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CHEMICALS AND BIOTECHNOLOGY COMMITTEE**

Case Studies for the Integrated Approaches for Testing and Assessment for Systemic Toxicity of Conazoles Using a Read-Across Approach, Eleventh Review Cycle (2025)

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Foreword

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

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

This case study illustrates practical use of IATA and was submitted to the 2025 review cycle of the IATA Case Studies Project. This case study was reviewed by the project team.

This case study is an illustrative example, and its publication as an OECD monograph does not translate into direct acceptance of the methodologies for regulatory purposes across OECD countries. In addition, this case study should not be interpreted as official regulatory decisions made by the authoring member countries.

Table of contents

Acknowledgements	3
Foreword	5
Executive Summary	9
Abbreviations and acronyms	10
Introduction	12
1 Regulatory Purpose/Context of use	14
2 Endpoint/Biology/Response addressed	17
3 Description of a Workflow	19
4 Scientific rationale/basis for IATA workflow	25
5 Defined domain of applicability	27
6 Description of Modules	29
6.1. Hazard	29
6.2. Toxicokinetics	29
6.3. Exposure	30
7 Description of information sources	31
7.1. Hazard	31
7.2. Toxicokinetics	31
8 Data Selection/gathering	36
8.1. In vivo data	36
8.2. High-throughput assays	37
8.3. Additional NAM assays	40
8.4. Kinetics assessment	43

9 Data Evaluation	45
10 Data integration and interpretation	46
11 Measures of Reliability	48
12 Expert judgement + alternative interpretations	50
13 Overall uncertainty	53
14 Evaluations and conclusions	54
15 Learnings and Lessons	55
References	59
Annex I. Reporting Individual Information Sources	62
1. Name of the information source	62
2. Relevance of the information source	65
3. Description	66
4. Response(s) measured	76
5. Prediction model	78
6. Metabolic competence	99
7. Status of development, standardization, validation, strengths and weaknesses	101
8. Technical limitations and limitations with regard to applicability	109
9. References	109
Annex II. Template for IATA Case Studies on Chemical Grouping (Read-across) ⁵²	113
1. Purpose	113
2. Hypothesis for the category approach	114
3. Source chemicals/category members	115
4. Justification of data gap filling	116
5. Uncertainty	119
6. References	132
Annex III. Reporting IATA Outcomes	134
1. Name of the chemical	134
2. Regulatory Purpose	136
3. Description of IATA framework, including all information sources used, and modules included in the specific approach	136
4. Data review	139
5. Data integration and interpretation	142
6. PHH transcriptomics analysis	159
7. H295R steroidogenesis assay	172
8. Developmental Neurotoxicity (DNT) assays	173
9. Physiologically based Kinetic (PBK) modelling	181
10. Expert judgement	187
11. Outcomes and Conclusions	191
12. Uncertainties in the specific workflow followed	195

13. Discussion of other interpretations (if applicable)	197
14. References	198

Supporting Information 200

FIGURES

Figure 1. Schematic representation of ASPA version v3.0	20
Figure 2. Initial steps in the ASPA workflow as followed for the NAM-based read-across assessment of conazole compounds in this case study	22
Figure 3. Dendrogram representing structure-based clustering of conazoles and metabolites in the case study using (a) Morgan and (b) MACCS fingerprints. The distance was calculated using Tanimoto coefficient	23
Figure 4. ASPA workflow for hazard estimation of compounds as applied to conazoles in this case study	32
Figure 5. ASPA workflow for toxicokinetics assessment, as applied to the compounds in this case study	34
Figure 6. Metabolite Workflow in ASPA illustrating the evaluation of compounds with metabolites	35
Figure 7. (a) Hierarchical clustering and (b) Tanimoto similarity of SCs, metabolites, positive controls and negative controls from the TC (difenoconazole) assessed by high throughput assays based on the LEC	40
Figure 8. The transcriptomics point of departure for every gene in the most sensitive module for each conazole compounds	41

Note: Only figures from the main sections are included in this list. Figures in the annexes are not listed.

TABLES

Table 1. <i>Compounds used in the case study. TC: Target Compound, SC: Source Compounds, MET: Metabolites, POS: positive controls, NEG: negative control.</i>	14
Table 2. PoDs and corresponding unbound plasma concentrations of SCs used as pesticides	37
Table 3. Lowest effective concentration (LEC) of the conazole compounds tested in CALUX® assays.	38
Table 4. Lowest effect concentration (LEC) of the conazole compounds tested in ToxProfiler assays	39
Table 5. Lowest effective concentration (LEC) of the conazole compounds in CPP assay	39
Table 6. LEC of the conazole compounds tested in the H295R steroidogenesis assays	42
Table 7. LEC of the conazole compounds tested for the developmental neurotoxicity (DNT) using A) UKN2, B) UKN4 and C) UKN5 assays	43
Table 8. The total and unbound maximal plasma concentration (µM) at steady state for all SCs and TC used in the case study	44
Table 9. Comparison of the LECs associated with biological activity across all in vitro assays for TC and SCs	47
Table 10. Comparison of NAM derived LOAEL with allometrically scaled in vivo LOAEL	50

Note: Only tables from the main sections are included in this list. Tables in the annexes are not listed.

Executive Summary

This case study explored the applicability of NAM (New Approach Methodology) data in a read-across scenario to predict the systemic toxicity and lowest observed adverse effect level (LOAEL) of the target compound (TC), difenoconazole following a sub-chronic exposure. Eight other conazoles were used as source compounds (SCs) along with the metabolites, hydroxyitraconazole and 1,2,4-triazole. The Alternative Safety Profiling Algorithm (ASPA) workflow, developed within the EU-project RISK-HUNT3R, was followed to structure the case study. The molecular similarity assessment using hierarchical clustering with Morgan and MACCS fingerprints showed that the TC was closest to the SC propiconazole. Additionally, Morgan fingerprints revealed a notable similarity between the TC and epoxiconazole, supporting their inclusion in the read-across analysis. The in vitro assays including Chemically Activated Luciferase-gene Expression (CALUX®), ToxProfiler, Cell Painting PLUS (CPP), H295R steroidogenesis, developmental neurotoxicity assays and transcriptomics in primary human hepatocytes (PHHs) were used to derive lowest effect concentrations (LEC) of TC and SCs. The similarity between TC, propiconazole and epoxiconazole was supported by the in vitro assay results as well. The known biology of conazoles including effect on mitochondria, activation of stress response pathways and steroid pathways was captured in the in vitro assays. The lowest toxicologically relevant effective concentrations (LEC) from the in vitro assays were selected as the point of departure (PoD) for the TC and SCs. Reverse dosimetry using PBK modelling predicted external dose corresponding to the PoDs (NAM-based LOAELs). Two read-across approaches were evaluated to predict the LOAEL of TC: a nearest neighbour approach and a conservative worst-case approach. The LOAEL for the TC using three nearest neighbours (propiconazole, epoxiconazole and cyproconazole) was predicted to be 6.21 mg/kg bw/day while the worst-case approach, conservatively set it to 2.71 mg/kg bw/day, corresponding to the lowest LOAEL among the neighbours (epoxiconazole). Both estimates were within a two-fold range of the animal-based LOAEL for TC (4.88 mg/kg bw/day), demonstrating the accuracy of the NAM-based read-across assessments. Moreover, the NAM based LOAEL predicted for the TC in this case study was 0.43 mg/kg bw/day, approximately ten-fold lower than the in vivo-based value, highlighting the conservative nature of the NAM based estimate. Overall, this case study highlights the accuracy and utility of a NAM based read across approach, while also demonstrating the use of ASPA workflow to structure it transparently and in a scientifically relevant manner.

Abbreviations and acronyms

Abbreviation	Definition
ADI	Acceptable Daily Intake
ADME	Absorption, Distribution, Metabolism, And Excretion
AOP	Adverse Outcome Pathway
AR	Androgen Receptor
ASPA	Alternative Safety Profiling Algorithm
AUC	Area Under the Curve
BMCs	Benchmark Concentrations
BMD	Benchmark Dose
BMDL	Benchmark Dose Lower Confidence Limit
BMDU	Benchmark Dose Upper Confidence Limit
BP	Biological Processes
CAR	Constitutive Androstane Receptor
CALUX®	Chemical Activated LUCiferase gene eXpression
CEP	Chemical Effect Predictor
CLP	Classification, Labelling, And Packaging
Cmax	Maximal Concentration achieved for an external dose
CPM	Counts Per Million
CPP	Cell Painting Plus
DARs	Draft Assessment Reports
DEG	Differentially Expressed Gene
DMSO	Dimethyl sulfoxide
ECHA	European Chemical Agency
EFSA	European Food Safety Authority
EPAA	European Partnership for Alternative Approaches to Animal Testing
ER	Estrogen Receptor
ER stress response	Endoplasmic Reticulum Stress Response
ESRE	Estrogen Response Element
FDR	False Discovery Rate
GFP	Green Fluorescent Protein
GR	Glucocorticoid Receptor
hOED	human Oral Equivalent Dose
HLM	Human Liver Microsome
IATA	Integrated Approaches to Testing and Assessment
IVIVE	In Vitro to In Vivo Extrapolation
KE	Key Event

kNN	K-Nearest Neighbour
LEC	Lowest Effect Concentration
LOAEL	Lowest Observed Adverse Effect Level
MACCS	Molecular Access Systems
MIE	Molecular Initiating Event
MoA	Mode Of Action
NAM	New Approach Methodology
NGRA	Next-Generation Risk Assessment
NOAEL	No Observed Adverse Effect Level
normAUC	Normalized Area Under the Curve
NRF2	Nuclear Factor Erythroid 2-Related Factor 2
OECD	Organisation for Economic Co-Operation and Development
PBK	Physiology-Based Kinetic (Modelling)
PHHs	Primary Human Hepatocytes
PI	Propidium Iodide
PoD	Point Of Departure
PPAR	Peroxisome Proliferator-Activated Receptor
PR	Progesterone Receptor
PXR	Pregnane X Receptor
QSAR	Quantitative Structure-Activity Relationship
RAR	Retinoid Acid Receptor
RDT	Repeated-Dose Oral Toxicity
SCs	Source Compounds
SMILES	Simplified Molecular-Input Line-Entry System
TC	Target Compound
TRb	Thyroid Hormone Receptor Beta
VIVD	Virtual In Vitro Distribution (Model)

Introduction

Background, endpoint(s) addressed, type of workflow/approach

The assessment of chemical risks to human health and the environment is a critical aspect of modern regulatory science, aimed at ensuring safety and minimizing adverse effects. There are strong scientific, ethical, and legislative reasons to shift from traditional approaches using *in vivo* animal studies to new approach methodologies (NAMs). However, a NAM-based assessment and testing strategy for systemic toxicity is not yet available. Therefore, there is a need for case studies to gain confidence in NAM-based approaches and increase their acceptance in regulatory risk and hazard assessment. In this case study, performed in the context of the European Union Horizon 2020 project RISK-HUNT3R, we demonstrate the applicability of NAMs for assessing shared toxicodynamic as well as toxicokinetic properties within a read-across assessment. The NAM-based data is used to derive the lowest-observed-adverse-effect level (LOAEL) of the target compound (TC), i.e. difenoconazole, and the source compounds (SCs). Furthermore, read-across is used to confirm that the biological activity of the SCs is captured by the high-throughput methods used in this case study, providing confidence in the NAM-based assessment.

The read-across approach uses a well-known group of conazoles to demonstrate the applicability of NAM data (EFSA Panel, 2009). Guidance on conducting a read-across is documented in the RAAF, the read-across assessment framework (*Read-Across Assessment Framework (RAAF)*, 2017). In this case study, the selection of the compounds for the read-across assessment was based on the shared MoA and availability of the data. The use of NAMs is particularly valuable for assessing conazoles given shared toxicophore, along with the structural diversity within this chemical class. By leveraging NAMs, including *in vitro* assays, computational modelling, and high-throughput screening, this study aims to provide a robust framework for evaluating the systemic toxicity of compounds in a read-across context. One conazole (difenoconazole) was selected as (hypothetical) TC, while all other conazoles in the case study served as SCs (**Table 1**).

The widespread use of conazoles and persistent nature and mobility of their metabolite 1,2,4-triazole (Arp, Hale, Schliebner, & Neumann, 2023) has raised concerns about potential contamination of drinking water and concerns about their adverse effects on human health. As triazole and imidazole derivatives, conazoles function primarily by inhibiting the biosynthesis of ergosterol, an essential component of fungal cell membranes, thereby exhibiting broad-spectrum antifungal activity (Ghannoum & Rice, 1999). More specifically, conazoles inhibit ERG11 (CYP51) in fungus which catalyzes the conversion of lanosterol to ergosterol. Inhibition of ERG11 leads to lanosterol depletion and disruption of fungal membrane integrity (Ghannoum & Rice, 1999).

Typically, assessment of the potential adverse health effects of a plant protection product in Europe requires in-depth toxicity testing of the active substances therein, involving mainly *in vivo* testing like the

sub-chronic repeated dose toxicity test (OECD TG 409¹)². The data-rich SCs in this read-across case study show primarily adverse liver effects at the lowest observed effect concentration in relevant *in vivo* studies. This leads to the read-across hypothesis that also the TC, difenoconazole, will cause liver toxicity.

High-throughput *in vitro* assays showed a shared biological response pattern. Physiologically based kinetic modelling as well as *in vitro* to *in vivo* extrapolation (IVIVE) was applied to compare kinetic properties of the TC and the SCs and to predict a PoD using NAMs. The existing *in vivo* data from the SCs, together with *in vitro* data generated through NAMs, were integrated into a read-across framework to predict the LOAEL for the TC.

The findings contribute to the evolving landscape of chemical risk assessment by highlighting the effectiveness of NAMs in regulatory assessments like read-across. The approach aligns with the principles of next-generation risk assessment (NGRA), which integrates NAMs and read-across approaches to develop more predictive, efficient, and ethical safety evaluations. NGRA emphasizes the use of human-relevant data, advanced computational models, and high-throughput screening technologies to better predict human health outcomes and environmental impacts.

¹ OECD (1998), *Test No. 409: Repeated Dose 90-Day Oral Toxicity Study in Non-Rodents*, OECD Guidelines for the Testing of Chemicals, Section 4, OECD Publishing, Paris, <https://doi.org/10.1787/9789264070721-en>.

² EC (2009). Regulation (EC) no 1107/2009 of the European Parliament and of the Council of 21 October 2009 concerning the placing of plant protection products on the market and repealing Council Directives 79/117/EEC and 91/414/EEC. 1-50.

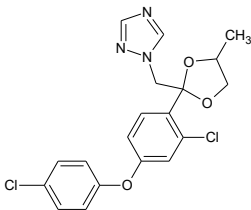
1 Regulatory Purpose/Context of use

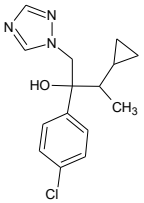
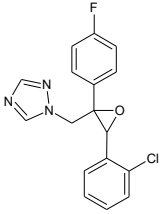
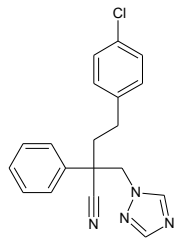
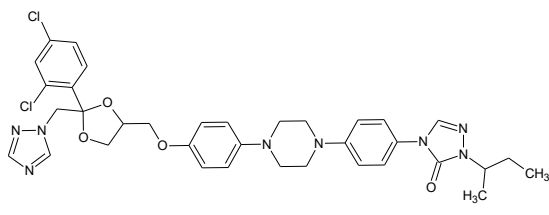
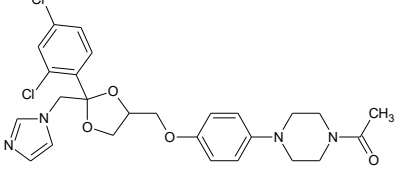
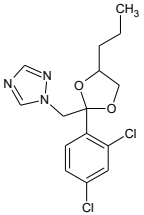
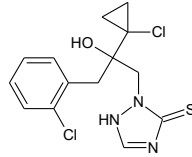
Difenoconazole is a broad-spectrum triazole fungicide which is used by spraying, seed coating as well as directly applying to the roots. It is used for the protection of vines, fruits, wheat and other agricultural plants from powdery mildew, black rot, leaf spot and other fungal infections.

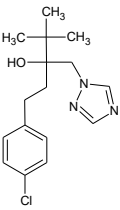
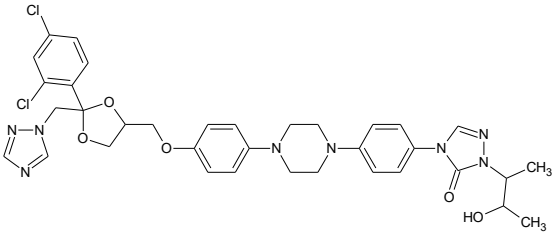
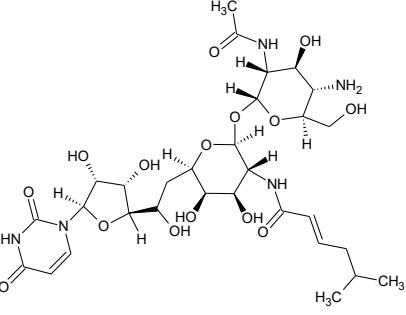
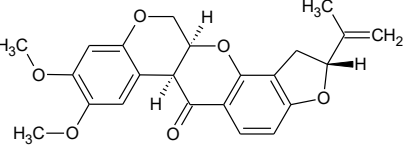
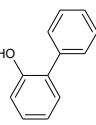
The case study assumes that the intended agricultural use of the TC difenoconazole as an active substance in pesticide requires prior approval within the EU on the basis of demonstrated safe use. A read-across approach is used to estimate the LOAEL for a sub-chronic exposure for the TC difenoconazole. For the purpose of this case study, we assume that difenoconazole does not have any relevant data (e.g. a 90-day repeated dose toxicity study) that is usually used for the derivation of a PoD for a plant protection product.

NAM data are used to examine whether difenoconazole has similar toxicological properties, in particular mechanistic and kinetic characteristics, compared to other data-rich conazoles. For both the TC and SCs, IVIVE was applied to predict LOAELs based on PoDs identified *in vitro* assays. Furthermore, a read-across assessment using *in vivo* data for the SCs was conducted to study whether the PoDs derived from NAM-based data are sufficiently conservative to capture all observed toxicities of the compounds in subchronic oral repeated dose studies. These findings support the conclusion that the PoD derived for the TC is comparable in reliability to the LOAEL obtained from *in vivo* assessments. As conazoles can be metabolized to form toxicologically active metabolites, a NAM-based risk assessment of some metabolites is also carried out. The two main potential metabolites of difenoconazole that will be considered in the risk assessment will be the hydroxy metabolite and 1,2,4-triazole (**Table 1**).

Table 1. Compounds used in the case study. TC: Target Compound, SC: Source Compounds, MET: Metabolites, POS: positive controls, NEG: negative control.

CAS	Name	Structure	Molecular weight (g/mol)	Type
119446-68-3	Difenoconazole		406.3	TC

94361-06-5	Cyproconazole		291.77	SC
133855-98-8	Epoxiconazole		329.8	SC
114369-43-6	Fenbuconazole		336.8	SC
84625-61-6	Itraconazole		705.6	SC
79156-75-5	Ketoconazole		531.4	SC
60207-90-1	Propiconazole		342.2	SC
178928-70-6	Prothioconazole		344.3	SC

107534-96-3	Tebuconazole		307.8	SC
288-88-0	1,2,4-triazole		69.1	MET
112559-91-8	Hydroxyitraconazole		721.6	MET
11089-65-9	Tunicamycin		717.7	POS
83-79-4	Rotenone		394.4	POS
90-43-7	2-Phenylphenol		170.2	NEG

2 Endpoint/Biology/Response addressed

As systemic toxicity of difenoconazole is being assessed in this case study, all the hazard effects predicted by the *in silico* tools, observed in the *in vitro* assays and/or known through the existing *in vivo* data of source compounds will be taken into consideration. A conservative estimate of the dose at which hazard activity begins to emerge will be established, serving as a protective threshold against adverse effects that may occur at higher exposures.

The *in vivo* data of SCs and *in silico* tools predict that difenoconazole causes liver toxicity and effects indicating endocrine disruption (**Annex II Table A2.4., Annex III Figure A3.3., Table A3.7.**). The *in vivo* studies collected in this case study demonstrate that the liver is the main target organ after repeated oral exposure, showing primarily hypertrophy, followed by fatty degeneration and necrosis at higher doses. Several publications associate this liver toxicity with the activation of the nuclear receptors FXR, CAR and PXR (Goetz & Dix, 2009). It is discussed that receptor activation leads to CYP enzyme induction, impairment of lipid and steroid homeostasis, and an increase in cell proliferation (Goetz & Dix, 2009; Heise et al., 2018; Hester et al., 2012). Prolonged, chronic cell proliferation is discussed as one reason for conazoles to cause liver cancer (Hester et al., 2012). Therefore, the systemic toxicity of the conazoles was primarily evaluated through the assessment of their effects on the liver and potential impacts on endocrine disruption were also evaluated.

The liver toxicity by conazoles could be manifested through various mechanisms including mitochondrial disruption, oxidative stress and/or aberration of relevant genes and pathways (Vlasveld et al., 2024). CALUX®, ToxProfiler, cell painting assays, and transcriptomics analysis in PHHs were used to identify this effect. Conazoles are also known to be endocrine disruptors. The H295R steroidogenesis³ (OECD TG456) assay was used to predict concentrations at which conazoles cause endocrine disruption. It is anticipated that this assay, along with transcriptomic analyses, can capture relevant endocrine-related signaling pathways and biological responses. Furthermore, by comparing NAM-derived PoDs with those obtained from *in vivo* studies, we evaluated the reliability of high-throughput NAMs and confirmed that NAM-derived PoDs are sufficiently conservative to capture the spectrum of conazole-induced toxicities.

The Alternative Safety Profiling Algorithm (ASPA) workflow developed within the RISK-HUNT3R project was followed for the risk assessment of the TC difenoconazole. The ASPA, “the Alternative Safety Profiling Algorithm”, is a broad-purpose risk assessment workflow that allows the integration of all types of information required for next-generation risk assessment (NGRA) (Leist et al., 2025). A major applicability domain is at present to ensure the protection of human health from systemic toxicity through repeated exposure. It uses a weight-of-evidence approach to (at least partially) substitute for the information requirements currently met by guideline animal studies. The ambition for ASPA in its final form is to provide

³ OECD (2025), *Test No. 456: H295R Steroidogenesis Assay*, OECD Guidelines for the Testing of Chemicals, Section 4, OECD Publishing, Paris, <https://doi.org/10.1787/9789264122642-en>

information equivalent to e.g. subchronic (28- and 90-day) repeated dose toxicity studies (TG 407⁴, TG 408⁵), developmental neurotoxicity studies (TG 426⁶), or carcinogenicity studies (TG 451⁷). The ASPA aims to provide a bioactivity PoD and to translate this by IVIVE into a measure that equates to a NO(A)EL (No Observed (Adverse) Effect Level) or LOAEL. Such output data (combined with measures of their uncertainty) may be used within prioritization approaches, risk assessments based on margins of exposure (MoE) and potentially for health-based guidance values and classification.

⁴ OECD (2025), *Test No. 407: Repeated Dose 28-day Oral Toxicity Study in Rodents*, OECD Guidelines for the Testing of Chemicals, Section 4, OECD Publishing, Paris, <https://doi.org/10.1787/9789264070684-en>.

⁵ OECD (2025), *Test No. 408: Repeated Dose 90-Day Oral Toxicity Study in Rodents*, OECD Guidelines for the Testing of Chemicals, Section 4, OECD Publishing, Paris, <https://doi.org/10.1787/9789264070707-en>.

⁶ OECD (2007), *Test No. 426: Developmental Neurotoxicity Study*, OECD Guidelines for the Testing of Chemicals, Section 4, OECD Publishing, Paris, <https://doi.org/10.1787/9789264067394-en>.

⁷ OECD (2018), *Test No. 451: Carcinogenicity Studies*, OECD Guidelines for the Testing of Chemicals, Section 4, OECD Publishing, Paris, <https://doi.org/10.1787/9789264071186-en>.

3

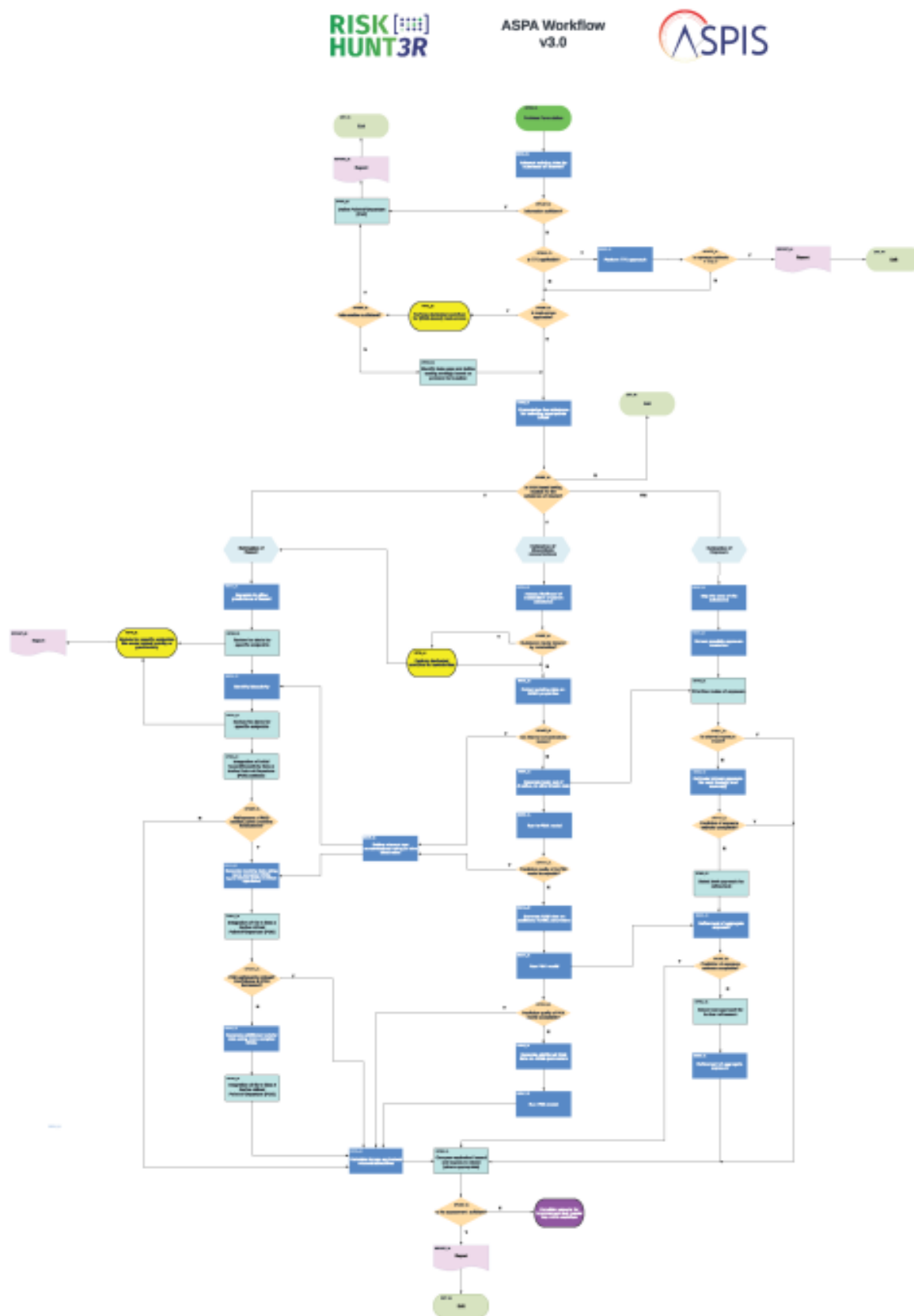
Description of a Workflow

The purpose of ASPA is to provide guidance to end-users for putting NGRA into practice, both for the generation and the assessment of data. ASPA has been developed as part of the RISK-HUNT3R project which aims at providing *ab initio* solutions for animal-free risk assessment of chemical substances. Within the RISK-HUNT3R case studies (as the one presented here) are conducted to assess the applicability of the ASPA workflow. During this process, the ASPA is modified and improved concerning better and more detailed guidance as well as concerning the granularity and connections of steps (actions, data generation tasks and decisions). *Vice versa*, the processing of case study data along the ASPA workflow is used to identify remaining data and knowledge gaps. The current overarching structure of ASPA, i.e. version 3.0, is depicted in Figure 1.

Given that the workflow should be applicable for regulatory decision-making, it comprises the standard elements for chemical safety assessment. Following a detailed problem formulation, ASPA defines how to collect data on a compound in the domains of hazard, toxicokinetics and exposure. Within each of these three domains, ASPA proposes a tiered approach on how to use NAMs. It also provides guidance on generating data, and how to assess the data. The tiered assessment aims to reduce uncertainty and to increase the accuracy of the NAM-based approach. It typically starts with low-cost and generic approaches (e.g. *in silico* models), which are subsequently refined by using increasingly complex and resource-demanding models and assays. The tiering of approaches is aimed at reducing residual uncertainty and providing higher specificity. One of the underlying concepts is to provide a mechanistic rationale for the findings that allows a plausible extrapolation towards adverse effects *in vivo*. The assessment and decision criteria of this tiered assessment are context-dependent, and after each tier ASPA will assess if the so far provided NAM approach is sufficient to address the problem formulation (Figure 2).

The workflow starts with the problem formulation which in this case study is to predict the LOAEL of difenoconazole (TC), using read-across. The workflow identifies that read-across is applicable to this set of compounds; however, the existing data is not sufficient to predict the LOAEL of TC with low uncertainty (**Figure 2**). Therefore, additional NAM-based data was generated to refine the assessment and reduce uncertainty.

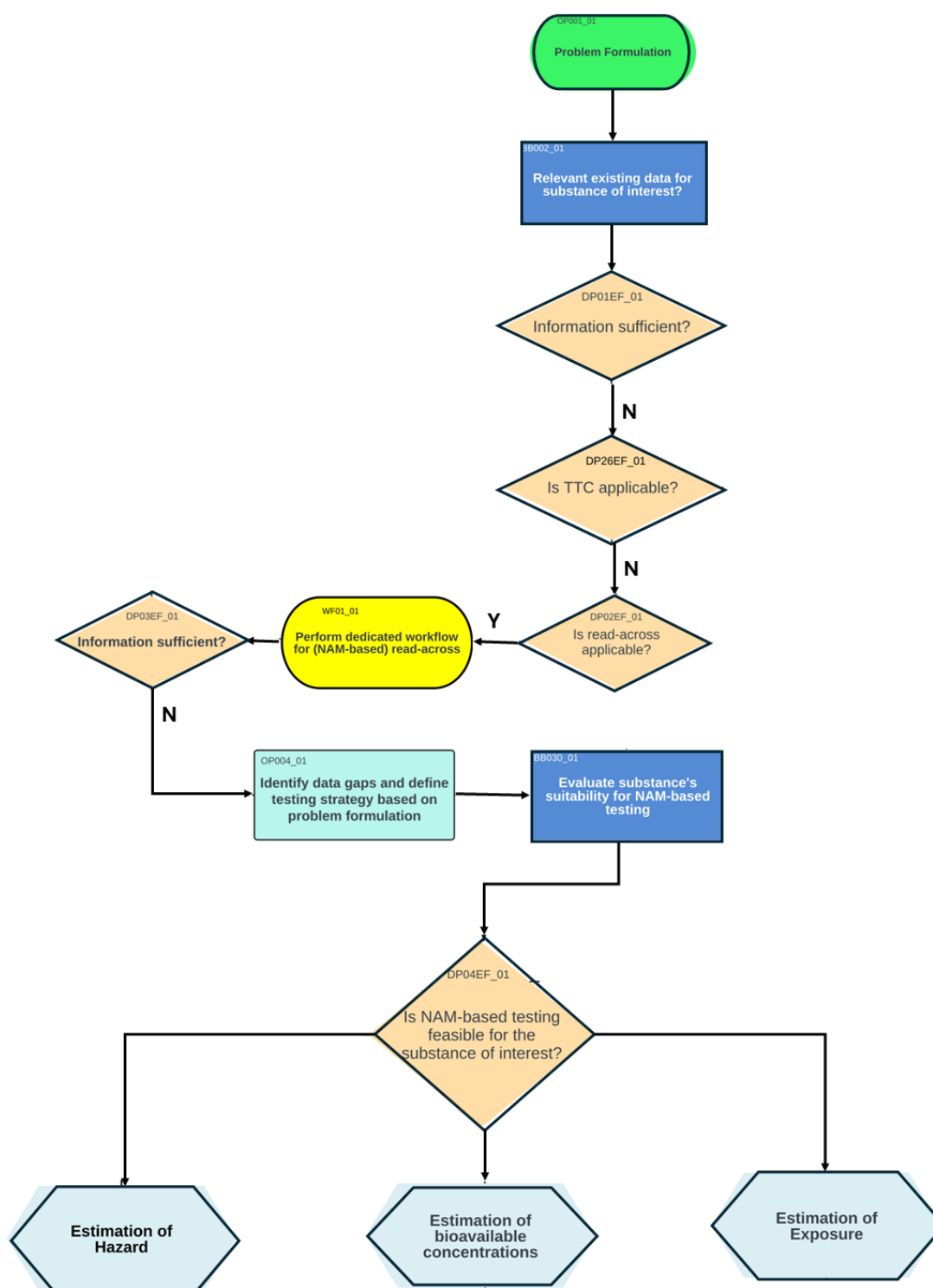
Figure 1. Schematic representation of ASPA version v3.0



Note: The problem formulation outlines the regulatory context of the NAM-based assessment, as well as its goals. After initial steps of collecting available data and deciding on whether they are sufficient for risk assessment or whether read-across is applicable the ASPA workflow continues with three major assessment pillars (Hazard, ADME and Exposure).

Note: The high-resolution version is available at: <https://www.oecd.org/content/dam/oecd/en/topics/policy-sub-issues/assessment-of-chemicals/case-studies-iaa-systemic-toxicity-conazoles/alternative-safety-profiling-algorithm-version-3-0.pdf> :

Figure 2. Initial steps in the ASPA workflow as followed for the NAM-based read-across assessment of conazole compounds in this case study

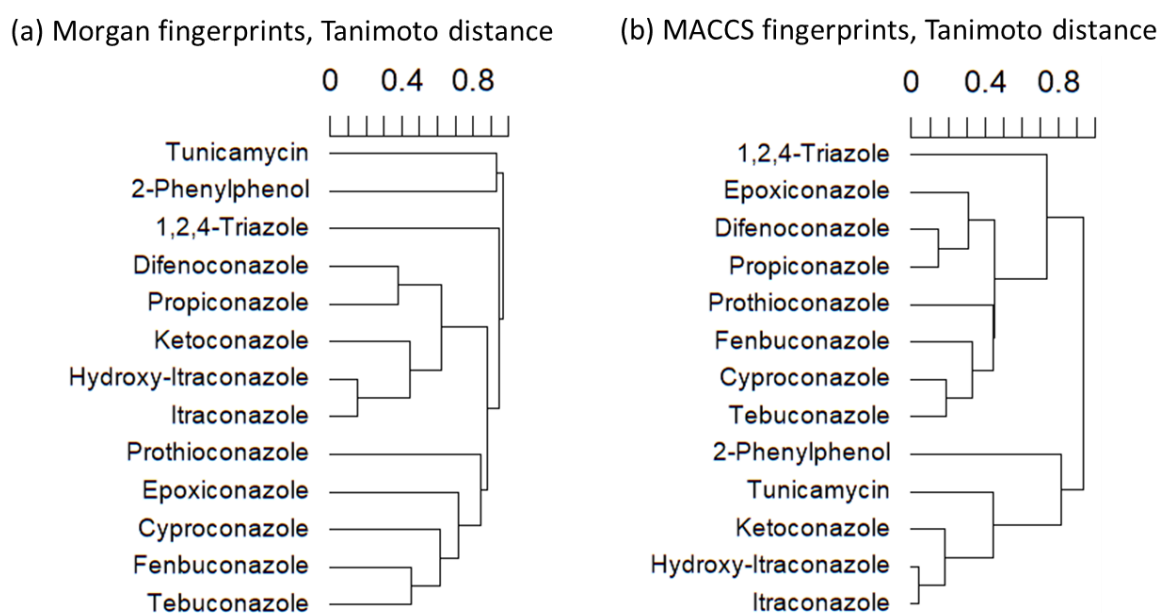


Note: The figure outlines early-stage data gathering, compound grouping, and evaluation strategies for NAM-based assessment.

Note: The high-resolution version is available at: <https://www.oecd.org/content/dam/oecd/en/topics/policy-sub-issues/assessment-of-chemicals/case-studies-iata-systemic-toxicity-conazoles/alternative-safety-profiling-algorithm-version-3-0.pdf>

For the read-across assessment, the workflow developed in the EU Horizon 2020 project EU-ToxRisk was followed (Escher et al., 2019). Read-Across begins with problem formulation, which involves identifying data gaps, determining the endpoints to be predicted, outlining exposure scenarios and defining levels of uncertainty. This approach continues with the characterization of TC, which relies on its known structural information. All available and relevant information from experimental and *in silico* data is compiled to develop a read-across hypothesis for TC. The initial selection of the SCs is usually based on their structural similarity or shared substructures (*Read-Across Assessment Framework (RAAF)*, 2017)., which also considers data availability (**Figure 3**). During this step, the similarity in toxicological profile within the SCs could be analyzed, e.g. common *in vivo* ADME properties, bioactivity, and mechanisms underlying toxicity, if known⁸. This could define the concerning toxicity profile and establish the exclusion criteria of the dissimilar SCs. It is assumed that compounds that share similar structure(s) may exhibit similar mode(s) of action and therefore cause similar hazard(s). However, the dimension of similarity is diverse, which also could also include properties such as mechanistic and metabolite similarity. Integrating this information could refine the selection of the SC. In this case study, we considered conazoles with data available from Draft Assessment Reports (DAR) and Regulatory Assessment Reports (RAR) available on the EFSA consultation page that included *in vivo* information on the shared metabolite.

Figure 3. Dendrogram representing structure-based clustering of conazoles and metabolites in the case study using (a) Morgan and (b) MACCS fingerprints. The distance was calculated using Tanimoto coefficient



Additional NAM data was generated to provide further information regarding the toxicodynamic and kinetic properties of the SCs and to fill data gaps in the read-across assessment. As a first step, the suitability of the compounds considered for NAM-based testing was evaluated. This was assessed using the physicochemical properties of the compounds. The case study compounds were considered stable in the medium typically used for the *in vitro* assays used in this case study. The compounds are not volatile, are soluble in DMSO and can permeate the cells. Hence, NAM-based testing was considered feasible for the compounds. This was followed by hazard, toxicokinetics and exposure assessments of the compounds.

⁸ OECD (2025), *Guidance on Grouping of Chemicals, Third Edition*, OECD Series on Testing and Assessment, No. 418, OECD Publishing, Paris, <https://doi.org/10.1787/b254a158-en>.

Assessments of hazard, toxicokinetics and exposure are inter-linked at several levels (Figure 1). For example, the assessment of ADME properties provides an estimate of the *in vitro* relevant test concentration to be used in the hazard modules, while exposure assessment will guide the definition of the most appropriate routes and thus the choice of test methods (hazard) and parametrization of runs of the physiologically-based kinetic (PBK) model. These modules are discussed in detail in Section 6.

4

Scientific rationale/basis for IATA workflow

A number of case studies demonstrating the applicability of NGRA approaches for regulatory risk assessment have been published over the years by OECD. Moreover, various efforts have been made worldwide to define the process of NGRA, including the design of new concepts to integrate exposure and hazard assessment models and to link this to the use of data from NAMs (Nicholas Ball et al., 2022; Berggren et al., 2017; Pastoor et al., 2014). However, a comprehensive operationalization of NGRA concepts at a high level of granularity is limited thus far. Therefore, there is a strong need for a detailed workflow, to serve as guidance on both data generation and data interpretation. RISK-HUNT3R, together with colleagues from ASPIS and a large panel of additional experts and stakeholders, aims at developing such a workflow. This workflow, called ASPA (Alternative Safety Profiling Algorithm), comprises a wide set of elements/ modules relevant for chemical risk assessment: toxicokinetic modelling and *in vitro* to *in vivo* extrapolation (IVIVE) approaches, hazard identification and characterization modules, exposure and risk assessment. ASPA is considered fit for purpose for this case study as it includes all modules of assessment relevant to this case study including read-across, metabolites, kinetics and hazard.

The scientific rationale of ASPA is built on three fundamental concepts:

First, it is assumed that the internal exposure to a compound is a major determinant for its hazard. For instance, compounds without any significant internal exposure are considered to have no hazard, and compounds with high (possibly persistent or accumulating) internal exposure are considered candidates for a high hazard (to be assessed within ASPA). The ADME, exposure and hazard pillars provide increasing levels of detailed information on this issue.

Second, ASPA assumes that NAMs can be used to characterize the spectrum of bioactivities of a test compound and that NAM-based testing can be designed comprehensively, so that no activity relevant for toxicity is minimised (avoidance of false negatives). In identifying potentially relevant bioactivities, ASPA does not aspire to identify every bioactivity. Instead, the goal is to identify at least one of the most potent bioactivities. This means that there may be unidentified bioactivities (acceptable in the ASPA process), but none of them would be more potent than the one with the highest potency identified. This is in line with a so-called “protective” risk assessment approach, i.e. identifying the highest concentration/dose or exposure level that does not result in an adverse effect. This implies that in many instances the exact adverse effect will not be predicted (e.g. liver toxicity vs kidney toxicity).

Third, ASPA assumes that it is possible, on the basis of generating NAM data as well as by interpreting these data on the background of biological and toxicological knowledge (e.g. using the AOP database or ontology maps of human physiological function), to link certain bioactivities to adverse effects at the level of the whole organism (human). Thus, an important module of ASPA follows up on bioactivities, eliminates false positives or those that are not toxicologically relevant, and then provides plausibility to the remaining ones. The latter step involves providing a mechanistic rationale, i.e. why a certain compound activity (e.g.

a disturbed or activated process at a certain exposure concentration) leads to adverse health effects. This involves (but is not limited to) AOPs and AOP networks, conform OECD guidance⁹.

ASPA is to a large degree technology-agnostic and information-focused. This means that ASPA defines the type of information required to be generated at various steps of the workflow, but it leaves open to the user which types of NAMs or approaches are used to generate the information. The level of information required by ASPA is determined by the problem formulation and may differ largely between e.g. a classification and labelling problem, a risk assessment of a plant protection product or a preliminary hazard characterization of a potential contaminant in a production process. Instead, ASPA shows how the different modules are interlinked, build upon each other, and lead through defined decision points to the ultimate goal, e.g. a risk assessment. The entry into ASPA usually leads to first insights into the properties of a substance to be assessed and a general overview of exposure situations (information on bioactivity, rough toxicokinetics characterization, likely exposure routes). In subsequent tiers, information is added and refined, while uncertainties are reduced.

In this case study high-throughput *in vitro* assays were used to characterize the toxicity of TC and the SCs and support the read-across assessment. The major benefits of using a high-throughput approach lie in its ability to generate data much faster and more efficiently than traditional animal-based testing or conventional *in vitro* assays. High-throughput assays enable simultaneous testing of a large number of chemicals or biological samples across multiple endpoints, significantly reducing the time and resources required for hazard or risk assessment. Once the assay platforms are properly established and optimized, they tend to be highly reproducible due to their automation, standardized protocols, and reduced human handling. This reproducibility enhances the reliability and comparability of results across laboratories and experiments. Furthermore, the substantial data output of such NAMs increases statistical power and enables a systematic characterization of uncertainty - encompassing reliability, precision, reproducibility, and human relevance - which collectively underpins the robustness of the results in this case study.

To demonstrate the utility of this approach, we included results from replicated assays (CALUX®, ToxProfiler, CPP and transcriptomics) which confirm that high-throughput experimental data are consistent and reproducible across multiple runs. Such reproducibility and efficiency make high-throughput assays not only suitable for rapid screening but also valuable for mechanistic studies that inform adverse outcome pathways and predictive toxicology models. As a result, high-throughput methods are increasingly gaining consideration among regulatory authorities and within chemical and pharmaceutical industries, as part of a battery of NAMs to replace or reduce animal testing while improving human-relevant risk assessment (Cable et al., 2024; Middleton et al., 2022; Najjar et al., 2024; Ouedraogo et al., 2022; Wood et al., 2024). Informed by the *in vivo* data for the SCs, we also assessed the compounds by H295R steroidogenesis and DNT assays.

⁹ OECD (2017), *Guidance Document for the Use of Adverse Outcome Pathways in Developing Integrated Approaches to Testing and Assessment (IATA)*, OECD Series on Testing and Assessment, No. 260, OECD Publishing, Paris, <https://doi.org/10.1787/44bb06c1-en>.

5 Defined domain of applicability

ASPA is an operationalization of NGRA. It thus has the typical limitations and applicability areas known for other NGRA approaches. The technical applicability is highly compound-dependent. Most NAMs used for hazard identification and characterization require compounds that are soluble in cell culture media and that do not evaporate too easily. Some adaptations are possible to extend the technical applicability. For instance, volatile compounds may be tested in specialized closed devices or by exposure via an air-liquid interface; highly lipophilic compounds may be tested by use of passive dosing devices or in the presence of high concentrations of carrier proteins; acidic compounds may be tested in appropriate buffers, testing of unstable compounds may be performed under continuous flow conditions and be supported by analytical methods that indicate the actual concentration of the compound remaining.

