diferences	High	Tanimoto similarity, physico-chemical properties	Moderate
AE C.4	Consistency of effects in the data matrix	Mechanistic plausibility, metabolism and TK similarity	Moderate	ToxCast, metabolism/TK data other data	Low
AE C.5	Reliability and adequacy of the source studies	Quality and consistency of data	Moderate	*In vivo* studies	Low
AE C.6	Bias that influences the prediction	None (a broad category was created)	Low	No strategy required	Low
AE 4.1	Compounds the test organism is exposed to	Consistency in the *in vivo* effects and potency data	Moderate	Consistency of finding and potency of *in vivo* data	Low
AE 4.2	Common underlying mechanism: qualitative aspects	Robustness of the supporting data sets	Moderate	All supporting data	Low
AE 4.3	Common underlying mechanism: quantitive aspects	Robustness of the supporting data sets	Moderate	All supporting data	Low
AE 4.4	Exposure to other compounds/metabolites	Weight of Evidence (WoE) supporting the prediction	Moderate	Databases, literature	Low
AE 4.5	Occurrence of other effects than covered by the hypothesis	WoE supporting the prediction	Moderate	Consider the *in silico* profiling results, NAM and other existing data	Low

550

551
552
553 *3.4 Domain of the Category*

554 The applicability domain of the triazole category is complex due to the size of the category and its relative
555 structural diversity. The basis for the category is two-fold i.e. in terms of chemistry, the presence of the
556 triazole functionality and in terms of toxicology, the common mechanism of action leading to hepatotoxicity.
557 The members of the category show good structural similarity in terms of the Tc defined by the PubChem
558 features (higher than 0.75 for 9 out of 14 source substances and higher than 0.6 for the remaining
559 substances), which is a useful initial determinant but not strictly definitive to confirm category membership.
560 Using the information in Table 2, the physico-chemical domain demonstrates good consistency – with a
561 relatively small range in log P (between 3-4) and molecular weights (approximately 300 Da), as is to be
562 expected for bioactive molecules. Thus, any new molecule proposed to join the category should meet these
563 initial domain criteria as well as have evidence of a similar mechanism of action.

564 It is worth mentioning that while the category was defined and data collected for the triazoles, similar NOAEL
565 values, ADME properties and metabolic features were confirmed for the imidazoles (e.g. imazalil, prochloraz,
566 triflumizole, amongst others) that together with triazoles forms the group of the azoles. Therefore, data (not
567 shown) indicate that the results and conclusion of this analysis could be extended to the imidazoles and
568 azoles in general.

569 4. **Discussion**

570 Read-across is crucial to regulatory toxicology as it illustrates how it may be possible to move from chemical
571 assessments based on animal testing to assessments by interpolation within a toxicologically relevant and
572 mechanistically plausible assessment. The present read-across case study for the group of triazoles was
573 supported by publicly available data, and analysed both through the ECHA RAAF framework and
574 consideration of uncertainties as strategies to confirm the similarity hypothesis. The key uncertainties were

established and were reassessed as further relevant *in vitro*, *in silico* and other existing data were added. As discussed previously (Schultz et al. 2019), the answer to whether the uncertainties are acceptable for a defined read-across situation could depend on specific requirements, for example, risk management or relevant legislation. Therefore, it is important to state the acceptable level of uncertainty as part of the problem formulation when starting the read-across process and throughout it to assure that uncertainty has reached an acceptable level at the process conclusion. If the level of uncertainty is too high for the decision context, additional information should be provided. It is important to note that the current state of the art of assessing and quantifying read-across uncertainties is rather subjective. This study applied some criteria (stated in Section 2.5) that have been developed from various other studies, most notably that of Schultz et al (2019). Whilst the anchoring to experimental data and, if required, in silico data, provides a more objective scheme for assigning uncertainties, it is acknowledged that there is still an overwhelming requirement for harmonisation and better quantification of this aspect of read-across.

In our analysis, the use of ECHA RAAF, combined with relevant questions as described by Schultz et al. (2019), proved to be an efficient way of determining the uncertainties. The category was constructed initially in terms of structural similarity, i.e. the triazole group, and the availability of high-quality toxicity data. The uncertainties associated with these two criteria alone are not sufficient to support a read-across within the category, especially one that could support a regulatory submission or risk assessment as defined by our Problem Formulation. Therefore, a more detailed analysis of the data and information was required, especially of how to reduce uncertainties further.