Many of the *in silico* models have a low performance for metals, polymers, and organometal compounds. This also applies to most of the ADME modelling approaches. Concerning exposure (and the conversion of external to internal exposure), ASPA can in theory deal with multiple exposure routes, even when combined. However, some can be dealt with more easily (lower uncertainty, less need for resources) than others. For instance, inhalation or dermal exposure to compounds that show a high barrier (lung or skin) metabolism is posing more problems than oral exposure of compounds with 100% bioavailability. Some compounds also have technical limitations concerning interference with analytical endpoints. Each IATA case study will highlight the technical limitations of the applied NAM models, like interference of auto fluorescent compounds etc. For all *in silico* models used, information will be given on an indication of their chemical and biological applicability domain.

The biological applicability of ASPA depends on the resources available for the assessment. While known *in vitro* methods can cover (identify, quantify) virtually all biological targets, the availability of methods and the expertise to run and interpret them may be limited. Moreover, it is likely that availability is going to change (improve) over time. For instance, concerning endocrine disruption, presently there are good and relatively cheap methods available for the estrogen, androgen and steroidogenesis-interrupting compounds (e.g. H295R assay used in this case study), while an assay battery for thyroid effects is under development and not all control systems of energy metabolism have been covered. A second example comes from the area of carcinogenicity, where genotoxic carcinogens can be predicted with high certainty, while a battery for the prediction of non-genotoxic carcinogens is still under construction and evaluation. At present, the information requirements for some toxicity areas have not been implemented in ASPA. This applies e.g. to bone marrow toxicity, hematotoxicity and immunotoxicity, and to some organs (e.g. reproductive or cardiac). This is not necessarily a fundamental problem of NGRA, but it is rather due to the early implementation stage of ASPA. The early version focusses on exemplary core areas that are described within the case study descriptions.

A special subchapter within the biological (especially toxicological) applicability of ASPA refers to compounds that undergo metabolism or specific (active) accumulation in some tissues. ASPA has been designed to deal with metabolite-dependent toxicity. However, the performance of the respective ASPA modules has not been validated. It is possible that certain forms of metabolism, or toxicity due to certain metabolites remain undetected. ASPA has also been designed to test compounds for affinity to various transport systems, and it also provides suggestions for the use of certain NAM that expresses toxicologically relevant transporters (e.g. the megalin system in kidney cells or the dopamine transporter

in dopaminergic neurons). However, it has not been stringently tested, how well these predictions and NAM would model the transport-dependent toxicity.

Based on the above, metals and polymers are clearly outside the applicability domain of ASPA. For all other compounds, the definition is not as stringent. They may, provided the relevant NAMs and resources are present, be used for ASPA evaluation. However, working at the limit of an applicability domain, or working with compounds for which the applicability is not clearly validated, would mean that the uncertainty of predictions is higher. ASPA was considered suitable for this case study as it encompasses key components of risk assessment—hazard, toxicokinetics, and exposure—while also incorporating read-across and metabolism, which are essential for this analysis.

The feasibility of testing conazoles in NAMs was assessed using biokinetics analysis carried out using the physicochemical properties of the compounds. Compound loss by binding to the *in vitro* system including cellular proteins, plastic and by vaporization in the air was accounted for when selecting the test concentrations for each *in vitro* assay. The physicochemical properties of all the conazoles are within the applicability domain of the VIVD model (Fisher, Siméon, Jamei, Gardner, & Bois, 2019) used for predicting (free concentration in medium)/(nominal concentration) ratio. Itraconazole, ketoconazole and difeniconazole have $\log P_{ow} > 4$ which is towards the upper limit of the performance of VIVD model (Fisher et al., 2019).

The compounds are within the applicability domain of the models used to build PBK modelling. The oral route of exposure was considered for all compounds as with 100 % absorption in the gut, it is the most conservative estimate. To decide the testing concentrations, two complementary approaches were considered. In the first approach, the highest testing concentration was just below cytotoxicity. In the second approach, for conazole pesticides used as SCs in this case study, allometrically scaled LOELs were used as dose to predict the unbound concentration in plasma by PBK modelling. Assuming that this unbound concentration will also cause toxicity *in vitro* assays, biokinetic modelling was carried out to predict the nominal concentration needed to achieve this free concentration in the medium. Finally, this concentration was included in the concentration ranges used in the *in vitro* assays. By integrating both approaches, the testing range included concentrations just below cytotoxic levels as well as those predicted by PBK modelling. The specific approach used in each *in vitro* assay is outlined in the **Annex I, Section 5.3**.

The source compounds are known to cause liver toxicity and endocrine disruption. To ensure this biology is captured in the *in vitro* assays, reporter gene CALUX® assays (U2OS), high throughput stress reporter assays ToxProfiler (HepG2), Cell Painting Plus assays (HepG2), transcriptomics (PHH), H295R steroidogenesis assays and developmental neurotoxicity assays (DNT) were carried out. The details on the methods on each assay are available in **Annex I (Sections 1-5)** and the results in **Annex III (Section 5-8)**.

6 Description of Modules

The major modules of ASPA are (i) the problem formulation (with a subsequent weight-of-evidence evaluation of already available data); (ii) the read-across module; (iii) the risk assessment and reporting module, and the three major data generation and integration pillars for (iv) hazard, (v) ADME/toxicokinetics and (vi) exposure. More details are given here on modules iv-vi.

6.1. Hazard

The assessment of hazard starts with a module that uses a broad panel of methods to identify bioactivities affected by the test compound. Some of these methods may trigger alerts for defined traditional toxicological endpoints: genotoxicity, skin sensitization and acute toxicity (topical or systemic). These may be followed up further (at present not the focus of the core ASPA workflow). If the problem formulation focuses on systemic repeat-dose toxicity (as in the presented case study) the initial module produces data on the basis of QSAR models, some docking models, panels of receptor activation assays, a battery of stress pathway assays, various cytotoxicity assays and high content methods such as CPP and transcriptomics (Figure 4). These assays are run in a mode to ensure high sensitivity, but not high specificity. This has been evaluated in a parallel case study in RISK-HUNT3R (IATA case study on prioritization and screening of compounds). Many (all *in vitro* approaches) provide concentration-bioactivity data sets and allow for the definition of PoDs for various bioactivities. The ASPA might be exited at this stage for instance, if no relevant (relative to expected internal exposure levels) bioactivity is identified.

In most cases, compounds will be further examined to reduce uncertainties, follow up on alerts, eliminate false positives, and generate a plausible link from bioactivity to an adverse outcome. This might imply the use of more complex NAM, e.g. to assess later key events of a putative AOP. The NAMs applied for this purpose are (i) dependent on the specific problem formulation (including the compound under evaluation) and (ii) on the alerts generated in the first module. This flexible and tiered approach may also involve iteration loops to generate increasingly plausible toxicity information that is eventually robust enough to define a PoD that can be clearly related (a mechanistic rationale, such as an AOP) to an adverse effect on the organism level, and that can be used as input for IVIVE to predict a corresponding equivalent dose.

6.2. Toxicokinetics

As in the hazard module, the data generation in the ADME module proceeds in a tiered manner (Figure 5). In the first tier, an estimate of plasma concentrations of the orally ingested test compound will be generated using a generic, relatively simple physiologically based kinetic (PBK) model. The conservative assumptions that the compound is 100% bioavailable and that it is only cleared renally (filtration, not by metabolism) will lead to a potential overestimate of internal exposure, and data on tissue levels will not be available. *In silico* predictions (e.g. on protein binding) are used to parameterize the model with compound specific input data. Where required, more refined models will be generated and used in tiers 2+3: the PBK model uncertainty is reduced by the generation of experimental data on the test compounds ADME properties (barrier crossing/transport, metabolism protein binding, blood-plasma distribution etc.). Also, the PBK model structure may be adapted to cover more tissues and life stages (e.g. fetus within mother or

children/aged subjects) and to include physiological processes like enterohepatic circulation, renal reabsorption, biliary excretions etc. Finally, also the genetic and phenotypic variance in human subpopulations can be considered in the high tier ASPA modules.

For the assessment of compounds that can form metabolite/s, a dedicated metabolite workflow has also been developed within ASPA (Figure 6). This workflow is initiated by silico predictions or classification schemes like Extended Clearance Classification System (ECCS) informing of metabolite formation and is integrated into the ADME assessment, contributing to the overall hazard evaluation. All compounds in this case study were classified as ECCS Class II, indicating metabolism as the predominant route of clearance. Due to the absence of metabolite standards, only qualitative estimations of metabolite formation could be derived from the hepatocyte-based clearance assays.

6.3. Exposure

This module is not applied in the submitted case studies. External exposure was included to predict how much 1,2,4-triazole will theoretically be formed from the conazoles. For the conazoles that are used as pesticides, external exposure was incorporated using the acceptable daily intake (ADI) set by EFSA (**Annex II, Table A2.5.**). For the pharmaceutical conazoles, ketoconazole and itraconazole, the therapeutic dose known to cause liver toxicity (200 mg/day) was used to estimate potential internal exposure (Lewis, Zimmerman, Benson, & Ishak, 1984; Lou et al., 2011). This approach assumes that higher doses would likely be constrained by hepatotoxicity, thereby limiting further exposure.

7 Description of information sources

The information generated in each of the assessment pillars has various levels. It goes from data generation (computational and experimental) over data integration (within one pillar; across pillars) to the generation of a toxicological rationale (plausible chain of events from measured molecular initiating events or key events) to an adverse outcome in accordance with the relevant concentrations of a compound in plasma, tissues or inside certain target cell types. The process is complex and compound-dependent and constitutes the core of the presented case studies. Below, only a very brief overview is given.

7.1. Hazard

Examples of *in silico* data generation tools are the OECD QSAR toolbox¹⁰, Danish (Q)SAR toolbox¹¹ and the Chemical Effect Predictor¹². Examples of generation of bioactivity data are an array of CALUX® assays, stress reporter cell lines from ToxProfiler or transcriptomics data from PHH. Additionally, H295R steroidogenesis assay and DNT assays were done to screen the effect of conazoles on endocrine disruption and developmental neurotoxicity, respectively. Several examples of higher tier follow up assays have been given in previous OECD case studies e.g. on imidacloprid or in publications (Magel et al., 2024).

7.2. Toxicokinetics

For the *in silico* module, generally all models relevant to the problem formulation can be applied (this might e.g., models available in the OECD QSAR toolbox, OPERA or ADMET Predictor).

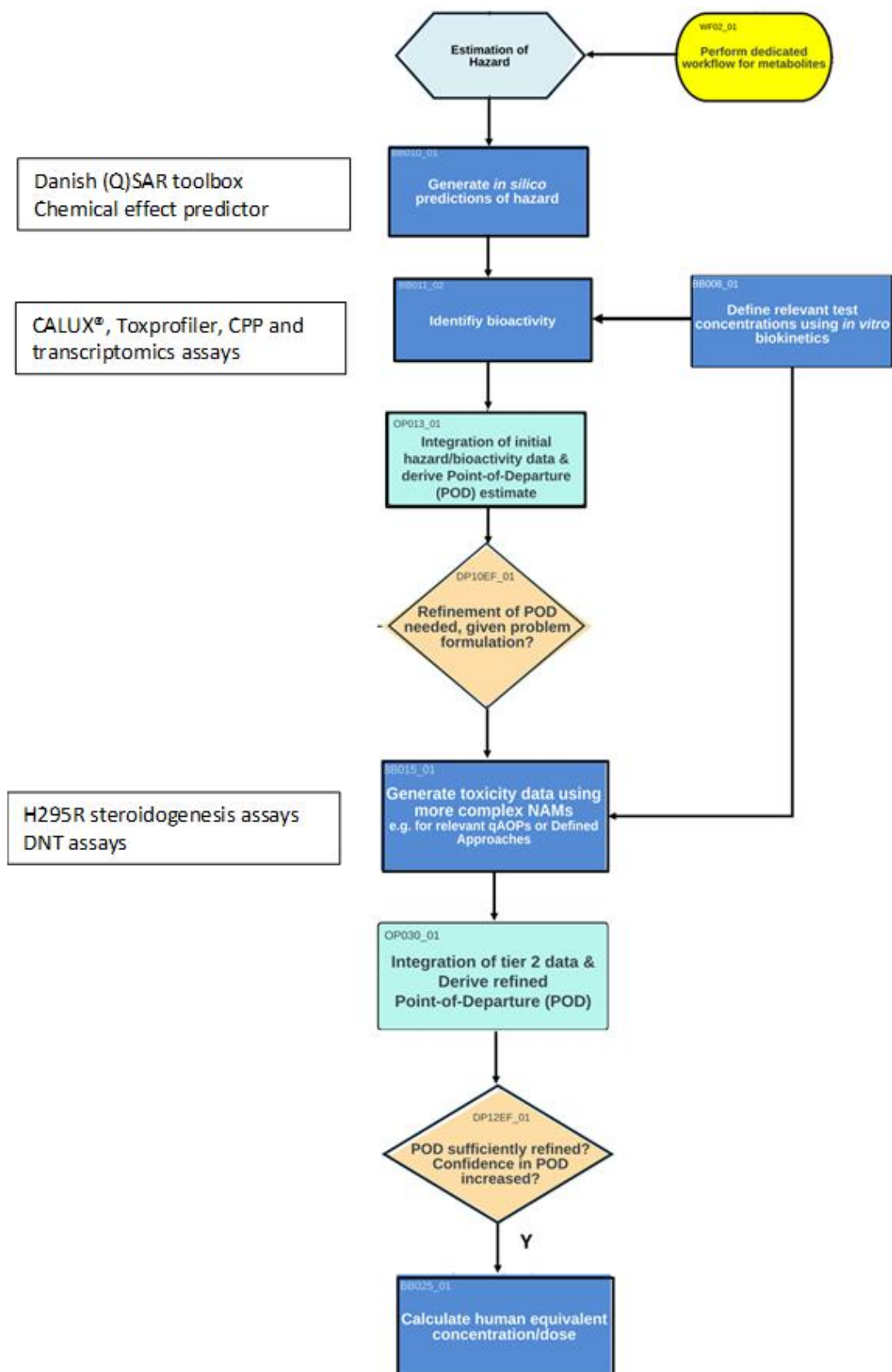
In the first tier of *in vitro* testing, permeability through relevant barrier models is performed like Caco-2 (intestinal) or Calu-3 (respiratory). Different assays are proposed to address metabolic clearance in liver or barrier organs e.g. microsomes, primary human cells, liver S9 mix etc.. The selection of these assays does neither prescribe nor preclude any NAM to be used. The model has to be relevant for the regulatory question outlined in the problem formulation. For the follow-up testing, each case study applies assays relevant for the specific problem formulation and alerts generated. Care has to be taken that the assessed substance is within the applicability domain of the assays (irrespective of whether *in silico* or *in vitro*).

¹⁰ <https://qsartoolbox.org/>

¹¹ Danish (Q)SAR Database, Research Group for Chemical Risk Assessment and GMO, National Food Institute, Technical University of Denmark, <http://qsar.food.dtu.dk>.

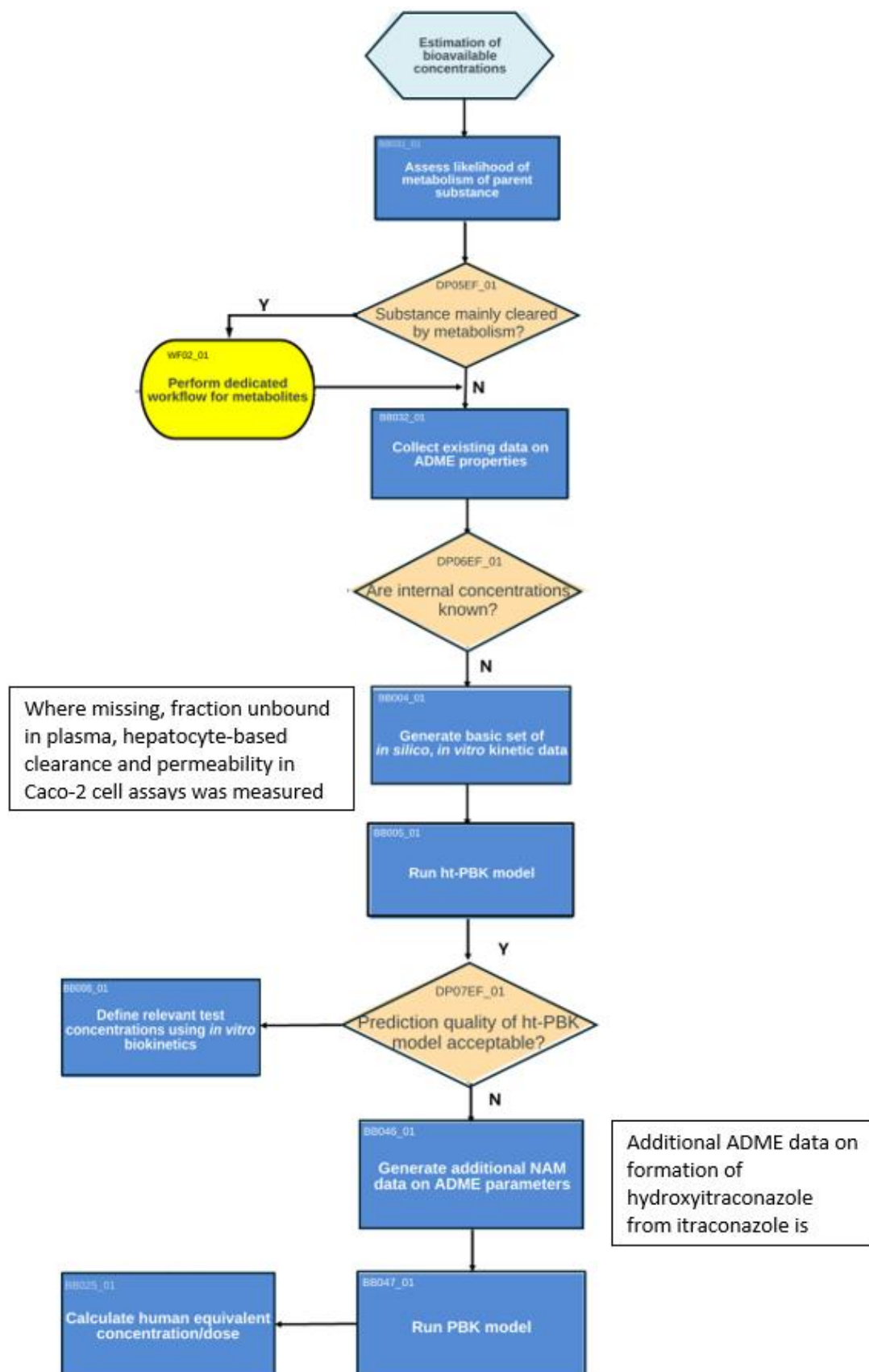
¹² <https://www.medbioinformatics.com/>

Figure 4. ASPA workflow for hazard estimation of compounds as applied to conazoles in this case study



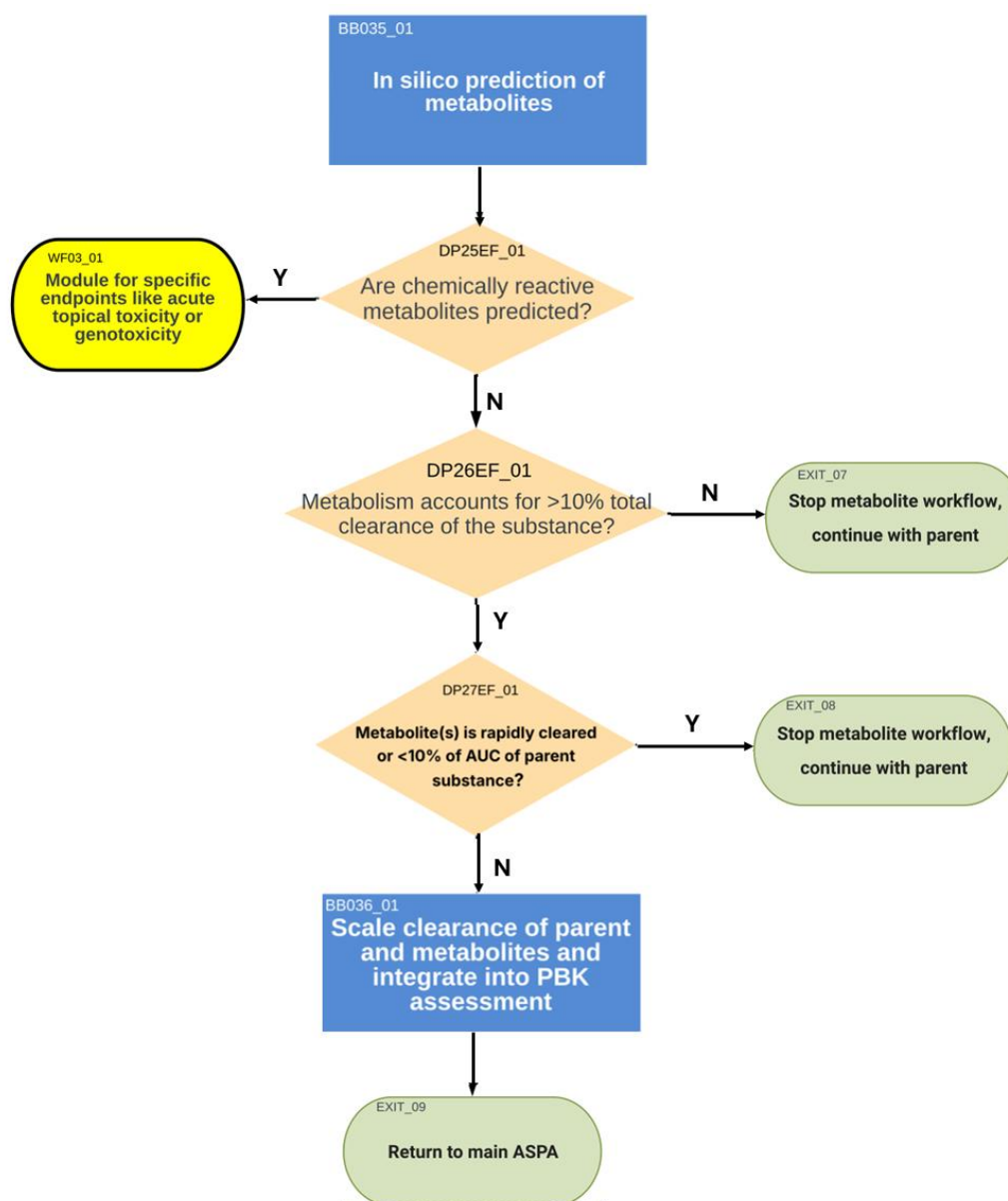
Note: The high-resolution version is available at: <https://www.oecd.org/content/dam/oecd/en/topics/policy-sub-issues/assessment-of-chemicals/case-studies-iaa-systemic-toxicity-conazoles/alternative-safety-profiling-algorithm-version-3-0.pdf>

Figure 5. ASPA workflow for toxicokinetics assessment, as applied to the compounds in this case study



Note: The high-resolution version is available at : <https://www.oecd.org/content/dam/oecd/en/topics/policy-sub-issues/assessment-of-chemicals/case-studies-iaa-systemic-toxicity-conazoles/alternative-safety-profiling-algorithm-version-3-0.pdf>.

Figure 6. Metabolite Workflow in ASPA illustrating the evaluation of compounds with metabolites



Note: The workflow includes key stages such as metabolite identification, profiling, and qualification, integrated with risk assessment.

Note: The high-resolution version is available at : <https://www.oecd.org/content/dam/oecd/en/topics/policy-sub-issues/assessment-of-chemicals/case-studies-iaa-systemic-toxicity-conazoles/alternative-safety-profiling-algorithm-v.1.2-metabolite-workflow.jpeg>

8 Data Selection/gathering

8.1. In vivo data

Repeated dose studies, ranging from subchronic to chronic exposure duration, conducted in rats or mice via oral routes were identified from the Draft Assessment Reports (DAR) and Regulatory Assessment Reports (RAR) available on the EFSA consultation page¹³. Adverse effects per target organ, gender and dose were entered into the relational database RepDose (Bitsch et al., 2006) using a controlled vocabulary/ontology. Relevant *in vivo* studies were available for the source compounds cyproconazole, epoxiconazole, fenbuconazole, propiconazole, prothioconazole, and tebuconazole (Table 2). The extended version of these studies, along with other supporting evidence, are available in **Annex II Table A2.4**. Two subchronic and two chronic *in vivo* studies are also available for the target compound difenoconazole. These *in vivo* studies are used to compare the read-across prediction.

Additionally, for pharmaceutical triazoles, including itraconazole and ketoconazole, information was retrieved from FDA Pharmapendium studies¹⁴, where available. For metabolites, including 1,2,4-triazole and hydroxymetabolites, information was gathered from dossiers of parent substances, when available¹. Specifically, studies on 1,2,4-triazole were obtained from the tebuconazole DAR, which also summarized studies conducted on 1,2,4-triazole. The "Conclusion on Pesticides" published by EFSA was also referenced for additional insights¹⁵.

The pesticides containing triazole moieties, or referred to as conazole pesticides, are known to cause liver toxicity. The PoDs including LOAEL and NOAEL in this assessment were used to guide the selection of relevant testing concentration range for the *in vitro* assays. The endpoints for repeated dose toxicity are depicted in Table 2. These studies were derived from rat and mice studies via oral administration. Additional supporting evidence from studies of both longer and shorter durations is provided separately in **Annex II Table A2.4**. For each of the PoD, the allometric scaling was applied using **Equation 1**. The allometric factor for the rats was set to 4, and mice to 7 based on allometric scaling factors in ECHA guidance on information requirements and chemical safety assessment¹⁶.

$$\text{Allometric LOAEL (mg/kg bw/d)} = \text{LOAEL (mg/kg bw/d)} / \text{factor} \quad \text{Equation 1}$$

¹³ <https://www.efsa.europa.eu/en/calls/consultations>

¹⁴ <https://www.pharmapendium.com/welcome?status=grey>

¹⁵ <https://www.efsa.europa.eu/en/corporate/pub/publicationscatalogue13>

¹⁶ Guidance on information requirements and chemical safety assessment Chapter R.8. Characterisation of dose [concentration]-response for human health 2. edition. Helsinki: European Chemicals Agency (ECHA) 2012. Table R. 8-3 Allometric scaling factors for different species as compared to humans (https://echa.europa.eu/documents/10162/17224/information_requirements_r8_en.pdf/e153243a-03f0-44c5-8808-88af66223258)

Table 2. PoDs and corresponding unbound plasma concentrations of SCs used as pesticides

Chemical Name	Duration, Route, Species, Strain	LOAEL (mg/kg bw/d)	Allometric LOAEL (mg/kg bw/d)	Concentration unbound plasma (µM)*	Target Organ	Descriptions of the main effects at LOAEL	References
Difenoconazole	91, feed, mouse, Crl:CD-1	34.2	4.88	0.06	liver	Liver: rel. weight ↑	Cox, R (1987), Difenoconazole DAR (2009)
Cyproconazole	91, feed, rat, Wistar	23.8	5.95	5.02	liver	Body weight gain ↓ Liver: abs. and rel weight ↑, lobular patternization ↑	Skinner, C (1986) Cyproconazole RAR (2006)
Epoxiconazole	91, feed, mouse, B6C3F1	19	2.71	0.50	liver	Liver: hypertrophy ↑, abs. weight ↑	BASF RegDoc 91/10855, Epoxiconazole RAR (2019)
Fenbuconazole	91, feed, rat, Crl:CD BR	25.3	6.32	0.24	liver	Liver: vacuolization ↑, hypertrophy ↑, rel. weight ↑	Bernacki, H., Hazelton, G. (1988) Fenbuconazole DAR (2005)
Propiconazole	91, feed, mouse, Crl:CD-1 (ICR)BR	71	10.14	0.47	liver	Liver: hypertrophy ↑, rel. weight ↑	Potrepka, R., Turnier, J. (1991) Propiconazole dRAR (2017)
Prothioconazole	91, gavage, mouse, CD-1	100	14.29	0.54	liver	Liver: cytoplasmic granular structure ↑, lobular pattern ↑, fatty degeneration ↑, hypertrophy ↑	Wirmitzer, U. (1999) Prothioconazole DAR (2004) Prothioconazole RAR (2018)
Tebuconazole	91, feed, rat, Wistar	34.8	8.7	1.38	liver, adrenal glands	Adrenal gland: vacuolization ↑ Liver: weight ↑	Bomhard, E.; Schilde, B. (1986) Tebuconazole DAR (2007)
1,2,4-triazole	Multi-generation, feed, rat, Wistar	30.9	7.7	260.74	sperm	F0 & F1 Sperm: morphology changes ↑	JMPR (2008), Difenoconazole RAR (2019)

Note: Associated adverse effects observed at LOAELs are also indicated.

* Predicted using human PBK models at allometrically-scaled LOAELs. See Section 8.4 for more information

8.2. High-throughput assays

For the *in vitro* assessments, all data, including cytotoxicity across various cell types, were generated within the project. The compounds were tested for a range of concentrations in CALUX®, ToxProfiler, CPP, transcriptomics in PHH, H295R steroidogenesis and DNT-specific UKN2, UKN4 and UKN5 assays. The lowest effect causing free media concentrations (LEC) in these assays is shown in Table 3 and Table 5. This chapter contains the main summary of the results from the high-throughput assays used in the analysis. More detailed results are presented in the **Annex III Section 5**.

8.2.1 CALUX® assay

The CALUX® assay panel consisting of 23 reporter gene assays was utilized to observe the specific interaction between the compounds and specific nuclear receptors alongside cellular signaling pathways (van der Burg, 2013). Conazole compounds along with the positive and negative controls were analyzed using the CALUX® assays, which represent the cytotoxicity, AP1, androgen receptor (AR), estrogen receptor (ER), estrogen response element (ESRE), glucocorticoid receptor (GR), nuclear factor erythroid 2-related factor 2 (NRF2), P21, progesterone receptor (PR), peroxisome proliferator-activated receptors (PPARs), pregnane X receptor (PXR), retinoic acid receptor (RAR), and thyroid receptor beta (TRb). The tested conazole compounds were observed to be active in the AR antagonist (AR-) assay with various potencies. Additionally, itraconazole was observed to be active in PR-. Itraconazole, ketoconazole and prothioconazole showed response to TRb-, which were not observed in other conazole compounds. The metabolites hydroxyitraconazole and 1,2,4-triazole were not active in the assays related to nuclear receptors. In summary, the TC showed similar biological activity to SCs cyproconazole, epoxiconazole, propiconazole, fenbuconazole and tebuconazole in terms of shared actives, but showed dissimilar biological activity to SCs itraconazole, ketoconazole and prothioconazole (Table 3).

Table 3. Lowest effective concentration (LEC) of the conazole compounds tested in CALUX® assays.

Compound	Cytotox	AP1	AR+	AR-	ER+	ER-	ESRE	GR+	GR-	NRF2	P21	PR+	PR-	PPARα+	PPARα-	PPARγ+	PPARγ-	PXR	RAR	TRb+	TRb-
Difenoconazole	3.148			0.099					0.005				0.164		0.249	0.069		0.576	0.256		
Cyproconazole	76.91			2.472					2.338				4.988						8.308		
Epoxiconazole				0.084					0.004				0.512					5.369	1.256		
Fenbuconazole				1.015									0.051						3.041		
Hydroxyitraconazole	0.461																				
Itraconazole				0.051					0.003						0.053			0.071			0.016
Ketoconazole				0.143					0.022	0.693			0.119					3.414			0.103
Propiconazole				0.063					0.146				0.094					0.553			
Prothioconazole	52.04	282.3		6.472	8.563		256.4			234			3.284		9.744		9.732	18.3	5.348		31.73
Tebuconazole				0.064						2.66			1.276					0.432			
1,2,4-triazole																					
Rotenone	0.131			6E-05		129			3E-05				1E-04		0.033			0.018			0.029
Tunicamycin	33			0.007		0.047	0.003		0.021				0.004		0.302			0.321			0.016
2-Phenylphenol			0.083		16.54				1.526		0.001					0.017	0.003		58.35	10.72	17.49

Agonistic assay

Antagonistic assay

Cell viability assay

Concentration

highest

lowest

Note: LEC represents the estimated free media concentration (µM) corresponding to the point of departure for each assay.

8.2.2 ToxProfiler assay

The ToxProfiler assays consist of seven panels of stable HepG2 BAC GFP reporter cell lines to test the activation of various chemically induced stress pathways. The assays cover oxidative stress (SRXN1-GFP), cell cycle stress (p21-GFP), endoplasmic reticulum stress (CHOP-GFP), autophagy (LC3-GFP), ion stress (MT1X-GFP), protein stress (HSPA1B-GFP), and inflammation (ICAM1-GFP) (Ter Braak et al., 2024).

Most of the tested compounds showed stress responses in cell cycle stress p21-GFP assays with different potency ranges. The glucose and galactose ratio and their impact on cytotoxicity give indication to the mitochondrial toxicity and autophagy. Here, most of the conazole compounds, except hydroxyitraconazole, itraconazole, the metabolite 1,2,4-triazole and tunicamycin showed indication of mitochondrial toxicity. In general, consistent with the patterns observed in the CALUX® assays (Table 3), the TC, difenoconazole, exhibited biological responses showing similar hits to the SCs propiconazole and epoxiconazole, and moderately similar to cyproconazole and tebuconazole (Table 4).

Table 4. Lowest effect concentration (LEC) of the conazole compounds tested in ToxProfiler assays

Compounds	Oxidative stress	ER stress	Cell cycle stress p21-GFP	Ion stress	Protein Stress	Autophagy	Inflammation	Cytotoxicity	Cytotoxicity
	SRXN1-GFP	CHOP-GFP		MT1X-GFP	HSPA1B-GFP	LC3-GFP	ICAM1-GFP	Glucose	Galactose
Difenoconazole	0.63	0.99	0.56			4.15		2.82	0.63
Cyproconazole	83.86	180.99	163.52	189.38		83.86			169.11
Epoxiconazole	32.97	32.97	23.00			32.97		96.22	38.34
Fenbuconazole		29.94	17.47	29.94				63.88	29.94
Hydroxyitraconazole			0.05			0.02			
Itraconazole			0.10			0.02			
Ketoconazole	0.57	0.82	0.57	5.43		0.19		5.43	1.90
Propiconazole	9.55	7.16	8.35			33.65		30.07	14.32
Prothioconazole	76.86	107.25	61.67	107.25	107.25	263.66		205.57	107.25
Tebuconazole	14.86	27.49	12.38			25.26			26.01
1,2,4-triazole									
Rotenone		0.02	0.04					0.11	0.02
Tunicamycin	2	0.4				4			
2-Phenylphenol	39.09	109.09	84.09					110.91	107.27

Note: LEC represents the estimated free media concentration (μM) corresponding to the point of departure for each assay.

8.2.3 Cell Painting Plus (CPP) assay

CPP integrates results from several cellular components, such as DNA, RNA, lysosomes, actin, the Golgi apparatus, mitochondria, and the endoplasmic reticulum (ER) and general cell morphology (von Coburg et al., 2025). Similar as CALUX® and ToxProfiler results, the metabolite 1,2,4-triazole was not active in CPP assays. Itraconazole, difenoconazole and ketoconazole were more potent than other conazole compounds, while cyproconazole was least potent of all the conazoles (Table 5). All the conazoles showed relatively stronger effects on mitochondria, indicating the mode of liver toxicity by this class of compounds. This differs from the Nrf2-CALUX® and ToxProfiler oxidative stress responses corresponding to mitochondrial effect, which may be attributed to the distinct biological targets and mechanisms assessed by each assay type. The overall potency of TC (showed as low median BMC) is high in the CPP assay.

Table 5. Lowest effective concentration (LEC) of the conazole compounds in CPP assay

Compound	Actin	DNA	ER	Generic	Golgi	Lyso	Mito	RNA
Difenoconazole	0.50	0.20	0.51	0.32	0.41	0.29	0.33	0.37
Cyproconazole	76.51	52.07	81.11	64.74	135.31	109.62	6.13	119.21
Epoxiconazole	30.60	13.84	19.83	9.11	21.42	28.92	5.31	16.77
Fenbuconazole	13.45	14.02	15.16	10.75	13.40	15.56	1.72	19.32
Hydroxyitraconazole	0.26	0.13	0.26	0.18	0.04	0.04	0.20	0.29
Itraconazole	0.43	0.35	0.83	0.55	0.30	0.32	1.06	0.35
Ketoconazole	1.76	1.32	1.68	0.33	0.51	0.50	0.02	1.32
Propiconazole	10.53	7.02	14.38	6.29	10.28	15.87	0.70	12.28
Prothioconazole	29.49	34.22	34.73	34.07	21.12	34.67	6.14	34.33
Tebuconazole	19.84	22.19	17.23	12.98	14.46	21.12	8.88	21.66
1,2,4-triazole								
Rotenone	0.00058	0.00058	0.00058	0.00058	0.00058	0.00208	0.00058	0.00058
Tunicamycin	4.88	3.92	1.37	0.61	1.21	1.32	0.7	1.22
2-Phenylphenol	31.87	39.17	37.48	37.89	41.13	42.68	25.43	40.59

Note: LEC is the free media concentration (μM) corresponding to the median BMC of organelles for the conazole compounds tested in the CPP assays

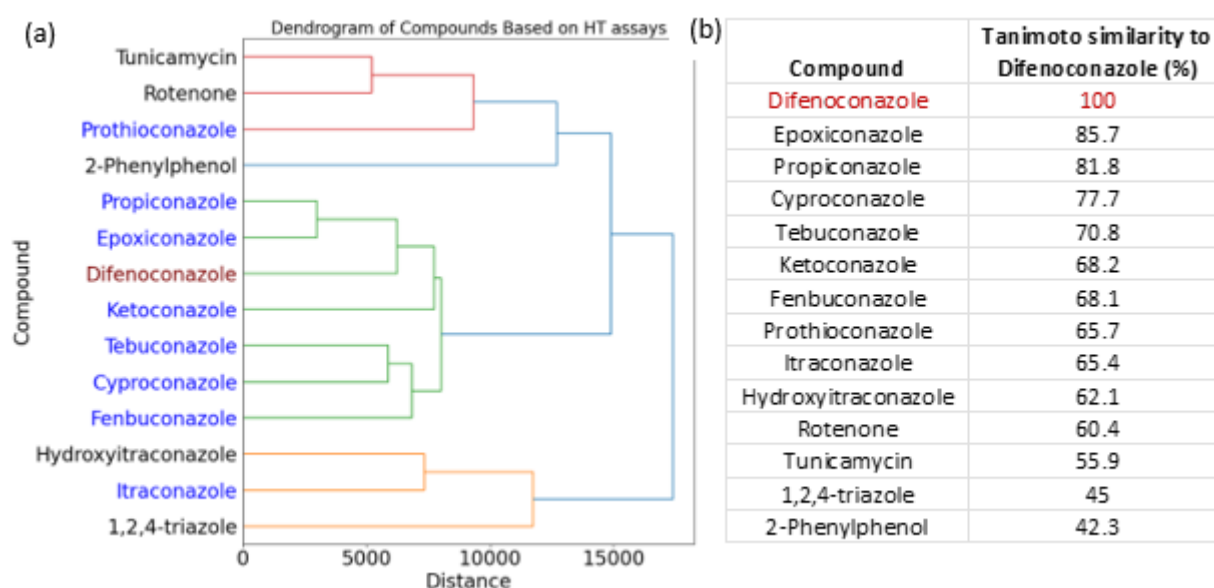
8.2.4 Combined analysis of CALUX®, ToxProfiler and CPP assays

The response observed in high-throughput assays CALUX®, ToxProfiler and CPP were integrated, and the clustering analysis was performed using the LEC (Table 3-Table 5) to identify relationships between

effect profiles and compound potency. Hierarchical clustering was performed using Ward's method and visualized as dendrogram (Figure 7a). The clustering showed the grouping between the TC, difenoconazole, together with the subcluster propiconazole and epoxiconazole. Additionally, the similarity calculation according to Tanimoto method was conducted using the LEC as input. Epoxiconazole, followed by propiconazole and cyproconazole were revealed as the top three of the most similar substances (Figure 7b).

Overall, all conazole compounds including the TC difenoconazole, alongside the SCs, propiconazole, epoxiconazole, ketoconazole, tebuconazole, cyproconazole, and fenbuconazole are clustered separately from the metabolites, the controls and prothioconazole. Prothioconazole has a structural feature of a triazolinethione ring, distinct from the other conazoles. In the results from high-throughput assays (Table 3-Table 5), prothioconazole consistently showed lower potency in comparison to other conazoles. In summary, the subcluster formed by propiconazole, epoxiconazole, and difenoconazole in the structural similarity analysis (Figure 3) was largely preserved in the clustering based on high-throughput assay data (Figure 7a).

Figure 7. (a) Hierarchical clustering and (b) Tanimoto similarity of SCs, metabolites, positive controls and negative controls from the TC (difenoconazole) assessed by high throughput assays based on the LEC



8.3. Additional NAM assays

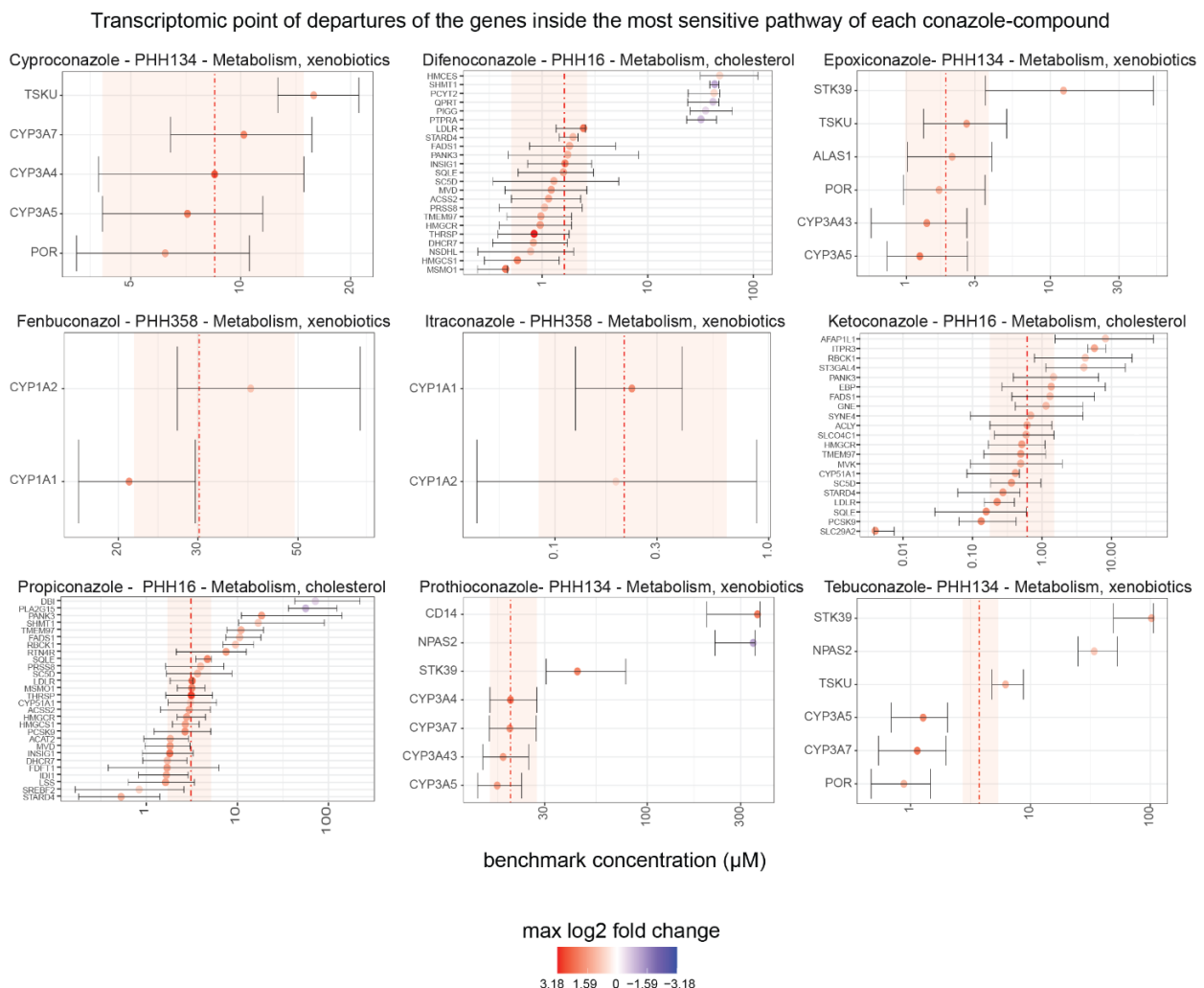
8.3.1 PHH transcriptomics

The effects of conazoles on PHHs were studied using transcriptomic endpoints, with a subsequent assessment of the biological effects based on the enrichment analysis of the PHH TXG-MAPr pathway based on the results from transcriptomic data (Callegaro et al., 2021). By employing this method, co-regulated genes, known as modules, were detected. Each module is evaluated based on the log₂ fold change values associated with them. The main analysis findings comprised aggregated differentially

expressed genes (DEGs), counts of both upregulated and downregulated genes, AUC calculations for module scores, and BMC values for modules that showed significant responses (**Annex III Section 6**). Overall, the most sensitive pathway of the TC difenoconazole was cholesterol metabolism. This pathway was also shared with the ketoconazole and propiconazole (Figure 8). While for other conazoles, e.g. cyproconazole, epoxiconazole, and tebuconazole, the xenobiotics metabolism was the most sensitive pathway.

Based on these findings, the transcriptomics analysis revealed response similarities among conazoles. Our results indicated that conazoles activate shared pathways, such as cholesterol and xenobiotic metabolism, though their effectiveness in perturbing related genes varies. Combining transcriptomics endpoints with a gene network-based pathway enrichment approach can identify common pathways among conazoles, emphasizing the similarities between SCs and TC-difenoconazole, thus supporting the read-across concept.

Figure 8. The transcriptomics point of departure for every gene in the most sensitive module for each conazole compounds



Note: The dots indicate the BMC values with the bars showing the range between upper and lower limits. The color indicates the maximum log2 fold for the genes.

8.3.2 H295R steroidogenesis assay

The H295R steroidogenesis assay (OECD TG456) is an *in vitro* test system designed to investigate effect of chemicals on the biosynthesis of steroid hormones. This assay uses the NCI-H295R human adenocarcinoma cell line, which maintains the necessary enzymatic components for synthesizing major steroid hormones, including 17 β -estradiol and testosterone. This case study assessed the endocrine-disrupting potential of conazoles and their metabolites hydroxyitraconazole and 1,2,4-triazoles. The findings revealed that all active compounds exerted a downregulatory effect on androgen and estrogen pathways. Notably, difenoconazole and 1,2,4-triazole did not show any androgen-disrupting activity, while prothioconazole and 1,2,4-triazole did not exhibit any estrogen-disrupting activity (Table 6).

Table 6. LEC of the conazole compounds tested in the H295R steroidogenesis assays

Compound	Highest non-cytotoxic concentration in H295R (μ M)	Androgens			Estrogens		
		LEC (μ M)	$\nearrow/\searrow$ hormone levels	max fold (average 2)	LEC (μ M)	$\nearrow/\searrow$ hormone levels	max fold (average 2)
Difenoconazole	3.33	-	$\searrow$	0.75	0.24	$\searrow$	0.65
Cyproconazole	33.3	30.11	$\searrow$	0.07	1.65	$\searrow$	0.22
Epoxiconazole	33.3	1.31	$\searrow$	0.13	2.38	$\searrow$	0.65
Fenbuconazole	10	1.48	$\searrow$	0.15	1.48	$\searrow$	0.58
Hydroxyitraconazole	1	0.06	$\searrow$	0.17	0.06	$\searrow$	0.63
Itraconazole	10	0.06	$\searrow$	0.28	0.56	$\searrow$	0.44
Ketoconazole	3.33	0.12	$\searrow$	0.14	0.12	$\searrow$	0.15
Propiconazole	10	5.60	$\searrow$	0.31	1.02	$\searrow$	0.27
Prothioconazole	33.3	9.72	-	0.63	-	-	0.86
Tebuconazole	10	1.90	$\searrow$	0.08	1.81	$\searrow$	0.24
1,2,4-triazole	100	-	-	0.92	-	-	0.92



Note: LEC were estimated free media concentrations (μ M) from the lowest observed effect concentration (LOEC). The LOECs were defined as the lowest tested concentration at which hormone levels increased to ≥ 1.50 fold or decreased to ≤ 0.66 fold relative to the solvent control, which are also the interpretation criteria set in OECD TG456 (OECD, 2025). The direction of change (up $\nearrow$ /down $\searrow$) is indicated in the hormone level trend column. For conazoles that did not meet the LOEC criteria but exhibited a consistent upward or downward trend exceeding thresholds used in earlier TG456 guideline versions (≥ 1.20 fold increase or ≤ 0.66 fold decrease), the trend in hormone level variation is also reported.

8.3.3 Developmental Neurotoxicity (DNT) assays

This case study utilized three assays from the Developmental Neurotoxicity (DNT) test battery, consisting of UKN2, UKN4 and UKN5. These were used to evaluate the effects of the conazole compounds on vital neurodevelopmental processes. Together, these assays provide information on the potential developmental neurotoxicity of the tested compounds by targeting the key cellular events. UKN2 (the cMINC Neural Crest Cell Migration Assay) assesses the impact of compounds on the migration and cytotoxicity of neural crest cells, crucial for the development of the peripheral tissues. UKN4 (NeuriTox Neurite Outgrowth of CNS Neurons Test) evaluates the neurite outgrowth in central nervous system neurons using human mesencephalic cells (LUHMES). UKN5 (PeriTox Neurite Outgrowth of PNS Neurons Test) investigates the neurite outgrowth in peripheral neurons with focus on peripheral nervous system development. For all the assays, the ratio between the target assays and cytotoxicity is the defining criteria for determining the specific hits (DNT positive).

For the DNT assays, prothioconazole and itraconazole which impaired the neurite outgrowth in the peripheral nervous system development with itraconazole being more potent than prothioconazole (Table 7). It is noteworthy that the PoDs derived from DNT assays are higher than that observed for LEC from CALUX® assays.

Table 7. LEC of the conazole compounds tested for the developmental neurotoxicity (DNT) using A) UKN2, B) UKN4 and C) UKN5 assays

A) UKN2 assays			
UKN2 assays	Total	Biokinetics	Free
Compounds	BMC25 (M) (μM)	Ratio (Nominal/Free media)	BMC25 (LEC) (M) (μM)
Difenoconazole	6.8	1.85	3.67
Fenbuconazole	11.5	1.08	10.67
Tebuconazole	57.5	1.22	47.04
B) UKN4 assays			
UKN4 assays	Total	Biokinetics	Free
Compounds	BMC25(NA) (μM)	Ratio (Nominal/Free media)	BMC25 (LEC) (NA) (μM)
Itraconazole	1	19.9	0.05
Ketoconazole	55	1.99	27.58
C) UKN5 assays			
UKN5 assays	Total	Biokinetics	Free
Compounds	BMC25(NA) (μM)	Ratio (Nominal/Free media)	BMC25 (LEC) (NA) (μM)
Itraconazole	1.4	21.67	0.06
Prothioconazole	16	1.01	15.78

Note: The nominal BMC25 for DNT assays converted to free media concentrations using biokinetics analysis were considered as the LEC for a measurable effect. Abbreviations: NA: Neurite outgrowth, M: Migration.

8.4. Kinetics assessment

For the kinetic assessment, established sources of calculated physicochemical properties and available ADME data were used. This included information from PubChem, ChEMBL (Zdravil et al., 2024), the CompTox Chemistry Dashboard (Williams et al., 2017), and the EPI Suite database (EPA., 2025). All the information sources used in the case study have been described in the **Annex I Section 5.3.** and **Annex III Section 9.** Briefly, permeability data in Caco-2 cells were generated, and where unavailable, experimental values for fraction unbound in plasma and intrinsic clearance in PHH were obtained as part of the project. Clearance in primary enterocytes was evaluated; however, none of the conazoles exhibited measurable clearance in enterocytes. These parameters were used to build PBK models in Simcyp V24 (Jamei et al., 2009). Multiple dose simulations were run to predict maximal total and unbound plasma concentrations at a steady state. Simulations for all the conazoles were run in healthy volunteers in Simcyp

V24 for repeat dosing every 24 hours for 15 days at a dose of 10 mg/kg bw/day (**Annex III, Figure A3.18. to Figure A3.27.**). The predicted total plasma $C_{\max}$ at steady state for the TC was within 3-fold of the predicted total plasma $C_{\max}$ at steady state of SCs itraconazole, propiconazole, fenbuconazole, tebuconazole and ketoconazole, while it was higher than 3-fold for SCs epoxiconazole, cyproconazole and prothioconazole. However, the unbound $C_{\max}$ for prothioconazole was ~3-fold of the TC due to low fraction unbound value (Table 8). These differences in predicted unbound $C_{\max}$ were considered when comparing relative toxicity of the SCs and carrying out reverse dosimetry and comparing NAM-based PODs into human oral equivalent (hOED) dose. The TC had both predicted total and unbound plasma $C_{\max}$ within 4-fold for propiconazole and fenbuconazole. It is noteworthy that the SC propiconazole clustered with the TC difenoconazole in structural-based clustering (Figure 3) high-throughput *in vitro* assay based clustering (Figure 7a) and AUC-correlation based clustering in PHH transcriptomics analysis as well (**Annex III, Figure A3.8.**).

A fully validated minimal PBK model of itraconazole-hydroxyitraconazole capsule incorporating enzyme kinetics, is available in Simcyp. This model was used to predict itraconazole and hydroxyitraconazole concentration-time profile. Details of all individual methods and sources are provided in the **Annex I Section 5.3**.

Table 8. The total and unbound maximal plasma concentration (μM) at steady state for all SCs and TC used in the case study

Compound	Dose (mg/kg bw/day)	Plasma $C_{\max}$ (μM)	Unbound plasma $C_{\max}$ (μM)	Allometric LOAELs (mg/kg bw/day)	Unbound plasma $C_{\max}$ at allometric LOAEL (μM)
Difenoconazole	10	12.65	0.12	NA*	NA*
Cyproconazole	10	78.81	8.44	5.95	5.02
Epoxiconazole	10	62.30	1.83	2.71	0.50
Fenbuconazole	10	20.00	0.37	6.32	0.24
Itraconazole	10	8.30	0.13	2.50	0.01
Ketoconazole	10	29.33	0.50	2.50	0.12
Propiconazole	10	9.47	0.47	10.14	0.47
Prothioconazole	10	114.02	0.38	14.29	0.54
Tebuconazole	10	23.05	1.59	8.70	1.38

Note: A red/yellow/green color scale has been applied to indicate plasma concentrations at toxic doses, with red representing the lowest concentrations, yellow indicating intermediate levels, and green the highest.

Note: * *Difenoconazole is the TC so it is assumed that in vivo LOAEL data is not available for this compound.*

9

Data Evaluation

In the *in silico* assessments, the outcomes are only considered when the compounds are within the applicability domain (**Annex III Section 3, Figure A3.3., Table A3.2.**). In the *in vitro* analysis, appropriate controls and replicates are included to reduce uncertainty in the assessment. The details of these are provided in **Annex I Section 3 and 5**, and where available in ToxTemp format ^{17,18,19}. The interindividual variability in internal exposure is accounted by including multiple healthy individuals in PBK modelling. Further, sensitivity analysis was carried out to assess the uncertainty due to compound-specific input parameters in the model outcome (**Annex III Figure A3.28**).

¹⁷ [Annex I CALUX method.pdf](#)

¹⁸ [Annex I Toxprofiler method.pdf](#)

¹⁹ [Annex I CPP method.xlsx](#)

10 Data integration and interpretation

All the outcomes from *in vitro* assays (CALUX®, ToxProfiler, CPP, transcriptomics in PHHs H295R steroidogenesis and DNT assays) were considered to derive toxicologically relevant PoDs. The final PoD for each compound was the lowest free (unbound) media concentrations, associated with biological activity across all *in vitro* assays. This value represents a conservative estimate of the lowest concentrations eliciting effects (Table 9). It is important to note that individual *in vitro* assays frequently measure multiple features—such as distinct transcriptomic modules or morphological profiles—each relevant to the compound's biological activity. A PoD was derived for each of these features, and a probabilistic approach was then employed to characterize the uncertainty across all features, thereby determining an overall PoD for the assay that systematically captures the most sensitive feature.

The LC-MS analysis for metabolite detection showed that the conazoles form hydroxy metabolites after incubation in PHH and can also form it in the liver (**Appendix III**). These metabolites could not be quantified due to the absence of reference standards of hydroxy metabolites of each conazoles. The high-throughput *in vitro* data, transcriptomics in PHHs and existing data indicate that hydroxy itraconazole has similar toxicity as the parent compound (**Tables 3-7, Figure 7a, Annex III Figure A3.7.**). The high throughput assays and plated PHHs have limited metabolic capacity and may overpredict the toxicity of the parent compound. Therefore, we assume that PoDs predicted by the NAMs in this case study capture toxicity of both parent and hydroxy metabolite of the conazoles.

Another metabolite of concern is 1,2,4-triazole, which is known to be associated with craniofacial alterations in prenatal developmental toxicity studies (European Food Safety et al., 2022). Given the high toxicity associated with this metabolite, its potential contribution to the overall risk posed by parent conazoles is of particular concern. Therefore, as part of the risk assessment for conazoles, we also aimed to evaluate the potential toxicity arising from the formation of 1,2,4-triazole. No toxicity of 1,2,4-triazole was observed in any of the *in vitro* assay used in this case study. 1,2,4-triazole showed some effect in PHH transcriptomics assay but did not pass the filtering criteria. The PBK modelling of 1,2,4-triazole at 4 mg/kg dose only with renal excretion accounted by mechanistic kidney (MechKim) model in Simcyp (Neuhoff et al., 2013) shows that 1,2,4-triazole will be completely excreted from the body within 24 hours (**Figure A3.29. in Annex III**).

Evidence for the *in vivo* formation of 1,2,4-triazole from conazoles is limited, and the quantity formed, if any, has not been well characterized. 1,2,4-triazole could not be detected in LC-MS analysis of supernatant of conazoles incubated with PHH. To predict how much 1,2,4-triazole can theoretically be formed from parent conazoles, simulations were carried out, assuming 100% conversion of the parent conazole to its metabolite incorporated using human liver microsome based clearance predicted from ADMET predictor. The acceptable daily intake (ADI) of each conazoles set by EFSA were used as the dose for PBK modelling of conazoles. This is a highly conservative estimate as:

- (a) Only renal excretion of 1,2,4-triazole is considered
- (b) 100 % conversion of conazole to 1,2,4-triazole is assumed

- (c) ADI of conazoles is considered as the input dose. In real situation, the intake of conazole will most likely be lower than ADI.

The analysis showed that even with this very conservative estimate, the unbound concentration corresponding to allometrically-scaled *in vivo* LOAEL²⁰ was predicted as 6000 times higher than the highest concentration of 1,2,4-triazole that can be formed by these set of conazoles (**Figure A3.30. in Annex III**).

Therefore, it was assumed that the NAM-based assessment of parent compound toxicity captured the metabolites as well. For all the SCs and TC, the lowest effective concentration was consistently observed in the CALUX® assays. These values were considered representative of the unbound concentrations potentially associated with *in vivo* toxicity and were designated as NAM-derived LOAELs. These concentrations were even lower than the lowest effect concentrations for H295R and DNT assays for all the compounds (Table 9).

Table 9. Comparison of the LECs associated with biological activity across all *in vitro* assays for TC and SCs

Compounds	CALUX®		Toxprofiller		CPP		Transcriptomics		H295R assay		DNT assays	
	LEC	Biology	LEC	Biology	LEC	Biology	LEC	Biology	LEC	Biology*	LEC	Biology
Difenoconazole	0.005	GR	0.563	Cell cycle stress	0.202	DNA	1.630	Metabolism, cholesterol	0.24	Estrogen ↘	3.67	UKN2
Cyproconazole	2.338	GR	83.857	Oxidative Stress	6.132	Mito-chondria	8.477	Metabolism, xenobiotic	1.65	Estrogen ↘	NA	Negative
Epoxiconazole	0.004	GR	23.001	Cell cycle stress	5.307	Mito-chondria	1.883	Metabolism, xenobiotic	1.31	Androgen ↘	NA	Negative
Fenbuconazole	0.051	PR	17.467	Cell cycle stress	1.715	Mito-chondria	30.216	Metabolism, xenobiotic	1.48	Androgen ↘ Estrogen ↘	10.67	UKN2
Itraconazole	0.003	GR	0.019	Auto-phagy	0.299	Golgi	0.211	Metabolism, xenobiotic	0.06	Androgen ↘	0.05	UKN4
Ketoconazole	0.022	GR	0.190	Auto-phagy	0.023	Mito-chondria	0.612	Metabolism, cholesterol	0.12	Androgen ↘ Estrogen ↘	27.58	UKN4
Propiconazole	0.063	AR	7.159	ER stress	0.701	Mito-chondria	3.089	Metabolism, cholesterol	1.02	Estrogen ↘	NA	Negative
Prothioconazole	3.284	PR	61.670	Cell cycle stress	6.137	Mito-chondria	20.105	Metabolism, xenobiotic	9.72	Androgen ↘	15.78	UKN5
Tebuconazole	0.064	AR	12.385	Cell cycle stress	8.884	Mito-chondria	3.734	Metabolism, xenobiotic	1.81	Estrogen ↘	47.04	UKN2

Note: The lowest of LEC (µM) across all *in vitro* assays were consistently observed in the CALUX® assays.

Note: ↘ indicates decrease of hormone concentration

Further, reverse dosimetry using the PBK model was carried out to predict an external dose, NAM-derived LOAEL, that will lead to unbound concentration in plasma in humans as the final PoD from the *in vitro* assessment (Table 10).

²⁰ HED Records Center Series 361 Science Reviews - File R124628, US EPA, 2006
https://www3.epa.gov/pesticides/chem_search/cleared_reviews/csr_PC-600011_7-Feb-06_a.pdf

11 Measures of Reliability

The same positive and negative control chemicals were included in high throughput assays to ensure the toxicity of the compounds is correctly captured in the assay. These have been described in **Annex I**. Along with this, assay specific controls were also included to confirm assay performance and reliability; these are documented in the ToxTemp report corresponding to each assay. The known biology of conazoles (Vlasveld et al., 2024) including effect on mitochondria, activation of stress response pathways and steroid pathways was captured in the *in vitro* assays (Table 9). These have been described in detail in **Annex III Section 5-8**.

In the similarity analysis supporting the reliability of the read-across approach, the structural correlation between the TC and the SCs was broadly reflected in the *in vitro* assay results as well. For example, the TC difenoconazole was clustered with the SC propiconazole and epoxiconazole in structure-based clustering (**Figure 3**) as well as *in vitro* assay-based clustering (**Figure 7a**). This convergence across multiple data streams strengthens the justification for read-across, as it suggests that the TC and SCs not only share key structural features—potentially indicative of similar modes of action—but also elicit comparable biological responses in high-throughput screening assays. The alignment between structural similarity and functional bioactivity enhances confidence that the toxicological properties of the SC can be used to predict those of the TC with a reasonable degree of certainty. The Tanimoto similarity based on the high-throughput assays revealed that epoxiconazole was 85.7% and propiconazole 81.8% similar to difenoconazole (**Figure 7b**).

The reliability of the *in vitro* data was assessed by comparing results across biological replicates to evaluate assay stability. For this purpose, biological replicates were tested simultaneously, each incorporating repeated measures. A statistical approach was implemented to quantitatively characterize the variability across these biological replicates, providing a robust measure of data consistency. A detailed discussion of the methodology and the results of this statistical analysis are provided in **Annex I** and **III**, respectively.

For the kinetics assessment of the compounds, published ADME parameters were supplemented with the *in vitro* data generated in the project. The fraction unbound of conazoles is less than 0.10 which could be difficult to accurately measure in the *in vitro* assays. The sensitivity analysis was carried out to see the effect of fraction unbound in plasma on the C_{max} of the TC, difenoconazole. The sensitivity analysis showed that the C_{max} is highly sensitive to fraction unbound in the region lower than 0.01 but is less sensitive to values between 0.01-0.10 (**Annex III Figure A3.28**). Note, the experimentally determined fraction unbound in plasma for all conazole compounds, except prothioconazole, was either 0.01 or more than 0.01 (**Annex I Table A1.6 – Table A1.15**). This indicates that, for most conazoles, small changes in fraction unbound in plasma above this threshold have a relatively minor impact on C_{max} predicted using PBK modelling.

We compared the C_{max} of ketoconazole predicted from PBK modelling with that observed for human for similar oral dose. The observed C_{max} of ketoconazole at an oral dose of 200 mg/day is observed to be 2 µg/ml (Van Tyle, 1984) while the PBK model developed using the parameters outlined in this case study predicted it as 2.13 µg/ml thus increasing our confidence in the model output. Further, another case study carried out in the RISK-HUNT3R project using 15 compounds showed that systemic clearance was either underpredicted (n=8) or was in the range (n=7) when scaled from clearance measured in primary human hepatocytes using PBK models developed using similar methods as in this case study (data not published).

Thus, even if the IVIVE may not be 100% accurate, the underprediction of clearance will only shift prediction of $C_{\max}$ and AUC on to the conservative side in the risk assessment of the chemicals.

12 Expert judgement + alternative interpretations

Assuming the unbound concentration in plasma will result in systemic toxicity, reverse dosimetry was used to derive hOED for all compounds for minimum LEC from all assays which is LEC in CALUX® assay (Table 9). This was compared to the *in vivo* LOAELs from rats and mice 90 days studies, which then allometrically scaled to human dose (mg/kg bw/day) for all the SCs. The lowest allometrically scaled *in vivo* LOAELs from each SC were selected as representative for *in vivo* LOAELs. The NAM-based LOAELs were either lower or similar to the *in vivo* LOAELs for all compounds except prothioconazole (Table 10). The predicted NAM-based LOAEL of prothioconazole was 6-times higher than allometrically scaled LOAEL. It is also to be noted, that the *in vivo* LOAEL for the prothioconazole was allometrically scaled from the mice study, which showed effects on liver at study LOAEL (allom. LOAEL: 14.29 mg/kg bw/d). In rat studies, the lowest dose causing effects were seen at much higher dose (allom. LOAEL: 125 mg/kg bw/d) with notably kidney as one of the prominent target organs in rats (**Annex II Table A2.4.**). Further, prothioconazole is structurally different from the TC and other SCs, containing a triazolinethione moiety instead of the standard triazole ring. It is also known that prothioconazole metabolizes into prothioconazole-desthio, a more toxic metabolite. However, it remains unclear whether this metabolic conversion occurred in the cellular systems used in this study, as it was not specifically characterized. Interestingly, prothioconazole also exhibited the highest LOAEL among the SCs in both NAM-based and *in vivo* assessments (Table 10).

Table 10. Comparison of NAM derived LOAEL with allometrically scaled *in vivo* LOAEL

Compound	Minimum LEC from all <i>in vitro</i> assays (µM)	Unbound C _{max} at 10 mg/kg/day (µM)	NAM-derived LOAELs (mg/kg bw/day)	<i>In vivo</i> allometric LOAELs (mg/kg bw/day)
Difenoconazole	0.005	0.12	0.43	4.88
Cyproconazole	2.338	8.44	2.77	5.95
Epoxiconazole	0.004	1.83	0.02	2.71
Fenbuconazole	0.051	0.37	1.37	6.32
Itraconazole	0.003	0.13	0.21	2.5
Ketoconazole	0.022	0.5	0.44	2.5
Propiconazole	0.063	0.47	1.33	10.14
Prothioconazole	3.284	0.38	86.41	14.29
Tebuconazole	0.064	1.59	0.4	8.7

Note: The TC difenoconazole is marked in bold. A green–yellow–red colour scale highlights LOAEL values, where red indicates lower LOAELs and therefore higher potency.

The comparison between NAM-derived and *in vivo* LOAELs for the SCs supports confidence in the read-across approach. For most SCs, the NAM-derived LOAELs were lower than their respective *in vivo* LOAELs, indicating a conservative nature of the NAM-based assessments (Table 10).

A two-step approach was used for read-across analysis. In first step, hierarchical clustering of SCs and TCs based on structural information was employed as a screening tool to identify and prioritize potential read-across candidates for the TC, difenoconazole. This approach serves primarily as a preliminary screening method to highlight substances which require further consideration. Propiconazole and epoxiconazole were shown to be structurally similar to difenoconazole based on the MACCS fingerprints and Tanimoto distance (Figure 3). However, structural similarity alone is not sufficient to conclude on the read-across candidate (N. Ball et al., 2016; Escher et al., 2019). A next step of integrating SC evaluation is essential to identify (dis)similarity, therefore refining the candidate selection. In this study, we integrated NAM data to evaluate the mechanistic aspect of the compounds and support read-across as a second step. The clustering based on the high-throughput NAM results refined the initial hypothesis by providing more information that the SCs propiconazole and epoxiconazole are not only structurally but also mechanistically similar to the TC (Figure 7a and b). The next step, which is data-gap filling (here LOAEL of TC) can be conducted using regular pattern, nearest neighbour or worst-case approach (RAAF, 2017, Escher et al., 2019). In this case study, we considered both the nearest neighbour and the worst-case approach to derive the LOAEL of the TC. These are discussed below:

- (a) The LOAEL value for TC was predicted using a *k*-nearest neighbour (*k*-NN) model based on a Tanimoto similarity matrix derived from high-throughput assay response profiles. We used three compounds with the highest similarity scores and available LOAEL values, which included epoxiconazole, propiconazole and cyproconazole (Figure 7a). The predicted LOAEL was calculated as a similarity-weighted average of the neighbours' LOAEL values (**Equation 2**):

$$LOAEL_{pred} = (\sum(S_i * LOAEL_i)) / (\sum(S_i)) \quad \text{Equation 2}$$

where S_i represents the Tanimoto similarity between the TC and neighbour i , calculated from the high-throughput assay response profiles, and $LOAEL_i$ is the allometrically scaled LOAEL for neighbour i (Table 10). The allometric LOAEL from this approach was predicted to be **6.21 mg/kg/day** which is 1.3 times of the actual allometric LOAEL for difenoconazole (4.88 mg/kg bw/day) (Table 2).

- (b) An alternative interpretation would be to use a worst-case approach to predict the LOAEL of TC. Both structural similarity and high-throughput NAM assessments indicated that propiconazole and epoxiconazole were the closest analogues to the TC. However, as epoxiconazole exhibited lower NAM-derived and *in vivo* LOAEL of the two SCs, the *in vivo* LOAEL for TC difenoconazole was inferred using epoxiconazole. Consequently, using this approach, an allometric LOAEL of **2.71 mg/kg bw/day** was established for the TC difenoconazole, matching that of epoxiconazole. When compared with the actual *in vivo* allometric LOAEL for difenoconazole (4.88 mg/kg bw/day) (Table 2), the predicted value of 2.71 mg/kg/day is lower but still within a two-fold range, supporting the conservative yet reliable nature of the read-across estimate.

Another alternative and scientifically justified approach would be to adopt the NAM-derived LOAEL for the TC as the final PoD. Despite the qualitative alignment of NAM-derived LOAELs among the SCs, no consistent fold-difference was observed when comparing these values with their respective *in vivo* allometric LOAELs. Epoxiconazole had the lowest NAM-derived LOAEL, consistent with its low *in vivo* LOAEL, alongside itraconazole and ketoconazole. However, the NAM-based LOAEL for epoxiconazole was 100 times lower than the *in vivo* value, reinforcing the conservative tendency of NAMs. Similarly, for propiconazole—clustered with the target compound (TC) across all assessments—the NAM-derived LOAEL was 10 times lower than the *in vivo* LOAEL. Additionally, the PBK model built using *in vitro* clearance data tends to underestimate systemic clearance (Jones et al., 2022). This limitation introduces uncertainty in the reverse dosimetry process, which may partly explain the lower LOAEL values derived from NAMs compared to those observed *in vivo*. Given this pattern, it is reasonable to expect that the NAM-derived LOAEL for the TC would also reflect a conservative estimate. In this case, the NAM-derived

LOAEL is 0.43 mg/kg bw/day (Table 10), which is 10-times lower than the *in vivo* allometric LOAEL of 4.88 mg/kg bw/day (Table 2).

The NAM-derived LOAELs were generally conservative and did not show a consistent fold difference relative to the corresponding allometrically scaled *in vivo* LOAELs for the SCs. Therefore, the use of a read-across approach based on NAM-derived similarity represents a pragmatic strategy for estimating missing toxicity value. The analysis presented above supports this conclusion, as both read-across approaches applied in this study predicted the LOAEL for TC within a two-fold range of its experimentally derived, allometrically scaled LOAEL. This level of agreement indicates that similarity in NAM bioactivity profiles can provide a reasonable basis for estimating *in vivo* toxicity thresholds within this chemical space.

13 Overall uncertainty

The overall uncertainty associated with this read-across assessment is considered low, justified by the following five reasons. First, the assessment integrates data from both the TC and SCs, and the toxicological relevance between them has been validated through a comprehensive read-across similarity analysis and is supported by historical evidence (Table 2). Second, multiple NAMs covering a broader biological space were utilized in a high-throughput format, and these assays have consistently and reliably captured the known biological activity of interest induced by both the TC and SCs. Therefore, the results from this battery of NAMs provide strong support for predicting systemic toxicity, and the inclusion of more specialized assays is not deemed necessary for this endpoint. Third, for all NAM assays included in this assessment, assay reliability was quantitatively characterized through two key measures: the consistent inclusion of negative and positive controls, and a rigorous analysis of variability across biological replicates. Fourth, the LOAELs derived from the NAM data in this assessment are lower than corresponding *in vivo* LOAELs (Table 10), providing robust, conservative estimates that enhance the protection of public health. Fifth, the confidence in these estimates is high because the PoDs were derived using an established benchmark dose (BMD) modeling methodology, which incorporated an advanced statistical approach to characterize uncertainty (see **Annex I** for details).

14 Evaluations and conclusions

The IATA framework applied in this case study demonstrates that a NAM-based approach can support read-across for assessing the systemic toxicity of the TC and yield accurate and scientifically robust outcomes. Specifically, as the LOAEL derived from NAM data demonstrates a conservative estimate when compared to the LOAEL obtained from traditional *in vivo* subchronic studies, a read-across assessment using similarity derived from NAM-data presents a pragmatic approach. This underscores the reliability of NAMs in capturing biologically relevant effects, while being conservative and realistic in the risk assessment process. However, the level of confidence in such estimates depends on the relevance, coverage, and biological domain represented by the NAMs used in the analysis. Importantly, the use of structurally similar SCs and mechanistically relevant NAMs enhances both the precision and contextual relevance of the assessment, particularly when integrated into high-throughput screening frameworks. This approach strengthens confidence in non-animal methodologies as viable alternatives for regulatory decision-making. Moreover, the assessment showcases the practical implementation of the ASPA workflow, illustrating how structured integration of structural, toxicokinetic, and toxicodynamic data from NAMs can support regulatory decision-making in a transparent and reliable manner.

15 Learnings and Lessons

The OECD IATA Case Studies illustrate the application of New Approach Methods (NAMs), including Integrated Approaches for Testing and Assessment (IATAs), in specific regulatory contexts. To support knowledge-sharing and reuse of experience across jurisdictions, the OECD IATA Case Study Project includes structured expert review and discussion of each submission.

Following discussion at the OECD IATA Case Study Project meeting in November 2025, it was agreed that key learnings and lessons arising from the review of each case study would be documented directly within the individual case study reports. This approach aims to enhance transparency, usability, and practical relevance, and to facilitate understanding of strengths, uncertainties, and considerations relevant to regulatory application.

The learnings from this case study, particularly regarding considerations for selecting source (SCs) and assessing the validity of the read-across approach, are described below.

The outcome and reliability of a read-across assessment are fundamentally dependent on the choice of source compounds (SCs) used to predict properties or hazards of a target compound (TC). Consequently, the identification and justification of appropriate SCs constitute one of the most critical steps in any read-across strategy. Poorly selected SCs can undermine the scientific credibility of the assessment, whereas a well-reasoned selection enhances confidence in the predicted outcomes.

Broadly, two overarching considerations govern the identification of suitable SCs: (i) the similarity between the SCs and the TC with regard to endpoint relevant properties, and (ii) the availability, relevance, and quality of endpoint data within the SCs for which a read-across is required.

Assessment of Similarity

Similarity assessment is central to read-across and should be evaluated in a multifaceted manner. While structural similarity is typically the starting point, existing regulatory and scientific guidance emphasizes that simple numerical similarity metrics, such as Tanimoto coefficients or Dice similarity scores, should not be applied in isolation. These metrics, although useful for initial SC identification and filtering, do not adequately capture the induction of toxicological effects which are based on toxicodynamic and toxicokinetic properties.

Several guidance documents have been developed to carry out read-across (ECETOC, 2012²¹; ECHA, 2008²²; OECD, 2014²³) or to evaluate read-across in systematic manner (ECHA, 2017²⁴). Additional relevant data should be carefully considered for a robust assessment. The current EFSA guidance on read-across outlines how different types of data can be combined to support read-across steps (EFSA Scientific Committee, 2025²⁵), which includes in vitro assays, in silico data and metabolite information. Also, the most recent guidance on Grouping of Chemicals from OECD 418²⁶ incorporated New Approach Methodologies (NAMs) including high-throughput assays, (Q)SAR models, omics and AOP to support the similarity assessment and read-across rationale (OECD, 2025).

A robust similarity assessment should integrate multiple dimensions, such as:

- **Chemical structure and functional groups**, focusing on reactive moieties, substituents, and structural alerts relevant to toxicity.
- **Physicochemical properties**, such as molecular weight, lipophilicity (e.g., logP), ionization state (pKa), solubility, and volatility, which influence absorption, distribution, and exposure within the body.
- **Chemical–biological interactions**, including binding affinities, reactivity with biological macromolecules, and interaction with transporters or enzymes.
- **Metabolism**, including identification of (shared) metabolites and proposed metabolic pathways.
- **Toxicity**, such as shared toxicological properties of SCs in the endpoint studies, or a consistent trend. This analysis might reveal similar most critical target organs or even adverse effects, at comparable lowest observed adverse effect levels. The analysis of shared toxicological properties is often difficult due to confounding factors like testing of different species, strains, routes in studies which differ with regard to dose selection and dose spacing.

Although the underlying principle of read-across is that structurally similar compounds are more likely to elicit similar toxicological effects, this assumption must be substantiated by additional lines of evidence. In particular, compounds with subtle structural differences may diverge in activity if those differences substantially affect reactivity, metabolism, or target interaction.

Role of Mechanistic Information and Mode of Action (MoA)

Beyond structural considerations, mechanistic similarities, especially similarity in mode of action (MoA) or adverse outcome pathways (AOPs), provides a strong scientific foundation for read-across. Compounds that perturb the same biological targets, operate through the same key events, or trigger similar

²¹ ECETOC (2012). Category approaches, Read-across, (Q)SAR. Technical report (TR) No. 116. <https://www.ecetoc.org/wp-content/uploads/2014/08/ECETOC-TR-116-Category-approaches-Read-across-QSAR.pdf>

²² ECHA (2008). Guidance on information requirements and chemical safety assessment. Chapter R.6: QSARs and grouping of chemicals. https://www.echa.europa.eu/documents/10162/17224/information_requirements_r6_en.pdf/77f49f81-b76d-40ab-8513-4f3a533b6ac9

²³ OECD (2014). *Guidance on Grouping of Chemicals*. OECD Series on Testing and Assessment, No. 80. OECD Publishing, Paris. doi: <https://doi.org/10.1787/9789264085831-en>

²⁴ ECHA (2017). Read-Across Assessment Framework (RAAF). doi: <https://doi.org/10.2823/619212>

²⁵ EFSA, Bennekou, S. H., Allende, A. et al. (2025). Guidance on the use of read-across for chemical safety assessment in food and feed. EFSA J 23, e9586. doi: <https://doi.org/10.2903/j.efsa.2025.9586>

²⁶ OECD (2025), *Guidance on Grouping of Chemicals, Third Edition*, OECD Series on Testing and Assessment, No. 418, OECD Publishing, Paris, <https://doi.org/10.1787/b254a158-en>

downstream toxicological pathways may be considered similar even when structural similarity is moderate rather than high.

In this context, combining structural similarity with mechanistic evidence often offers a more defensible and transparent read-across rationale than reliance on either alone. Mechanistic understanding helps to explain why similarity is expected to translate into comparable biological effects, thereby strengthening confidence in the read-across prediction.

In addition to any preexisting data available, NAMs can be an alternative to strengthen read-across rationale, with many implemented specifically to address the MoA identification. The inclusion of NAMs to inform the mechanistic understanding has also been discussed in some reports in the last years (ECHA, 2022²⁷; ECHA 2023²⁸).

Consideration of Metabolism and Biotransformation

Metabolism represents a particularly important, and often decisive, dimension of similarity. Biotransformation can either decrease or increase toxicity, and structurally similar parent compounds may behave very differently if they give rise to distinct metabolites.

The identification of common metabolites between the SCs and the TC represents an overlap between chemical and biological similarity. This is especially relevant when metabolites increase or cause additional toxicity than the parent compound.

Key considerations when using metabolic similarity include:

- Focusing on Phase I metabolites (e.g., oxidation, reduction, hydrolysis), reactive intermediates and similar known metabolites as these are more likely to be toxicologically relevant than late-stage metabolites.
- Identifying active or toxic metabolites, under the assumption that shared metabolite profiles may result in similar toxicological outcomes.
- Ensuring that metabolism data are derived from relevant species, particularly those used in toxicological studies or are of human relevance.

Data Availability and Practical Constraints

In addition to scientific similarity, practical considerations related to data availability strongly influence SC selection. Read-across is inherently data-driven; therefore, SCs must have sufficiently robust and relevant datasets to support hazard or risk prediction.

When a large number of potential SCs are identified, a pragmatic refinement step is often necessary. This may involve prioritizing or retaining only those compounds for which:

- High-quality toxicity data are available for relevant endpoints;
- Metabolic information exists, preferably in common species;
- Exposure, toxicokinetic, or mechanistic data are sufficient to support biological plausibility.

Such prioritization helps streamline the assessment while maintaining scientific rigor, ensuring that the final set of SCs is both biologically relevant and evidence-rich.

Overall Weight-of-Evidence Consideration

²⁷ ECHA (2022). Minutes of the 67th Meeting of the Management Board (mb/m/34/2022 final).

²⁸ ECHA (2023a). The use of alternatives to testing on animals for the REACH Regulation. Fifth report under Article 117(3) of the REACH Regulation. doi:10.2823/805454

Ultimately, the validity of a read-across approach depends on a transparent and coherent weight-of-evidence evaluation that integrates structural, physicochemical, metabolic, mechanistic, and toxicological information. The selection of SCs should be clearly justified, and uncertainties—such as gaps in metabolism data or mechanistic understanding—should be explicitly acknowledged and, where possible, addressed.

By adopting a systematic and multifactorial approach to SC selection, the read-across assessment can achieve greater robustness, credibility, and regulatory acceptance.

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Annex I. Reporting Individual Information Sources

1. Name of the information source

This case study utilized high-throughput and medium-throughput NAMs, including:

1.1. Caco-2 permeability assays

The method assesses the transepithelial permeability over a filter-grown monolayer of Caco-2 cells, a cell line that has been shown to correspond closely to the *in vivo* human small intestinal epithelium. The method is considered a gold standard in the pharmaceutical industry.

1.2. Chemically Activated Luciferase eXpression (CALUX®) Reporter Gene Assays

The CALUX® assay panel consists of 23 reporter gene assays and aimed to evaluate the interaction between test compounds with specific nuclear receptors and cellular signaling pathways (van der Burg, 2013). The assays utilized the reporter cell lines, modified to express luciferase. This method allows to measure receptor activation or inhibition through luminescence. In this case study on conazoles, various test compounds were evaluated using the CALUX® assays, which cover a wide range of targets, including cytotoxicity, AP1, androgen receptor (AR), estrogen receptor (ER), estrogen response element (ESRE), glucocorticoid receptor (GR), nuclear factor erythroid 2-related factor 2 (NRF2), P21, progesterone receptor (PR), peroxisome proliferator-activated receptors (PPARs), pregnane X receptor (PXR), retinoic acid receptor (RAR), and thyroid receptor beta (TRb).

1.3. Cell Painting PLUS (CPP) Assays

Cell Painting PLUS (CPP) is a high-throughput phenotypic profiling (HTPP) method that labels nine different cellular compartments, including lysosomes (Lyso), actin cytoskeleton (Actin), cytoplasmic, plasma membrane, nucleolar ribonucleic acid (RNA), mitochondria (Mito), nucleus (DNA), Golgi apparatus (Golgi) and the endoplasmic reticulum (ER), using seven fluorescent dyes. The resulting images are then analyzed to extract information about the phenotypical changes occurring after substance exposure (E. von Coburg et al., 2025). The analysis focuses on key features like morphology, symmetry, compactness, distribution, intensity, texture, and nucleoli number following substances exposure.

1.4. Chemical effect predictor (CEP)

The Chemical Effect Predictor (CEP), a machine learning model powered by data extracted from a Knowledge Graph (KG) for characterizing hazards caused by drugs/chemicals in humans.

1.5. Danish (Q)SAR Database²⁹

The Danish (Q)SAR Database is a freely available online repository of *in silico* predictions for more than 650,000 substances from more than 200 (Q)SAR freely available, commercial and DTU-developed models. The (Q)SAR models include endpoints for physico-chemical properties, environmental fate, bioaccumulation,

²⁹ Danish (Q)SAR Database, Research Group for Chemical Risk Assessment and GMO, National Food Institute, Technical University of Denmark, <http://qsar.food.dtu.dk>.

eco-toxicity, absorption, metabolism, endocrine / molecular activity and toxicity. The DTU models are developed in the following software Leadscope Predictive Data Miner from Leadscope Inc., CASE Ultra from MultiCASE Inc. and SciQSAR from Lhasa LLC. In many cases, the same training set was modelled in all three systems. Predictions are in these cases included from all three models, and a battery call is made based on the three predictions, requiring at least two of the three predictions to agree and be within the defined applicability domain to receive a battery call in domain. Most of the models used for the Danish (Q)SAR Database are documented in QMRFs (QSAR Model Reporting Format), available in the system. For individual substances, (Q)SAR predictions can be downloaded in a report (RTF format) compatible with Microsoft Word and OpenOffice. The Danish (Q)SAR Models is a part of the Danish (Q)SAR Database website in which user-defined chemical structures can be predicted in real-time in Leadscope versions of DTU-developed models and more detailed predictions retrieved in the (Q)SAR Prediction Reporting Format (QPRF).

1.6. Enterocyte metabolism

This method uses primary human jejunal enterocytes isolated from tissue samples donated from patients undergoing gastric bypass surgery. The cells are used in suspension to study the intrinsic clearance (CL_{int}) of a compound. Intrinsic clearance reflects the inherent ability of enterocyte enzymes to metabolize a chemical, independent of blood flow or other systemic factors. This value can be extrapolated (*in vitro* to *in vivo* extrapolation) to predict intestinal clearance *in vivo* using biokinetic models.

1.7. Intrinsic metabolic clearance assays and *in vitro* metabolite profiling

This assay uses cryopreserved primary human hepatocytes (liver parenchymal cells) isolated from patient donors in suspension or plated format to study the intrinsic clearance (CL_{int}) of a compound. Intrinsic clearance reflects the inherent ability of liver enzymes to metabolize a drug, independent of blood flow or other systemic factors. This value can be extrapolated (*in vitro* to *in vivo* extrapolation) to predict hepatic clearance *in vivo* using biokinetic models.

1.8. PBK and *in vitro* biokinetics modelling

PBK modelling uses physicochemical (PC) and absorption, distribution, metabolism and excretion (ADME) parameters of a compound to predict the internal concentration of a compound in the body followed by an external dose. Certara's Simcyp simulator was used for PBK modelling and workflow similar to OECD guidance document for physiologically based kinetics (PBK) modelling (OECD 2021) was followed³⁰. The biokinetics modelling was carried out using virtual *in vitro* distribution (VIVD) (Fisher, Siméon, Jamei, Gardner, & Bois, 2019) from Certara to predict ratio of nominal to free media concentration in *in vitro* assays. It uses PC properties of the compounds to predict compound lost by binding to the plastic and partitioning into air, cells and other media components in the *in vitro* assays.

1.9. ToxProfiler Stress Reporter Assays

The ToxProfiler assay, developed by Toxys B.V., features a panel of seven stable HepG2 BAC GFP reporter cell lines designed to investigate the activation of various chemically induced stress pathways. These pathways include oxidative stress (SRXN1-GFP), cell cycle stress (p21-GFP), endoplasmic reticulum stress (CHOP-GFP), autophagy (LC3-GFP), ion stress (MT1X-GFP), protein stress (HSPA1B-GFP), and inflammation (ICAM1-GFP) (Ter Braak et al., 2024). In addition to assessing cellular stress, mitochondrial toxicity is evaluated using the glucose/galactose medium switch principle (Ter Braak et al., 2024).

³⁰ OECD (2021), *Guidance document on the characterisation, validation and reporting of Physiologically Based Kinetic (PBK) models for regulatory purposes*, OECD Series on Testing and Assessment, No. 331, OECD Publishing, Paris, <https://doi.org/10.1787/d0de241f-en>.

1.10. Whole genome targeted RNA sequencing-TempO-Seq® in PHH and PHH TXG-MAPr pathway enrichment analysis

Whole genome targeted RNA sequencing using TempO-Seq is a high-throughput platform that assesses around 16,000 genes with approximately 20,000 probes. The sequencing measurements were obtained from cryoconserved 10 donor pools of primary human hepatocytes (PHH). This sequencing technology relies on the specific annealing of the specific probes to each targeted gene measuring the number copies of these genes and expressing the values as the count values. Gene expression is quantified as count values to derive (log₂) fold change values to control in treated primary human hepatocytes (PHH) after 24-hour exposure. Additionally, the PHH-TXG-MAPr pathway enrichment platform was utilized to explore cellular responses using log₂ fold change results from transcriptomic data (Callegaro et al., 2021). Key analysis results included aggregated differentially expressed genes (DEGs), counts of upregulated and downregulated genes, AUC derivation for module scores, and BMC values for significant responding modules.

1.11. H295R steroidogenesis assay

The H295R steroidogenesis assay (OECD TG456)³¹ is a mechanistic *in vitro* test system designed to evaluate the effect of chemicals on steroid hormone biosynthesis. It utilises the human adenocarcinoma cell line NCI-H295R, which retains the enzymatic machinery necessary for the production of main steroid hormones, including 17 β -estradiol and testosterone. Upon exposure of this cell line to a test chemical, the effect on steroidogenesis can be assessed by quantifying the amount of 17β-estradiol (E2) and testosterone (T) produced by the cells and comparing this to cells exposed to vehicle control only. At BDS, the quantification of E2 and T is performed using the ERα CALUX® and AR CALUX® bioassays, respectively (Nikopaschou et al., 2023). The goal of the assay according to the OECD guideline is to provide a YES/NO answer with regard to the potential of a chemical to induce or inhibit the production of T and E2; however, it is also possible to report quantitative results by determining a lowest observed effect concentration (LOEC).