Considering the amount of available data and homogeneity of information, uncertainty of quality and consistency of data were initially assessed as moderate. To evaluate the quantitative differences of the repeated exposure values (NOAEL) among the compounds, an empirical observation described in the OECD QSAR Toolbox Tutorial 33 (RAAF Scenario 6, available from https://qsartoolbox.org/wp-content/uploads/2020/04/Tutorial_33_Example-illustrating-RAAF_Scenario_6.pdf) was employed. This

599 stated that variation in the toxicity is not expected when it is less or equal to 1 log unit. The toxicity of the
600 compounds in the present study were within this range, therefore confirming the consistency of data and in
601 the potency and effects.

602 Table 11 confirms that a similarity hypothesis for such a large category has moderate-high uncertainty on
603 the RAAF AE considered, which could be unacceptable for most regulatory purposes. One efficient means of
604 reducing uncertainty is to add in further lines of evidence (Benfenati et al., 2019; Escher et al., 2019; Gadaleta
605 et al., 2020). In this example, NAM and other existing data were sought from the literature or publicly
606 available sources. Experimental data or *in silico* estimations were considered suitable for inclusion, with the
607 intention being to reduce uncertainties in areas encompassing structural similarity, metabolism,
608 toxicokinetics and mechanism of action among target and source substances – alongside relation of chemical
609 structures to biological activity. These data are summarised in Tables 2 and 6-10. The concept of
610 incorporating biological data from *in vitro* assays into read-across assessment is not novel. Several authors
611 have pointed out that knowledge of chemical descriptors may not be sufficient to understand the effects of
612 chemicals on human health, so biological descriptors from cell-based and small model organism assays
613 should be used to further characterise chemicals and inform risk assessment (Ball et al., 2016; Chesnut et al.,
614 2018; Escher et al., 2019). The results summarised in Table 11 demonstrate that confidence in the read-
615 across prediction was enhanced by providing mechanistic information, appropriate toxicokinetic properties
616 in the form of ADME, relevant *in vitro*, *in silico* and high throughput screening (HTS) data together with
617 appropriate experimental data for the compounds. The overall uncertainty in the predictions, while initially
618 high-to-moderate, was reduced to low-to-moderate with the addition of available NAM data.

619 It is acknowledged that for this case study, the triazoles were extensively tested in conventional animal
620 experiments to identify target organs, toxic effects and NOAELs for the derivation of reference values.
621 Although initial toxicity studies seldom describe the detailed molecular mechanisms involved, later and more
622 detailed studies identified relevant biological pathways perturbed by triazoles and suggested an interaction

623 with hepatic nuclear receptors as the underlying mode of action leading to hepatotoxicity (Tully et al., 2006).
624 In rodent toxicity studies, the liver was identified as the main target organ of adverse azole action with clear
625 involvement of several CYP enzymes and nuclear receptors (Tully et al., 2006; Goetz & Dix 2009; Heise et al.,
626 2018). A mechanistic study postulated a nuclear receptor mediated, non-genotoxic mode of action for the
627 development of liver tumours in tetraconazole-treated mice in which the initiating event was the activation
628 of the CAR to the appearance of hepatocellular tumours. For the source substances, such as cyproconazole,
629 epoxiconazole, myclobutanil, propiconazole, tebuconazole, triadimenol and triadimefon, *in vivo* activation
630 of CAR, PXR and AHR was demonstrated to cause several adverse liver effects such as liver enzyme induction,
631 hepatocellular hypertrophy, liver enlargement and eventually cancer development (Tully et al., 2006; Martin
632 et al., 2007; Goetz & Dix 2009). ToxCast data have been found to be useful to support read-across hypotheses
633 (Punt et al., 2020) and this information was crucial to underpin the arguments relating to the mechanistic
634 basis for read-across and assisted the search for relevant NAM data as reported in Tables 9 and 10.

635 A useful framework to investigate mechanistic toxicology and thus demonstrate common toxic effects is the
636 Adverse Outcome Pathway (AOP) concept (Ankley et al., 2010). This requires the identification of a Molecular
637 Initiating Event (MIE) and intermediate Key Events (KE) linked causally to the target endpoint. Repeat dose
638 systemic organ toxicity is currently an endpoint of considerable interest for AOP development, with the liver
639 being the main concern due to its function on chemical metabolism (Vinken, 2015; Peffer et al., 2018). The
640 nuclear receptors investigated in this study, namely AHR, CAR, PXR, LXRα, FXR and RARα have been described
641 to be involved in AOP related to hepatotoxic effects, such as hepatic steatosis and cholestasis, both processes
642 described as AOP (Vinken, 2015; Mellor et al., 2016). Although the target substance tetraconazole was active
643 in all tested nuclear receptors, the source triazoles were not as active. Knebel et al. (2019) suggest the nuclear
644 receptor PXR as a central player in a multi-receptor response induced by triazole fungicide and indeed PXR
645 activity has been observed in all triazoles here studied, except for penconazole. According to Knebel et al.
646 (2019), the triazole compounds did not seem to act exclusively at PXR, but rather through activation of

several nuclear receptors, thereby inducing a complex gene expression response, as also suggested by Tully et al. (2006) and Marx-Stoelting et al. (2020).