1.12. DNT assays

In this case study, three assays from the Developmental Neurotoxicity (DNT) test battery (Blum et al., 2023)—UKN2, UKN4, and UKN5 were used to assess the effects of conazole compounds and five additional control compounds. Each assay targets a critical neurodevelopmental process:

UKN2 – The cMINC Neural Crest Cell Migration Assay (UKN2 Test): Assesses the impact of compounds on the migration of neural crest cells, which are essential for the development of various peripheral tissues and structures.

UKN4 – NeuroTox Neurite Outgrowth of CNS Neurons Test (UKN4 Test): Uses LUHMES cells (a human-derived CNS neuron model) to evaluate compound effects on neurite outgrowth in central nervous system neurons.

UKN5 – PeriTox Neurite Outgrowth of PNS Neurons Test: Investigates how compounds influence neurite outgrowth in peripheral neurons, focusing on peripheral nervous system development.

These assays collectively provide insight into the potential developmental neurotoxicity of the tested substances by targeting key cellular events involved in neurodevelopment.

1.13. Whole genome targeted RNA sequencing-TempO-Seq® in RPTEC/TERT1

Whole-transcriptome targeted RNA sequencing using the TempO-Seq® platform was employed to assess gene expression changes in human renal proximal tubular epithelial cells (RPTEC/TERT1). The TempO-Seq

³¹ OECD (2025), *Test No. 456: H295R Steroidogenesis Assay*, OECD Guidelines for the Testing of Chemicals, Section 4, OECD Publishing, Paris, <https://doi.org/10.1787/9789264122642-en>.

assay measures approximately 19,000 genes using target-specific probe pairs. Sequencing data were obtained from confluent RPTEC/TERT1 cultures maintained under serum-free conditions and exposed for 24 hours to sets of conazole compounds.

This sequencing technology relies on the specific annealing of probes to targeted transcripts, enabling precise quantification of transcript abundance as digital count values. Gene expression data were normalized, and differential expression analysis was performed using DESeq2, with results expressed as $\log_2$ fold change values relative to vehicle controls (DMSO or medium-only). The resulting differential expression profiles were used to identify significantly affected pathways associated with nephrotoxicity, such as oxidative stress, apoptosis, and mitochondrial dysfunction. Tunicamycin and rotenone served as mechanistic control compounds to confirm expected stress-response activation in RPTEC/TERT1 cells.

1.14. Uncertainty analysis

The uncertainty analysis in this assessment uses elements from the established uncertainty characterization framework within the ASPA workflow (RISKHUNT3R, 2025a, 2025b), which aims to systematically consider the combined impact of sources of uncertainty, with options to reduce and refine the characterisation of overall uncertainty.

2. Relevance of the information source

The *in vitro* NAM test systems, along with *in silico* methods, were selected for this case study to encompass a wide range of biological processes.

Reporter genes and general cellular processes were included into the testing to cover a broad mechanistic space. The CALUX reporter panel tested the activation of PPAR, PXR, AhR, GR and LXR. Oxidative stress was tested using the Nrf2-CALUX reporter assays. Endoplasmic reticulum (ER) stress was measured using ESRE-CALUX reporter assays. The nuclear hormone receptor assays from CALUX like ER α , Anti-ER α , AR, anti-AR, PR, Anti-PR, Anti-GR, TR β , Anti-TR β are related to endocrine activity; RAR, and the stress- and other pathway assays Hif1 α to hypoxia; TCF to the wnt-pathway; AP-1 to cell cycle control, NF κ B to inflammatory response and p21 and p53 to DNA damage responses (van der Burg, 2013).

ToxProfiler is a human *in vitro* assay that combines a panel of stable fluorescent reporter cell lines. It utilizes live cell confocal imaging to quantify eight toxicological relevant endpoints: oxidative stress (SRXN1-GFP), ER stress (CHOP-GFP), cell cycle stress (p21-GFP), ion stress (MT1X-GFP), protein stress (HSPA1B-GFP), autophagy (LC3-GFP), inflammation (ICAM1-GFP) and mitochondrial toxicity (glu/gal medium switch). It has been recently shown that concentration response modeling and point-of-departure estimation for each reporter protein allowed for chemical potency ranking and revealed the primary toxicological MoA of chemicals. Furthermore, the assay could effectively group chemicals based on their shared toxicity signatures and link them to specific toxicological targets, e.g. mitochondrial toxicity and genotoxicity, and different human pathologies, including liver toxicity and cardiotoxicity (Ter Braak et al., 2024).

CELL PAINTING PLUS (CPP) uses fluorescent dyes to stain cells and evaluate the phenotypic state of major organelles and components (Seal et al., 2025; E. von Coburg et al., 2025). The seven fluorescent dyes label nine different subcellular compartments and organelles, including the plasma membrane, the actin cytoskeleton, cytoplasmic RNA, nucleoli, lysosomes, nuclear DNA, the endoplasmic reticulum, mitochondria, and the Golgi apparatus. CPP captures around 1,000 morphological features, including size, shape, texture, and intensity. While the morphological alterations are not directly associated with adverse outcomes, they largely reflect the response of cells to chemically induced perturbations.

Whole transcriptome analyses are performed in a dose dependent way in primary human hepatocytes. This cellular model was chosen because of its relatively high metabolic capacity in comparison to other liver models like HepG2 cells or HepaRG cells. 10 donor pools are tested to reduce donor specific variability in

responses. Liver is known to be one of the most frequently observed target organs in preclinical *in vivo* studies (Batke, Aldenberg, Escher, & Mangelsdorf, 2013) and its transcriptomics are used to capture early changes on the gene level. The TXGMAPr is an R-Shiny-based implementation of weighted gene co-expression network analysis (WGCNA) obtained from the Primary Human Hepatocytes (PHH) TG-GATes dataset. Well-known stress response pathways are captured in the modules. Moreover, the transcriptomic responses were also analysed in RPTEC/TERT1 cells. Both PHH and RPTEC/TERT1 cells were compared to assess the effects of conazoles across different cell types. Additionally, as the *in vivo* data of the SCs and *in silico* tools give indication of the potential endocrine disrupting properties of the SCs, H295R steroidogenesis and developmental neurotoxicity (DNT) assays were conducted to capture relevant endocrine related signalling pathways and responses.

It is noted that a direct adversity is not measured in these assays. Furthermore, they typically use a single application of the compounds followed by an exposure period of 24h. The *in vitro* models also do not involve all required cell types to e.g. mimic typical reactions like an inflammatory process or tissue remodeling like fibrosis. The *in vitro* test battery does, however, cover a wide range of early MIEs and KEs, which can be used to infer the adverse outcome.

PBK models assess the level of exposure in humans at doses at which liver toxicity has been reported for the source compounds in humans or animals and compare these with the toxic concentrations in the *in vitro*-assays in RISK-HUNT3R NAM battery. This information is being used within the context of a read across approach to assess the human oral equivalent dose than can lead to liver toxicity effect by difenoconazole based on information for the source compounds and predict LOAEL of difenoconazole. In this context of use, a PBK model is not intended to precisely characterize the PK processes but to represent an interpretation of the available data by addressing the relationships between an external dose and internal tissue, plasma³².

3. Description

A relevant concentration range per assays was determined. Each *in vitro* assay is performed according to standardized protocol. This study used a set of 16 compounds which consisted of: nine conazoles (cyproconazole, difenoconazole, epoxiconazole, fenbuconazole, itraconazole, ketoconazole, propiconazole, prothioconazole, and tebuconazole), two metabolites (1,2,4-triazole and hydroxyitraconazole), four positive controls (Rotenone, Chloroquine bis(phosphate), Tunicamycin, Chlorpyrifos) and one negative control (2-Phenylphenol). All chemicals were purchased at the highest purity available. A target top concentration of 0.1 M was aimed at for the entire compound set, provided that they were soluble in DMSO or water. The stock solutions were aliquoted and stored at -20°C until used. The nominal to free media concentration in *in vitro* assays was carried out using virtual *in vitro* distribution (VIVD) (Fisher et al., 2019) from Certara.

3.1. Caco-2 permeability assays

Caco-2 permeability measurements of conazoles and their metabolites were performed essentially as described (Hubatsch, Ragnarsson, & Artursson, 2007). In addition, bound compound was extracted from the plastic of the donor chamber at the end of the experiment using 60% acetonitrile. At the same time compound accumulated in the Caco-2 cell layer was extracted using 60% acetonitrile. The assay is commonly performed at 1-10 µM concentration to ascertain that the concentration of the compound tested is below the *K_m* of relevant transporters. The method is available in the RISK-HUNT3R methods database in ToxTemp format³³.

³² <https://www.who.int/publications/i/item/9789241500906>

³³ <https://risk-hunt3r.net/test-methods/protocol/217/ UU1a ADME Caco NA 1 • RISK-HUNT3R>

3.2. CALUX® reporter gene assays

The measurement was carried out according to DB-ALM Protocol 197 (Automated CALUX reporter gene assay procedure)³⁴. Tested concentrations are provided in Annex_I_CALUX_concentrations.xlsx. Reporter cells were exposed to the test compounds, previously dissolved in DMSO, for 24h and at 0.1% or 1% (v/v) according to the procedure described in the DB-ALM protocol 197 “Automated CALUX reporter gene assay procedure”. The LEC (lowest effect concentration) were derived per assay and based on the vehicle control responses. For nuclear receptor agonist assays, the LEC was defined as the PC10 concentration in LogM, which is the concentration where the test compound causes an activation effect equal to 10% of the maximum effect elicited by the test's reference compound. For nuclear receptor antagonist assays, the LEC was defined as the PC20 concentration, which is the concentration where the test compound causes an antagonist effect equal to 20% of the maximum antagonist effect elicited by the test's reference compound. For the stress pathway related assays which typically do not show sigmoidal dose-response curves, the LEC was defined as the FI 1.5 concentration, which is the concentration where the test compound elicits pathway activation 1.5-fold above baseline.

3.3. Cell painting assays³⁵

Cell Painting PLUS Cell Culture and Staining

Cells from passages 3-4 were seeded in 40 µl culture medium at a density of 5,500 HepG2 cells/well in 384-well PhenoPlates pre-coated with 0.1 mg/ml collagen type I. Cells were grown for 24 h followed by compound treatment. At this step, 10 µl of exposure medium containing the reference compounds was added to the 40 µl culture medium. Cells were then exposed to test compounds for 24 h with a maximum background DMSO concentration in both compound-treated and solvent control wells of 0.4%. Live-cell staining included aspiration of exposure medium to ~ 15 µl, addition of 15 µl 2X staining solution containing LysoBrite NIR Fluorescence (1:250 in staining solution (buffer A containing dye); 1:500 in well) and MitoTracker Orange CMTMROS (1 µM in staining solution; 0.5 µM in well) (both in LysoBrite NIR Fluorescence buffer B containing 1% BSA; 1:2 in well), and incubation for 30 min at 37 °C and 5% CO₂. Fixation of cells included no aspiration of the staining solution but direct addition of 30 µl 2X fixation solution containing 8% PFA (in PBS; 4% in well), and incubation for 20 min at RT protected from light. Permeabilization and fixed cell staining of cells was performed simultaneously and included the complete aspiration of the fixation solution, washing three times with 100 µl PBS, aspiration to ~ 15 µl, addition of 15 µl 2X permeabilization and fixed cell staining solution containing Triton X-100 (0.2% in staining solution; 0.1% in well), Alexa Fluor Plus 405 Phalloidin (66 nM in staining solution; 33 nM in well), and SYTO 14 Green Fluorescent Nucleic Acid Stain (0.6 µM in staining solution; 0.3 µM in well) (in PBS containing 1% BSA; 0.5% in well), and incubation for 30 min at RT protected from light. Finally, plates were washed three times with 100 µl PBS without final aspiration. After imaging, elution of cycle 1 dyes included washing three times with 100 µl deionized water, aspiration to ~ 15 µl, addition of 40 µl 1.375X elution buffer containing 0.5 M L-Glycine and 1% SDS (pH 2.5) (in deionized water), and incubation for 10 min at RT. The elution buffer was stored at 4 °C and used within one month. Finally, plates were washed three times with 100 µl deionized water and three times with 100 µl PBS without final aspiration. To check for successful elution of cycle 1 dyes, nine random control wells were imaged with identical setting as used in cycle 1. Immediately after elution, the second fixed cell staining included aspiration of PBS to ~ 15 µl, addition of 15 µl 2X fixed cell staining solution containing Hoechst33342 (8 µM in staining solution; 4 µM in well), Wheat Germ Agglutinin Alexa Fluor 488 Conjugate (2.6 µg/ml in staining solution; 1.3 µg/ml in well), and Concanavalin A Alexa Fluor 647 Conjugate (20 µg/ml in staining solution; 10 µg/ml in well)

³⁴

https://jeodpp.jrc.ec.europa.eu/ftp/jrc-opendata/EURL-ECVAM/datasets/DBALM/LATEST/online/DBALM_docs/197_P_Automated%20CALUX.pdf

³⁵ [Annex I CPP method.xlsx](#)

(in PBS containing 1% BSA; 0.5% in well), and incubation for 30 min at RT protected from light. Finally, plates were washed three times with 100 µl PBS without final aspiration.

Cell Painting PLUS Image and Data Analysis

Image analysis was performed on single image planes using the integrated Harmony software (Revvity Inc.) and customized image analysis sequences for CPP. After data cleaning and assignment of wells to treatment conditions, all extracted features of each individual cell were standardized to the median and median absolute deviation (MAD) of the pooled DMSO solvent control cell population from the corresponding plate (24 wells/plate) to derive for each feature a robust z-score per cell. Next, those cell-level feature data were aggregated to the well-level by calculating for each feature the median of cell-level robust z-scores per well. For each feature, Benchmark concentration was calculated using tcplfit2 with five different curve-fitting models ("cnst", "hill", "poly1", "poly2", "pow"). BMR was set as default 1.349*SD (corresponding to approximately 1 interquartile range (IQR) for normal distributed data). Curves with no BMDL/BMDU or hitcall probability ≤ 0.9 were considered as inactive. BMCs higher than maximal tested non-toxic concentration were also defined as inactive. If BMC was smaller than the lowest tested concentration, it was adjusted to a modified lowest concentration as shown previously (Nyffeler et al., 2021) according to equation 1.

$$\log_{10}(BMC^{LOW}) = \log_{10}(\text{lowest tested conc.}) - 0.5 \quad \text{Equation 1}$$

3.4. CEP analysis

described in 5.1.

3.5. Danish (Q)SAR Database and Models websites

described in 5.2.

3.6. Enterocyte metabolism

The method is available in the RISK-HUNT3R methods database as ToxTemp format³⁶. Jejunal samples were obtained from patients undergoing Roux-en-Y gastric bypass surgery at Elisabethssjukhuset, Uppsala, Sweden (ethical permit EPM 2023-01524-01). All donors have given written informed consent. Tissue samples were rinsed with ice cold oxygenated Krebs-Ringer buffer and the mucosa was isolated from underlying muscle and connective tissue layers. Small samples of mucosa were isolated and kept for proteomic analysis and snap frozen in liquid nitrogen. Jejunal samples were kept at -150°Celsius until shipment to SIGNATOPE. In this respect the tissue is coming from obese individuals and so enzyme levels may not reflect those in adults in the normal weight range. However, the subjects present with comparatively mild obesity without metabolic syndrome and type II diabetes. Global proteomic studies by UU (unpublished) found little correlation between expression levels of ADME related proteins in more or less obese subjects indicating that the expression levels may be close to the average human population.

Enterocytes were isolated from jejunal mucosa samples using a gentle non-enzymatic protocol. Mucosal samples were incubated in Ca^{2+} free Krebs-Ringer solution, 30 min on ice followed by 60 min at 37 degrees Celsius with shaking at 300 rpm. Finally, samples were vortexed 10 x 10 s at medium speed to dislodge the epithelial layer. Subepithelial tissue was removed, and the cell suspension strained twice through a cell strainer (100 µm followed by 70 µm). Viable enterocytes were isolated from erythrocytes and dead cells by Percoll gradient centrifugation (performed twice). This yielded a suspension of enterocytes with high viability and yield (> 85% viable cells, > 35 million cells per gram mucosa).

Enterocytes (10^6 cells/ ml) were incubated with conazoles or their metabolites at a concentration of 1 µM in DMEM (21063-029, Gibco) at 37C with shaking. Samples were extracted at time points from 0 to 120 min

³⁶ <https://risk-hunt3r.net/test-methods/protocol/290/> UU2a ADME ENT Intest CLint 1 • RISK-HUNT3R

and immediately quenched with ice cold acetonitrile. Samples were then analyzed by UPLC-MSMS (see below).

UPLC-MSMS analysis

Analysis of conazole concentrations in experimental samples was performed by ultra-performance liquid chromatography (Acquity Waters, Milford, MA USA) coupled with triple quadrupole mass spectrometer Xevo TQ-S micro (Waters, Milford, MA, USA). UPLC-MS/MS methods are presented in **Table A1.1**. Method 1 was used for difenoconazole, itraconazole, tebuconazole, and 1,2,4 - triazole. Method 2 was used for epoxiconazole, fenbuconazole, propiconazole, prothioconazole, cyproconazole, and ketoconazole. Multiple reaction monitoring (MRM) parameters are presented in **Table A1.2**. Warfarin (6 nM) was used as an internal standard. Compound concentrations were calculated using the TargetLynx software (Waters).

Table A1.1. UPLC and MS/MS conditions. ESI = electrospray ionization

Parameter	Method 1	Method 2
Column	Acquity BEH C18 (2.1 x 100 mm, 1.7 µm)	Column: Acquity BEH C18 (2.1 x 50 mm, 1.7 µm)
Mobile phase	A: 0.1 % formic acid in water B: ACN	A: 0.1 % formic acid in water B: CAN
Stepwise gradient	initial 98%A; 1.0 min – 98%A; 2.5 min – 2%A; 3.5 min – 2%A; 4.0 min – 98%A; 5.0 min - 98%A; 6.0 min - 98%A	initial 95%A; 1.0 min – 95%A; 1.5 min – 2%A; 4.0 min – 2%A; 4.3 min – 95%A; 5.0 min - 95%A
Flow	0.4 ml/min	0.4 ml/min
Column temperature	30°C	30°C
Injection volume	1 µl (5 µl for triazole)	1 µl
Ionization	ESI positive mode	ESI positive mode
Capillary voltage	2.5 kV	4.0 kV
ESI source temperature	140°C	140°C
Desolvation gas (N ₂) flow	800 l/h	800 l/h
Desolvation temperature	600°C	600°C

Table A1.2. MRM parameters. MRM = multiple reaction monitoring, IS = internal standard

Compound	MRM transition	Cone voltage (V)	Collision energy (eV)
Cyproconazole	292.20 > 70.00	20	20
	292.20 > 125.00		
Difenoconazole	406.00 > 251.00	20	25
Epoxiconazole	330.10 > 121.00	30	21
Fenbuconazole	337.10 > 125.00	30	30
Hydroxyitraconazole	721.10 > 408.30	20	40
Itraconazole	705.10 > 392.20	20	35
Ketoconazole	531.20 > 489.10	20	30
Propiconazole	342.10 > 69.10	30	20
	342.10 > 159.00		
Prothioconazole	344.00 > 326.00	20	10
Tebuconazole	308.20 > 70.00	20	20
1,2,4-Triazole	69.90 > 43.00	30	15
Rotenone	395.00 > 192.00	20	20
	395.00 > 213.00		
Warfarin IS	309.10 > 163.13 309.10 > 251.20	12	15

Calculations

Cellular accumulation was determined by calculating the K_p using Equation 2.

$$Kp = \frac{C_{cell,60}}{C_{donor,60}} \quad \text{Equation 2}$$

Where $C_{cell, 60}$ is the concentration of compound in the cell layer at $t = 60$ min and $C_{donor, 60}$ is the concentration of compound in the donor chamber at $t = 60$ min.

Mass balance was calculated using Equation 3.

$$\text{Mass balance} = \frac{n_{acc,60} + n_{don,60} + n_{cell,60} + n_{wash,60}}{n_{don,0}} \quad \text{Equation 3}$$

Where $(n_{acc,60} + n_{don,60} + n_{wash,60} + n_{cell,60})$ is the amount of compound in the acceptor chamber, donor chamber, bound to plastic, and in the cells at $t = 60$ min. $n_{don,0}$ is the amount of compound in the donor chamber at $t = 0$ min.

P_{app} was calculated using Equation 4.

$$P_{app} = \frac{dQ}{dt} \frac{1}{A * C_{don,0}} \quad \text{Equation 4}$$

where dQ/dt is the steady state flux, A is the area of the transwell filter, and $C_{don, 0}$ is the concentration of compound in the donor chamber at $t = 0$ min.

3.7. Intrinsic metabolic clearance assays and *in vitro* metabolite profiling

The hepatocytes are exposed to the test chemical by incubation in culture medium, preparing replicate samples to enable the interaction to be terminated after appropriate intervals, e.g., from time zero to two hours (e.g., 0, 15, 30, 60, 90, 120 mins). In the assay a 10 donor pool of human hepatocytes was used (BIOIVT, Lot ZCX). Determination of the supernatant chemical concentration with time allows calculation of any depletion rate occurring due to metabolism and/or other biotransformation processes. Here, UPLC and MS were used to quantify the metabolites.

Table A1.3. UPLC and MS/MS conditions. ESI = electrospray ionization

Parameter	Method 1
Column	Acquity BEH C18 (2.1 x 50 mm, 1.7 µm)
Mobile phase	A: 0.1 % formic acid in water B: CAN
Stepwise gradient	initial 98%A; 3 min – 98%A; 8 min – 0%A; 10 min – 0%A; 10.2 min – 98%A; 12 min - 98%A
Flow	0.4 ml/min
Column temperature	40°C
Injection volume	1 µl
Ionization	ESI positive mode
Capillary voltage	0.7 kV
ESI source temperature	120°C
Desolvation gas (N ₂) flow	800 l/h
Desolvation temperature	400°C

3.8. PBK and biokinetics modelling

described in 5.3.

3.9. ToxProfiler stress reporter assays

The measurement was carried out according to ToxTemp standardized protocol³⁷. Tested concentrations are provided in Annex_I_ToXProfiler_concentrations.xlsx. ToxProfiler reporter cells were seeded in 384-well Phenoplates with 9000 cells/well/40µL DMEM medium. Cells were exposed to seven concentrations of the test compounds for which the DMSO concentrations in the assay medium were kept constant at 0.4% (v/v). Using live cell confocal imaging (Operetta CLS, Perkin Elmer) the reporter responses were quantified with 4 channels (HOECHST; nuclei, GFP; stress response activation, DPC; cellular borders, Propidium Iodide (PI); cytotoxicity). Using segmentation pipelines (Harmony, Revvity) the endogenously regulated reporter responses were quantified in the relevant subcellular localisation with a single cell resolution. Data was plate-wise min-max normalised using reference conditions included on each plate. Concentration response modelling was used to automatically derive a point of departure (PoD). This PoD is the lowest concentration with a significant stress response. A PoD was also derived from the cytotoxicity (PI) concentration response curves. If the ratio of these PoDs in the galactose:glucose conditions was larger than 2 and the ER stress (CHOP-GFP) reporter was significantly activated, the compound was considered a mitochondrial toxicant.

3.10. Whole genome targeted RNA sequencing-Tempo-Seq® in PHH and PHH TXG-MAPr pathway enrichment analysis^{9,10}

PHH culture

PHH (10 donor pool) is thawed from a frozen vial in thawing medium (BioIVT). The cells are suspended and spun down at 1000 rpm for 5 mins. The supernatant is aspirated, and the cells are re-suspended in the plating medium (BioIVT). The cells are plated in the collagen coated 96 well plate (Corning) with the density of 70.000 cells/well. After 6 hours of plating, the medium is replaced with the maintenance medium (BioIVT) containing 5000mg/ml - 5000u/ml PEN-STREP. The exposure is performed 24 hours after thawing. The cells are placed in the incubator at 37°C, 5% CO₂. The protocol is provided in RISKHUNT3R database as ToxTemp format³⁸.

Exposure and sample collection

The medium is refreshed with 50 µL of new maintenance medium. The compounds are prepared reaching 2x higher desired concentration (9 concentration levels) in the maintenance medium. The compounds are added to the plate (50 µL) and the plates are incubated for 24 hours at 37°C, 5% CO₂. The exposure is performed for 3 biological replicates. After 24 hours, the exposure medium is aspirated, and the cells are washed with DPBS 1x. In the meantime, the TempoSeq enhance lysis buffer (ELB) is prepared by diluting the buffer with DPBS 1:1. The 1x ELB buffer is added (50µL) to each well and the plates are incubated at 37°C, 5% CO₂ for 10 minutes. After the incubation, the plates are sealed with aluminum seal and stored at -80°C until further processes (shipment, library preparation, RNA sequencing). The processes for RNA sequencing are performed by BioClavis LTD, United Kingdom.

Differential expressed genes analysis

The count values are retrieved in tabulated format. The total count for each sample (library size) is used to filter the low-quality samples (library size < 1.000.000). The low-expressed probes are excluded using the adjusted R-ODAF criteria (Verheijen et al., 2022) - the probe should have minimum 3 (CPM) normalized value in at least one replicate and this criterion should apply in at least one tested condition. The raw-count values from the probes measuring the same genes are summed. The data were normalized using count per million (CPM) approaches and the samples with low median normalized-count distribution (lower than 10x average median distribution) are excluded. The calculation of the log2 fold change is performed using

³⁷ <https://risk-hunt3r.net/test-methods/protocol/227>

³⁸ <https://risk-hunt3r.net/test-methods/protocol/297/>

DESeq2 function³⁹ by comparing the treated samples with the DMSO samples from the same plates. The function is applied for a subset of the dataset for every compound (with DMSO control). The differentially expressed genes are defined with the condition of adjusted p-value (FDR) < 0.05 and absolute log2 fold change > 0.5.

PHH TXG-MAPr pathway enrichment analysis

The tabulated log2 fold change values are uploaded to the PHH TXG-MAPr. Upon data upload, the platform provides the new eigengene scores of the modules based on the log2 fold change values of the uploaded data. These scores reflect the modulation of the gene networks that are also annotated with cellular responses, biological process, and molecular functions. Using these eigengene scores, we can further evaluate the magnitude of these responses improving the biological relevance and insight of the transcriptomics outcomes.

3.11. H295R steroidogenesis assay

The H295R steroidogenesis assay was conducted based on OECD TG456 (2025)⁴⁰, including the MTT cytotoxicity assay measurement. To determine the effect of the test chemicals on steroidogenesis, H295R cells were seeded in 48 wells plates in assay medium, incubated for 24h and exposed for 48h to a serial dilution of the compound in triplicate (0.1% v/v, for a compound prepared in DMSO) after which the medium was harvested and the MTT cytotoxicity assay performed. To quantify the levels of estrogens and androgens produced by the H295R cells after exposure, the assay medium was diluted and analysed on the ER α and AR CALUX® bioassays as described previously (CALUX® bioassays). Changes in hormone levels compared to a vehicle control (DMSO) exposure indicate that certain enzymes involved in steroidogenesis were being affected by the test compound. To eliminate false positive results in the H295R-CALUX® read-out, (anti-) ER α CALUX®, (anti-) AR CALUX® and Cytotox CALUX® activities were taken into account. This is done to rule out that the activity observed in the H295R-CALUX® read out is caused by carry over of ER α or AR active compounds and not by changes in hormone levels in the H295R cells or compounds cytotoxicity in Cytotox CALUX® cells.

3.12. DNT assays

The cMINC Neural Crest Cell Migration Assay (UKN2 Test)

This *in vitro* test method uses human neural crest cells (NCCs) generated from induced pluripotent stem cells (iPSC). It assesses disturbances of NCC migration during fetal development. The number of migrated NCCs, as well as cell viability are measured simultaneously using high content imaging. Thereby the processes of cell migration and cell death are measured in cells exposed to potential toxicants for 24 h. The data of this method are meant to predict developmental disorders and malformations e.g. neural tube defects or craniofacial malformations caused by compound exposure during fetal development. The method has a well-established prediction model, but it has not undergone formal validation, and it has not been part of a ring trial. It has been used in the screening of several medium-sized compound libraries. According to the readiness criteria as published (Bal-Price et al., 2018) the neural crest cell migration assay obtained the readiness score A-. A detailed description of the assay is available in the ToxTemp format⁴¹ and SOPs are available on DB Alm.

The NeuriTox Neurite Outgrowth of CNS Neurons Test (UKN4 Test)

³⁹ <https://www.rdocumentation.org/packages/DESeq2/versions/1.12.3/topics/DESeq>

⁴⁰ OECD (2025), *Test No. 456: H295R Steroidogenesis Assay*, OECD Guidelines for the Testing of Chemicals, Section 4, OECD Publishing, Paris, <https://doi.org/10.1787/9789264122642-en>.

⁴¹ <https://risk-hunt3r.net/test-methods/protocol/232/>

This *in vitro* test method is based on human neurons (LUHMES cells) at a stage of neurite growth. It assesses (a) disturbances in the development of the nervous system/brain structures, and (b) direct damage to the adult nervous system, by exposure to toxicants. The neurite area (which serves as indirect measurement of neuronal interconnectivity) of stained differentiating neurons as well as cellular viability are measured simultaneously using high content imaging. The processes of neurite outgrowth and cell viability are assessed. The data of this method are meant to predict (a) developmental disorders in children caused by compound exposure during fetal development, and (b) damage to the developing nervous system, in particular to dopaminergic parts of the nervous system. The method has not undergone formal validation and has not been part of a ring trial. It predicts some aspects of neurotoxicity, but not all aspects covered by an *in vivo* neurotoxicity study (TG424). It has been used in the screening of medium-sized compound libraries, has undergone some mechanistic evaluation, and has been linked to AOP id3 in aopwiki.org (parkinsonian motor deficits). According to the readiness criteria as published by (Bal-Price et al., 2018) the NeuroTox assay obtained the readiness score A. A detailed description of the assay is available in the ToxTemp format⁴² and SOPs are available on DB Alm.

The PeriTox Neurite Outgrowth of PNS Neurons Test (UKN5 Test)

This *in vitro* test method is based on human iPSC-derived immature dorsal root ganglia (iDRG) neurons at a stage of neurite growth. It assesses (a) disturbances in the development of the (peripheral) nervous system, and (b) direct damage to the peripheral nervous system, by exposure to toxicants. The neurite area (which serves as indirect measurement of neuronal interconnectivity) of stained differentiating neurons, as well as cellular viability are measured simultaneously using high content imaging. The processes of neurite outgrowth and cell death are measured. The data of this method are meant to predict (a) developmental disorders in children caused by compound exposure during fetal development, and (b) damage to the developed nervous system, in particular to the peripheral nervous system. The method has not undergone formal validation and has not been part of a ring trial. It predicts some aspects of neurotoxicity, but not all aspects covered by an *in vivo* neurotoxicity study (TG426). It has been used in the screening of medium-sized compound libraries, has undergone some mechanistic evaluation, and has been linked to AOP-279 (AOPwiki ID) / ETR09N (EU-ToxRisk AOP task ID) (Peripheral neuropathy caused by microtubule interacting drugs). According to the readiness criteria as published by (Bal-Price et al., 2018) the PeriTox assay obtained the readiness score A-. A detailed description of the assay is available in the ToxTemp format⁴³ and SOPs are available on DB Alm.

3.13. RPTEC/TERT1 culture and sequencing

RPTEC/TERT1 Culture

Human renal proximal tubular epithelial cells (RPTEC/TERT1; Evercyte GmbH, Vienna, Austria) were maintained under serum-free conditions in DMEM/F12 medium supplemented with defined growth factors at 36.5 °C and 5 % CO₂. Cells were seeded in 96-well plates (Corning) and cultured until confluence was reached. Confluent monolayers were maintained for at least five days to ensure contact inhibition and differentiation prior to exposure. All experimental conditions were performed in three biological replicates. The detailed RPTEC/TERT1 culture and maintenance procedure is described in (Aschauer et al., 2015).

Exposure and Sample Collection

Cells were exposed for 24 hours to the test compounds (conazoles and control substances) in a 96-well plate format. Final DMSO concentrations ranged between 0.1 % and 0.4 %. Positive control compounds included tunicamycin (7.5 µM) and rotenone (0.025 µM), representing ER stress and mitochondrial stress, respectively. Following exposure, the medium was aspirated, and cells were washed once with 1× DPBS. The TempO-

⁴² <https://risk-hunt3r.net/test-methods/protocol/235/>

⁴³ <https://risk-hunt3r.net/test-methods/protocol/237/>

Seq® Enhance Lysis Buffer (ELB) was diluted 1:1 in DPBS, and 50 µL of the resulting 1× ELB was added per well. Plates were incubated for 10 minutes at 37 °C and 5 % CO₂, sealed with aluminium film, and stored at –80 °C until shipment. Library preparation and TempO-Seq® sequencing were performed by BioClavis Ltd. (Glasgow, United Kingdom) following the manufacturer's standard protocol.

Sequencing and Data Processing

The TempO-Seq® assay enables whole-transcriptome targeted RNA sequencing, quantifying approximately 19,000 genes. Sequencing was performed by BioClavis Ltd. (Glasgow, United Kingdom). This technology relies on the hybridisation of oligonucleotide probe pairs to RNA molecules, followed by ligation of correctly matched probes, amplification, and sequencing, resulting in digital gene expression counts per well.

Raw count values were provided in tabulated format following sequencing. Gene expression quantification was based on read counts corresponding to each targeted transcript. Normalisation, differential gene expression analysis, and pathway enrichment analysis were performed as described in Section 3.10.

Quality Control and Acceptance Criteria

Assay acceptance was determined based on expected transcriptomic responses from mechanistic control treatments. Tunicamycin induced canonical unfolded protein response (UPR) genes (e.g., HSPA5, ATF4, DDIT3), while rotenone upregulated oxidative stress-related markers (HMOX1, ATF3, DDIT4). A ≥ 2-fold induction relative to vehicle controls confirmed assay performance.

Biological replicate correlations were evaluated, and outlier samples were excluded from further analysis. Vehicle controls (e.g., 0.1 % DMSO) were verified to show no pathway activation beyond the historical range observed in medium and vehicle-only controls.

3.14. Uncertainty analysis

The uncertainty analysis is performed by identifying sources of uncertainty and evaluating the combined impact of these sources on the overall uncertainty in the answer to the assessment question. This is done by breaking the assessment into parts. Strengths and weaknesses with inputs and methodologies are evaluated for each information source. A description of the parts is provided below, with detailed results available in Annex III.

Selection of the tested compounds: Uncertainty was qualitatively evaluated through a similarity analysis between the target and source compounds, supported by a systematic review of historical evidence (see section 10 & 11 and Table 2 in the main text).

Benchmark Dose (BMD) modelling: Uncertainties in benchmark concentrations were quantitatively evaluated using established benchmark dose modeling, with different approaches and platforms for different NAMs: CALUX® (using a non-parametric dose-response modelling), CPP (using U.S. EPA tcplfit2, Sheffield 2025⁴⁴), and Transcriptomics (using BMD express 3.0). Uncertainty in the estimated benchmark concentrations from the BMD modelling was summarised by the mid estimate, lower and upper bounds of uncertainty intervals (95% probability intervals), as well as other information about BMD results across feature categories (lack of response trend, poor BMD model fit, low activation).

Derivation of overall Point of Departure: The overall PoD from an *in vitro* NAM was derived by aggregating results across different feature categories (a term introduced here to generalize the approach across the three NAMs, where the categories are CALUX®/reporter gene bioassays, CPP/cell morphology channels and Transcriptomics/modules). The uncertainty characterisation framework proposes a probabilistic overall PoD, which computes the probability that at least one feature category occurs at a given concentration, by assigning a normal distribution to the Log10 to each category with the estimated mid value of the BMD as

⁴⁴ <https://cran.r-project.org/web/packages/tcplfit2/tcplfit2.pdf>

the mean and the calculated standard deviation as parameters. The probability that at least one biological effect is activated at concentration c is calculated as:

$$P_{overall}(c) = 1 - \prod_{i=1}^J \left(1 - \Phi \left(\frac{c - m_i}{s_i} \right) \right) \quad \text{Equation 5}$$

where $\Phi()$ is the probability function for the normal distribution. This formulation derives from the statistical principle that the probability of at least one event occurring equals one minus the probability that no events occur. An uncertainty analysis can use the full probabilistic point of departure, or derive a conservative value (pPOD) as the concentration where the overall probability reaches a specified threshold $P_{threshold}$:

$$pPOD = \min\{c: P_{overall}(c) \geq P_{threshold}\} \quad \text{Equation 6}$$

This approach provides a statistically robust foundation for deriving protective concentration levels that account for multiple endpoints and their associated uncertainties. For comparative purposes, we calculated three alternative POD metrics: the minimum lower bound of the estimated benchmark concentrations across all categories and the 5th percentile of the benchmark concentrations values. There is little difference between the minimum of the lower bounds and the pPoD based on a threshold of 2.5%, when there is one feature category that is most sensitive to the compound.

Reliability in NAM-based PoDs: In addition to the qualitative description at every NAM, reliability was evaluated quantitatively for each *in vitro* NAM using data containing biological replicates, each containing repeated measures, followed by the NAM specific approaches of benchmark dose modelling. Between replicate variabilities in NAM-based benchmark concentrations as well as overall PoD were quantified as the standard deviation in Log10 of the benchmark concentration of results across biological replicates, using a statistical analysis with replicate as random effects and where applicable compound or NAM-specific feature as fixed effects:

$$y_{ij} = \beta_0 + \beta_1 \times \text{Compound}_i + \text{Replicate}_j + \varepsilon_{ij} \quad \text{Equation 7}$$

$$\varepsilon_{ij} \sim N(0, s_{ij})$$

$$\text{Replicate}_j \sim N(0, \tau)$$

where y_{ij} and s_{ij} are the point estimate and the standard deviation of the benchmark concentration (Log10 BMC) per replicate $j=1, \dots, J$ and per compound, $i = 1, \dots, N$. The standard deviation s_{ij} was calculated from viewing the upper and lower benchmark concentrations as a 95% probability interval symmetric around the mid value using the equation $s = (\text{Log10[BMDU]} - \text{Log10[BMDL]}) / (2 \times 1.96)$. The standard deviation of the replicates random effect τ indicates the variability of results across biological replicates. The larger the standard deviation, the lower robustness of the *in vitro* assay. This statistical analysis was performed for CALUX, CPP and transcriptomics assays using the brms package (Bürkner, 2018) in R software (R Core Team, 2025)⁴⁵. Results are provided in Annex III.

Exposure modelling: Uncertainty due to between human variability is considered in the PBK modelling by predicting concentrations for different human donors and summarizing the 5th, 50th and 95th quantiles. Uncertainty in PBK modelling can be considered by considering the impact of plausible values on model parameters on predicted exposure, using sensitivity analysis (sufficient level of refinement to conclude in this case study) or a full probabilistic approach (RISKHUNT3R 2025a & 2025b).

Characterisation of overall uncertainty by expert judgement: The final step of an uncertainty analysis was done by considering the impact of any additional sources of uncertainty on the conclusion. First, qualitatively by evaluating the importance of each source on the answer to the assessment question and then quantitatively by expressing level of certainty representing expert's confidence in the conclusion on a subjective probability scale (EFSA 2018, RISKHUNT3R 2025a & 2025b). This was applied by selecting

⁴⁵ <https://cran.r-project.org/doc/manuals/r-release/fullrefman.pdf>

uncertainty factors for the overall PoDs. When that was done, the experts (authors of this case study) were more than 95% certain that the derived NAM-based LOAEL in human equivalent doses is a human-relevant threshold for difenoconazole.

4. Response(s) measured

4.1. Caco-2 permeability assays

The measurement of the apparent permeability constant, P_{app} , is based on the rate of compound accumulation in the receiver chamber normalized for initial donor concentration and cell surface area (Hubatsch et al., 2007). The P_{app} value can be fed into PBK models to generate a human fraction absorbed after oral dosing.

4.2. Chemically Activated Luciferase eXpression (CALUX®) Reporter Gene Assays

The response measured in the CALUX assay is the induction of luciferase enzyme activity within the reporter cells. This induction is a direct consequence of the test item binding to and activating the target nuclear receptor or interacting with the studied pathway. Only dilutions that did not show any signs of cytotoxicity (relative induction in the cytotoxicity CALUX® bioassay > 80%) were used for final evaluation of analysis results. In general, relative light units (RLUs) were analyzed as follows. For agonist assays, background values (exposure to DMSO only) were subtracted, and the maximum activity of the assay's reference compound was set to 100%. For antagonist assays, the basal signal (exposure to fixed concentration of agonist only) was set to 100%, and maximum inhibition elicited by a reference antagonist was set to 0%. Activity of the samples was scaled accordingly, resulting in expression of the data as "percentage of maximum reference response". Dose-response curves were fitted (4-parameters logistic curve) and graphs generated using the statistical software package GraphPad Prism V5.03. Using these graphs, the PoD (as lowest effect concentrations, LEC) were determined; PoD is defined as the lowest concentration where the compound significantly activates the assay, which is set to 10% of the maximum reference compound activity for agonist assays, and 20% of the maximum reference compound activity for antagonist assays.

4.3. Cell Painting Plus (CPP) Assays

BMCs from the 894 morphological profiles are extracted as described in 3.3. The median BMC from calculated BMC of all features belonging to specific organelle were summarized (Nyffeler et al., 2021).

4.4. Chemical Effect Predictor (CEP)

described in 5.1.

4.5. Danish (Q)SAR Database

described in 5.2.

4.6. Enterocyte metabolism

In the assay the disappearance of parent compound and/or appearance of metabolites over time are measured to generate an intrinsic clearance value, CL_{int} , expressed in $\mu\text{L}/\text{min}/\text{million cells}$. In PBK modeling this value can give the fraction escaping metabolism in the intestinal wall (f_{gut}).

4.7. Intrinsic metabolic clearance assays and *in vitro* metabolite profiling

Intrinsic clearance CL_{int} expressed in $\mu\text{L}/\text{min}/\text{million cells}$ and protein binding (F_u) in %. For *in vitro* metabolite profiling, LC-MS was used, and the areas were calculated to quantify the metabolite.

4.8. PBK and biokinetics modelling

PBK modelling was used to predict concentration-time profile of compounds within plasma and other tissues following an external dose. Biokinetics model predicted free media concentration of a compound for a nominal concentration after partitioning into air, cells and plastic (also see 5.3.).

4.9. ToxProfiler Stress Reporter Assays

GFP concentration response plots were generated from which PoDs are calculated using a previously described software package “Modelpod” (Ter Braak et al., 2024). In short, the curves were fitted using a LOESS (Local Estimated Scatterplot Smoothing) regression and the intersect of the fit with the critical effect size (CES) was defined as the PoD. The CES is reporter-dependent and therefore a sensitive and reliable PoD for each ToxProfiler measurement was established.

4.10. PHH TXG-MAPr pathway enrichment analysis

Transcriptomics point of departure (tPOD) values are derived using BMD express 3.0. Prior to the BMD express processing, the tabulated log2 normalized CPM count data is provided containing the expression values of the genes for each compound with the DMSO from the same plate. The datasets are then processed with the set parameters: benchmark ratio (BMR) type: standard deviation, BMR factor: 1.021 (5%), applied models: Hill, Power, Exponential 3, Linear, Poly2. Williams trend test is also applied to each gene to evaluate the significance of dose-response pattern (p-value > 0.05). The best BMD, BMDL, and BMDU values based on the BMD express output are used for the further data analysis. The benchmark concentration values following several criteria are selected for functional enrichment analysis:

- Best fitPValue > 0.01
- Best BMD value/Best BMDL value < 20
- Best BMD value > 10% of lowest tested concentration
- Best BMDU value/Best BMDL value < 50
- Best BMD value/Best BMDL value > 1.01
- Passing Williams trend test
- Differentially expressed

Functional enrichment analysis is performed leveraging the gene networks-based enrichment from PHH-TXG-MAPr. The valid BMC values from the genes are grouped together according to the module memberships (gene network membership) and the median values of the BMD, BMDL, and BMDU are derived representing the BMD, BMDL, and BMDU values of the corresponding modules.

4.11. H295R steroidogenesis assay

For the H295R assay, the quantities of estrogens and androgens produced by the H295R cells are measured using the ER α or AR CALUX® as a read-out, respectively. The luminescence measured in the dilutions of the tested sample was normalized to the Estradiol (reference compound ER α CALUX®) or DHT (reference AR CALUX®) response in the CALUX® assay and then re-normalised to the estrogenic/androgenic activity of H295R cells treated with the solvent control (DMSO). Finally, the amount of estrogens and androgens produced by the H295R cells exposed to the test compound was divided by the amount of estrogens and androgens produced by the solvent control-exposed cells. Only dilutions that did not show any signs of activity carry-over, cytotoxicity in H295R, according to an MTT cytotoxicity test, were used for final evaluation of analysis results. Results are reported as the “lowest observed effect concentration” (LOEC), which is defined as the first datapoint that exceeds the activity threshold defined in the OECD guideline (0.66-fold decrease/1.5-fold increase in hormone levels).

4.12. DNT assays

The cMINC Neural Crest Cell Migration Assay (UKN2 Test)

Human embryonic stem cells (H9 line) were differentiated into neural rosettes and FACS-sorted for positive expression of the NCC marker HNK-1 and negative for expression of DII1 (Nyffeler et al., 2017). NCC were seeded in wells containing silicon stoppers (Platypus Technologies, Madison, WI, US) to create a cell free area. One day after seeding the stoppers were removed and the cells could migrate into the cell free area. After 24 hours of migration the cells were exposed to chemicals for additional 24 hours before the migration was assessed by imaging on a high content microscope (Cellomics ArrayScanVTI, Thermo Fischer). Quantification of the migration was performed using a software tool (freely accessible at <http://invitrotox.unikonstanz.de/>).

The NeuroTox Neurite Outgrowth of CNS Neurons Test (UKN4 Test)

Lund human mesencephalic cells (LUHMES) were differentiated into dopaminergic like neurons for 48 h (Delp et al., 2018). Cells were then replated at a low density and differentiation was continued for 24 h in the presence of chemicals. Cells were stained with Calcein AM and Hoechst 33342 before imaged with a high-content imaging ArrayScan VTI microscope (Cellomics, Waltham, MA, USA) to assess neurite area. For neurite area determination, an automated algorithm was used, which calculates the area of the cell soma and subtracts this area from all calcein-positive pixels imaged.

The PeriTox Neurite Outgrowth of PNS Neurons Test (UKN5 Test)

Human sensory neurons derived from human embryonic stem cells (WA09 Line) were obtained from WiCell (Madison, WI, USA) (Delp et al., 2018). Cells were seeded at low density and exposed to chemicals for 24 h, subsequently stained with Calcein AM and Hoechst 33342 and imaged with a high-content imaging ArrayScan VTI microscope (Cellomics, Waltham, MA, USA) to assess neurite area. For neurite area determination, an automated algorithm was used, which calculates the area of the cell soma and subtracts this area from all calcein-positive pixels imaged.

RPTEC/TERT1 Culture and TempO-Seq® Analysis

The transcriptomic response was defined as changes in mRNA abundance across approximately 19,000 genes following exposure to conazole compounds or control treatments. Gene expression levels were quantified as digital read counts per gene obtained from the TempO-Seq® assay. Differential expression ($\log_2$ fold change) between treated and vehicle control samples was calculated using DESeq2. Pathway activation or inhibition was subsequently inferred from enrichment analyses based on the sets of differentially expressed genes.

5. Prediction model

5.1. Chemical Effect Predictor (CEP)

The Chemical Effect Predictor (CEP) is a machine learning model powered by data extracted from a Knowledge Graph (KG) for predicting hazard concern caused by drugs/chemicals in humans. The KG was inspired by the multiscale interactome (Ruiz, Zitnik, & Leskovec, 2021) and it is composed of 4 types of nodes: Drugs/chemicals (N=24,789), Diseases/phenotypes (N=6,890), Proteins (N=20,882) and Biological processes (BP) (N=6,926). Those 4 types of nodes are associated among them based on different databases. Drug/Chemical-Protein associations are extracted from ChEMBL (Gaulton et al., 2017), Disgenet provides the relationships between diseases and proteins (Pinero et al., 2020), protein-protein interactions are obtained from Intact (Kerrien et al., 2012) and Protein-BP and BP-BP associations are derived from Gene Ontology Resource (Harris et al., 2004). All in all, the KG consists of 59,487 nodes and ~1,5M edges, representing the action of the compound through the intricate network of proteins and their associated biological processes to the disease phenotype. Based on the KG representing biological relationships,

different variables describing the association between drugs/chemicals and diseases were extracted for training a machine learning predictive model based on a random forest algorithm. For training and evaluating a model that will predict the potential adverse effects of a drug/chemical on human health data, the gold standard was generated based on data obtained from OnSIDES⁴⁶ (Tanaka et al., 2025). CEP trained on these data achieved an AUC-ROC of 0.84.

For each query compound, predictions for all the diseases (N=6,890) were executed and ranked in descending order based on their prediction values ranging from 1 to 0, with values close to 1 indicating the highest probability of the query compound causing the specific adverse effect. To extract robust signals for the prioritization of specific health effect concerns Disease Set Enrichment Analysis (DSEA) approach was designed inspired by Gene Set Enrichment Analysis (GSEA)(Subramanian et al., 2005). This approach considered the classification of diseases in higher-order disease classes and the ranked list of predicted diseases based on the prediction value. DSEA calculates an enrichment score (ES) of each disease class for the query compound based on Kolmogorov-Smirnov statistic, which reflects the degree to which the diseases in a given disease class are overrepresented at the higher prediction values of the ranked disease predictions. Significance is estimated based on sample randomization. In this analysis, for each compound, disease classes with p-adjusted values below 0.5 were considered as significantly enriched and those disease classes with ES>0 were selected.

5.2 Danish (Q)SAR Database

The compounds were predicted in the Danish (Q)SAR Models, accessible e.g. via the Danish (Q)SAR Database, accessed on 17-05-2024. The SMILES strings were used to import structure information for the compounds into the system. The compounds were predicted in all the models for endocrine receptors, genotoxicity/cancer, skin sensitization, skin irritation and respiratory sensitization (**Table A1.4.**). Original results are provided in Appendix I and the summarized results in Annex III.

Table A1.4. Model used for the (Q)SAR prediction of conazole compounds, information adapted from Danish QSAR Models documentation in the QMRF template⁴⁷

Model	N in training set	Sensitivity/Specificity/Concordance (%)	Modelling software
Not ready biodegradability (POS=Not Ready)	735	87.3/85.2/86.4/-	Leadscope
Cytochrome P450 2D6 (CYP2D6) substrates (human clinical data)	746	60.0/89.4/80.1/-	Leadscope
Cytochrome P450 2C9 (CYP2C9) substrates (human clinical data)	736	30.0/89.6/75.4/-	Leadscope
Maximum recommended daily dose (MRDD) in humans ≤ 2.69 mg/kg -2bw/d	1222	78.6/82.5/80.7/-	Leadscope
Severe skin irritation in rabbit	836	79.5/81.7/80.6/-	Leadscope
Respiratory sensitisation in humans	80	91.7/95.5/93.9/-	Leadscope
Estrogen Receptor α binding (human <i>in vitro</i>) ALL	802	75.2/90.1/84.7/-	Leadscope
Estrogen Receptor α binding (human <i>in vitro</i>) Balanced	595	83.7/89.0/86.3/-	Leadscope
Estrogen Receptor α activation (human <i>in vitro</i>)	481	73.1/86.6/80.7/-	Leadscope
Estrogen Receptor activation (<i>in vitro</i> , CERAPP data)	1420	78.5/96.7/-/87.6 (2*5-fold cross-validation)	Leadscope
Androgen Receptor inhibition (human <i>in vitro</i>)	874	51.7/91.2/80.4/-	Leadscope
Androgen Receptor binding (<i>in vitro</i> , CoMPARA data)	1662	80.4/93.3/-/86.9 (2*5-fold cross-validation)	Leadscope

⁴⁶ <https://github.com/tatonetti-lab/onsides>

⁴⁷ <https://qsarmodels.food.dtu.dk/download/QMRF/QMRF.html>

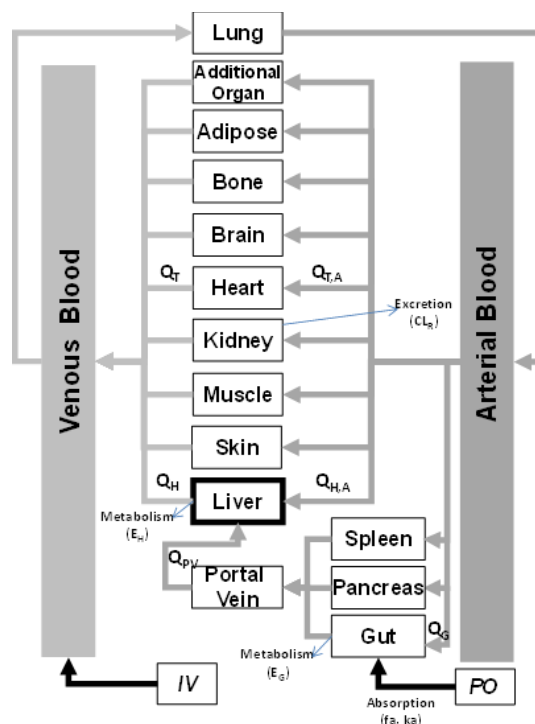
Androgen Receptor activation (<i>in vitro</i> , CoMPARA data)	1659	76.3/99.3/-/87.8 (2*5-fold cross-validation)	Leadscope
Androgen Receptor inhibition (<i>in vitro</i> , CoMPARA data)	1525	81.5/92.2/-/86.9 (2*5-fold cross-validation)	Leadscope
Arylhydrocarbon (AhR) Activation – Rational final model	4625	75.4/87.0/-/80.8 (2*5-fold cross-validation)	Leadscope
Arylhydrocarbon (AhR) Activation – Random final model	4625	86.4/91.5/-/88.9 (2*5-fold cross-validation)	Leadscope
Thyroperoxidase (TPO) inhibition QSAR1 (rat <i>in vitro</i>)	877	72.4/89.0/-/80.6 Ext. validation: 79.7/90.8/-/85.3	Leadscope
Thyroperoxidase (TPO) inhibition QSAR2 (rat <i>in vitro</i>)	1519	75.6/89.8/-/82.7	Leadscope
Pregnane X receptor binding (human <i>in vitro</i>)	631	80.4/80.4/80.4/-	Leadscope
Pregnane X Receptor (PXR) Binding (Human <i>in vitro</i>) NEW	1504	86.6/98.5/92.6 (2*5-fold cross-validation)	Leadscope
Pregnane X Receptor (PXR) Activation (Human <i>in vitro</i>)	2176	89.1/98.6/-/93.9 (2*5-fold cross-validation)	Leadscope
Pregnane X Receptor (PXR) Activation (Rat <i>in vitro</i>)	2330	86.5/97.4/-/92.0 (2*5-fold cross-validation)	Leadscope
CYP3A4 Induction (Human <i>in vitro</i>)	2271	86.7/98.2/-/92.5 (2*5-fold cross-validation)	Leadscope
Constitutive Androstane Receptor (CAR) activation at max. 20 µM	924	72.2/93.5/-/82.8 (2*5-fold cross-validation)	Leadscope
Constitutive Androstane Receptor (CAR) activation at max. 50 µM	1903	78.4/91.4/-/84.9 (2*5-fold cross-validation)	Leadscope
Constitutive Androstane Receptor (CAR) inhibition at max. 20 µM	1408	58.4/97.1/-/77.8 (2*5-fold cross-validation)	Leadscope
Constitutive Androstane Receptor (CAR) inhibition at max. 50 µM	1870	72.4/91.6/-/82.0 (2*5-fold cross-validation)	Leadscope
Bacterial reverse mutation test (Ames test in <i>S. typhimurium in vitro</i>)	4102	84.3/85.7/84.9/-	Leadscope
	600	74.6/75.2/74.9/-	Leadscope
Chromosome aberrations in CHL cells (<i>in vitro</i>)			
Mutations in thymidine kinase locus in mouse lymphoma cells (<i>in vitro</i>)	555	85.1/83.8/84.4/-	Leadscope
Mutations in HGPRT locus in CHO cells (<i>in vitro</i>)	239	81.7/78.4/80.5/-	Leadscope
Unscheduled DNA synthesis (UDS) in rat hepatocytes (<i>in vitro</i>)	415	74.1/70.1/72.4/-	Leadscope
Syrian hamster embryo (SHE) cell transformation (<i>in vitro</i>)	363	71.6/76.5/74.5/-	Leadscope
Sex-linked recessive lethal (SLRL) test in <i>Drosophila m. in vivo</i>)	367	79.1/80.3/79.6/-	Leadscope
Micronucleus test in mouse erythrocytes (<i>in vivo</i>)	357	64.1/77.6/72.3/-	Leadscope
Dominant lethal mutations in rodents (<i>in vivo</i>)	191	61.5/80.4/71.8/-	Leadscope
Sister chromatid exchange in mouse bone marrow cells (<i>in vivo</i>)	265	88.6/95.9/94.0/-	Leadscope
Comet assay in mouse (<i>in vivo</i>)	286	86.6/80.8/83.1/-	Leadscope
Liver specific cancer in rat or mouse (<i>in vivo</i>)	320	35.6/88.6/69.3/-	Leadscope

5.3. PBK modelling

The aim of the read across case study was to estimate the concentrations of nine conazoles in this case study in human plasma. To accomplish this a full body physiologically based kinetic (PBK) model (**Figure**

A1.1.) was built for cyproconazole, difenoconazole, epoxiconazole, fenbuconazole, ketoconazole, propiconazole, prothioconazole and tebuconazole. As the clearance in primary human hepatocytes could not be measured for itraconazole, the fully validated model in Simcyp was used for itraconazole and hydroxyitraconazole. PBK model was also developed for the common metabolite 1,2,4-triazole.

Figure A1.1. A physiologically based pharmacokinetic model.



Note: Q_H , $Q_{H,A}$, Q_{PV} , Q_G , $Q_{T,A}$ and Q_T are blood flows in the hepatic vein, hepatic artery, hepatic portal vein, gut and blood flows into and out of the other tissue (T) compartments, respectively; E_G and E_L are the fractions undergoing first pass metabolism in the gut and liver, respectively; CL_R is the renal clearance; f_a and k_a are the fraction absorbed and the first order absorption rate constant, respectively.

In constructing the PBK models for conazoles in the CS the following aspects were considered as suggested in the WHO PBK guidance.

- The critical effect caused by the chemicals after repeated dose was liver toxicity demonstrated primarily as hypertrophy and necrosis at higher doses.
- Several publications associate liver toxicity of conazoles with the activation of the nuclear receptors FXR, CAR and PXR. The activation of these receptors leads to CYP enzyme induction, impairment of lipid and steroid homeostasis and an increase in cell proliferation. Prolonged, chronic cell proliferation is discussed as one reason for conazoles to cause liver cancer.
- The *in vivo* dose–response relationship for the critical effect was based on published studies in EFSA reports for cyproconazole, epoxiconazole, fenbuconazole, propiconazole, prothioconazole and tebuconazole and for drug labels and publications of itraconazole and ketoconazole.
- All the compounds were assumed to have the same mechanism of action as outlined in the read across documentation with potential interaction activation of nuclear receptors and disruption of CYP homeostasis leading to liver toxicity.
- Both parent compound and its hydroxymetabolite are the toxic moieties.

- Based on *in silico* predictions and experimental observations, metabolism is thought to be the major clearance pathway of conazoles in this case study and the clearance in human subjects was predicted using an *in vitro* – *in vivo* extrapolation approach using intrinsic clearance data generated in human hepatocytes.
- The physiology (i.e. tissue weights and blood flow rates) of the species of interest were the default values in the Simcyp human simulator (V24).

Specific aims of the modelling exercise were

- To develop a PBK model to predict internal exposure of conazoles in the case study
- To perform IVIVE and compare the concentrations at which toxic effects are observed *in vitro* assays with the concentrations corresponding to LOAELs for the source compounds in this read across
- To examine the inter-individual variability in the simulated exposure of difenoconazole in humans
- To predict the toxicity-inducing dose of difenoconazole by extrapolating toxic concentrations identified in high-throughput *in vitro* assays

Distribution

In the human simulator inter-individual variability in tissue distribution is accounted for through relationships between tissue volume and age, sex, weight and height (4). The *in vivo* volume of distribution at steady state (V_{ss}) is predicted using Equation 8 (Sawada, Hanano, Sugiyama, Harashima, & Iga, 1984).

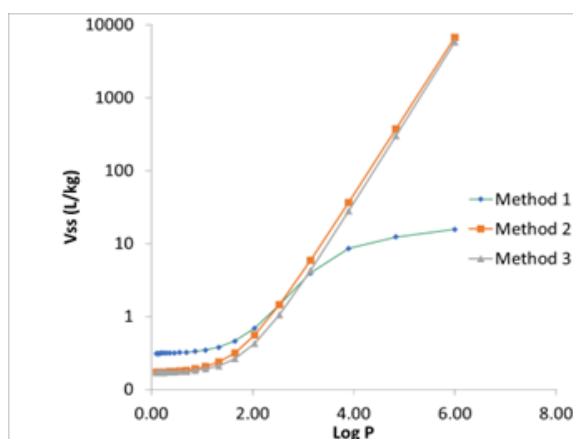
$$V_{ss} = \left(\sum V_T * P_{T:p} \right) + (V_e * E:P) + V_p \quad \text{Equation 8}$$

Where V is the fractional body volume (L/kg) of a tissue (T), erythrocytes (e), and plasma (p), E:P is the erythrocyte:plasma drug concentration ratio and $P_{T:p}$ is the partition coefficient of drug between tissues and plasma components. Three methods are available for prediction of $P_{T:p}$. The first method was reported by Poulin and Thiel (Poulin & Thiel, 2002) as corrected by Berezhkovskiy (Method 1) (Berezhkovskiy, 2004). Method 1 uses the physicochemical properties of the compounds (pKa and log P) together with *in vitro* information (B/P and fu) to predict partitioning into the tissues with the assumption that tissues and plasma are mixtures of lipids, water and proteins with a global pH of 7.4.

The second method was developed by Rodgers and Rowland (Method 2) (Rodgers & Rowland, 2006). The latter splits the tissue water volume into intra- and extracellular components, along with the addition of a tissue acidic phospholipid fraction. These equations take explicit account of the extent of ionisation of a compound at the pH of the compartment concerned and have been shown to improve the prediction of tissue:plasma partition coefficients, and consequently V_{ss} , for strong bases. The Rodgers and Rowland method was further extended by the science team at Simcyp to account for the effect of differences in membrane potential in the different tissues on compound distribution (Method 3). Method 3 is only available in the human PBK models and also allows for the distribution of compounds into specific subcellular organelles to be modelled. Method 3 also forms the basis for the *in vitro* distribution biokinetic model. The LogP and pKa values are important for predicting the tissue affinity of the compound and its volume of distribution within the body. This is illustrated in **Figure A1.2.** where the relationship between LogP and V_{ss} are shown for a neutral compound with varying values of LogP assumed. In this case the fu in plasma was kept a constant value of 0.1. As LogP increases, the V_{ss} increases and when LogP is > 4 the V_{ss} increases dramatically especially when Method 2 (Rodgers & Rowland, 2006) or 3 (Fisher et al., 2019) is used. This highlights the importance of having robust estimates of LogP and understanding the limitations of the different methods for predicting V_{ss} . At high LogP, Method 2 and 3 tends to overpredict V_{ss} , this limitation can be overcome by calculating a lipid binding scalar. In this exercise, when Log P was > 4 the lipid binding scalar approach was utilised. These mechanistic predictions assume non-saturating conditions prevail for all

binding processes, drug transport is via passive processes (i.e. no active transport), and each tissue has a well-stirred distribution limited by blood perfusion (i.e. perfusion limited not permeability limited).

Figure A1.2. Relationship between LogP and Vss for a neutral compound ($f_u = 0.1$) with three different prediction methods of tissue:plasma partition coefficients.



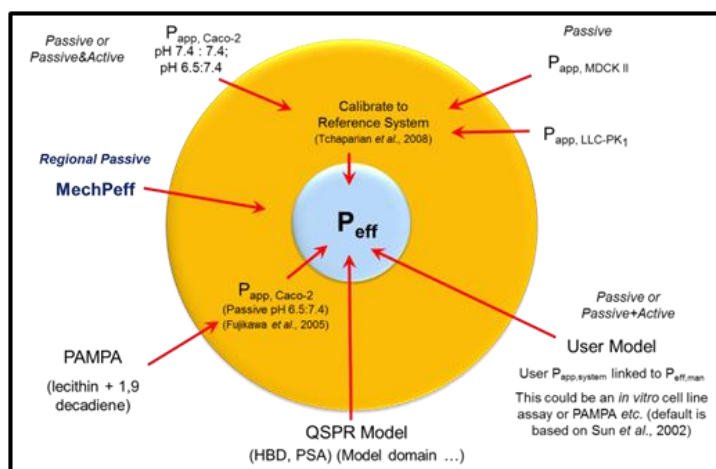
Perfusion and permeability limited distribution

Lipophilic drugs diffuse rapidly across the capillary membrane into tissue interstitial fluid such that blood flow to the tissue is the rate-limiting step in uptake. This is described as perfusion-limited distribution and is implemented in all tissues represented in the PBK model (**Figure A1.1.**). In addition, an option is provided within the Simcyp Simulator to allow for permeability-limited uptake, simulating both passive diffusion in parallel with active uptake and efflux in specific organs such as the liver, kidney (human only), intestine and brain. In these models the tissue is divided into compartments representing vascular, extracellular and intracellular fluid spaces - with distribution between these spaces defined as a dynamic process. The 'permeability-limited' models in the liver, brain and kidney are only available when Method 2 or 3 are selected to predict $P_{T:p}$. Given the lipophilic nature of conazoles, perfusion limited models were used for all tissues in the simulations presented here.

Oral Absorption

For drugs in solution, several absorption models are available within the Simcyp Simulator including a first-order absorption model, a compartmental absorption transit (CAT) model (Yu & Amidon, 1998) and the advanced dissolution absorption metabolism (ADAM) model (Jamei, Turner, et al., 2009). Simulation of the absorption of drugs from solid dosage forms requires use of the ADAM model.

The effective permeability in humans, $P_{eff,man}$ (jejunal), can be measured using the Loc-i-gut methodology and can be used in simulations to describe the absorptive processes in the intestine (Nilsson, Fagerholm, & Lennernas, 1994). For novel investigation drugs and many marketed agents and all industrial chemicals measured values of $P_{eff,man}$ (jejunal) are not available and therefore several methods can be used within the Simcyp Simulator to predict $P_{eff,man}$ (jejunal). These are based on data obtained with cell lines (such as Caco-2, MDCK-II or LLC-PK₁ cells) (Sun et al., 2002), PAMPA or from a QSPR model based upon physicochemical properties (PSA and HBD, (Winiwarter et al., 1998)) or by using the mechanistic permeability (MechPeff) model (**Figure A1.3.**). The regional permeability (seven small intestine segments plus colon) is assumed to be the same by default but can be modified by the user. The regional distributions of drug metabolising enzymes and efflux transporters such as P-gp and BCRP are also incorporated, allowing simulation of the effects of efflux transport and metabolism on drug absorption (**Figure A1.3.**).

Figure A1.3.: Input options to predict P_{eff} and subsequently absorption within the Simcyp Simulator

Note: The P_{app} values measured in Caco-2 cells at pH 7.4:7.4 were used to predict P_{eff} for conazoles in this study. The P_{app} values measured in Caco-2 cells at pH 7.4:7.4 were used to predict P_{eff} for conazoles in this study.

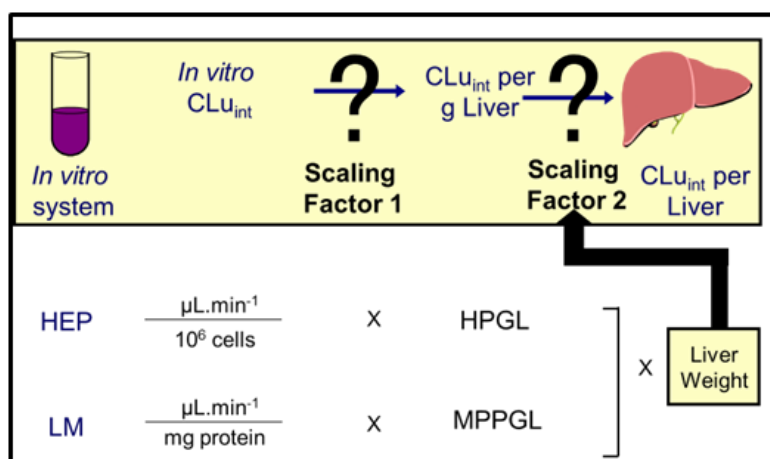
The $MechP_{eff}$ Model is a fully mechanistic model that predicts passive intestinal permeability using a compound's physicochemical parameters such as LogP, pKa, molecular weight and compound type. The intestinal absorption for all conazoles was predicted using Caco-2 cell permeability measured within this project.

Metabolic Clearance

Elimination of a compound can be characterized by various inputs of clearance such as intravenous or oral clearance (CL_{iv} or CL_{po}), whole organ metabolic clearance via hepatocytes (CL_{int} ; $\mu\text{L}/\text{min}/10^6$ cells), liver and intestinal microsomes (CL_{int} ; $\mu\text{L}/\text{min}/\text{mg}$) or incubation with intestinal slices (CL_{int} ; $\mu\text{L}/\text{min}/\text{g}$ of intestine).

Hepatic metabolic clearance

On a general basis the *in vivo* hepatic metabolic clearance is predicted using *in vitro-in vivo* extrapolation (IVIVE) as shown schematically in **Figure A1.4.** followed by scaling for the specific metabolising tissue blood flow (liver in this case) and fraction of unbound drug in blood.

Figure A1.4. Schematic of IVIVE for scaling of *in vitro* intrinsic clearance available for the Simcyp simulator

For the human simulator clearance PBK models for conazoles were predicted using metabolic intrinsic clearance ($CL_{int, hep}$) data generated in cryopreserved human hepatocytes. *In vitro* $CL_{int, hep}$ was scaled up to the *in vivo* CL_{int} ($CL_{int, u, H}$) according to Equation 9.

$$CL_{int, u, H} = \frac{CL_{int, hep}}{f_{u, hep}} * Uptake * HPGL \text{ Scaling Factor} * Liver \text{ Weight} \quad \text{Equation 9}$$

$$* 10^{-6} * 60$$

Where $f_{u, hep}$ is the fraction unbound of the compound in the hepatocyte incubation, HPGL is the number of hepatocytes per gram of liver, uptake was assumed to be only due to passive processes in these simulations (set =1), and 10^{-6} and 60 are to adjust units from $\mu\text{L}/\text{min}$ per 10^6 cells to L/h in the whole liver (Equation 10, Equation 11). Correction for non-specific protein binding is important for IVIVE (Brown, Chadwick, & Houston, 2007; McGinnity, Berry, Kenny, Grime, & Riley, 2006). $f_{u, hep}$ was predicted from an estimate of the fraction unbound in microsomal incubations ($f_{u, mic}$) as described by Kilford et al (Kilford, Gertz, Houston, & Galetin, 2008).

$$f_{u, hep} \text{ (acidic and neutral)} = \frac{1}{1 + 125 * V_R * 10^{0.072 * \log D_{7.4}^2 + 0.067 * \log D_{7.4} - 1.126}} \quad \text{Equation 10}$$

$$f_{u, hep} \text{ (bases)} = \frac{1}{1 + 125 * V_R * 10^{0.072 * \log P^2 + 0.067 * \log P - 1.126}} \quad \text{Equation 11}$$

Where V_R is V_{cell}/V_{inc} ratio, where V_{cell} is the cell volume and V_{inc} the incubation volume and $\log D_{7.4}$ and $\log P$ as descriptors of lipophilicity. The hepatic clearance (CL_H) in humans was calculated from the whole liver scaled *in vivo* CL_{int} using the well stirred model (Equation 12).

$$CL_H = \frac{Q_H * CL_{int, u, h} * f_{u_B}}{Q_H + (CL_{int, u, h} * f_{u_B})} \quad \text{Equation 12}$$

Excretion

It is possible to account for the excretion of unchanged drug in the kidney or via the biliary system in the PBK models. This was accounted for conazoles using $f_u * \text{GFR}$ as the clearance rate (L/h), where f_u is the fraction unbound in plasma and GFR is the glomerular filtration rate in L/h.

General description of equations for eliminating and non-eliminating organs

Each compartment within the full body PBK model was initially described as a perfusion limited model with representative equations for an eliminating and non-eliminating organ as shown below. The equations describing the behaviour of the compound within the intestine following oral absorption are as described by Jamei et al (Jamei, Turner, et al., 2009). The basic principles for a PBK model outlined on P19 of the WHO guidance on PBK modelling were adhered to. Namely

- the mixing of the chemical in the effluent blood from the tissues is instantaneous and complete.
- blood flow is unidirectional, constant and non-pulsatile; and
- the presence of chemicals in the blood does not alter the blood flow rate

In non-eliminating tissues the compound concentration (C) at a given time (t) is defined as follows:

$$\frac{dC_{tissue}}{dt} = \frac{Q_{tissue}}{V_{tissue}} * (C_{ab} - \frac{C_{tissue}}{\frac{P_{tissue:p}}{BP}}) \quad \text{Equation 13}$$

Where Q = blood flow to the tissue, V = tissue volume, C_{ab} = arterial blood concentration, BP = blood to plasma ratio and $P_{Tissue:p}$ – partition coefficient of drug between tissues and plasma.

In the liver the following equation is applied:

$$\frac{dC_{liver}}{dt} = \frac{1}{V_{liver}} ((Q_{liver} - Q_{pv}) * C_{ab} + Q_{pv}C_{pv} - \frac{Q_{liver}C_{liver}}{\frac{P_{liver:p}}{BP}} - \frac{fu}{BP} * \frac{Clu_{int}}{\frac{P_{liver:p}}{BP}} * C_{liver}) \quad \text{Equation 14}$$

Where Q_{liver} = sum of blood flow to the liver by the hepatic artery and hepatic portal vein, Q_{pv} = hepatic portal blood vein flow, C_{pv} = hepatic portal compound vein concentration, fu = fraction unbound in plasma, Clu_{int} = intrinsic clearance.

Within each simulated human subject, the sum of the tissue blood flow rates (excluding the lung) is equal to cardiac output. In line with accepted mammalian physiology the lung also receives a blood flow equal to total cardiac output. Tissue volumes and blood flow rates are within the documented range for each species and age group (pediatric versus adult) considered (Jamei, Dickinson, & Rostami-Hodjegan, 2009; Johnson, Bonner, Tucker, Turner, & Jamei, 2018; Musther et al., 2017).

Population data

Predictions of plasma drug exposure, clearance and other parameters such as fraction metabolised by a particular pathway were made for virtual populations of healthy volunteers. Each population is generated using values and formulae describing demographic, anatomical and physiological variables. Thus, to assess clearance predictions in a specific population, data are required for the population variables as well as for the *in vitro* metabolism/transport of the test drug and its observed clearance in the population of interest. The parameter values within the Simcyp Simulator for creating a virtual healthy volunteer population (population, physiological parameters including liver volume and blood flows, enzyme abundances) have been described previously (Jamei, Dickinson, et al., 2009).

Physiology data used in the healthy human population PBK populations

Table A1.5. Mean and range of physiological values generated in a population of 100 Healthy adults using an age range of 20-50 as input.

Parameter	Mean value	Range
Age (y)	29.6	20 – 48
Weight (kg)	72.45	45 – 118
Height (cm)	168.4	149 – 189
Cardiac output (L/h)	321.8	252 – 416
Serum albumin (g/L)	45.7	38 – 60

The PBK models in the case study used predicted log P values from ChemAxon (CXlogP) and experimental estimates of the fraction unbound in plasma sourced from the htk R package. For epoxiconazole and prothioconazole, these fractions were experimentally measured. The effect of varying protein binding values on unbound and total compound concentrations was analyzed during model construction, revealing that while this uncertainty affects total concentrations, it has minimal impact on unbound concentrations. Since the unbound (free) concentration drives toxicity, this uncertainty is not significant for toxicological exposure assessments of the two compounds. Additionally, the blood-to-plasma ratios for all compounds were predicted using the QSPR model in Simcyp, showing comparable blood cell to plasma partitioning.

Absorption

For oral absorption simulations the intestinal permeability of all conazoles was predicted from the Papp measured in the Caco-2 cells. This leads to a high predicted fraction absorbed after oral exposure assuming that there are no solubility limitations.

Distribution

The volume of distribution at steady state (V_{ss}) of was predicted using Method 3 in Simcyp.

Metabolic Clearance

The clearance of conazoles was simulated in a population of 100 healthy human subjects (aged 20-50; 50% female) using the available *in vitro* metabolism data and blood binding data as inputs to the model.

Data and the corresponding source and/or reference used in the simulation of conazoles compound file are shown below. No clearance in intestinal cells (primary enterocytes) was observed for the compounds in this case study.

Table A1.6. Parameter used for the simulation of cyproconazole

Parameter	Value	Method/Comment	Source
Compound Name	Cyproconazole		
Mol Weight (g/mol)	291.78		https://pubchem.ncbi.nlm.nih.gov
Compound type	Neutral		Compound: CYPROCONAZOLE (CHEMBL2131954) - ChEMBL
LogP	2.85		Compound: CYPROCONAZOLE (CHEMBL2131954) - ChEMBL
TPSA ($\text{\AA}^2$)	50.94		https://pubchem.ncbi.nlm.nih.gov
Hydrogen bond donors	1		https://pubchem.ncbi.nlm.nih.gov
f_u	0.1086	Assumed that albumin is the major binding protein	Integration of dosimetry, exposure, and high-throughput screening data in chemical toxicity assessment - PubMed
B/P	0.8356		httk database
V_{ss} (Subs)(L/Kg)	2.42	Method 3	Predicted in Simcyp
f_a	0.99986		Predicted in Simcyp
k_a (h^{-1})	4.932		Predicted in Simcyp
f_{uGut}	1		Assumed
P_{app} Caco-2 cells (10^{-6} cm/s)	41.05		Experimentally measured in the project
Clint ($\mu\text{l}/\text{min}/10^6$ cells)	1.54		Integration of dosimetry, exposure, and high-throughput screening data in chemical toxicity assessment - PubMed
		Human hepatocytes	
Hepatocyte binding (f_u hep)	0.78		Calculated using Kilford method (Kilford et al., 2008).

Table A1.7. Parameter used for the simulation of difenoconazole

Parameter	Value	Method/Comment	Source
Compound Name	Difenoconazole		
Mol Weight (g/mol)	406.27		https://pubchem.ncbi.nlm.nih.gov
Compound type	Neutral		Compound: DIFENOCONAZOLE (CHEMBL466426) - ChEMBL
LogP	4.86		Compound: DIFENOCONAZOLE (CHEMBL466426) - ChEMBL

TPSA (Å ²)	58.4		https://pubchem.ncbi.nlm.nih.gov
Hydrogen bond donors	0		https://pubchem.ncbi.nlm.nih.gov
fu	0.01	Assumed that albumin is the major binding protein	Assessing Toxicokinetic Uncertainty and Variability in Risk Prioritization - PubMed
B/P	0.94	Predicted	Predicted in Simcyp
Vss (Subs)(L/Kg)	10.968	Method 3	Predicted in Simcyp
Lipid binding scalar	0.26		Predicted in Simcyp
fa	0.99		Predicted in Simcyp
ka (h ⁻¹)	3.1876		Predicted in Simcyp
fu _{Gut}	1		Assumed
P _{app} Caco-2 cells (10 ⁻⁶ cm/s)	21.86		Experimentally measured in the project
Clint (µl/min/10 ⁶ cells)	30.6		Assessing Toxicokinetic Uncertainty and Variability in Risk Prioritization - PubMed
Hepatocyte binding (fu heps)	0.17		Calculated using Kilford method (26)

Table A1.8. Parameter used for the simulation of epoxiconazole

Parameter	Value	Method/Comment	Source
Compound Name	Epoxiconazole		
Mol Weight (g/mol)	329.76		https://pubchem.ncbi.nlm.nih.gov
Compound type	Neutral		Compound: EPOXICONAZOLE (CHEMBL486029) - ChEMBL
LogP	3.74		Compound: EPOXICONAZOLE (CHEMBL486029) - ChEMBL
TPSA (Å ²)	43.24		https://pubchem.ncbi.nlm.nih.gov
Hydrogen bond donors	0		https://pubchem.ncbi.nlm.nih.gov
fu	0.028		Experimentally measured in the project
B/P	0.92		Predicted in Simcyp
Vss (Subs)(L/Kg)	11.206	Method 3	Predicted in Simcyp
fa	0.9995		Predicted in Simcyp
ka (h ⁻¹)	3.8134		Predicted in Simcyp
fu _{Gut}	1		Assumed
P _{app} Caco-2 cells (10 ⁻⁶ cm/s)	48.86		Experimentally measured in the project
Clint (µl/min/10 ⁶ cells)	2.7		Experimentally measured in the project
Hepatocyte binding (fu heps)	0.54		Calculated using Kilford method (26)

Table A1.9. Parameter used for the simulation of fenbuconazole

Parameter	Value	Method/Comment	Source
Compound Name	Fenbuconazole		
Mol Weight (g/mol)	336.83		https://pubchem.ncbi.nlm.nih.gov
Compound type	Neutral		Compound: FENBUCONAZOLE (CHEMBL1879687) - ChEMBL
LogP	4.35		Compound: FENBUCONAZOLE (CHEMBL1879687) - ChEMBL
TPSA (Å ²)	54.5		https://pubchem.ncbi.nlm.nih.gov
Hydrogen bond donors	0		https://pubchem.ncbi.nlm.nih.gov

fu	0.019		Assessing Toxicokinetic Uncertainty and Variability in Risk Prioritization - PubMed
B/P	0.85		Predicted in Simcyp
Vss (Subs)(L/Kg)	7.64		Predicted in Simcyp
Lipid binding scalar	0.489		Predicted in Simcyp
fa	0.99		Predicted in Simcyp
ka (h ⁻¹)	3.606		Predicted in Simcyp
fu _{Gut}	1		Assumed
P _{app} Caco-2 cells (10 ⁻⁶ cm/s)	45.07		Experimentally measured in the project
Clint (μl/min/10 ⁶ cells)	21.7		Assessing Toxicokinetic Uncertainty and Variability in Risk Prioritization - PubMed
Hepatocyte binding (fu heps)	0.32		Calculated using Kilford method (26)

Table A1.10. Parameter used for the simulation of ketoconazole

Parameter	Value	Method/Comment	Source
Compound Name	Ketoconazole		
Mol Weight (g/mol)	531.4		https://pubchem.ncbi.nlm.nih.gov
Compound type	Diprotic base		Dollery, C. Therapeutic Drugs (1999)
pKa1	2.940		Dollery, C. Therapeutic Drugs (1999)
pKa2	6.510		Dollery, C. Therapeutic Drugs (1999)
LogP	4.19		Compound: KETOCONAZOLE (CHEMBL157101) - ChEMBL
TPSA (Å ²)	69.06		Compound: KETOCONAZOLE (CHEMBL157101) - ChEMBL
Hydrogen bond donors	0		Compound: KETOCONAZOLE (CHEMBL157101) - ChEMBL
fu	0.0172		Measured in EUTR project
B/P	0.69		Measured in EUTR project
Vss (Subs)(L/Kg)	8.95		Predicted in Simcyp
fa	0.99		Predicted in Simcyp
ka (h ⁻¹)	2.2751		Predicted in Simcyp
fu _{Gut}	1		Predicted in Simcyp
P _{app} Caco-2 cells (10 ⁻⁶ cm/s)	23.18		Experimentally measured in the project
Clint (μl/min/10 ⁶ cells)	3.8		Measured in EUTR project
Hepatocyte binding (fu heps)	0.38		Calculated using Kilford method (26)

Table A1.11. Parameter used for the simulation of propiconazole

Parameter	Value	Method/Comment	Source
Compound Name	Propiconazole		
Mol Weight (g/mol)	342.23		https://pubchem.ncbi.nlm.nih.gov
Compound type	Neutral		Compound: PROPICONAZOLE (CHEMBL560579) - ChEMBL
LogP	4.33		Compound: PROPICONAZOLE (CHEMBL560579) - ChEMBL
TPSA (Å ²)	49.17		Compound: PROPICONAZOLE (CHEMBL560579) - ChEMBL
Hydrogen bond donors	0		Compound: PROPICONAZOLE (CHEMBL560579) - ChEMBL
fu	0.05		Assessing Toxicokinetic Uncertainty and Variability in Risk Prioritization - PubMed
B/P	1.45		Predicted in Simcyp
Vss (Subs)(L/Kg)	22.833	Method 3	Predicted in Simcyp
Lipid binding scalar	0.5016		Predicted in Simcyp

fa	0.99881		Predicted in Simcyp
ka (h ⁻¹)	3.1436		Predicted in Simcyp
fu _{Gut}	1		Assumed
P _{app} Caco-2 cells (10 ⁻⁶ cm/s)	36.97		Experimentally measured in the project
Clint (μl/min/10 ⁶ cells)	13.9		Experimentally measured in the project
Hepatocyte binding (fu _{heps})	0.33		Calculated using Kilford method (26)

Table A1.12. Parameter used for the simulation of prothioconazole

Parameter	Value	Method/Comment	Source
Compound Name	Prothioconazole		
Mol Weight (g/mol)	344.27		https://pubchem.ncbi.nlm.nih.gov
Compound type	Neutral		Compound: PROTHIOCONAZOLE (CHEMBL2251446) - ChEMBL
LogP	3.47		Compound: PROTHIOCONAZOLE (CHEMBL2251446) - ChEMBL
TPSA (Å ²)	53.84		Compound: PROTHIOCONAZOLE (CHEMBL2251446) - ChEMBL
Hydrogen bond donors	2		Compound: PROTHIOCONAZOLE (CHEMBL2251446) - ChEMBL
fu	0.0034		Measured in the project
B/P	0.58		Predicted in Simcyp
V _{ss} (Subs)(L/Kg)	0.423		Predicted in Simcyp
fa	0.99265		Predicted in Simcyp
ka (h ⁻¹)	1.9765		Predicted in Simcyp
fu _{Gut}	1		Assumed
P _{app} Caco-2 cells (10 ⁻⁶ cm/s)	18.92		Experimentally measured in the project
Clint (μl/min/10 ⁶ cells)	32.6		Experimentally measured in the project
Hepatocyte binding (fu _{heps})	0.63		Calculated using Kilford method (26)

Table A1.13. Parameter used for the simulation of tebuconazole

Parameter	Value	Method/Comment	Source
Compound Name	Tebuconazole		https://pubchem.ncbi.nlm.nih.gov
Mol Weight (g/mol)	307.82		Compound: TEBUCONAZOLE (CHEMBL487186) - ChEMBL
Compound type	Neutral		Compound: TEBUCONAZOLE (CHEMBL487186) - ChEMBL
LogP	3.69		Compound: TEBUCONAZOLE (CHEMBL487186) - ChEMBL
TPSA (Å ²)	50.94		Compound: TEBUCONAZOLE (CHEMBL487186) - ChEMBL
Hydrogen bond donors	1		Compound: TEBUCONAZOLE (CHEMBL487186) - ChEMBL
fu	0.07		Incorporating High-Throughput Exposure Predictions With Dosimetry-Adjusted In vitro Bioactivity to Inform Chemical Toxicity Testing Toxicological Sciences Oxford Academic

B/P	1.3591		Predicted in Simcyp
Vss (Subs)(L/Kg)	11.63		Predicted in Simcyp
fa	0.99892		Predicted in Simcyp
ka (h ⁻¹)	3.2151		Predicted in Simcyp
fu _{Gut}	1		Assumed
P _{app} Caco-2 cells (10 ⁻⁶ cm/s)	38.19		Experimentally measured in the project
Cl _{int} (μl/min/10 ⁶ cells)	7.3		Experimentally measured in the project
Hepatocyte binding (fu heps)	0.56		Calculated using Kilford method (26)

Table A1.14. Parameter used for the simulation of itraconazole and hydroxyitraconazole

Parameter	Compound	Reference	Compound	Reference
Compound Name	Itraconazole		hydroxyitraconazole	
Mol Weight (g/mol)	705.6		721.7	https://pubchem.ncbi.nlm.nih.gov
Compound type	Monoprotic Base	Avdeef (2012)	Monoprotic Base	
pKa	4.28	Avdeef (2012)	4.28	Assumed same as parent
LogP	4.47	Avdeef (2012)	4.47	Assumed same as parent
TPSA (Å ²)	101	https://pubchem.ncbi.nlm.nih.gov	121.02	https://pubchem.ncbi.nlm.nih.gov
Hydrogen bond donors	0	https://pubchem.ncbi.nlm.nih.gov	1	https://pubchem.ncbi.nlm.nih.gov
fu (assumed albumin as main binding protein)	0.016	Ishigam et al. (2001)	0.016	Assumed same as parent
B/P	0.58	Heykants et al. (1989)	0.58	Assumed same as parent
Vss (Subs)(L/Kg)	2.5085	Predicted; Method 1	1.305	Predicted; Method 1
fa	0.59	Fitted; Van Peer et al. (1989)	0.809	Predicted using PSA and HBD model
ka (h ⁻¹)	0.6	Fitted; Van Peer et al. (1989)	0.518	Predicted using PSA and HBD model
fu _{Gut}	0.016	=Plasma fu	0.016	=Plasma fu
CYP1A1 Clint (μl/min/pmol)	55	Nominal value to form the metabolite under conditions where CYP3A4 is saturated		
CYP3A4	V _{max} =0.065 pmol/min/pmol K _m =0.0039 μM	Isoherranen et al. (2004)	V _{max} =0.13 pmol/min/pmol K _m =0.0027 μM	Isoherranen et al. (2004)

PBK modelling was also carried out to predict how much 1,2,4 triazole can theoretically be formed from parent conazoles for pesticides used in this case study assuming 100 % of parent conazole converts to 1,2,4-triazole. The unbound HLM based clearance from ADMET predictor was used in Simcyp to predict conversion rate of parent conazole to 1,2,4-triazole. The mechanistic kidney (MechKim) model in Simcyp (Neuhoff et al., 2013) was used to account for renal excretion of 1,2,4-triazole. No other clearance was considered for 1,2,4-triazole.