In rodents, an AOP correlating CAR activation and hepatotoxicity was described by Peffer et al. (2018). However, this AOP stresses that such a response was not confirmed in various mammalian species, including humans. Lake (2018) compared a series of effects in rodents and humans to evaluate the relevance of this mode of action for human hepatotoxicity. This study found evidence both in rodents and humans of three key events: CAR activation, CYP2B induction and liver hypertrophy, but not for the mitogenic effect of CAR activators leading to liver tumours in rodents. Corroborating this evidence, CAR and CYP2B6 activity were detected in human cells for all compounds here analysed (with the exception of bitertanol).

In addition, CYP enzymes responsible for the metabolism of many exogenous compounds have been consistently reported as being related to the hepatotoxic effects of azole compounds in rodents and humans (Martin et al., 2007; Goetz & Dix 2009; Marx-Stoelting et al., 2020). The series of triazoles investigated in this case study showed activity for CYP1A1, CYP1A2, CYP2B6 CYP2C9, CYP2C19 and CYP3A4 enzymes. These results are a clear confirmation of hepatotoxicity as a mechanistic hypothesis for the azoles, and of the homogeneity of the category.

While triazoles are known to induce of hepatic CYPs through activation of nuclear receptors, several studies report inhibition of certain CYP enzymes in endocrine target tissues (Zarn et al. 2003; Trosken et al. 2004; Goetz and Dix, 2009). As some CYPs are essential for the biosynthesis of cholesterol or steroid hormones, triazoles are reported to interfere in these pathways leading to disturbances in the biosynthesis of estradiol or testosterone. Disruption of both steroid and testosterone homeostasis accompanied by reproductive toxicity could be the result of changes in gene expression leading to increased steroidogenesis in the testis and decreased steroid liver metabolism (Goetz and Dix, 2009). Additionally, triazoles are reported to disturb the metabolism of progesterone, androgens and estrogens, as well as to antagonise steroid hormone receptors such as the androgen (AR) and estrogen receptor (ER) (Zarn et al. 2003; Trosken et al. 2004;

671 Kjaerstad et al., 2010). Taken together, conazoles might have multiple modes of action in regard to affecting
672 reproductive development. Evidences have been presented by *in vivo* rodent studies (Menegola et al 2005),
673 as well as *in vitro* zebrafish assays (Teng et al 2018). Specific teratogenic effects were observed in *Xenopus*
674 embryos (Groppelli et al 2005) and rodents (Menegola et al 2005). The mechanisms of toxicity proposed
675 involve imbalance in the endogenous retinoic acid metabolism in embryonic tissues leading to different
676 malformations (Menegola et al 2005; Marotta and Tiboni, 2010) and blocking of cardiac potassium channels
677 (Liu et al. 2017). Results from in silico DART profiler confirm all compounds as triazole derivatives to present
678 reproductive and developmental toxic potential and that there is no dissimilarity between the target and
679 source compounds. Similarly, the predicted ER binding properties for tetraconazole and source substances
680 are generally consistent and could, if required, be supported by further NAM data.

681 Numerous potential mechanisms underlying azoles toxicity have been put forward, including impairment of
682 glycolysis and fatty acid metabolism (Hermsen et al. 2012), alteration in cholesterol synthesis (Goetz and Dix,
683 2009), disruption in steroid biosynthesis and retinol metabolism (Marotta and Tiboni, 2010), alteration of
684 calcium signaling pathways and cardiac muscle contraction (Liu et al. 2017) and disruption of energy
685 metabolism (Teng et al. 2018). Combined, the ability of these compounds to interfere with hormone
686 synthesis and drug metabolism, as precursor to endocrine disruption, hepatotoxicity and developmental
687 toxicity, raises very serious concerns on human health. The AHR nuclear receptor has also been reported to
688 be involved in non-genotoxic carcinogenesis for a structurally diverse variety of chemicals (Benigni et al.,
689 2013). The mechanism of action of three alerts identified by the Toxtree profiler, including that of
690 halogenated benzenes, is mediated by AHR, which when complexed is capable of binding to the regulatory
691 region of several target genes, including genes coding for phases I and II biotransformation enzymes and/or
692 involved in regulation of development, proliferation and differentiation (Benigni et al., 2013). Interestingly,
693 using Toxtree profiler, 10 out of 15 compounds presented structural non-genotoxic alerts, nine of them
694 matched the halogenated benzenes alert.