Table A1.15. Parameter used for the simulation of 1,2,4,-triazole

Parameter	Value	Method/Comment	Source
Compound Name	1,2,4-triazole		
Mol Weight (g/mol)	69.007		https://pubchem.ncbi.nlm.nih.gov
Compound type	Monoprotic base		
pKa	2.49		Compound: 1,2,4-TRIAZOLE (ChEMBL15571) - ChEMBL
LogP	-0.58		https://pubchem.ncbi.nlm.nih.gov
TPSA (Å²)	41.6		Compound: 1,2,4-TRIAZOLE (ChEMBL15571) - ChEMBL
Hydrogen bond donors	1		Compound: 1,2,4-TRIAZOLE (ChEMBL15571) - ChEMBL
fu	0.92		Predicted in S+ ADMET predictor
B/P	0.67		Predicted in Simcyp
Vss (Subs)(L/Kg)	0.470	Method 3	Predicted in Simcyp
fa	0.99		Predicted in Simcyp
ka (h ⁻¹)	3.86		Predicted in Simcyp
fu _{Gut}	1		Assumed
Renal excretion			MechKim model (Neuhoff et al., 2013)

Table A1.16. HLM based clearance predicted by S+ ADMET predictor

Compound	HLM clearance unbound (µL/min/mg of protein)
Cyproconazole	26.13
Difenoconazole	72.63
Epoxiconazole	31.80
Fenbuconazole	85.83
Propiconazole	27.32
Prothioconazole	12.50
Tebuconazole	40.26

5.4. Biokinetics modelling

In this case study the biokinetics analysis of all conazoles, metabolites and control substances have been carried out using Virtual *in vitro* distribution (VIVD) model from Certara. The model is based on Fick-Nernst Planck equation to predict differential compound ionisation in culture medium and intracellular water. All the equations for predicting partitioning into the cell, plastic, air and media are available in the associated publication (Fisher 2019).

Assumptions of the model:

- The model assumes monolayer culture in wells or in round dishes.
- The steady state version of the model was used which accounts for cell membrane potential, pH, and extracellular binding to culture medium proteins, lipids, and plastics, but loss of compound through metabolic clearance or instability is considered negligible.
- The effect of transporters has not been accounted for in the model.
- The size of cells in the culture will maintain comparable size and composition as similar cells in human. E.g PHH will have comparable size and composition as hepatocytes in human liver.

- The binding to cellular acidic phospholipids is assumed to be relevant for significantly ionised basic compounds and so does not contribute to the intracellular binding of neutral compounds.
- The prediction of binding to components within serum is predominantly driven by logPow even with accounting for ionisation within the model framework. The following compound parameters are used in the model development (**Table A1.17.**):
- Molecular weight
- LogP or log K_{ow} (Octanal Water Partition coefficient)
- Compound type (Acidic, Neutral, Basic, Ampholyte)
- Blood to Plasma ratio
- Henry's Law constant ($\text{Pa}/\text{m}^3/\text{mol}$)
- Aqueous solubility (mgL^{-1})

The cell composition parameters used in the model development (**Table A1.18.**):

- Fraction of cell volume comprising intracellular water
- Fraction of cell volume comprising neutral lipid
- Fraction of cell volume comprising neutral phospholipid
- Fraction of cell volume comprising lysosomes
- Fraction of cell volume comprising mitochondria
- pH of intracellular water
- pH inside lysosomes
- pH inside mitochondria
- membrane potential of the cell (mV)
- membrane potential of the lysosome (mV)
- membrane potential of the mitochondria (mV)
- cellular concentration of acidic phospholipids (mg/g)
- Average diameter of a single cell (μM)
- Number of cells at the time of the assay

Culture conditions and parameters needed for the development of the model (**Table A1.19.**):

- pH of cell media culture
- fraction of cell culture media comprising fetal bovine serum
- pH of fetal bovine serum
- Mass of albumin in 1 litre of fetal bovine serum (g)
- Concentration of triacylglyceride in fetal bovine serum (mmol/L)
- Total volume culture media per well (μl)
- Diameter of a well of the culture vessel (mm)

- Total volume of a well of the culture vessel (µl)
- Culture temperature (°C)

Fraction unbound in the medium can be a user input or predicted using models within the tool:

- a) Human serum model
- FBS model (Rodgers and Rowland based prediction using input fetal bovine composition)
 - Fraction unbound in the medium for each compound was predicted within VIVD model using FBS model.

Table A1.17. Conazole input file for Biokinetics analysis

Name	Nominal Treatment Conc. (mol/L)	MW (g/mol)	logPow	Compound Type	pKa 1	pKa 2	BP	Henry's Law Constant (Pa/m ³ /mol)	Aqueous Solubility (mg/L)
Cyproconazole	1.00E-06	291.78	2.9	Neutral	0	0	0.84	7.19E-05	93
Difenoconazole	1.00E-06	406.27	4.3	Neutral	0	0	0.94	9.03E-07	15
Epoxiconazole	1.00E-06	329.76	3.44	Neutral	0	0	0.92	4.03E-05	7.1
Fenbuconazole	1.00E-06	336.8	3.23	Neutral	0	0	0.85	0.00842	3.77
Hydroxyitraconazole	1.00E-06	721.65	4.47	Monoprotic base	4.28	0	0.58	4.68E-22	0.334
Itraconazole	1.00E-06	705.6	4.47	Monoprotic base	4.28	0	0.58	1.28E-17	0.0005
Ketoconazole	1.00E-06	531.4	4.35	Diprotic base	2.94	6.51	0.69	5.66E-15	40

Propioconazole	1.00E-06	342	3.72	Neutral	0	0	1.45	0.000174	150
Prothioconazole	1.00E-06	344.27	2.37	Neutral	0	0	0.58	9.60E-08	300
Tebuconazole	1.00E-06	307.83	3.7	Neutral	0	0	1.36	1.47E-05	32
1,2,4-triazole	1.00E-06	69.007	-0.58	Neutral	0	0	0.68	0.155	424400
Rotenone	1.00E-06	394.42	4.1	Neutral	0	0	1	3.49E-05	1.13E-08
Tunicamycin	1.00E-06	816.9	-0.3	Neutral	0	0	1	5.85E-38	36.43
2-phenylphenol	1.00E-06	170.21	3.32	Neutral	0	0	1	0.00436	535.8

Table A1.18. Cell composition for different cells in the in vitro assays

Parameter	CALUX assay	Toxprofiler	Cell painting	PHH transcriptomics	H295R steroidogenesis	DNT (UKN2)	DNT (UKN 4)	DNT (UKN 5)
Fraction of cell volume comprising intracellular water	0.314	0.586	0.586	0.586	0.63	0.678	0.678	0.678
Fraction of cell volume comprising neutral lipid	0.074	0.0348	0.0348	0.0348	0.0284	0.051	0.051	0.051
Fraction of cell volume comprising neutral phospholipid	0.0011	0.0252	0.0252	0.0252	0.0111	0.0565	0.0565	0.0565
Fraction of cell volume comprising lysosomes	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
Fraction of cell volume comprising mitochondria	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
pH of intracellular water	7.12	7	7	7	7	7.12	7.12	7.12
pH inside lysosomes	4	4	4	4	4	4	4	4

pH inside mitochondria	8	8	8	8	8	8	8	8
membrane potential of the cell (mV)	-41	-70	-70	-70	-41	-41	-41	-41
membrane potential of the lysosome (mV)	10	10	10	10	10	10	10	10
membrane potential of the mitochondria (mV)	-120	-120	-120	-120	-120	-120	-120	-120
cellular concentration of acidic phospholipids (mg/g)	0.67	5.09	5.09	5.09	1.32	0.4	0.4	0.4
Average diameter of a single cell (μM)	20	30	30	30	20	12	10	12
Number of cells at the time of the assay	6666	9000	6000	70000	75000	20000	30000	25000

Table A1.18. Cell composition for different cells in the in vitro assays

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Fraction of cell volume comprising intracellular water	0.314	0.586	0.586	0.586	0.63	0.678	0.678	0.678
Fraction of cell volume comprising neutral lipid	0.074	0.0348	0.0348	0.0348	0.0284	0.051	0.051	0.051
Fraction of cell volume comprising neutral phospholipid	0.0011	0.0252	0.0252	0.0252	0.0111	0.0565	0.0565	0.0565
Fraction of cell volume comprising lysosomes	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
Fraction of cell volume comprising mitochondria	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
pH of intracellular water	7.12	7	7	7	7	7.12	7.12	7.12
pH inside lysosomes	4	4	4	4	4	4	4	4
pH inside mitochondria	8	8	8	8	8	8	8	8
membrane potential of the cell (mV)	-41	-70	-70	-70	-41	-41	-41	-41
membrane potential of the lysosome (mV)	10	10	10	10	10	10	10	10
membrane potential of the mitochondria (mV)	-120	-120	-120	-120	-120	-120	-120	-120
cellular concentration of acidic phospholipids (mg/g)	0.67	5.09	5.09	5.09	1.32	0.4	0.4	0.4
Average diameter of a single cell (µM)	20	30	30	30	20	12	10	12
Number of cells at the time of the assay	6666	9000	6000	70000	75000	20000	30000	25000

Table A1.19. Culture conditions for different assays

Parameters	CALUX assay	Toxprofiler	Cell painting	PHH transcriptomics	H295R steroidogenesis	DNT (UKN2)	DNT (UKN 4)	DNT (UKN 5)
pH of cell media culture	7.4	7.4	7.4	7.4	7.4	7.4	7.4	7.4
fraction of cell culture media comprising fetal bovine serum	0.105	0.1	0.05	0	0.025	0	0	0.0024
pH of fetal bovine serum	7	7	7	7	7	7	7	7
Mass of albumin in 1 litre of fetal bovine serum (g)	23.5	23.5	23.5	23.5	23.5	23.5	23.5	23.5
Concentration of triacylglyceride in fetal bovine serum (mmol/L)	0.52	0.52	0.52	0.52	0.52	0.52	0.52	0.52
Total volume culture media per well (µl)	100	50	100	100	1000	125	100	100
Diameter of a well of the culture vessel (mm)	2.67	3.26	3.26	6.4	11.37	6.4	6.1	6.4
Total volume of a well of the culture vessel (µl)	112	145	145	360	1700	360	390	360
Culture temperature (°C)	37	37	37	37	37	37	37	37

5.5. DNT assays

The cMINC Neural Crest Cell Migration Assay (UKN2 Test)

Three different models are used:

- prediction model for screening:
hit = inhibition of NCC migration while viability is not changed:
Migration $\leq$ 80% of DMSO control
Viability $\geq$ 90% of DMSO control
- prediction model for compound hazard evaluation:
hit confirmation testing: $EC_{10} \text{ Viability (V)} / EC_{25} \text{ Migration (M)} \geq 1.3 \rightarrow$ specific migration inhibitor of NCCs
- prediction model for borderline compounds:

A ratio of BMC10 Viability (V) / BMCL25 Migration (M) ≥ 1.3 is considered a borderline hit. In some scenarios the viability does not reach the BMC10 Viability (V) necessary for the ratio calculations. In this case the highest tested concentration (HTC) was used. A full overview with a schematic representation of the UKN4 prediction model can also be found in supplementary Figure 3 of Blum et al., 2022 (Blum, Masjosthusmann et al. 2023).

The NeuroTox Neurite Outgrowth of CNS Neurons Test (UKN4 Test)

Three different models are used:

1. prediction model for screening:

hit = decrease/increase in neurite area while viability is not changed (compare to narciclasine positive control): Neurite area $\leq 80\%$ of DMSO control (For early screening tiers 80% can be used as a more conservative filter than 75%) TEMP

Viability $\geq 90\%$ of DMSO control

2. prediction model for compound hazard evaluation:

hit confirmation testing; BMC25 Viability (V) / BMC25 Neurite Area (NA) $\geq 4 \rightarrow$ specifically neurotoxic

3. prediction model for borderline compounds:

A ratio of BMC25 Viability (V) / BMCL25 Neurite Area (NA) ≥ 4 is considered a borderline hit. In some scenarios the viability does not reach the BMC25 necessary for the ratio calculations. In this case the highest tested concentration (HTC) was used. A full overview with a schematic representation of the UKN4 prediction model can also be found in supplementary figure 3 of Blum et al., 2022 (Blum, Masjosthusmann et al. 2023).

The PeriTox Neurite Outgrowth of PNS Neurons Test (UKN5 Test)

Three different models are used:

1. prediction model for screening:

hit = decrease/increase in neurite area while viability is not changed (compare to narciclasine positive control):

Neurite area $\leq 75\%$ of DMSO control

Viability $\geq 90\%$ of DMSO control

2. prediction model for compound hazard evaluation:

hit confirmation testing; BMC25 Viability (V) / BMC25 Neurite Area (NA) $\geq 3 \rightarrow$ specifically neurotoxic

3. prediction model for borderline compounds:

A ratio of BMC25 Viability (V) / BMCL25 Neurite Area (NA) ≥ 3 is considered a borderline hit. In some scenarios the viability does not reach the BMC25 necessary for the ratio calculations. In this case the highest tested concentration (HTC) was used.

A full overview with a schematic representation of the UKN4 prediction model can also be found in supplementary figure 3 of (Blum et al., 2023) (Masjosthusmann et al., 2020).

6. Metabolic competence

6.1. Caco2 permeability assays – Caco-2 cells are not metabolically competent in most cases (exceptions exist for e.g. proteases, hydrolases). Permeability data generated does thus not factor in any metabolism of the compound in question in the gut wall.

6.2. CALUX® Reporter Gene Assays: U2OS cells are non-metabolizing

6.3. Cell Painting Plus (CPP) Assays: HepG2 cells have limited metabolic competence

6.4. Chemical Effect Predictor (CEP): not applicable

6.5. Danish (Q)SAR Database: not applicable

6.6. Enterocyte metabolism

Isolated primary human jejunal enterocytes have been demonstrated to show expression levels of phase I and II metabolic enzymes similar to the human *in vivo* jejunal epithelium. Metabolic experiments have shown measurable activity of several phase I (in particular CES and CYP3A4) and phase II (SULT:s and UGT:s) enzymes.

6.7. Intrinsic metabolic clearance assays and in vitro metabolite profiling: Hepatocytes (cells derived from liver tissue) contain the full complement of metabolizing enzymes, maintained within the intact cell. Hepatocytes therefore provide the best *in vitro* model for predicting *in vivo* metabolic hepatic clearance.

6.8. PBK and biokinetics modelling: not applicable

6.9. ToxProfiler Stress Reporter Assays: HepG2 cells have limited metabolic competence

6.10. PHH TXG-MAPr pathway enrichment analysis: Plated primary human hepatocytes have shown limited metabolic competence

6.11. DNT assays

The cMINC Neural Crest Cell Migration Assay (UKN2 Test)

No specific information available

The NeuriTox Neurite Outgrowth of CNS Neurons Test (UKN4 Test)

No specific information available

The PeriTox Neurite Outgrowth of PNS Neurons Test (UKN5 Test)

No specific information available

6.12. RPTEC/TERT1

RPTEC/TERT1 cells exhibit metabolic activity representative of human renal proximal tubules, including phase I and II enzyme functions relevant to xenobiotic metabolism. Their differentiated state supports oxidative metabolism and mitochondrial function. Although OAT1 and OAT3 transporters are absent, other transporters such as Na⁺/K⁺-ATPase and efflux pumps maintain epithelial polarity and barrier integrity. These cells provide a stable and reproducible model for assessing compound-induced transcriptional responses related to oxidative stress, mitochondrial dysfunction, and nephrotoxicity. For more information on the RPTEC/TERT1 metabolic competence refer to (Aschauer et al., 2013; Aschauer et al., 2015).

6.13. Uncertainty analysis:

Not applicable

7. Status of development, standardization, validation, strengths and weaknesses

This section describes the development status of the NAM test battery, also indicating the strengths and weaknesses of the information source, compared to existing similar non-testing or testing methods. Other aspects include the reliability of the methods within and between laboratories (if applicable), their predictive capacity and proprietary information.

7.1. Caco2 permeability assays

Strengths: Easy to use and gives reliable and reproducible permeability measurements for all compounds that can be analyzed. The method is based on an easily available cell line.

Weaknesses: Compounds with low solubility or high plastic binding can present problems. Our development of the method also measures compound accumulation in cells and compounds bound to plastic, improving Papp estimates and mass balance calculations. The method needs a reliable quantification method such as UPLC-MSMS, fluorescently or radioactively labeled compounds. Training to achieve high quality measurements takes some time.

Reliability: High with inter-lab variability. The method should be validated and calibrated in each lab using a compound set spanning from low to high absorption compounds.

Predictive capacity: High – is considered a gold standard in the pharmaceutical industry.

Proprietary aspects: None

7.2. CALUX® Reporter Gene Assays

Strengths:

- Direct measure of nuclear receptor-based activity which is crucial in the understanding in compounds' mode of action.
- Highly sensitive which allows determination of PoD at low concentrations
- High-throughput and efficient screening tool; assay is performed on an automated high-throughput screening platform and allows dose-response characterization over multiple concentrations (typically 12) and triplicate.

Weaknesses:

- Does not directly predict adverse outcomes. Although for well-characterized receptor-mediated effects (e.g., estrogenic), CALUX® results can show good correlation with *in vivo* studies.
- Rarely compounds may interfere with the luminescence read-out and produce false positives (e.g. stabilization). Such compounds are spotted through data analysis.

Reliability:

- Each test item is tested in triplicate; each plate includes a full dose-response of the reference compound and QA/QC is performed on every plate.
- All assays have standardized SOP. Technology has been successfully transferred to other labs (see literature), validation guidelines are available and some tests received OECD approval (ERa and AR CALUX; OECD TG455 and TG458).

Predictive capacity: Good predictor of receptor mediated effects for outcomes primarily driven by receptor/pathway activation/inhibition (e.g., estrogen receptor)

Proprietary aspects: CALUX® cell lines are developed, proprietary and licensed through BDS (CALUX® itself is a registered trademark).

7.3. Cell Painting PLUS (CPP) Assays

Strengths:

- Cell Painting (CP) is the widely utilized high-throughput phenotypic profiling (HTPP) method for multiplexed staining and analysis of subcellular compartments and organelles.
- The CPP assay enhances the original CP assay by separating channels and improving image analysis, leading to better organelle specificity and more diverse phenotypic profiles.
- CP assays were used to generate bioactivity profiles for over 1,000 industrial chemicals in U2OS osteosarcoma cells, which are included in the U.S. EPA CompTox Chemicals Dashboard.
- The JUMP-Cell Painting Consortium has created the largest dataset of images and phenotypic profiles for more than 135,000 pharmaceutical compounds and genetic modifications, including CRISPR-Cas9 interventions with CP assay.
- Future studies with larger compound libraries aim to further evaluate the predictive capacity of the CPP assay.

Weaknesses: the CPP assay does not directly predict adverse outcomes; it focuses on key events and modes of action (MOAs) such as mitochondrial, tubulin, and DNA toxicity.

Reliability:

- The CPP assay shows high robustness across different biological replicates.
- An inter-laboratory comparison is currently in progress, while the inter-laboratory reliability of the CP assay has been well-documented in existing literature (Bray et al., 2016; Nyffeler et al., 2023; Pahl et al., 2023; Wolff et al., 2025).

Predictive Capacity: CPP assays can predict various key events and MOAs but do not aim for direct predictions of adverse outcomes.

Proprietary Aspects: The CPP assay, along with its image and data analysis pipelines⁴⁸, is publicly available for all laboratories (Elena von Coburg et al., 2025).

Robustness:

- The correlation between the four biological replicates within one screen is around 0.8, which is very robust.
- Saccharin was used as a negative reference control, and cytochalasin D and rotenone were used as positive reference controls for each assay.
- So far, we have performed two CPP screens containing altogether 7 biological replicates with HepG2 cells under the same compound treatment conditions (24 hours). We compared the heatmap of these control compounds and reviewed the average pearson correlation score of the two positive reference controls, cytochalasin D and rotenone. Among all features, the correlation scores between different biological replicates were higher than 0.75.

7.4. Chemical Effect Predictor (CEP)

Strengths: The strong points of CEP are the following: First, the variables used for training are extracted from a KG built on relationships obtained from experimental data rather than predictions. Whenever possible, high-confidence relationships are only included. Second, the KG encompasses a wide range of disease endpoints, allowing the model to predict compound hazards across multiple endpoint types, many of them

⁴⁸ <https://zenodo.org/records/14982928>

not covered by alternative approaches, simultaneously. Third, by relying on bioactivity data extracted from ChEMBL, the model can evaluate the hazard of approximately 600,000 compounds. In cases where information for a given compound is not available in ChEMBL, additional data sources (binding data or target binding predictions) can be integrated.

Weaknesses: Regarding the weaknesses, data on the targeted proteins is necessary for being able to evaluate a specific compound. Furthermore, the model assumes that the chemical mode of action involves modulation of protein activity, which may not apply to all compounds. Moreover, the KG captures *generic* information on protein interactions and relations to biological functions but does not include *contextual* information such as the relevance of these interactions in specific organs, tissues, cell types, and/or developmental stages, or gender. This kind of information could be integrated into the network to obtain *context-specific networks*, but it is not currently included in this version of CEP. Finally, the scarcity of human toxicity data for chemical compounds remains a significant limitation when training the model. To address this, the model relies on the more abundant data from drug-induced adverse effects for training and evaluation.

Reliability (within and between laboratories): Not applicable

Predictive capacity: The CEP outputs the probability that a query compound is associated with a specific disease endpoint while simultaneously evaluating its association across all disease endpoints represented in the knowledge graph (KG). The CEP was trained using data from OnSIDES, achieving sensitivity of 0.78, specificity of 0.75, and an AUC-ROC of 0.84 on an independent test set from OnSIDES.

Proprietary aspects: The CEP tool is proprietary and protected by intellectual property rights held by Medbioinformatics Solutions SL.

7.5. Danish (Q)SAR Models

Strengths: The Danish (Q)SAR Models website offers free access to predict user-defined chemical structures in >60 DTU-developed models. Most of the models (except the ones where manuscripts are in preparation) have been documented in the (Q)SAR Model Reporting Format (QMRF, template version from the QMRF Editor software) which are available from the website. Upon user import of structure information (SMILES, MOL or drawing in editor), the structure is curated in the system to have same format as used for model development (specified in the QMRF, e.g. stripping off ions and neutralizing parent, checking for unacceptable atoms or multiple constituents). The Danish (Q)SAR Models website also offers generation and download of (Q)SAR Prediction Reporting Formats with details for a prediction for a substance in a given model. This report can be used for expert review and details alerts identified, closest neighbors in the training set, and the probability associated with the prediction^{49,50}.

Weaknesses: Their accuracy relies on high-quality data, and they may not be applicable to all chemical classes. All models will give wrong predictions in some cases, even also when predictions obtained are within the defined applicability domain (the system reports whether or not predictions are in the domain). Expert judgment is needed to understand their results.⁵¹

Reliability: All models have undergone robust leave-many-out cross-validation and the results for binary endpoints in terms of sensitivity, specificity etc. are given in the QMRF documentation⁸.

⁴⁹ https://qsar.db.food.dtu.dk/presentations/DK_DB_QSAR_2021.pdf

⁵⁰ Nikolov, N. G., Cross, K. P., Quigley, P., Dybdahl, M., Reffstrup, T. K., Abildgaard Rosenberg, S., & Wedebye, E. B. (2016). Danish (Q)SAR Models: A free online DTU QSAR predictor powered by Leadscope. Poster session presented at QSAR 2016 conference, Miami Beach, Florida, United States.

⁵¹ <https://qsar.db.food.dtu.dk/db/index.html>

Predictive capacity: For all models, the applicability domain within which the performance is measured in the validation is clearly defined and documented in the QMRFs⁹. The results presented in this case study were based on prediction output conducted on May 17th 2024.

Proprietary aspects: Free for everyone to use, works in a browser, no need to install programs, no plugins or add-ons required. All major browsers and operating systems are supported. Reproduction of information from the database is permitted provided the source is acknowledged as follows: “Danish (Q)SAR Database, Research Group for Chemical Risk Assessment and GMO, National Food Institute, Technical University of Denmark,, National Food Institute, Technical University of Denmark, <http://qsar.food.dtu.dk>.”

7.6. Enterocyte metabolism

Strengths: Expression and activity of metabolic enzymes closely mimicking the human jejunal epithelium.

Weaknesses: The method needs access to freshly isolated human jejunal tissue samples, which requires human ethical approval and easy access to human tissue by collaboration with surgical teams at a nearby hospital. We are investigating if cells can be cryopreserved after isolation which would simplify access to the enterocytes. The tissue samples derived from obese patients might not show the same expression levels of metabolizing enzymes as the average human population, however initial proteomic studies suggest that the differences to the average human population seem small.

Reliability: Under investigation. So far data seems highly reproducible with a variation that could be the result of inter-donor variation in enzyme activity.

Predictive capacity: In light of initial studies predictivity of human jejunal metabolism seems high.

Proprietary aspects: None

7.7. Intrinsic metabolic clearance assays and *in vitro* metabolite profiling

No officially adopted standard test method, however primary human hepatocyte metabolism is considered as the gold standard for prediction of *in vivo* human hepatic metabolism. The method has been used for several decades in the pharmaceutical industry.

Strengths:

- Use of human cells result in prediction of human liver metabolism including both phase I and phase II enzymes.
- Supports IVIVE for estimating *in vivo* hepatic clearance
- Multiple donor hepatocyte pools can be used to assess variability in the population.
- The commercial availability of cryopreserved primary human hepatocytes makes the material easily accessible.

Weaknesses:

- Limited viability of human hepatocytes in suspension or culture limits the determination of Clint for metabolically stable compounds.
- If extrahepatic metabolism is prominent for a compound this will not be captured by the method.

Reliability (within and between laboratories):

- Reliability and reproducibility for the method is high when using standardized protocols by skilled persons.
- Standardization of protocols allows comparison of data between labs with good reliability.

- Individual variability between hepatocyte donors introduces variability into results. This can be circumvented using hepatocyte pools derived from multiple donors (10-15 donors commonly used).

Predictive capacity: Clint from primary human hepatocytes is considered to be highly predictive of hepatic clearance.

Proprietary aspects: None

7.8. PBK and biokinetics modelling

PBK modelling

Strengths: PBK models offer mechanistic insights and allow for dynamic simulation of chemical behavior across biological systems. Different inbuilt populations in the simulation platform and route of exposure can be considered in the development of PBK models. Their ability to integrate diverse data types and parameters makes them particularly useful for IVIVE and risk assessment. The models predict concentration-time profiles in various tissues within the body on exposure to an external dose of a compound. It is therefore a powerful alternative to animal testing aligning with the principles of 3Rs.

Weaknesses: PBK model building requires detailed input data (e.g., measure of permeability to predict absorption in the gut, fraction unbound in plasma, metabolic rates). Many parameters may be estimated or poorly defined, affecting accuracy. For example, fraction unbound in plasma for all the conazoles is close to 0.1 to less than 0.1 which can be difficult to measure accurately by standard *in vitro* methods. Further, the model outputs can be sensitive to fraction unbound in plasma when it is less than 0.1. Also, PBK models can be difficult to develop and validate specially for chemicals where there is limited data available.

Reliability and Predictive capacity: The PBK models developed in this case study were not validated for all conazoles due to lack of human *in vivo* data. We compared the output of C_{max} of ketoconazole with that observed for human for similar oral dose. The observed C_{max} of ketoconazole at an oral dose of 200 mg is observed to be 2 µg/ml (Van Tyle, 1984) while the PBK model developed using the parameters outlined in this case study predicted it as 2.13 µg/ml thus increasing our confidence in the model output. Further, another case study carried out in the Riskhunt3R project using 15 compounds showed that systemic clearance was either underpredicted (n=8) or was in the range (n=7) when scaled from clearance measured in primary human hepatocytes using PBK models developed using similar methods as in this case study (data not published). Thus, even if the IVIVE may not be 100% accurate, the underprediction of clearance shifts prediction of C_{max} and AUC on to the conservative side in the risk assessment of the chemicals.

Proprietary aspects: None

Biokinetics modelling

Strengths: Biokinetics modelling leverages the physicochemical properties of compounds and the specific culture conditions to predict their distribution within *in vitro* assay systems. This approach eliminates the need for direct measurement of compound distribution, which can be both costly and time-consuming.

Weaknesses: Biokinetics model relies on the physicochemical properties of the compounds to predicts its partitioning into air, plastic, medium and cells. Thus, it is highly dependent on the inputs for model development including logPow and Henry's law constant.

Reliability and Predictive capacity: Biokinetics model used in this case study used published data to validate its output (Fisher et al., 2019). The output of the model is highly dependent on the inputs for the physicochemical properties of the compounds. We have used measured logPow values of all the compounds, except for prothioconazole where we could not find measured logPow value, to increase accuracy of the model output. As the model is static, it does not consider compound loss in the assay due to metabolism or degradation. We expect this is not an issue for the assays considered here, as the cells used in the assays have no or limited metabolic capacity.

Proprietary aspects: None

7.9. ToxProfiler Stress Reporter Assays

ToxProfiler, offered by Toxys, helps assess toxicological safety by measuring seven biomarker protein responses in human liver cells. Each biomarker is closely linked to its biological pathway, and the assay shows high reliability, supported by consistent results from reference compounds and a rescreening of ten compounds.

Here are some typical applications of ToxProfiler:

- Early-phase *in vitro* safety screening: It provides a quick and dependable way to generate detailed toxicological profiles, which is valuable for compound selection and development.
- Weight-of-evidence approach: ToxProfiler can be used as a follow-up to regulatory tests, giving insights into how compounds work and aiding hazard assessments.
- Read-across approaches: It offers mechanistic insights that help cluster chemicals with similar reactivity based on their toxic properties.
- Late-phase *in vitro* screening: The assay can clarify specific pathological outcomes seen in *in vivo* studies.

While the assay has been validated internally and detailed in a peer-reviewed publication (Ter Braak et al., 2024) , it has not undergone formal validation. The results from same compounds tested in 2020 and 2023/2024 show that the results from Toxprofiler are highly consistent. The results are available in ToxTemp.

7.10. PHH TXG-MAPr pathway enrichment analysis

Strength:

- PHH is the golden standard of the liver *in vitro* test system currently
- Transcriptomics measuring broader end points capturing multiple cellular responses
- TXG-MAPr reduces the number of the dimension from the transcriptomics end points (90% from ~14000 genes to 389 gene networks)
- TXG-MAPr enrichment provides the biological relevant score reflecting the modulation of the perturbed pathways/responses

Weakness:

- The response of the PHH is limited to the 10 donors (pooled)
- PHH showed to have lower metabolic capacity compared to fresh tissue
- Despite measuring broader responses, transcriptomics also introduces high noise that can influence the accuracy of the biological interpretation
- The scores of the modules (EGs) are the aggregated value from the log2 fold change of the genes inside a particular module. These values can inaccurately interpret the module activity when the log2 fold change values of the genes are not significant.

Reliability (within and between laboratories):

- Inter-laboratories variabilities of the PHH have been reported, this variability is to some extent reduced by using 10 donor pools
- Transcriptomics output also shows inter-laboratory variability

Predictive capacity: None

Proprietary aspects: The access to the PHH TXG-MAPr at this point is limited to RH3R consortium members. The user needs to request the credential before using this platform. The script to build TXG-MAPr can not be disclosed.

7.11. DNT Assays

Strength:

- Assays are well documented in the ToxTemp including performance parameters etc.
- SOPs are available at DB-Alm ECVAM database.

Weakness:

- (Neuro)development cannot be covered by single *in vitro* assays. To cover all relevant biological processes, a battery is required.
- Uncertainty remains to be defined.
- No formal OECD 34 validation has been done e.g. RING trials.

Predictive capacity:

A screen was performed in the context of the EFSA DNT test battery evaluation with 120 compounds (Masjosthusmann et al., 2020). From this screen the following performance indicators were obtained:

- Specificity of DNT IVB: 100% --> Also with cMINC, NeuroTox or PeriTox as standalone assay in 17 'tool negatives' tested (Masjosthusmann et al., 2020).
- Sensitivity of DNT IVB: 82.7% à With cMINC, NeuroTox and PeriTox combined in a full DNT battery and a selected set of 27 positive compounds with evidence for DNT (Masjosthusmann et al., 2020).

Details on additional screens, inter/intra lab performance etc. are available in the ToxTemp.

Proprietary aspects: The cells are not protected by patents or any other licences.

7.12. RPTEC/TERT1 Test Method for Transcriptomics

Strengths

- RPTEC/TERT1 is a well-characterised human renal proximal tubular epithelial model that reflects key structural and metabolic features of the kidney.
- The differentiated, contact-inhibited state supports oxidative metabolism and mitochondrial function comparable to primary human proximal tubular cells, enabling detection of stress-response and nephrotoxicity-related pathways.
- TempO-Seq® transcriptomics captures genome-wide expression responses (~16,000 genes), providing a comprehensive molecular overview. RPTEC/TERT1 cells show high stability and reproducibility for transcriptomic applications.
- The assay allows high-throughput profiling directly from cell lysates, minimising variability associated with RNA extraction.
- Mechanistic control compounds (tunicamycin and rotenone) confirm biological responsiveness of ER-stress and mitochondrial-stress pathways.

Weaknesses

- RPTEC/TERT1 cells express Phase I and II enzymes at levels closer to primary proximal tubular cells but lack uptake transporters such as OAT1 and OAT3, limiting representation of compound accumulation mechanisms *in vivo*.

- As a single immortalised cell line, the model does not account for donor variability or contributions from other renal cell types (e.g., podocytes, endothelial cells).

Reliability (within and between laboratories)

- RPTEC/TERT1 demonstrates stable morphology, viability, and gene-expression profiles across passages (75–90) under standardised culture and exposure conditions.
- The TempO-Seq® platform provides high technical reproducibility through direct lysis and probe-based detection, reducing operator variability.

Predictive Capacity

- The model reliably detects transcriptional perturbations associated with oxidative stress, mitochondrial dysfunction, apoptosis, and ER-stress pathways.
- It is suitable for mechanistic screening and nephrotoxicity-related mode-of-action studies but less appropriate for metabolism-driven toxicity prediction.

Proprietary aspects: The RPTEC/TERT1 cell line is a commercially available product under intellectual property protection by Evercyte GmbH. All intellectual property rights associated with the assay and the cell line are respected and managed according to the respective ownership agreements.

7.13. Uncertainty analysis

Strength:

- Applies the state-of-art uncertainty characterisation framework to evaluate the combined impact of source of uncertainty on the answer to an assessment question, with options to reduce or refine uncertainty.
- Provides quantitative characterisation of uncertainty from within- and across *in vitro* assays
- Provides quantitative characterisation of uncertainty from PBK modelling
- Provides a structured approach for characterising overall uncertainty by expert judgement supported by qualitative and quantitative methods.

Weakness:

- Assumes normal distribution to represent uncertainty in the log10 of benchmark concentration
- Characterisation of overall uncertainty depends on selected experts and the use of skilled facilitators for formal expert judgement

Reliability: Not applicable

Predictive capacity: Not applicable

Proprietary aspects:

The access to the uncertainty characterisation framework at this point is limited to RISKHUNT3R consortium members. The user needs to request the credential before accessing the framework guideline document.

8. Technical limitations and limitations with regard to applicability

8.1. Solubility and stability

The cellular models are limited to compound, which can be solubilized in the test medium, without the usage of solvents that interfere with the test system. This aspect was considered in a pretesting phase, in which stock solutions were prepared in either DMSO or water. The final *in vitro* tested concentrations did not contain DMSO contents higher than 0.1 or 0.2%. Insolubility or instability in plasma can cause technical issues as a sufficient concentration for analysis needs to be obtained for the experiment to be successful. Inherent instability of the molecule in plasma, buffer or medium used for the assay will lead to an overestimation of the hepatic clearance.

8.2. Plastic binding

A potential loss of the compounds based on plastic binding was considered within the calculation of the *in vitro* relevant dose. Most of the conazoles have logPow > 3 which reflects relative higher hydrophobicity. These compounds can bind to the plastic and the compound loss could be quantified using the VIVD model. The conversion ratio using VIVD model was used to correct the nominal concentration to free effective concentration.

8.3. Volatility

None of the cellular test systems in this case study is able to control the loss of volatile compounds. Therefore, (partly) volatile compounds, as confirmed by the VIVD model and the Henry coefficient, were not included in test set of chemicals.

8.4. Sensitivity

Analytical limitation relating to sensitivity, if analytical methods of sufficient quality and reproducibility are unavailable data generation will be challenging. LC-MS, or other analytical method needs to be developed for quantitative information.

8.5. Autofluorescence

None of the training set compounds is causing autofluorescence. This is an issue for the CALUX®, CPP as well ToxProfiler reporter gene assays. Such compounds are outside the applicability domain.

8.6. Nanoparticles

Dosing of particles requires a specific characterization of their agglomeration status of particles before and after dosing into the medium. This aspect is not addressed in this case study, and therefore, we assume that nanoparticles are currently outside the applicability domain of the presented approach.

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Annex II. Template for IATA Case Studies on Chemical Grouping (Read-across)⁵²

1. Purpose

This case study explores the applicability of NAM data in read-across approach. For this purpose, one target compound (TC) difenoconazole is selected. The hazard of this TC is to be addressed using a read-across approach, following subchronic repeated oral exposure. Therefore, we use a read-across approach to extrapolate the toxicity of difenoconazole from suitable subchronic repeated dose studies of relevant analogues. Eight other conazoles source compounds (SC) alongside common metabolite with vivo data have been identified. NAM data substantiate the read-across hypothesis by showing a similar profile with regard to toxicokinetic and toxicodynamic properties within the grouped compounds.

1.1. Target chemical definition

Difenoconazole is selected as hypothetical TC. For the purpose of this case study and as proof of concept, it is assumed that difenoconazole lacks sufficient data. Physicochemical data are listed below:

Name: Difenoconazole

CAS: 119446-68-3

Molecular formula: C₁₉H₁₇ClN₃O₃

Molecular weight, MW: 406.27 g/mol

Melting point, MP (purity): 82 – 83°C (99.3%)⁵²

Boiling point, BP (purity): 100.8°C (99.3%)⁵²

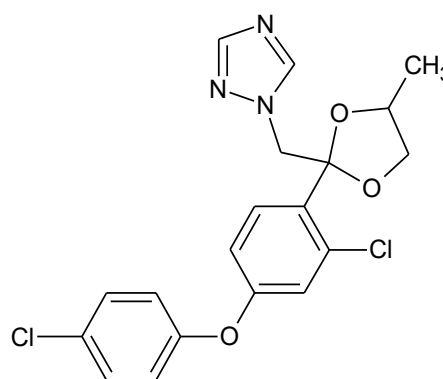
Vapor pressure, VP (purity): 3.32 x 10⁻⁸ Pa at 25°C (99%)⁵²

Henry's Law constant: 9 x 10⁻⁷ Pa m³ mol⁻¹ at 25°C⁵²

Water solubility, WS: 15 ± 1.3 mg/l at pH 7.2 and 25°C⁵²

logPow: 4.86⁵³

Dissociation constant, pka (purity): 1.07 ± 0.18 (99.3%)



Smiles:

C1COC(CN2C=NC=N2)(O1)C1=C(Cl)C=C(OC2=CC=C(Cl)C=C2)C=C1

⁵² EFSA (European Food Safety Authority), 2011. Conclusion on the peer review of the pesticide risk assessment of the active substance difenoconazole. EFSA Journal 2011;9(1):1967. [71 pp.]. doi:10.2903/j.efsa.2011.1967

⁵³ CX logP from chEMBL

Difenoconazole is the common name for 3-chloro-4-[(2RS,4RS;2RS,4SR)-4-methyl-2-(1H-1,2,4-triazol-1-ylmethyl)-1,3-dioxolan-2-yl]phenyl 4-chlorophenyl ether (IUPAC). It comprises a triazole ring, a dioxolane ring and two chlorophenyl rings.

The physicochemical properties indicate that difenoconazole has a low solubility in water (WS = 15 mg/l) and very low volatility (VP = 3.32×10^{-8} Pa) (EFSA, 2011). Its hydrophobicity is also reflected by high partition coefficient (logPow = 4.86), therefore indicating potential for bioaccumulation. Dissociation constant of difenoconazole is associated with the deprotonation of triazole ring. At physiological pH (e.g. pH = 7.2), difenoconazole is present in its neutral, non-ionized form.

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

The read-across is performed for the endpoint repeated dose toxicity (90 days) via oral application.

2. Hypothesis for the category approach

The Alternative Safety Profiling Algorithm (ASPA) workflow developed within the RISK-HUNT3R project was followed for the hazard assessment of difenoconazole. It is assumed that available data for TC, difenoconazole, is insufficient to support a standalone read-across assessment; therefore, NAM data are incorporated to strengthen the overall justification.

The information on conducting read-across is documented in read-across assessment framework (ECHA, 2017). In this case study, the selection of the compounds for this read-across was based on the shared MoA and availability of the data. This read-across will extrapolate a human relevant threshold for the fungicide difenoconazole using a NAM based testing and assessment approaches. The assessment will apply a category approach in which *in vivo* evidence from eight data rich source compounds (SCs) are used to define a read-across hypothesis. SCs (cyproconazole, epoxiconazole, fenbuconazole, hydroxyitraconazole, itraconazole, propiconazole, prothioconazole, tebuconazole) share a common “toxicophore” which is 1,2,4 triazole ring.

Available *in vivo* studies from SC show primarily liver toxicity as critical target for the category. Shared effects in oral rodent subchronic studies are liver toxicity indicating steatosis at the Lowest Observed Adverse Effect Levels (LOAELs), often progressing to liver necrosis at higher dosing (**Table A2.4.**). Several publications associate this liver toxicity with the activation of the nuclear receptors farnesoid X receptor (FXR), constitutive androstane receptor (CAR) and pregnane X receptor (PXR) (Goetz and Dix, 2009; Heise et al., 2018; Hester et al., 2012). It is discussed that receptor activation could lead to cytochrome (CYP) enzyme induction, impairment of lipid and steroid homeostasis and an increase in cell proliferation. Prolonged, chronic cell proliferation is discussed as one reason for conazoles to cause liver neoplasms. Moreover, many of the conazole compounds are known to cause cranio-facial alteration, indicating developmental toxicity. These were assessed and grouped as cumulative assessment group in 2022 (European Food Safety et al., 2022).

SCs also exhibit a similar trend in their physico-chemical properties (**Table A2.3.**). All SCs show very low Henry's law constants and VP, indicating that they are involatile. On average, the SCs also demonstrate relatively high logPow values (>3), indicating lipophilicity. The TC difenoconazole falls within this range and belongs to this category.

Therefore, NAM data from primarily high-throughput assays are utilized to provide evidence that the grouped SCs share similar toxicokinetic and toxicodynamic properties. The assays included in our *in vitro* test battery consist of CALUX®, Cell Painting Plus (CPP), ToxProfiler assays. Additionally, the results from the H295R and DNT assays, alongside transcriptomics from primary human hepatocytes (PHH) and RPTEC/TERT1 were included. *In silico* models such as chemical effect predictor (CEP) and the Danish QSAR Toolbox are presented.

Overall, this supports the read-across hypothesis that the TC difenoconazole is likely to exhibit a similar toxicological profile, including liver effects following repeated exposure in animals or humans. The read-across hypothesis suggests that the SCs and TC constitute a category and display comparable toxicological properties in terms of kinetics and dynamics. Based on this read-across hypothesis, a human-relevant threshold for TC could be derived through *in vitro* to *in vivo* extrapolation (IVIVE). This could be used to predict the NAM-based LOAEL in human equivalent doses. These are used to support the conclusion that the NAM-based LOAEL derived for the TC is comparable to allometric LOAEL obtained from *in vivo* assessments.

This case study also raises additional questions regarding the roles of metabolites. Conazoles containing a triazole ring undergo biotransformation to the metabolite 1,2,4-triazole, which is being evaluated for potential toxicological concerns. Hydroxy-itraconazole, a hydroxy metabolite of itraconazole, was included to assess whether this metabolite could behave similarly to the parent compounds. Ketoconazole was also included due to its different mode of action observed in *in vivo* studies, allowing us to compare how it differs from the other conazoles.

3. Source chemicals/category members

Target compound for this read-across approach is difenoconazole. For this, eight compounds consisting of parents alongside their metabolites were selected for the read-across evaluation. Six of these compounds (cyproconazole, epoxiconazole, fenbuconazole, propiconazole, prothioconazole and tebuconazole) generate 1,2,4-triazole as common metabolite. Hydroxy metabolites are suggested to be comparable to the parent compounds, e.g. hydroxyitraconazole. Therefore, three additional pharmaceutical azoles, which are antifungal compounds used to treat fungal infections. Ketoconazole, itraconazole and its primary metabolite hydroxyitraconazole were tested. These substances in this case studies are referred to as conazoles.

The main rationale of including the conazoles were based on shared toxicophore, common mode of action and shared common metabolite. The toxicophore, 1,2,4-triazole is responsible for the antifungal activity by inhibiting the CYP51 activity, leading to disruption of ergosterol biosynthesis and therefore membrane stability in fungi (Zarn et al., 2003). These effects induce concern of a potential disruption in human/animal sterol-based hormone synthesis. In *in vivo* repeated dose studies, conazole pesticides were causing liver toxicity, with effects ranging from changes in weight, enzyme activities, to more adverse histopathological findings progressing to steatosis effects (EFSA, 2009; Goetz and Dix, 2009).

1,2,4-triazole is not only the toxicophore of the conazole pesticides, but it also a common metabolite during their biotransformation in rat studies (DAR/RAR). Results from *in vivo* studies in DAR/RAR documents show the generation of 1,2,4-triazole in the six pesticide conazoles (RMS, 2005, 2006a, b, 2007, 2016). It is mostly generated in the later phases of metabolism round, and observed in urine, bile or faeces from rats, as well as plasma from pregnant rat (RMS, 2006c).

To assess the similarity among these SCs, two approaches are utilized. Structural similarity relative to the target compound is calculated.

Chemical similarity

Apart to the shared triazole ring, other parts of the structures from conazoles are diverse. They differ in molecular weight, as well as side chain and other functional substructures. Considering this diversity, similarity scores of all SCs relative to the TC were calculated using the MACCS fingerprint from RDKit (Landrum, 2010) and Tanimoto (Tanimoto, 1958) algorithm (**Table A2.3.**). Scores from SCs range from 37% (1,2,4-triazole) to 85% (propiconazole). Using the algorithm, compound closest to the TC is propiconazole (score 85%), followed by itraconazole (score 76%). Propiconazole share most similar substructures with TC, i.e. triazole ring, dioxolane ring and chlorophenyl. Overall, all conazoles show similarity averaging at 66.7%.

While the similarity scores provide some guidance, it is to be noted that all triazole conazoles in this exercise shared the same substructure, 1,2,4-triazole ring, which is the crucial part in the structure. Bemis and Murcko method of identifying the core structures can also be used to compare most similar SC (Bemis and Murcko, 1996; Landrum, 2010). Using this method, all SC and TC core structure are retained, while the non-essential groups are removed. Within the SC, TC difenoconazole shared 1,2,4-triazole ring substructure with cyproconazole, epoxiconazole, fenbuconazole, hydroxyitraconazole, itraconazole, propiconazole, prothioconazole, tebuconazole and the common metabolite 1,2,4-triazole. Moreover, TC difenoconazole shares most of its substructures with propiconazole, itraconazole and hydroxyitraconazole.

4. Justification of data gap filling

This section offers a summary of the data and assays employed for read-across evaluation, organized into three key sections to facilitate understanding and analysis:

- *In vivo* endpoint data:
Include summary from subchronic studies conducted in rats and mice, with focus over a duration of 13 weeks with oral exposure. The detailed summary of each study, alongside with proposed LOAELs is provided in **Table A2.4**.
- Toxicokinetic data – ADME properties (**Annex I**):
Physicochemical Properties and Other Molecular Descriptors: include data regarding the physicochemical properties, including logPow, water solubility and bioaccumulation potential and results from PBK and biokinetics modelling (**Annex I, Table A1.6. to Table A1.15.**).
- Toxicodynamic data – Hazard characterization by NAMs (**Annex III**):
Include results from CALUX® assays (**Annex III, Table A3.4.**), ToxProfiler reporter gene assays (**Annex III, Table A3.5.**) and CPP assays (**Annex III, Table A3.6.**). Additionally, analysis results from transcriptomics data from primary human hepatocytes (PHH) and RPTec/TERT1 were presented (**Annex III, Section 6**), alongside the H295R steroidogenesis assay (**Annex III, Section 7**) and developmental neurotoxicity assays (DNT) (**Annex III, Section 8**). *In silico* model from the Chemical Effect Predictor (CEP) (**Annex III, Figure A3.3.**) and Danish QSAR Toolbox⁵⁴ (**Annex III, Table A3.2.**) provide prediction results related to these compounds.
- Acceptable daily intake (ADI)
To obtain a highly conservative estimate of the theoretical formation of 1,2,4-triazole from parent conazoles, the ADIs established by EFSA for each parent compound were used as input doses in the PBK model (EFSA et al., 2022). Using this approach, the maximum concentration of 1,2,4-triazole that could potentially be generated under worst-case assumptions was predicted.

The full documentation of the *in silico* models and *in vitro* assays with all experimental data is provided in the Annex I (Description of test methods) the results are reported in Annex III (IATA Outcomes).

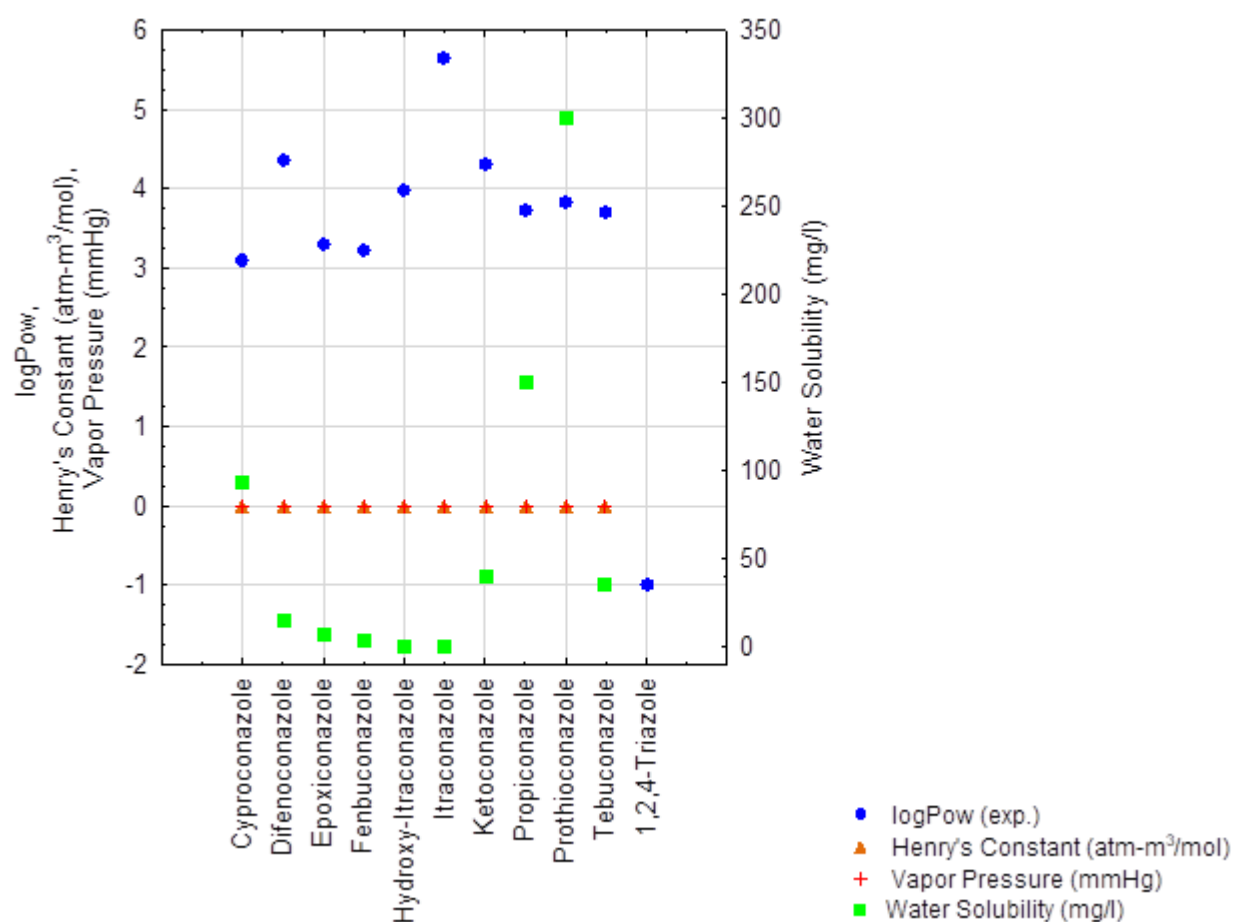
Physicochemical properties of the compounds

The octanol-water partition coefficient (logPow) values for the conazoles range from -1 for 1,2,4-triazole, followed by 3.09 (cyproconazole) to 5.66 (itraconazole). The differences in logPow among the conazole compounds are minimal, considering only the parent compounds. These logPow values indicate the lipophilicity of the compounds, suggesting their ability to partition into lipophilic phases or to diffuse across biological membranes. All conazoles fall within this lipophilic range. The TC difenoconazole is in comparison to other conazoles on the higher end, however still within this range, at 4.36. The Henry's law constants for the compounds range from 5.5×10^{-10} atm·m³/mol for cyproconazole to 4.9×10^{-6} atm·m³/mol for

⁵⁴ <https://qsarmodels.food.dtu.dk>

difenoconazole. Together with vapor pressures significantly below 1 atm (normal atmospheric pressure), this indicates that these substances are not volatile. Water solubility varies among the compounds, with values ranging from 0.0005 mg/L for itraconazole to 300 mg/L for prothioconazole. It is important to note that the water solubility of prothioconazole is influenced by the pH of the testing conditions. Specifically, prothioconazole solubility can range from 5 mg/L at pH 4, to 300 mg/L at pH 8, and up to 2000 mg/L at pH 9 (RMS, 2005). For this analysis, we assume neutral conditions and have used the value for pH 8. This highlights the significant impact that minor changes in pH can have on prothioconazole's water solubility. Overall, all compounds tend to exhibit insoluble to slightly soluble properties regarding water solubility. Itraconazole, with a solubility of 0.0005 mg/L, is at the lower end of this range, along with hydroxy-itraconazole, which has a solubility of 0.334 mg/L. The increase in solubility for hydroxy-itraconazole can be attributed to the presence of the hydroxyl group, which enhances solubility. Other conazoles fall within a range below 150 mg/L, indicating slight solubility properties. The target compound difenoconazole shows values within this range, which indicates the same category with other conazole compounds.

Figure A2.1. Overview of physicochemical properties of TC, SC and their metabolites



Note: Water solubility for 1,2,4-triazole is out of range (730000 mg/l) and therefore not shown.

In vivo endpoint data

In vivo studies were gathered for all compounds of the read-across group (Table A2.1. and Table A2.4.). Studies with oral route, conducted in rats or mice are compiled. Most of the compounds, have been evaluated in 90-day repeated dose toxicity (RDT) studies. Hydroxyitraconazole and ketoconazole are not listed in the

90-day RDT studies in the RepDose® database (Bitsch et al., 2006). However, ketoconazole has a 28-day study as supporting information (Shin et al., 2006).

Table A2.1. Number of studies available for SC and TC. Species: rats or mice, route of administration: oral (gavage, diet, drinking water), duration: subchronic (90-day studies)

CAS	Compound	Number of repeated dose toxicity studies (90-days)
94361-06-5	Cyproconazole	4
119446-68-3	Difenoconazole	2
133855-98-8	Epoxiconazole	5
114369-43-6	Fenbuconazole	3
112559-91-8	Hydroxyitraconazole	0
84625-61-6	Itraconazole	2
79156-75-5	Ketoconazole	0
60207-90-1	Propiconazole	2
178928-70-6	Prothioconazole	2
107534-96-3	Tebuconazole	1
288-88-0	1,2,4-Triazole	3

Note: Species: rats or mice, route of administration: oral (gavage, diet, drinking water), duration: subchronic (90-day studies).

Repeated dose toxicity: The *in vivo* studies collected in this case study demonstrate that liver is the main target organ after repeated dose exposure, showing primarily hypertrophy and progressing to necrosis at higher doses (summary from **Table A2.4.**).

Liver is identified as the primary target organ for most of the source compounds (SC) in repeated dose toxicity studies. These studies provide evidence that conazoles, including cyproconazole, epoxiconazole, fenbuconazole, itraconazole, propiconazole, prothioconazole, and tebuconazole, primarily affect the liver, with effects ranging from significant weight changes to necrosis and fatty accumulation. Prothioconazole particularly impacts both the liver and kidneys, with the kidney effects observed in male rats potentially being age-related. In contrast, ketoconazole shows a different mode of action by disrupting hormonal levels, leading to significant changes in reproductive hormones, such as an extended estrous cycle in females. Although the study on ketoconazole is based on a 28-day duration, this serves as evidence that it behaves differently presumably due to its unique imidazole structure, setting it apart from the other conazoles. Furthermore, 1,2,4-triazole indicates potential reproductive toxicity, especially in males, with signs such as germ cell degeneration in the epididymis and testes atrophy. In summary, the liver is consistently observed as the main target organ across most SCs, suggesting that the TC difenoconazole may exhibit similar toxicological effects.

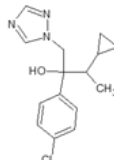
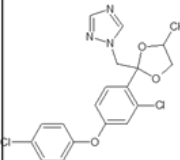
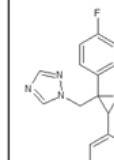
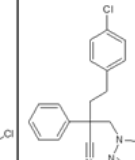
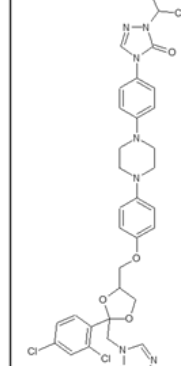
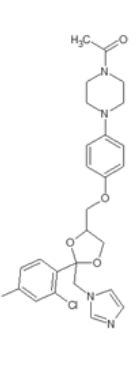
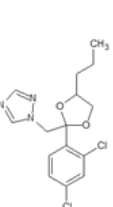
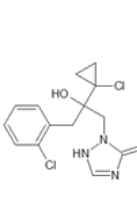
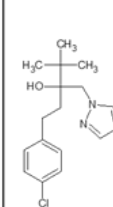
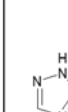
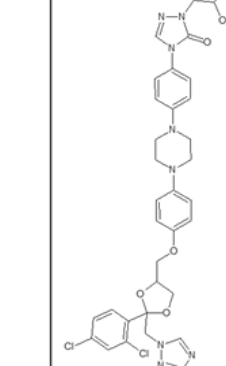
5. Uncertainty

Table A2.2 Uncertainty for the read-across approach with target compound difenoconazole

Factor	Uncertainty (low, medium, high)	Comment
Hypothesis used for the read across	Low	The conazole case study aims to explore the applicability of NAM data in read-across scenario to predict the systemic toxicity of target compound difenoconazole. Source compounds (SC) belong to same category due to the <i>in vivo</i> study indicating liver as main target organ. Studies were extracted from DAR/RAR from Rapporteur Member States (RMS) available at EFSA Public consultation website. This is considered to be reliable. Read-across hypothesis based on shared toxicological effect from SC <i>in vivo</i> data, supporting evidence available on relevant NAM methods
Structural similarity	Low	Shared toxicophore within grouped compounds.
Similarity of physico-chemical properties	Low	Consistent trend in category observed, potential for bioaccumulation if considered as one aspect for NAM testing
Similarity of toxicokinetics data	Low	<i>In vitro</i> data available to parametrize PBK model; physicochemical parameters in applicability domain of <i>in vitro</i> biokinetic model. Simulations were run for repeat doses to account for possible bioaccumulation
Similarity of other supportive data for hazard assessment	Low	Relevant NAM data selected to assess shared MoA within category and towards TC (see Annex III).
Number of analogues used for the read across	Low	Interpolation possible because of high number of source compounds.
Quality of the endpoint data used for the read across	Low	Many <i>in vivo</i> studies of high quality available for all source compounds. Studies were extracted from DAR/RAR from Rapporteur Member States (RMS) available at EFSA Public consultation website. There are small variation regarding the guideline for the studies, which is all indicated in the conclusion and executive summaries in the dossiers. Some studies were conducted according to guidelines or methods considered similar or equivalent to OECD guidelines. Some studies lack defined guidelines, or the guidelines are not specified. This served as supporting evidence. Hydroxy itraconazole and ketoconazole have few to no available <i>in vivo</i> rodent studies. Some ketoconazole studies are limited to 28 days, which may be insufficient for predicting longer-term effects. Our case study aims to assess toxicity at a subchronic level (90 days) for a better understanding of potential risks. Nevertheless, the results can still serve as supportive evidence.
Similarity of the endpoint data (among source chemicals)	Low	High concordance in shared <i>in vivo</i> toxicological profile
Concordance and weight of evidence of all data used for justifying the hypothesis	Low	NAM methods relevant for read-across hypothesis.
Overall uncertainty of the read-across	Low	

Note: Low – absence of conflicting evidence. Moderate – good evidence from primary line of data, few data gaps. High – some evidence, doubts can be raised about data gaps and relevance of approaches/data.

Table A2.3. Overview on target (TC) and selected source compounds (SC)

	SC 1	TC	SC 2	SC 3	SC 4	SC 5	SC 6	SC 7	SC 8	MET	MET
CAS	94361-06-5	119446-68-3	133855-98-8	114369-43-6	84625-61-6	79156-75-5	60207-90-1	178928-70-6	107534-96-3	288-88-0	112559-91-8
NAME	CYPROCONAZOLE	DIFENOCONAZOLE	EPOXICONAZOLE	FENBUCONAZOLE	ITRACONAZOLE	KETOCONAZOLE	PROPICONAZOLE	PROTHIOCONAZOLE	TEBUCONAZOLE	1,2,4-TRIAZOLE	HYDROXY-ITRACONAZOLE
STRUCTURE											
SMILES	<chem>CC(C1CC1)C(O)(CN1C=NC=N1)C1=CC=C(C1)C=C1</chem>	<chem>CC1COC(CN2C=NC=N2)(O1)C1=C(C1)C=C(C1)C=C(C1)C=C1</chem>	<chem>C1=CC=C(C1)C2C(O2)(CN3C=NC=N3)C4=CC=C(C4)F1</chem>	<chem>C1C1=CC=C(C1)C2C=CC(C2)N2C=NC=N2(C#N)C2=CC=C(C2)C=C1</chem>	<chem>CCC(C)N1C(=O)N(C=N1)C2=CC=C(C2)N3CCN(CC3)C4=CC=C(C4)OC5COC(O5)(CN6C=NC=N6)C7=C(C=C(C7)C1)C1</chem>	<chem>CC(=O)N1CCN(CC1)C2=CC=C(C2)OCC3COC(O3)(CN4C=NC=N4)C5=C(C=C(C5)C1)C1</chem>	<chem>CCCC1COC(CN2C=NC=N2)(O1)C1=C(C1)C=C(C1)C=C1</chem>	<chem>OC(CN1NC=NC1=S)(C1=C(C1)C=C(C1)C1)CC1</chem>	<chem>CC(C)(C)C(O)(CC1=CC=C(C1)C=C1)CN1C=NC=N1</chem>	<chem>N1C=NC=N1</chem>	<chem>CC(C)O)N1C(=O)N(C=N1)C2=CC=C(C2)N3CCN(CC3)C4=CC=C(C4)OCC5COC(O5)(CN6C=NC=N6)C7=C(C=C(C7)C1)C1</chem>
INCHI KEY	UFNOUKDBUIZYDE-UHFFFAOYSA-N	BQYJATMQXGBDHF-UHFFFAOYSA-N	ZMYFCFLJBGAQRS-IRXDYDNUSA-N	RQDJADAKIFFEKQ-UHFFFAOYSA-N	VHVFPQPYKVGDNFY-UHFFFAOYSA-N	XMAYWYJOQHxEE-K-UHFFFAOYSA-N	STJLVHWMYQXC-PB-UHFFFAOYSA-N	MNHVNIJQRIYDH-UHFFFAOYSA-N	PXMNMQRDXW-ABCY-UHFFFAOYSA-N	NSPMIYGKQJPB-QR-UHFFFAOYSA-N	ISJVOEOJQLKSJU-UHFFFAOYSA-N
MW [g/mol]	291.77	406.30	329.80	336.80	705.60	531.44	342.20	344.30	307.82	69.07	721.60
logPow (exp.)	3.09 ⁵	4.36 ⁴	3.23 ⁶	4.35 ³	5.66 ¹²	4.31 ⁷	3.72 ⁸	3.82 ⁹	3.7 ¹⁰	-1 ³	3.98 ¹²
logPow (chEMBL) ¹	2.85	4.86	3.74	4.35 ¹	4.47 ²	4.19	4.33	3.47	3.69	-0.41	4.47 ²
logPow (CompTox) ¹¹	2.9	4.3	3.44	3.23	4.47	4.35	3.72	2.37	3.7	-0.58	4.26
WS (mg/l)	93 (pH 7.1) ⁵	15 ± 1.3 (pH 7.2) ⁴	7.10 ⁶	3.77 ³	0.0005 ¹¹	40 ⁷	150 (pH 5.2) ⁸	5 (pH 4) ⁹ 300 (pH 8) ⁹ 2000 (pH 9) ⁹	36 (pH 7.2) ¹⁰	730000 ¹¹	0.334 ¹¹
SIMILARITY SCORE	68%	-	71%	60%	76%	72%	85%	57%	66%	37%	75%

- 1 CX LogPow (cHEMBL)
- 2 Avdeef, A. (2012) 'Solubility', in Absorption and drug development, pp. 251–318. doi: <https://doi.org/10.1002/9781118286067.ch6>
- 3 EFSA (European Food Safety Authority). 2010. Conclusion on the peer review of the pesticide risk assessment of the active substance fenbuconazole. EFSA Journal 2010; 8(4):1558, 64 pp. doi:10.2903/j.efsa.2010.1558
- 4 EFSA (European Food Safety Authority). 2011. Conclusion on the peer review of the pesticide risk assessment of the active substance difenoconazole. EFSA Journal 2011;9(1):1967. [71 pp.]. doi:10.2903/j.efsa.2011.1967
- 5 EFSA (European Food Safety Authority). 2010. Conclusion on the peer review of the pesticide risk assessment of the active substance cyproconazole. EFSA Journal 2010;8(11):1897. [73 pp.]. doi:10.2903/j.efsa.2010.1897
- 6 EFSA (European Food Safety Authority). 2008. Conclusion regarding the peer review of the pesticide risk assessment of the active substance epoxiconazole. EFSA Scientific Report (2008) 138, 1-80
- 7 Jacobs et al., 2016. Topical delivery of acyclovir and ketoconazole. Drug Delivery 2016, 23, 631– 641. DOI: 10.3109/10717544.2014.933283
- 8 Propiconazole RAR, 2016
- 9 EFSA (European Food Safety Authority). 2008. Conclusion regarding the peer review of the pesticide risk assessment of the active substance prothioconazole. EFSA Scientific Report (2007) 106, 1-98
- 10 EFSA (European Food Safety Authority). 2014. Conclusion on the peer review of the pesticide risk assessment of the active substance tebuconazole. EFSA Journal 2014;12(1):3485, 98 pp. doi:10.2903/j.efsa.2014.3485
- 11 <https://comptox.epa.gov/dashboard/chemical/properties>
- 12 <https://www.pharmapendium.com>

Note: SMILES are converted in MACCS fingerprint and similarity scores are calculated using Tanimoto method. SCs are given in alphabetical order.

Table A2.4. Summary of in vivo repeated dose toxicity studies with proposed LOAEL

CAS	NAME	SPECIES, STRAIN, NUMBER, GENDER	EXPOSURE	GUIDELINE (Y/N, GUIDELINE)	ADVERSE EFFECT SUMMARY		REFERENCE
					♂	♀	
94361-06-5	Cyproconazole	Rat Wistar 15 ♂/♀	90 d Feed 0, 20, 350, 700, 1400 ppm	Y OECD 408	350 ppm: adrenal fatty degeneration ↑, adrenal single cell necrosis ↑, bilirubin ↓, liver fatty degeneration ↑, liver hypertrophy ↑, rel. liver weight ↑, pituitary gland hypertrophy ↑, spleen hemosiderosis ↑ 700 ppm: BW ↓, BWG ↓, ALB/GLOB ↓, AST ↑, GLOB ↑, protein ↑, triglyceride ↑, BUN ↑, leukocytes ↑, lymphocytes ↑, prothrombin activity ↑, RDW ↑, HDW ↑, large unstained cells ↑, spleen extramedullary hematopoiesis ↓ 1400 ppm: GGT ↑, ALT ↑, food consumption ↓, eosinophils ↑, MCH ↑, MCV ↑, kidney hemosiderosis ↑, abs. liver weight ↑, rel. testes weight ↓, urine volume ↑ LOAEL (ppm): 350 LOAEL (mg/kg bw/d): 24.7 (♂) and 29.6 (♀) LOAEL (mmol/kg bw/d): 0.0847 (♂) and 0.1014 (♀) NOAEL (ppm): 20 NOAEL (mg/kg bw/d): 1.4 (♂) and 1.64 (♀)	350 ppm: ALB/GLOB ↓, bilirubin ↓, GLOB ↑, liver hypertrophy ↑, rel. liver weight ↑, spleen extramedullary hematopoiesis ↓ 700 ppm: adrenal ceroid deposit ↑, BW ↓, BWG ↓, GGT ↑, CHOL ↑, protein ↑, haematocrit ↓, MCV ↑, monocytes ↑, neutrophils ↑, RDW ↑, HDW ↑, kidney hemosiderosis ↑, abs. liver weight ↑, spleen hemosiderosis ↑, abs. and rel. spleen weight ↓ 1400 ppm: adrenal single cell necrosis ↑, AST ↑, AIP ↑, eosinophils ↑, leukocytes ↑, lymphocytes ↑, MCH ↑, large unstained cells ↑, leukocyturia ↑	Gersprach, R. (1999) Cyproconazole RAR (2006)

					NOAEL (mmol/kg bw/d: 0.0048 (♂) and 0.0056 (♀))		

					NOAEL (mmol/kg bw/d: 0.022 (♂) and 0.024 (♀))		
		Mouse CD-1 10 ♂/♀	90 d Feed 0, 5, 15, 300, 600 ppm	Y OECD 408	300 ppm: BW ↓, BWG ↓, food consumption ↓, rel. heart weight ↑, accentuated lobular pattern liver ↑, eosinophilic focus periportal liver ↑, liver necrosis ↑, abs. and rel. liver weight ↑, rel. spleen weight ↑ 600 ppm: eosinophilic focus centrilobular liver ↑, liver hypertrophy ↑, liver vacuolization ↑ LOAEL (ppm): 300 LOAEL (mg/kg bw/d): 43.8 (♂) and 70.2 (♀) LOAEL (mmol/kg bw/d): 0.1501 (♂) and 0.2406 (♀) NOAEL (ppm): 15 NOAEL (mg/kg bw/d): 2.2 (♂) and 3.2 (♀) NOAEL (mmol/kg bw/d): 0.0075 (♂) and 0.011 (♀)	300 ppm: food consumption ↓, liver necrosis ↑, liver vacuolization ↑, abs. and rel. liver weight ↑ 600 ppm: BW ↓, BWG ↓, abs. brain weight ↓, abs. heart weight ↓, accentuated lobular pattern liver ↑, eosinophilic focus centrilobular liver ↑	Warren, S., Skinner, C., Karapally, J. (1987) Cyproconazole RAR (2006)
119446-68-3	Difenoconazole	Rat CRL:SD 15 ♂/♀	90 d feed 0, 20, 200, 750, 1500 and 3000 ppm	Y OECD 408	200 ppm: BWG ↓, rel. liver weight ↑ 750 ppm: rel. liver weight ↑, erythrocytes ↓, GLOB ↓, haematocrit ↓ 1500 ppm: BUN ↑, liver hypertrophy ↑ 3000 ppm: abs. adrenal weight ↓, bilirubin ↓, BW ↓, BWG ↓, ketonuria ↑ LOAEL (ppm): 750 LOAEL (mg/kg bw/d): 51 (♂) and 66 (♀) LOAEL (mmol/kg bw/d): 0.1255 (♂) and 0.1624 (♀) NOAEL (ppm): 20 NOAEL (mg/kg bw/d): 13 (♂) and 17 (♀) NOAEL (mmol/kg bw/d): 0.0319 (♂) and 0.0418 (♀)	200 ppm: BWG ↓, rel. liver weight ↑ 750 ppm: ALT ↓, BW ↓, rel. liver weight ↑ 1500 ppm: bilirubin ↓, erythrocytes ↓, haematocrit ↓, haemoglobin ↓, liver hypertrophy ↑ 3000 ppm: CHOL ↑, rel. brain weight ↑	Cox, R. (1987) Difenoconazole RAR (2019)

		Mouse CRL:CD1 15 ♂/♀	90 d feed 0, 20, 200, 2500, 7500, 15000 ppm	Y OECD 408	200 ppm: liver hypertrophy ↑, rel. liver weight ↑ 2500 ppm: abs. and rel. liver weight ↑, BW ↓, BWG ↓, liver hypertrophy ↑, liver vacuolization ↑ 7500 ppm: mortality ↑ 15000 ppm: mortality ↑ LOAEL (ppm): 200 LOAEL (mg/kg bw/d): 34.2 (♂) and 45.2 (♀) LOAEL (mmol/kg bw/d): 0.0842 (♂) and 0.1112 NOAEL (ppm): 20 NOAEL (mg/kg bw/d): 3.3 (♂) and 4.6 (♀) NOAEL (mmol/kg bw/d): 0.0081 (♂) and 0.0113 (♀)	2500 ppm: abs. heart weight ↓, abs. and rel. liver weight ↑, abs. ovary weight ↓, adrenal necrosis ↑, BW ↓, BWG ↓, liver hypertrophy ↑, liver necrosis ↑, liver vacuolization ↑ 7500 ppm: mortality ↑ 15000 ppm: mortality ↑	Cox, R. (1987) Difenoconazole RAR (2019)
133855-98-8	Epoxiconazole	Rat Wistar 10 ♂/♀	90 d Feed 0, 30, 90, 270, 800 ppm	Y OECD 408	270 ppm: abs. and rel. adrenal weight ↓, liver hypertrophy ↑, spleen haemosiderosis ↑ 800 ppm: GGT ↑, abs. and rel. liver weight ↑ LOAEL (ppm): 270 LOAEL (mg/kg bw/d): 20 (♀) and 22 (♀) LOAEL (mmol/kg bw/d): 0.0606 (♀) and 0.0667 (♀) NOAEL (ppm): 90 NOAEL (mg/kg bw/d): 7 (♂) and 8 (♀) NOAEL (mmol/kg bw/d): 0.006 (♂) and 0.009 (♀)	270 ppm: liver hypertrophy ↑, spleen haemosiderosis ↑ 800 ppm: GGT ↑, CHOL ↑, abs. and rel. liver weight ↑	Schiling, K. (1991) Epoxiconazole RAR (2019)
		Rat Wistar 10 ♂/♀	90 d Feed 0, 500, 1000, 1500, 2000 ppm	Y OECD 408	500 ppm: abs. and rel. adrenal gland weight ↓ 1000 ppm: liver GGT ↑, food consumption ↓, thrombocytes ↓, rel. liver weight ↑ 1500 ppm: serum GGT ↑ 2000 ppm: CHOL ↑, abs. liver weight ↑ LOAEL (ppm): 500 LOAEL (mg/kg bw/d): 35 (♂) and 41 (♀) LOAEL (mmol/kg bw/d): 0.1061 (♂) and 0.1243 (♀) NOAEL: -	500 ppm: triglyceride ↑, abs. liver weight ↑, rel. liver weight ↑ 1000 ppm: serum and liver GGT ↑ 1500 ppm: adrenal gland deposits ↑, CHOL ↑ 2000 ppm: changes in adrenal cortex structure ↑, abs. adrenal gland weight ↓	Epoxiconazole RAR (2019)

	Mouse B6C3F1 10 ♂/♀	90 d Feed 0, 30, 90, 270 ppm	Y OECD 408	30 ppm: CHOL ↓, triglyceride ↓, rel. liver weight ↑ 90 ppm: ALB ↓, total protein ↓, liver hypertrophy ↑, abs. liver weight ↑ 270 ppm: ALT ↑, liver fatty degeneration ↑ LOAEL (ppm): 90 LOAEL (mg/kg bw/d): 19 (♂) and 25 (♀) LOAEL (mmol/kg bw/d): 0.0576 and 0.0758 (♀) NOAEL (ppm): 30 NOAEL (mg/kg bw/d): 6 (♂) and 9 NOAEL (mmol/kg bw/d): 0.0182 (♂) and 0.0273 (♀)	30 ppm: CHOL ↓, triglyceride ↓, rel. liver weight ↑ 90 ppm: abs. liver weight ↑ 270 ppm: ALB ↓, GLOB ↓, total protein ↓, liver hypertrophy ↑	BASF RegDoc 91/10855 Epoxiconazole RAR (2019)
	Mouse C57BL 10 ♂/♀	90 d Feed 0, 7.5, 125, 250, 500, 1000 ppm	Y OECD 408	125 ppm: CHOL ↓, triglyceride ↓, liver hypertrophy ↓, abs. and rel. liver weight ↑ 500 ppm: ALT ↑, liver eosinophilic structures ↑, liver fatty degeneration ↓ 1000 ppm: BW ↓ LOAEL (ppm): 125 LOAEL (mg/kg bw/d): 26 LOAEL (mmol/kg bw/d): 0.0612 NOAEL (ppm): 7.5 NOAEL (mg/kg bw/d): 2 NOAEL (mmol/kg bw/d): 0.006	125 ppm: CHOL ↓, triglyceride ↓, abs. and rel. liver weight ↑ 500 ppm: GGT ↑ 1000 ppm: ALT ↑, liver eosinophilic structures ↑, liver fatty degeneration ↓, liver hypertrophy ↓	BASF RegDoc No. 91/10908 Epoxiconazole RAR (2019)
	Mouse B6C3F1 10 ♂/♀	90 d Feed 0, 7.5, 15, 30 ppm	Y OECD 408	30 ppm: CHOL ↓, triglyceride ↓ NOAEL (ppm): >30) NOAEL (mg/kg bw/d): 7.3 NOAEL (mmol/kg bw/d): 0.0221		Epoxiconazole RAR (2019)

114369-43-6	Fenbuconazole	Rat CRL:CD BR 10 ♂/♀	90 d feed 0, 20, 80, 400, 1600 ppm	Y OECD 408	80 ppm: liver vacuolization ↑ 400 ppm: liver hypertrophy ↑, rel. liver weight ↑, thyroid hypertrophy ↑ 1600 ppm: BW ↓, BWG ↓, GGT ↑, CHOL ↑, triglyceride ↑, liver lobular pattern ↑, liver discoloration ↑ LOAEL (ppm): 400 LOAEL (mg/kg bw/d): 25.3 (♂) and 31.5 (♀) LOAEL (mmol/kg bw/d): 0.0751 (♂) and 0.0935 (♀) NOAEL (ppm): 80 NOAEL (mg/kg bw/d): 5.1 (♂) and 6.3 (♀) NOAEL (mmol/kg bw/d): 0.0151 (♂) and 0.0187 (♀)	400 ppm: liver hypertrophy ↑, liver vacuolization ↑, rel. liver weight ↑, thyroid hypertrophy ↑ 1600 ppm: abs. adrenal weight ↑, BW ↓, BWG ↓, rel. brain weight ↑, ALB ↑, GLOB ↑, ALB/GLOB ↑, GGT ↑, CHOL ↑, triglyceride ↑, BUN ↑, erythrocytes ↑, MCHC ↑, MCV ↑, thrombocytes ↑, abs. and rel. kidney weight ↑, liver lobular pattern ↑, liver discoloration ↑, abs. liver weight ↑	Bernacki, H., Hazelton, G. (1988) Fenbuconazole DAR (2005)
		Mouse CRL:CD-1(ICR)BR 10 ♂/♀	90 d Feed 0, 20, 60, 180, 540 ppm	Y OECD 408	180 ppm: AST ↑, rel. liver weight ↑ 540 ppm: ALT ↑, liver hypertrophy ↑, liver necrosis ↑ LOAEL (ppm): 540 LOAEL (mg/kg bw/d): 99 (♂) and 139 (♀) LOAEL (mmol/kg bw/d): 0.2939 (♂) and 0.4127 (♀) NOAEL (ppm): 180 NOAEL (mg/kg bw/d): 28.6 (♂) and 50.4 (♀) NOAEL (mmol/kg bw/d): 0.0849 (♂) and 0.1496 (♀)	540 ppm: liver hypertrophy ↑, liver vacuolization ↑, rel. liver weight ↑	Harris, J., Hazelton, G. (1988) Fenbuconazole DAR (2005)
		Mouse CD-1 10 ♂/♀	90 d Feed 0, 540, 1000, 3000, 10000 ppm	Y OECD 408	540 ppm: CHOL ↓, triglyceride ↓, liver enlargement ↑, liver hypertrophy ↑, liver vacuolization ↑, abs. and rel. liver weight ↑ 1000 ppm: abs. kidney weight ↓, liver necrosis ↑ 3000 ppm: BWG ↓, ALT ↑, potassium ↓ 10000 ppm: mortality ↑ LOAEL (ppm): 540 LOAEL (mg/kg bw/d): 87.1 LOAEL (mmol/kg bw/d): 0.2586 NOAEL: -	540 ppm: triglyceride ↓, liver enlargement ↑, liver hypertrophy ↑, liver vacuolization ↑, abs. and rel. liver weight ↑ 3000 ppm: CHOL ↓, potassium ↓, liver necrosis ↑ 10000 ppm: mortality ↑	Wolfe, G. (1990) Fenbuconazole DAR (2005)

84625-61-6	Itraconazole	Rat - 20 ♂/♀	90d Gavage 0, 10, 40, 160 mg/kg bw/d	Not mentioned; Supporting evidence	40 mg/kg bw/d: eosinophils ↓, erythrocytes ↓, haematocrit ↓, neutrophils ↓, lymphocytes ↑ 160 mg/kg bw/d: adrenal fatty degeneration ↑, bone growth ↓, mortality ↑, thymus macrophage ↑, LOAEL (mg/kg bw/d): 10 LOAEL (mmol/kg bw/d): 0.0141 NOAEL: -	10 mg/kg bw/d: CHOL ↑, circling disease ↑ 40 mg/kg bw/d: eosinophils ↓, erythrocytes ↓, haematocrit ↓, neutrophils ↓, lymphocytes ↑, heart weight ↑, kidney weight ↑, liver weight ↑ 160 mg/kg bw/d: mortality ↑, thymus weight ↑	Itraconazole NDA 22-484 (2009) Pharmapendium
		Rat - 20 ♂/♀	90 d Gavage 0, 5, 20, 80 mg/kg bw/d	Not mentioned; Supporting evidence	5 mg/kg bw/d: CHOL ↑, phospholipids ↑, kidney weight ↑ 20 mg/kg bw/d: adrenal and liver enlargement ↑, adrenal and liver weight ↑ LOAEL (mg/kg bw/d): 5 LOAEL (mmol/kg bw/d): 0.007 NOAEL: -	5 mg/kg bw/d: CHOL ↑, phospholipids ↑, adrenal and liver enlargement ↑, adrenal and liver weight ↑ 20 mg/kg bw/d: kidney weight ↑, lung foam cells ↑	Itraconazole NDA 22-484 (2009) Pharmapendium
		Rat - 20 ♂/♀	180 d Feed 0, 10, 40, 160 mg/kg bw/d	Not mentioned; Supporting evidence	10mg/kg bw/d: adrenal weight ↑, bone fractures ↑, CHOL ↑, kidney granulation ↑, liver weight ↑, lung weight ↑, spleen weight ↑ 40mg/kg bw/d: adrenal swelling ↑, bone growth ↓, teeth hypocellular pulp ↑ 160mg/kg bw/d: adrenal fibrosis ↑, adrenal pigmentation ↑, adrenal vacuolization ↑, mortality ↑, erythrocytes ↓, haematocrit ↓, haemoglobin ↓, thrombocytes ↓, kidney pigmentation ↑, kidney vacuolization ↑, lung foam cells ↑, lung foci ↑, lymph node swelling ↑, spleen swelling ↑, tongue foci ↑ LOAEL (mg/kw bw/d): 10 NOAEL: not determined-	10mg/kg bw/d: adrenal weight ↑, bone fractures ↑, CHOL ↑, CRET ↓, kidney granulation ↑, liver weight ↑, lung weight ↑, spleen weight ↑ 40mg/kg bw/d: adrenal swelling ↑, bone growth ↓, teeth hypocellular pulp ↑ 160mg/kg bw/d: adrenal fibrosis ↑, adrenal pigmentation ↑, adrenal vacuolization ↑, erythrocytes ↓, haematocrit ↓, haemoglobin ↓, thrombocytes ↓, kidney pigmentation ↑, kidney vacuolization ↑, lung foam cells ↑	Itraconazole NDA 22-484 (2009) Pharmapendium

65277-42-1 / 79156-75-5	Ketoconazole	Rat SD 10 ♂/♀	28d Gavage 0, 6.25, 25, 100 mg/kg bw/d	Y OECD 407 Supporting evidence	6.25 mg/kg bw/d: rel. seminal vesicle weight ↓ 25 mg/kg bw/d: testosterone ↓, E2 ↑, FSH ↑, LH ↑ 100 mg/kg bw/d: ALT ↑, T3 ↓, T4 ↓, TSH ↑, rel. adrenal weight ↑, rel. kidney weight ↑, rel. epididymis weight ↓, rel. liver weight ↑, rel. prostate weight ↓, spermatid retention ↑ LOAEL (mg/kg bw/d): 6.25 LOAEL (mmol/kg bw/d): 0.0118 NOAEL (mg/kg bw/d): - NOAEL (mmol/kg bw/d): -	6.25 mg/kg bw/d: rel. uterus weight ↓ 25 mg/kg bw/d: rel. adrenal weight ↑, rel. ovary weight ↑ 100 mg/kg bw/d: ALT ↑, BW ↓, MCHC ↓, E2 ↑, FSH ↑, LH ↑, T3 ↓, T4 ↓, TSH ↑, estrous cycle length ↑, rel. heart weight ↑, rel. kidney weight ↑, rel. liver weight ↑, rel. vagina weight ↑	(Shin et al., 2006)
60207-90-1	Propiconazole	Rat Tif:RAIf(SPF) 20 ♂/♀	90 d Feed 0, 240, 1200, 6000 ppm	Y OECD 408	6000 ppm: BWG ↓, BW ↓, GGT ↑, erythrocytes ↓, abs. adrenal weight ↓, abs. heart weight ↓, abs. kidney weight ↓, LOAEL (ppm): 1200 LOAEL (mg/kg bw/d): 77.59 LOAEL (mmol/kg bw/d): 0.2267 NOAEL (ppm): 240 NOAEL (mg/kg bw/d): 16.82 NOAEL (mmol/kg bw/d): 0.0491	1200 ppm: BWG ↓, BW ↓ 6000 ppm: GGT ↑, AP ↑, BUN ↑, erythrocytes ↓, haematocrit ↓, haemoglobin ↓, abs. heart weight ↓, abs. kidney weight ↓, spleen haemosiderosis ↑	Sachsse, K., Suter, P., Luetkemeier, H., et al. (1979) Propiconazole dRAR (2017)
		Mouse Cri:CD-1(ICR)BR 40 ♂	90 d Feed 0, 20, 500, 850, 1450, 2500 ppm	Y OECD 408	500 ppm: CHOL ↓, liver hypertrophy ↑, abs. and rel. liver weight ↑ 850 ppm: SDH ↑, liver discoloration ↑, liver enlargement ↑, liver necrosis ↑, liver vacuolization ↑ 1450 ppm: ALT ↑, liver lobular pattern ↑ 2500 ppm: liver mineralization ↑ LOAEL (ppm): 500 LOAEL (mg/kg bw/d): 71 LOAEL (mmol/kg bw/d): 0.2075 NOAEL (ppm): 20 NOAEL (mg/kg bw/d): 2.8 NOAEL (mmol/kg bw/d): 0.0081		Potrepka, R., Turnier, J. (1991) Propiconazole dRAR (2017)

178928-70-6	Prothioconazole	Rat Wistar 10 ♂/♀	90 d Gavage 0, 20, 100, 500 mg/kg bw /d	Y OECD 408	500 mg/kg bw/d: AST ↓, EH ↑, UDP-glucuronyltransferase ↑, CHOL ↑, BUN ↑, water intake ↑, kidney hyperbasophilia ↑, liver cytoplasmic granular structure ↑, liver hypertrophy ↑ LOAEL (mg/kg bw/d): 500 LOAEL (mmol/kg bw/d): 1.4522 NOAEL (mg/kg bw/d): 100 NOAEL (mmol/kg bw/d): 0.2904	500 mg/kg bw/d: AST ↓, EH ↑, CHOL ↑, triglyceride ↓, water intake ↑, liver cytoplasmic granular structure ↑, liver hypertrophy ↑, abs. and rel. liver weight ↑	Wirnitzer, U. (1999) Prothioconazole DAR (2004) Prothioconazole RAR (2018)
		Mouse CD-1 10 ♂/♀	90 d Gavage 0, 25, 100, 400 mg/kg bw/d	Y OECD 408	25 mg/kg bw/d: rel. liver weight ↑ 100 mg/kg bw/d: ECOD ↑, EROD ↑, ALD ↑, liver cytoplasmic granular structure ↑, liver lobular pattern ↑, liver fatty degeneration ↑, liver hypertrophy ↑ 400 mg/kg bw/d: ALB ↓, bilirubin ↓, protein ↓, liver enlargement ↑, liver vacuolization ↑, abs. liver weight ↑ LOAEL (mg/kg bw/d): 100 LOAEL (mmol/kg bw/d): 0.2904 NOAEL (mg/kg bw/d): 25 NOAEL (mmol/kg bw/d): 0.0726	25 mg/kg bw/d: ECOD ↑, EROD ↑, ALD ↑, GST ↑ 100 mg/kg bw/d: liver cytoplasmic granular structure ↑, liver lobular pattern ↑, liver hypertrophy ↑, rel. liver weight ↑ 400 mg/kg bw/d: bilirubin ↓, EH ↑, UDP-glucuronyltransferase ↑, CHOL ↑, liver enlargement ↑, liver fatty degeneration ↑, abs. liver weight ↑	Wirnitzer, U. (1999) Prothioconazole DAR (2004) Prothioconazole RAR (2018)
107534-96-3	Tebuconazole	Rat Wistar 10 ♂/♀	90 d Feed 0, 100, 400, 1600 ppm	Y OECD 408	400 ppm: BW ↓, CYP ↑, liver weight ↓ 1600 ppm: adrenal vacuolization ↑, N-Demethylase ↑ LOAEL (ppm): 400 LOAEL (mg/kg bw/d): 34.8 (♂) and 46.5 (♀) LOAEL (mmol/kg bw/d): 0.113 (♂) and 0.151 (♀) NOAEL (ppm): 100 NOAEL (mg/kg bw/d): 8.6 (♂) and 10.8 (♀) NOAEL (mmol/kg bw/d): 0.0279 (♂) and 0.035 (♀)	100 ppm: N-Demethylase ↓, CYP ↑, liver weight ↓ 400 ppm: adrenal vacuolization ↑ 1600 ppm: BW ↓, spleen haemosiderosis ↑	Bomhard, E.; Schilde, B. (1986) Tebuconazole DAR (2007)

288-88-0	1,2,4-Triazole	Rat Wistar 20 ♂/♀	90 d feed 0, 250, 500, 3000, 4000 ppm	Y OECD 408 (+ neurotoxicity)	3000 ppm: BW ↓, BWG ↓, abs. brain weight ↓, N-demethylase ↑, triglyceride ↓, fur coloured ↑, hypoactivity ↑, lacrimation ↑, moving uncoordinated ↑, poor general conditions ↑, tremor ↑, abnormal behaviour ↑, convulsions ↑, cerebellum degeneration ↑, nerve fibre degeneration ↑ LOAEL (ppm): 3000 LOAEL (mg/kg bw/d): 183 (♂) and 234 (♀) LOAEL (mmol/kg bw/d): 2.6495 (♂) and 3.3879 (♀) NOAEL (ppm): 500 NOAEL (mg/kg bw/d): 33 (♂) and 41 (♀) NOAEL (mmol/kg bw/d): 0.4778 (♂) and 0.5936 (♀)	3000 ppm: BW ↓, BWG ↓, abs. brain weight ↓, O-demethylase ↑, fur coloured ↑, hypoactivity ↑, lacrimation ↑, moving uncoordinated ↑, poor general conditions ↑, tremor ↑, abnormal behaviour ↑, convulsions ↑, cerebellum degeneration ↑, nerve fibre degeneration ↑	Wahle, B. S. (2004) Tebuconazole DAR (2006)
		Rat Wistar 15 ♂/♀	90 d feed 0, 100, 500, 2500 ppm	Y OECD 408	2500 ppm: BW ↓, BWG ↓, haematocrit ↓, haemoglobin ↓, MCH ↓, MCV ↓, liver fatty degeneration ↑ LOAEL (ppm): 2500 LOAEL (mg/kg bw/d): 212.3 (♂) and 266.7 (♀) LOAEL (mmol/kg bw/d): 3.0737 (♂) and 3.8613 (♀) NOAEL (ppm): 500 NOAEL (mg/kg bw/d): 37.9 (♂) and 54.2 (♀) NOAEL (mmol/kg bw/d): 0.5487 (♂) and 0.7847 (♀)	2500 ppm: BW ↓, BWG ↓, liver fatty degeneration ↑	Unpublished report (1979) Difenoconazole RAR (2019)
		Mouse CD-1 20 ♂/♀	90 d feed 0, 500, 1000, 3000, 6000 ppm	Y OECD 408	3000 ppm: BW ↓, BWG ↓, rel. liver weight ↑, testes apoptosis ↑ 6000 ppm: brain Purkinje cell loss ↑, abs. brain weight ↓, food consumption ↑, fur coloured ↑, piloerection ↑, tremor ↑, epididymis germ cell degeneration ↑, testes atrophy ↑, spermatid degeneration ↑, abs. testes weight ↓ LOAEL (ppm): 3000 LOAEL (mg/kg bw/d): 487 (♂) and 663 (♀) LOAEL (mmol/kg bw/d): 7.0512 (♂) and 9.5996 (♀) NOAEL (ppm): 1000 NOAEL (mg/kg bw/d): 161 (♂) and 487 (♀)	3000 ppm: BW ↓, BWG ↓, rel. liver weight ↑ 6000 ppm: abs. brain weight ↓	Wahle, B. S. (2004) Tebuconazole DAR (2006)

					NOAEL (mmol/kg bw/d): 2.3311 (♂) and 7.0512 (♀)		
		Rat Wistar- Hannover 30 ♂/♀	Multigeneration- study 0, 250, 500, 3000 ppm	Y OECD 416	F0 & F1 Sperm: morphology changes ↑		JMPR (2008), Difenoconazole RAR (2019)

Note: Abbreviations. AIP: acute intermittent porphyria, ALB: albumin, ALD: Aldrin Epoxidase, ALT: alanine transferase, AP: alkaline phosphatase, AST: aspartate transferase, BUN: blood urea nitrogen, BWG: body weight gain, BW: body weight, CHOL: cholesterol, CRET: creatinine, CYP: Cytochrome P450, E2: estradiol, ECOD: ethoxycoumarin deethylase, EROD: ethoxyresorufin O-deethylase, EH: epoxide hydrolase, FSH: follicular stimulating hormone, LH: luteinizing hormone, GGT: gamma glutamyl transferase, GLU: glucose, GLOB: globulin, GST: glutathione S-transferase, HDW: hemoglobin distribution width, MCH: mean corpus haemoglobin, MCHC: mean corpus hemoglobin concentration, MCV: mean corpus volume, MPV: mean platelet volume, RDW: red blood cell distribution width, SDH: sorbitol dehydrogenase, T3: triiodothyronine, T4: thyroxin, TSH: thyroid stimulating hormone, abs.: absolute, rel.: relative

Table A2.5. Acceptable daily intakes of conazole compounds (EFSA et al., 2022)

CHEMICAL NAME	ADI (mg/kg bw/d)	Reference
Cyproconazole	0.02	(EFSA, 2010a)
Difenoconazole	0.01	(EFSA, 2011)
Epoxiconazole	0.008	(EFSA, 2008)
Fenbuconazole	0.006	(EFSA, 2010b)
Itraconazole	pharmaceutical	
Ketoconazole	pharmaceutical	
Propiconazole	0.04	(EFSA, 2017)
Prothioconazole	0.036	(EFSA, 2025)
Prothioconazole-desthio	0.01	(EFSA, 2025)
Tebuconazole	0.03	(EFSA, 2014)
1,2,4-triazole	0.023	(EFSA, 2018)
Hydroxyitraconazole	pharmaceutical metabolite	

Note: Data were available for conazoles registered as plant protection products. Insufficient occurrence data were reported for 1,2,4-triazole.