It is suggested that non-genotoxic alerts might be linked to species/sex/strain specific effects for certain classes (Benigni et al., 2013). This might be the case for the other alert detected in four compounds analysed here: the substituted n-alkylcarboxilic acid, which is mediated by peroxisome proliferators. The human relevance of the hepatocarcinogenic action of the peroxisome proliferators observed in rodents is subject of intensive debate (Benigni et al., 2013). Relevance of the two genotoxic alerts detected by the *in silico* profilers may also be low. According to the EFSA database specifically for the pesticide active substance and their metabolites, comprising the main genotoxicity endpoints (EFSA, 2014b), the triazoles selected in our study are non-genotoxic. Ross et al. (2009), studying triadimefon, myclobutanil and propiconazole confirm the non genotoxicity of these compounds.

Whilst well-studied, the molecular mechanisms and respective AOP for the azoles in humans are currently not well understood. Considering the activity of several nuclear receptors in most triazoles (same effect in the imidazoles), it could be hypothesised that azole compounds are involved in more than one pathway of hepatotoxicity leading to a range of toxic effects, including a non-genotoxic common mechanism of chronic liver toxicity. More information would be necessary to take this hypothesis into account.

Although the present read-across case study was based on a data rich group of compounds in which a significant amount of information could be mined resulting in high quality and consistent results, it clearly demonstrated that NAM data could be applied for a particular purpose. In a more realistic, data-poor scenario, where there are wide gaps among chemicals and few experimental data are available, the enhanced difficulty of read-across is acknowledged. In such a case, NAM will become vital for read-across justifications, the need for them being led by the analysis of uncertainties and informed by the needs of regulatory acceptance. Schultz and co-workers (2017a) reported a successful case study showing how to integrate NAM data in the read-across hypothesis where there were relatively few experimental data to predict the 90-day NOAEL for a category of aliphatic alcohols. The existing *in vitro* data and *in silico* predictions on nuclear receptor binding supported the read-across hypothesis of no activity associated with

a specific mode of action in the category, allowing for an eventual integration of the new data into the assessment in a qualitative manner. Other alternatives, if NAM data were lacking in such a scenario, would be to fill the data gaps with some targeted experimentation. In this sense, NAMs should constitute tests performed with the specific goal of reducing the uncertainties and demonstrating and justifying the read-across hypothesis.

Identifying the best practices for using biological profiling tools to support the similarity of compounds in read-across could be the key to good predictions of a proposed endpoint. Considering the limitations of *in vivo* tests, the future trend is to develop tools that increase predictivity and reliability by combining read-across and NAM to reduce uncertainty in the prediction. As the confidence in non-animal data increases, its exclusive use for category formation and data gap filling in the case of lack of *in vivo* data could lead to read-across application totally based on *in vitro/in silico* data.

We believe that this read-across case study supported by NAM data is a robust example, with usable publicly available resources, that can contribute to increase the consistency of read-across assessments. The read-across value of 2.5 mg/kg bw/d for tetraconazole is consistent and acceptable as compared to the published experimental value of 4 mg/kg bw/d (EFSA, 2008c). The study of more read-across cases is necessary in order to build confidence in this new approach and better expand the use of read-across for regulatory toxicology.

5. Conclusions

To conclude, this read-across study was performed in a well defined category, which included physico-chemical properties, *in vivo* toxicity, metabolism, structural similarity and differences, allowing for domains to be defined and limitations to be understood. It was demonstrated that uncertainties initially raised could be reduced by the inclusion of NAM. In addition, the importance of the use of a framework as ECHA RAAF and the AE contained therein, complemented with specific questions concerning uncertainties was demonstrated to understand the gaps and increase the confidence that can be placed in read-across.

Although a data rich group of compounds with a strong mechanistic basis was analysed, it was clearly demonstrated that NAM and other existing data available from publicly available resources, could be applied for a particular purpose. It is acknowledged that most read-across studies will not be so data rich or mechanistically robust, therefore some targeted experimentation would be required to fill the data gaps. In this sense, NAMs should constitute new (non-animal) experimental tests performed with the specific goal of reducing the uncertainties and demonstrating the read-across hypothesis.

Conflicts of Interest

No authors declare any conflicts of interest.

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