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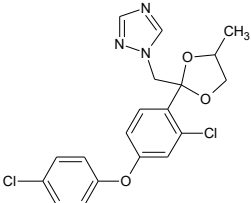
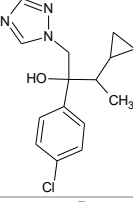
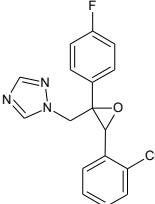
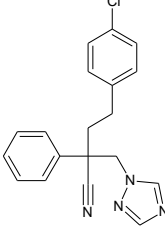
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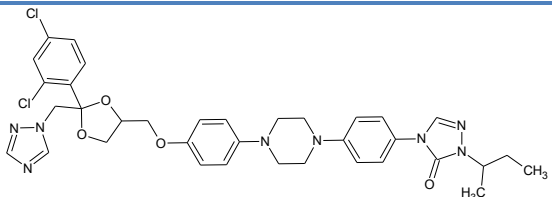
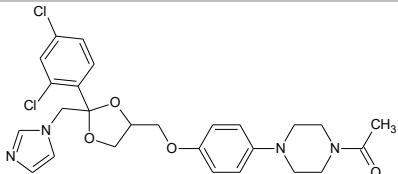
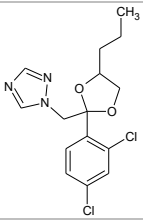
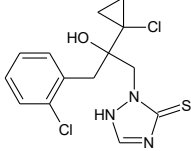
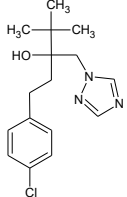
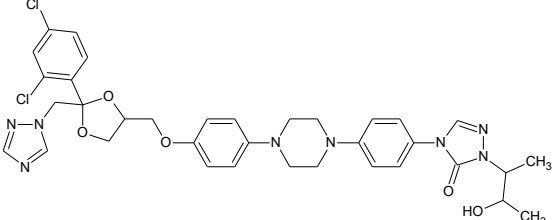
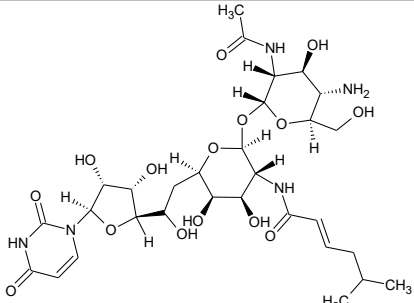
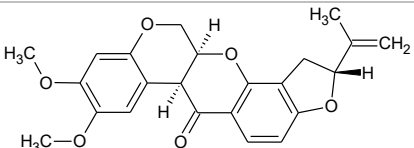
Annex III. Reporting IATA Outcomes

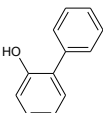
1. Name of the chemical

The conazole case study analyses a total of 14 compounds. This case study explores the applicability of NAM data in a read-across scenario. This consists of one target compound (TC) difenoconazole, for which hazard is to be addressed using read-across following subchronic repeated oral exposure. The relevant analogues or source compounds (SC) were selected based on their shared toxicophore, the triazole ring. Physicochemical properties are provided, along with subchronic oral *in vivo* studies from all SCs (Annex II). Additionally, two metabolites (hydroxyitraconazole and 1,2,4-triazole), two positive controls (rotenone and tunicamycin) and one negative control (2-phenylphenol) are included **Table A3.1**.

Table A3. 1. Compounds used in the conazole case study. TC: Target Compound, SC: Source Compounds, MET: Metabolites, POS: positive controls, NEG: negative control.

CAS	Name	Structure	Molecular weight (g/mol)	Type
119446-68-3	Difenoconazole		406.3	TC
94361-06-5	Cyproconazole		291.77	SC
133855-98-8	Epoxiconazole		329.8	SC
114369-43-6	Fenbuconazole		336.8	SC

84625-61-6	Itraconazole		705.6	SC
79156-75-5	Ketoconazole		531.4	SC
60207-90-1	Propiconazole		342.2	SC
178928-70-6	Prothioconazole		344.3	SC
107534-96-3	Tebuconazole		307.8	SC
288-88-0	1,2,4-triazole		69.1	MET
112559-91-8	Hydroxy-itraconazole		721.6	MET
11089-65-9	Tunicamycin		717.7	POS
83-79-4	Rotenone		394.4	POS

90-43-7	2-Phenylphenol		170.2	NEG
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2. Regulatory Purpose

The conazole case study uses a read-across approach to extrapolate a human relevant threshold for one target compound, which is the fungicide difenoconazole. This approach would traditionally require the assessment of a 90-day repeated dose toxicity study with oral exposure.

The read-across approach applies NAMs to assess a shared toxicological mode of action and further an *in vitro* to *in vivo* extrapolation (IVIVE) to derive a human relevant threshold. A group of eight conazole SCs was selected based on their shared 1,2,4 triazole ring substructure with the TC. Their available *in vivo* animal studies with repeated oral application showed that liver is the primary target organ of all conazole SCs. Shared effects in oral rodent (sub)chronic studies at the Lowest Observed Adverse Effect Levels (LOAELs) are related to liver steatosis, often progressing to liver necrosis at higher dosing (**Annex II, Table A2.4.**). This leads to the read-across hypothesis that also difenoconazole will induce liver toxicity after repeated oral exposure.

Therefore, NAM data from *in vitro* assays are utilized to provide evidence that the grouped SCs share similar toxicokinetic and toxicodynamic properties. The assays included in *in vitro* testing battery consisting of CALUX® (van der Burg, 2013), Cell Painting PLUS (von Coburg et al., 2025), ToxProfiler assays (Ter Braak et al., 2024), transcriptomics analysis from primary human hepatocytes (PHHs) (Callegaro et al., 2021), H295R steroidogenesis assay and DNT assays. Additionally, the results from *in silico* models such as CEP⁵⁵ and the Danish (Q)SAR Toolbox⁵⁶ are presented.

This supports the read-across hypothesis that the TC difenoconazole is likely to exhibit a similar toxicological profile, including liver effects following repeated exposure in animals or humans. The read-across hypothesis suggests that the SCs and TC constitute a category and display comparable toxicological properties in terms of kinetics and dynamics. Based on this read-across hypothesis, a human-relevant threshold for TC could be derived through IVIVE. This could correspond to an NAM-based LOAEL in human equivalent doses.

3. Description of IATA framework, including all information sources used, and modules included in the specific approach

The IATA submission applies the ASPA workflow for the hazard assessment of difenoconazole within a read-across approach. For this case study, we assume that there is no relevant *in vivo* data available for the hazard assessment of difenoconazole. We also assume that the existing data of other conazoles is not sufficient to carry out read-across assessment of difenoconazole.

With these assumptions, we generated NAM-based *in silico* and *in vitro* data for all the case study compounds. The read-across workflow follows the one published by the EUTOXRISK project, in which structural data are used to define an initial set of source compounds and toxicokinetic and dynamic data from NAM assay are used to prove a similar toxicological properties leading to the same adverse outcomes (Escher et al., 2019).

⁵⁵ <https://www.medbioinformatics.com/>

⁵⁶ <https://qsardb.food.dtu.dk/db/index.htm> |

The ASPA workflow version 3 was used for hazard assessment (ASPA_v3.pdf). The workflow starts with the problem formulation (in this case read-across, see **Annex II**), and once a data gap has been identified (see assumptions, (Escher et al., 2019)), the workflow enters into the NAM based assessment of hazard, kinetics and exposure (Figure A3.1). The first question evaluates the applicability of a NAM based approach by asking “Is NAM-based testing feasible for the compound of interest”. All compounds in this case study, based on their physicochemical properties, are assumed to be i) stable in the medium of *in vitro* assays, ii) DMSO and water soluble and iii) not volatile. Based on this analysis, the grouped compounds are considered suitable for NAM-based *in vitro* testing using exposure regimens in the assay protocols described in **Annex I**.



Within the read-across approach a tiered testing and assessment is followed to determine a shared mode of action. The tiered assessment starts with the evaluation of chemical similarity to then include data on shared biological activity. Biological similarity is initially evaluated using evidence from *in silico* models, before progressing to the shared *in vitro* profiles. The results from *in silico* models such as CEP⁵⁷ and the Danish (Q)SAR Toolbox⁵⁸ are presented. NAM data from primarily high-throughput assays are utilized to provide evidence that the grouped SCs and the TC share similar toxicokinetic and toxicodynamic properties. Transcriptomics data from PHHs were used to address liver toxicity (Callegaro et al., 2021). Additional human *in vitro* models are used to broaden the mechanistic space of the *in vitro* testing battery. Therefore, also two different reporter gene assays, namely CALUX® (van der Burg, 2013) and ToxProfiler (Ter Braak et al., 2024) as well as Cell Painting PLUS (von Coburg et al., 2025) were tested. Informed by the *in vivo* data for the SCs, we also assessed the compounds by H295R steroidogenesis and developmental neurotoxicity (DNT) assays (European Food Safety, Anagnostopoulos, et al., 2022). This tiered approach aims to reduce uncertainty by integrating more relevant information. *In vitro* to *in vivo* extrapolation used the free media concentrations from lowest effect concentrations (LEC) from active assays to derive a human equivalent free plasma concentration, which is used as *in vitro* Point of Departures (PoDs).

Vice versa forward dosimetry, using physiologically based kinetic (PBK) model, evaluated the bioavailable plasma concentrations of all data rich SCs starting from LOAEL values reported in *in vivo* animal studies with (sub)chronic exposure, as well as acceptable daily intake values published by EFSA (European Food Safety et al., 2024; European Food Safety, Carrasco Cabrera, & Medina Pastor, 2022).

Thus, beside showing similarity with the grouped compounds with regard to toxicodynamic and kinetic properties, a human-relevant threshold for TC is derived through IVIVE. This threshold is used to derive an NAM-based LOAEL in human equivalent doses. Both values were compared to assess the conservativeness, or safety margin, between the *in vitro* derived and *in vivo* derived human plasma concentrations of the data rich SCs. Two read-across approaches were evaluated to predict the LOAEL of TC: a nearest neighbour approach and a conservative worst-case approach.

4. Data review

Compound information

Compound information includes chemical name, CAS registration number, chemical structure in form of SMILES structures. The compounds were selected by considering their shared toxicophore and shared toxicological profile based on *in vivo* rodent studies. Physicochemical properties were extracted from experimental data, where available, and from high quality databases, including CompTox⁵⁹ and chEMBL⁶⁰. Both experimental and predicted values were presented in **Annex II Figure A2. 1. and Table A2. 3.** Overall, the differences in physicochemical properties among the conazole parent compounds are minimal. This indicates similarity in hydrophobicity, volatility and water solubility. LogPow of all parent conazole compounds fall within range 3-6, indicating that the conazoles are hydrophobic. Henry's law constants together with vapor pressure far below 1 atm indicate the non-volatile behaviour of the compounds. Water solubility of the compounds falls below 150 mg/l indicating slight solubility properties. The target compound (TC) difenoconazole falls within the range for physicochemical properties, which indicate the same category with other conazoles. The information regarding read-across, along the information on

⁵⁷ <https://www.medbioinformatics.com/>

⁵⁸ <https://qsardb.food.dtu.dk/db/index.html>

⁵⁹ <https://comptox.epa.gov/dashboard/>

⁶⁰ <https://www.ebi.ac.uk/chembl/>

physicochemical properties and outcomes of *in vivo* literatures are documented in **Annex II Table A2.3 and A2.4.**

In vivo data

In vivo repeated dose toxicity studies in rats and mice were extracted from high quality databases such as ToxRef (Martin, Judson, Reif, Kavlock, & Dix, 2009; Martin, Mendez, et al., 2009; Watford et al., 2019) and RepDose® (Bitsch et al., 2006), Pharmapendium⁶¹ and compound dossiers extracted from EFSA consultation websites⁶², where available. Route of the administration is oral, covering diet and gavage studies. Most of the compounds have been evaluated in 90-days studies. This includes cyproconazole, difenoconazole, epoxiconazole, fenbuconazole, itraconazole, propiconazole, prothioconazole, tebuconazole and 1,2,4-triazole. There are no subchronic (90-days) studies available for hydroxyitraconazole and ketoconazole. However, there are 28-day studies available for ketoconazole, which serves as supporting information. The studies were conducted according or equivalent to OECD guidelines (OECD 408)⁶³, if not, the guideline used for that specific study was entered (870.31, EU method B.26). Studies without guidelines or without sufficient study design information were acknowledged as supporting evidence. Specifically, point of departure (PoD) values, i.e. Lowest Observed Adverse Effect Level (LOAEL) from oral repeated dose were determined by considering the proposed LOAEL by authors. For the purpose of this case study, all available effects were collected where the findings were declared toxicologically relevant and test treatment related. LOAEL from rat and mice studies were allometric scaled to compare to human relevant dose, with factor 4 and 7, respectively. This was conducted according to ECHA guidance on information requirements and chemical safety assessment⁶⁴. Forward dosimetry using human PBK models were used to predict unbound plasma concentration for allometrically-scaled LOAELs. These values were compared with PoDs from NAMs and derived LOAEL values. The information of the *in vivo* studies are provided in **Annex II Table A2.4.** Shared effects in oral rats and mice subchronic studies are liver toxicity at the Lowest Observed Adverse Effect Levels (LOAELs), with effects ranging from significant weight changes to necrosis and fatty degeneration at higher dosing (**Annex II Table A2.4.**). In contrast, ketoconazole administration resulted in different kind of mode of action, with observed effects including disrupted hormone levels. Based on literature, this could be associated with the nuclear receptors FXR, CAR and PXR activation (Goetz & Dix, 2009; Heise et al., 2018; Hester et al., 2012). Receptor activation could impact the CYP enzyme induction, impairment of lipid and steroid homeostasis and an increase in cell proliferation. Prolonged, chronic cell proliferation is discussed as one reason for conazoles to cause liver neoplasms. As liver is consistently observed as main target organ the read-across hypothesis in this case study is that also the TC difenoconazole will have similar toxicological properties and will cause liver effects after repeated subchronic application.

Acceptable daily intake (ADI)

To obtain a highly conservative estimate of the theoretical formation of 1,2,4-triazole from parent conazoles, the ADIs established by EFSA for each parent compound were used as input doses in the PBK model (European Food Safety, Carrasco Cabrera, & Medina Pastor, 2022). Using this approach, the maximum concentration of 1,2,4-triazole that could potentially be generated under worst-case assumptions was predicted. EFSA combined occurrence data of the conazoles from approximately 30 EU Member States

⁶¹ <https://www.elsevier.com/de-de/products/pharmapendium>

⁶² <https://www.efsa.europa.eu/en/calls/consultations>

⁶³ OECD (2025), *Test No. 408: Repeated Dose 90-Day Oral Toxicity Study in Rodents*, OECD Guidelines for the Testing of Chemicals, Section 4, OECD Publishing, Paris, <https://doi.org/10.1787/9789264070707-en>.

⁶⁴ Guidance on information requirements and chemical safety assessment (November 2012). https://echa.europa.eu/documents/10162/13632/information_requirements_r8_en.pdf/e153243a-03f0-44c5-8808-88af66223258

between 2020 and 2022 with adult food consumption surveys from 15 EU Member States. Values were available for compounds registered as plant protection products (European Food Safety et al., 2024; European Food Safety, Carrasco Cabrera, et al., 2022). There was no data available from EFSA regarding pharmaceutical conazoles such as itraconazole, ketoconazole and hydroxyitraconazole. Insufficient occurrence data were reported for 1,2,4-triazole. The ADI reported by EFSA ranges from 0.006 mg/kg bw/d (fenbuconazole) to 0.04 mg/kg bw/d (propiconazole) (**Annex II Table A2.5.**). The information on the *in vivo* studies is also provided in **Annex II Table A2.4.**

In vitro data

In this case study, *in vitro* NAM testing was conducted to provide the additional evidence for the shared toxicokinetic and toxicodynamic properties from the selected compounds. The assays were conducted according to in-house protocol, published protocol or well documented ToxTemp. The detailed information of the assays is available in **Annex I Section 3.** A relevant concentration range per assays was determined by identifying the concentration that affects the cytotoxicity in each of the test set. CALUX® (van der Burg, 2013), ToxProfiler (Ter Braak et al., 2024), Cell Painting PLUS (CPP) (von Coburg et al., 2025) were used to assess the hazard profile and similarity of the selected compounds. Compounds were tested in a total of 23 CALUX® assay panels, nine ToxProfiler assays and eight organelles in CPP assays. CALUX® assay panels tested the response of each compound to cytotoxicity, AP-1 related to cell cycle control. The nuclear hormone receptor assays related to endocrine activity were tested, this includes ER and anti-ER, AR and anti-AR, PR, and anti-PR, GR and anti-GR, TRβ and anti-TRβ. The endoplasmic reticulum (ER) stress was tested using the ESRE reporter assays. Oxidative stress was measured using NRF2. DNA damage responses were tested using assays for P21. Hypoxia related pathways are tested using RAR. Other tested assays include PPARα, anti-PPARα, PPARγ, anti-PPARγ, PPARγ, anti-PPARγ and PXR. ToxProfiler assays covered nine relevant endpoints including oxidative stress, ER stress, cell cycle stress, ion stress, protein stress, autophagy, inflammation and cytotoxicity in glucose and galactose. CPP assays include morphological alteration in cells including actin, DNA, ER, generic, golgi, lysosomes, mitochondria and RNA. Each of the tested assays represents a feature to analyse the similarity.

Moreover, several assays were additionally conducted to study the conazoles in other test systems. Transcriptomics were performed in PHHs in a dose-dependent manner to capture the changes related to substances on the gene level. For this test, 10 donor pools were used to reduce donor specific variability. H295R steroidogenesis assays were also conducted in NCI-H295R cell lines, as well as developmental neurotoxicity assays conducted in UKN cell lines to study the neurite outgrowth and neural crest cell migration.

Conazoles containing a triazole ring can undergo biotransformation to the metabolite 1,2,4-triazole, which is being evaluated for potential toxicological concerns. Hydroxy-itraconazole was also included as it showed similar effects to its parent compound itraconazole. Therefore, the assays also tested the response of these compounds. Moreover, the buildup of metabolites within human hepatocytes from these conazole compounds were quantified. The *in vitro* metabolite profiling detected hydroxy-metabolites, however, not 1,2,4-triazole (**Appendix III**).

In silico models

In silico models use the available structural information and help to predict potential effects and guide the subsequent testing approach mentioned above. The detailed information to the *in silico* models is provided in **Annex I.** Chemical Effect Predictor (CEP)⁶⁵ is a machine learning model, which integrates the extracted data from EFSA, ECHA CLP, and human evidence from literature to characterize potential hazard caused by compounds. CEP covers diseases such as blood, lymphatic, cardiac, endocrine, psychiatric, gastrointestinal, hepatobiliary, immune system, metabolism, nutrition, neoplasm, nervous system, renal,

⁶⁵ <https://www.medbioinformatics.com/>

respiratory and reproductive systems disorder. The results of CEP on conazole compounds showed concerns in hepatobiliary disorders, along with neoplasms, endocrine and reproductive disorders. Danish (Q)SAR Toolbox⁶⁶ used over 50 models to predict the alerts for conazole compounds. This includes endocrine-related models, hormones, nuclear receptors, mutagenicity, skin effects, neoplasms etc.. The documentation in the international QMRF format is available. PBK models were constructed for cyproconazole, difenoconazole, epoxiconazole, fenbuconazole, itraconazole, ketoconazole, propiconazole, prothioconazole and tebuconazole. The input parameters used for PBK and biokinetics models are described in **Annex I Table A1.6.-A1.17**. Sources from high quality database such as PubChem⁶⁷, chEMBL⁶⁸, httk database (Pearce, Setzer, Strope, Wambaugh, & Sipes, 2017), along with prediction tools such as SimCyp⁶⁹ were used for the simulation. The PBK model was built by considering WHO PBK guidance⁷⁰. Simulated toxicokinetic data using PBK modelling showed the relatively rapid absorption from conazole compounds. Maximum plasma concentration (C_{max}) was achieved below one hour of dosing (T_{max}).

5. Data integration and interpretation

Eight conazoles, hydroxyitraconazole and 1,2,4-triazole were used to assess toxicity causing concentration of difenoconazole and predict its LOAEL using a read-across approach. Structural similarity is assessed as first indication of shared properties within the grouped compounds, following the idea that shared structural properties might cause similar toxicokinetic and toxicodynamic effects in the human organism.

Hierarchical clustering was carried out to see a structural correlation between the TC and SCs. The molecular structures represented using SMILES were used as input for the conazoles and were converted to RDKit Mol objects. Morgan fingerprints (radius = 2) were generated for each compound using RDKit (version 2022.09.5). Tanimoto similarity was calculated pairwise between all compound fingerprints to generate a similarity matrix. Hierarchical clustering was performed on this matrix using complete linkage, as implemented in the `scipy.cluster.hierarchy.linkage` function (SciPy version 1.10.1) (Figure A3.2a).

Molecular similarity analysis was also performed using molecular access systems (MACCS) structural keys to evaluate the relationships among the compounds. Binary MACCS fingerprints were generated for each molecule, and Tanimoto coefficient were computed to quantify dissimilarity between the fingerprint vectors. The resulting distance matrix was subsequently used for hierarchical clustering and dendrogram visualization, providing insights into the structural relatedness of the compounds (Figure A3.2b).

The clustering analysis using Morgan fingerprints showed that the TC, difenoconazole shared closest similarity to the TC, propiconazole (Figure A3.2a). The other TCs, cyproconazole, epoxiconazole, fenbuconazole and tebuconazole are also clustered with the TC. Hydroxyitraconazole and itraconazole cluster together, which is expected as hydroxyitraconazole is a hydroxyl metabolite of itraconazole. These along with ketoconazole are clustered with cyproconazole, difenoconazole, epoxiconazole, fenbuconazole, propiconazole and tebuconazole. Prothioconazole and 1,2,4-triazole are clustered together but separately from other conazoles. It could be because prothioconazole is less similar to other conazoles because it contains a triazolinethione ring instead of a triazole. Ketoconazole is also different in structure as it has an imidazole ring in place of a triazole ring.

⁶⁶ <https://qsardb.food.dtu.dk/db/index.html>

⁶⁷ <https://pubchem.ncbi.nlm.nih.gov>

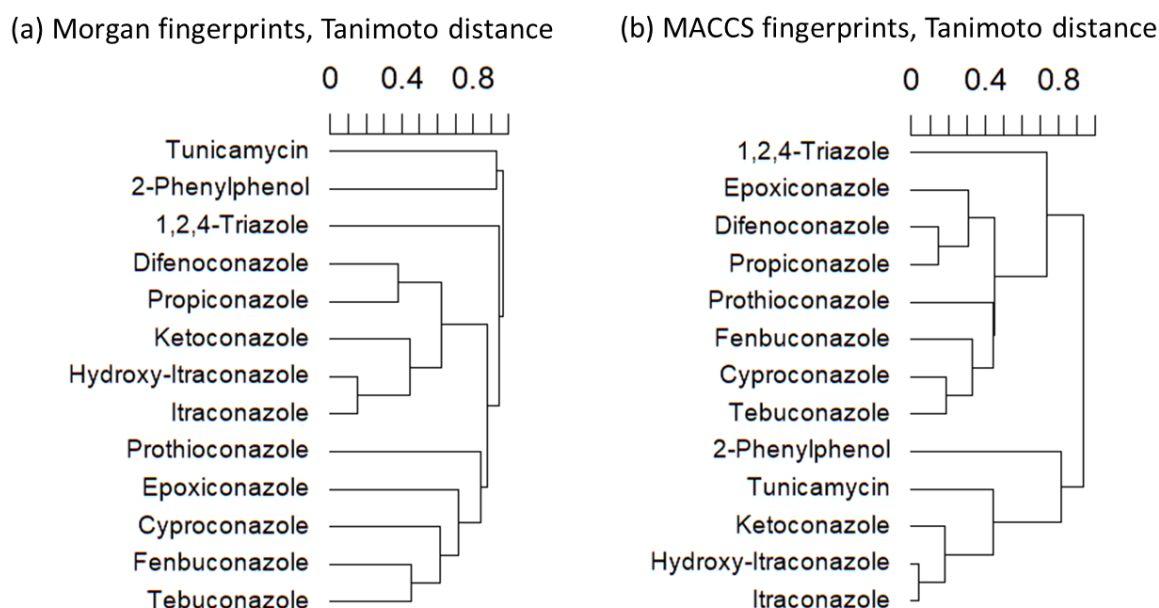
⁶⁸ <https://www.ebi.ac.uk/chembl/>

⁶⁹ <https://www.certara.com/software/simcyp-pbpbk/>

⁷⁰ <https://www.who.int/publications/i/item/9789241500906>

In the analysis using the MACCS fingerprints as well a similar pattern in clustering was observed (Figure A3.2b). The TC, difenoconazole and propiconazole cluster together similar to that observed in the Morgan fingerprints-based clustering. Hydroxyitraconazole and itraconazole cluster together followed by ketoconazole. The other conazoles cluster together but separately from 1,2,4-triazole. Overall, the clustering pattern reveals structural similarities shared between the conazoles and also differences in the side chains and aromatic substitutions. These differences may have implications in the biological activity of the conazoles, which are analysed in the following tiers.

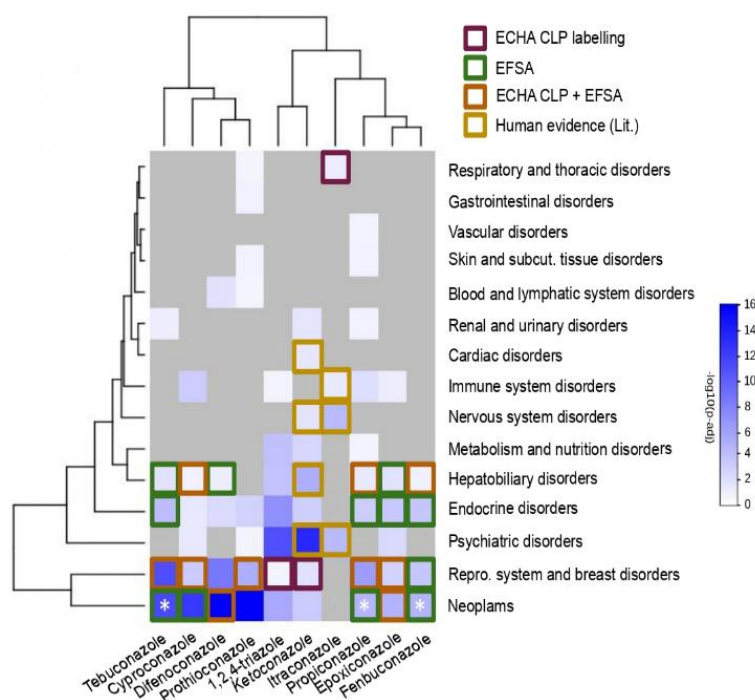
Figure A3. 2. Dendrogram representing structure-based clustering of conazoles and metabolites in the case study using (a) Morgan and (b) MACCS fingerprints



Note: The distance was calculated using Tanimoto coefficient.

The structural analysis of the conazoles was followed by the analysis of existing data derived from in silico models. Disease set enrichment analysis (DSEA) (Pinero et al., 2020) is an in silico approach which use a knowledge base on protein-protein interactions to evaluate associate diseases (Figure A3.3). The analysis of existing data of the target compounds showed close similarity in biological activity of fenbuconazole, epoxiconazole and propiconazole. In another subcluster, TC difenoconazole is clustered with prothioconazole which is further clustered with cyproconazole and tebuconazole. All conazoles except itraconazole show enrichment in endocrine disorders and reproductive system and breast disorders. Tebuconazole, cyproconazole, difenoconazole, ketoconazole, propiconazole, epoxiconazole and fenbuconazole have shown evidence of hepatobiliary disorders. Thus, while structural similarity is partially reflected in the Chemical Effect Predictor analysis, notable variations in clustering patterns are also observed. These discrepancies may be due to genuine structural differences or from incomplete evidence regarding specific biological activities.

Figure A3. 3. Heatmap of the $-\log_{10}(p\text{-adj})$ values of DSEA analysis above $-\log_{10}(0.5)$ for the 10 compounds



Note: Boxed legends on the upper right show the overlap of enriched disease classes with EFSA and ECHA CLP labelling reports, and literature evidence on humans. Neoplasm evidence from EFSA marked with an asterisk (*) is based on evidence from animal data.

The Danish (Q)SAR toolbox provides predictions on biological activity of the compounds based on the chemical structure of the compound. The models were run for all the endpoints available but not all the predictions are in the applicability domain of the model (as indicated by “out” table), only in domain data were assessed (Table A3.2). The antagonistic effect of conazoles on androgen receptor activity was predicted for all conazoles. Similarly, retinoic acid receptor (RAR) inhibition at any concentration was predicted for all conazoles except for prothioconazole, tebuconazole and fenbuconazole where it is inconclusive and outside applicability domain. Conazoles can cause liver toxicity by inhibiting constitutive androstane receptor (CAR) and/or activating pregnane X-receptor (PXR). This activity was predicted for the TC difenoconazole as well as some SCs including epoxiconazole, hydroxyitraconazole, itraconazole, propiconazole and ketoconazole. It was either negative or inconclusive for cyproconazole, prothioconazole, tebuconazole and fenbuconazole.

None of the conazoles were predicted to induce CYP3A4 or activate CAR, as they were either outside the applicability domain of the models or were predicted to be negative. The TC, difenoconazole, was predicted to inhibit RAR, interact with NIS (sodium iodide symporter), inhibit PPAR γ , cause PXR activation and predict positive for Syrian Hamster Embryo Cell Transformation *in vitro* assay. Overall, the effects predicted for difenoconazole were characteristic of typical conazole toxicities. The metabolite hydroxyitraconazole did not show exclusive positive predictions in any model beyond those already predicted positive for itraconazole. 1,2,4-triazole falls out of the applicability domain of several models and was not predicted positive in any of the remaining evaluated models.

Table A3. 2. The following table provides results for all the models that were positive for one or more conazoles

Model	Difenoconazole	Cyproconazole	Epoxiconazole	Itraconazole	Propiconazole	Prothioconazole	Ketoconazole	Tebuconazole	Fenbuconazole	Hydroxyitraconazole	1,2,4-Triazole	Rotenone	Tunicamycin	2-phenylphenol
AR antagonism ^{a)}	INC_OUT	POS_IN	NEG_IN	POS_IN	POS_OUT	NEG_IN	POS_IN	INC_OUT	INC_OUT	POS_IN	INC_OUT	NEG_IN	NEG_IN	POS_IN
AR binding ^{b)}	POS_IN	POS_IN	POS_IN	POS_IN	POS_IN	INC_OUT	POS_IN	POS_IN	POS_IN	POS_IN	NEG_IN	POS_IN	NEG_IN	NEG_IN
AR inhibition ^{b)}	POS_IN	POS_IN	POS_IN	POS_IN	POS_IN	POS_IN	POS_IN	POS_IN	POS_IN	POS_IN	NEG_IN	POS_IN	NEG_IN	NEG_IN
NIS (higher sensitivity)	POS_IN	POS_IN	POS_IN	POS_IN	POS_IN	INC_OUT	POS_IN	POS_IN	POS_IN	INC_OUT	INC_OUT	POS_IN	NEG_IN	INC_OUT
NIS (higher specificity)	POS_IN	POS_IN	POS_IN	POS_IN	POS_IN	INC_OUT	POS_IN	POS_IN	POS_IN	INC_OUT	INC_OUT	POS_OUT	NEG_IN	INC_OUT
PPAR γ inhibition (10 μ M) ^{a)}	INC_OUT	NEG_IN	INC_OUT	POS_IN	NEG_IN	NEG_OUT	POS_IN	NEG_IN	NEG_IN	POS_IN	INC_OUT	POS_IN	POS_IN	NEG_IN
PPAR γ inhibition (any concentration) ^{a)}	POS_IN	INC_OUT	POS_OUT	POS_IN	POS_OUT	NEG_OUT	POS_IN	INC_OUT	POS_OUT	POS_IN	INC_OUT	POS_IN	POS_IN	INC_OUT
RAR Inhibition (10 μ M) ^{a)}	NEG_OUT	NEG_IN	INC_OUT	POS_IN	INC_OUT	NEG_IN	POS_IN	NEG_IN	NEG_IN	POS_IN	INC_OUT	POS_IN	POS_IN	NEG_IN
RAR Inhibition (any concentration) ^{a)}	POS_IN	POS_IN	POS_IN	POS_IN	POS_IN	INC_OUT	POS_IN	INC_OUT	INC_OUT	POS_IN	INC_OUT	POS_IN	POS_IN	NEG_IN
PXR binding ^{a)}	INC_OUT	NEG_IN	INC_OUT	POS_IN	INC_OUT	NEG_OUT	POS_IN	NEG_OUT	INC_OUT	INC_OUT	INC_OUT	NEG_IN	NEG_IN	NEG_IN
PXR binding, new model ^{a)}	POS_IN	NEG_OUT	INC_OUT	POS_IN	POS_IN	INC_OUT	POS_IN	INC_OUT	INC_OUT	POS_OUT	INC_OUT	INC_OUT	NEG_IN	NEG_IN
PXR activation ^{a)}	INC_OUT	INC_OUT	POS_IN	POS_IN	INC_OUT	INC_OUT	POS_IN	INC_OUT	NEG_IN	INC_OUT	NEG_IN	NEG_OUT	NEG_IN	NEG_IN
PXR activation ^{c)}	POS_IN	NEG_IN	POS_IN	POS_IN	POS_IN	INC_OUT	POS_IN	NEG_IN	NEG_IN	POS_IN	INC_OUT	POS_IN	NEG_IN	NEG_IN
CYP3A4 induction ^{a)}	INC_OUT	INC_OUT	INC_OUT	INC_OUT	POS_OUT	INC_OUT	POS_OUT	INC_OUT	INC_OUT	INC_OUT	INC_OUT	INC_OUT	NEG_IN	NEG_IN
CAR activation (50 μ M) ^{a)}	INC_OUT	POS_OUT	INC_OUT	INC_OUT	INC_OUT	NEG_IN	POS_OUT	NEG_IN	NEG_IN	INC_OUT	INC_OUT	INC_OUT	NEG_IN	INC_OUT
In vitro carcinogenicity ^{d)}	POS_IN	INC_OUT	POS_IN	POS_IN	INC_OUT	INC_OUT	INC_OUT	INC_OUT	INC_OUT	INC_OUT	INC_OUT	POS_IN	NEG_IN	POS_OUT
Liver specific cancer	INC_OUT	POS_IN	POS_IN	INC_OUT	POS_IN	INC_OUT	INC_OUT	INC_OUT	INC_OUT	INC_OUT	INC_OUT	POS_OUT	INC_OUT	NEG_IN
Severe Skin Irritation	NEG_IN	POS_IN	NEG_OUT	INC_OUT	INC_OUT	INC_OUT	INC_OUT	POS_IN	NEG_IN	INC_OUT	INC_OUT	INC_OUT	NEG_IN	POS_OUT
Similarity to difenoconazole (shared assays)	1 (10)	0.83 (7)	1 (7)	1 (9)	1 (7)	1 (1)	1 (8)	0.8 (6)	0.89 (6)	1 (5)	0 (2)	1 (7)	0.36 (10)	0 (5)

Note: Compounds with positive predictions that fall outside the model's applicability domain are not highlighted. Individual outputs for all the conazoles are also provided in the Appendix I.

Note: a) human in vitro, b) human in vitro, CoMPARA data, c) rat in vitro, d) Syrian Hamster Embryo Cell (SHEC) Transformation

The *in silico* models provided some evidence on shared properties within the grouped compounds, as TC difenoconazole and SCs epoxiconazole and propiconazole showed very similar model predictions consistent with that shown by structure-based clustering. Nonetheless, the scattered picture, the low number of relevant models were not considered conclusive to assess the read-across hypothesis. The toxicological properties of the compounds were therefore in the next tier also evaluated by high-throughput *in vitro* assays.

Cell Viability

The cytotoxicity assays were conducted for the cells used in the *in vitro* assays including the U2OS (CALUX®), HepG2 (ToxProfiler) and PHH (Transcriptomics). In the U2OS cells, cytotoxicity was observed for 4 of the 11 tested conazoles and for 2 of the 3 tested controls (**Table A3.3**). These include difenoconazole, cyproconazole, prothioconazole, hydroxyitraconazole, and the positive controls rotenone and tunicamycin. Other conazole compounds, alongside the metabolite 1,2,4-triazole and the negative control 2-phenylphenol exhibit no cytotoxic effects at highest tested dose (1000 µM).

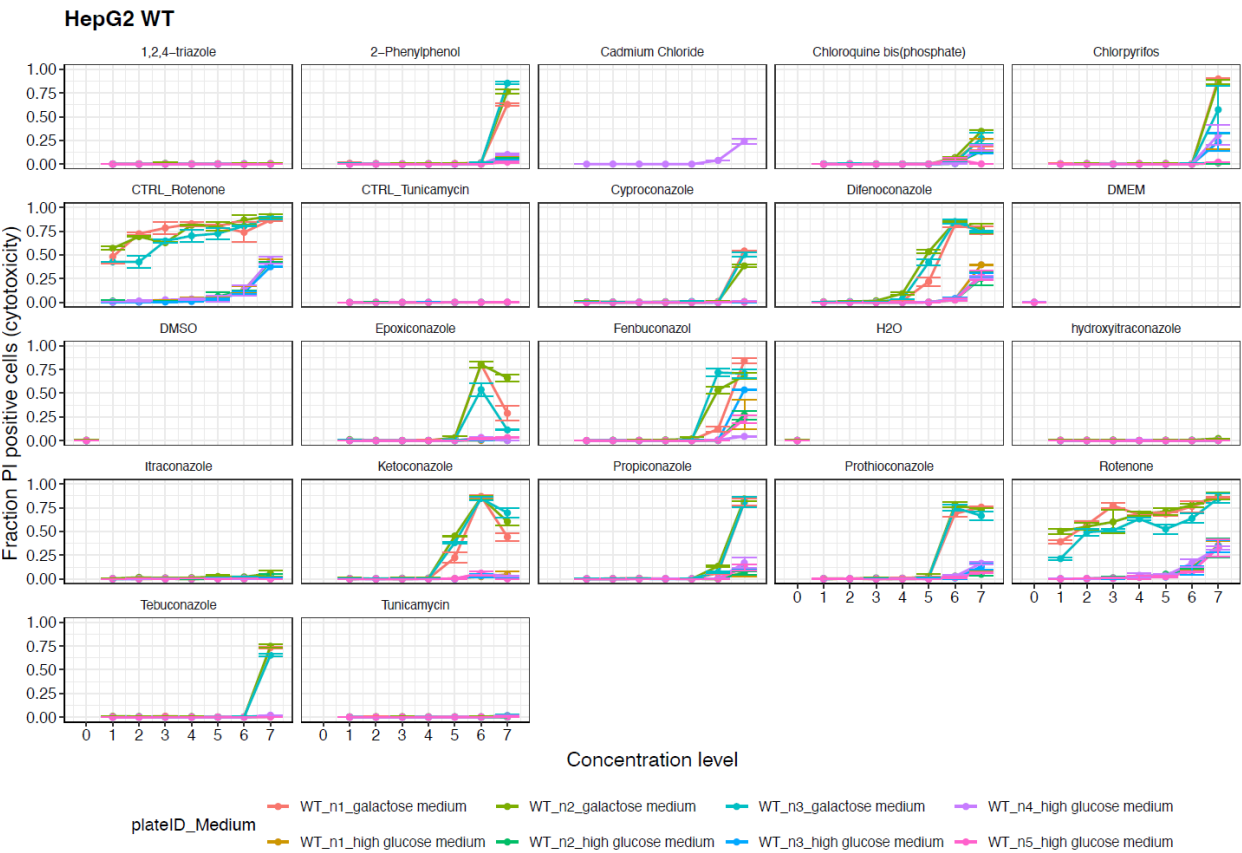
Table A3.3. Cytotoxicity of U2OS test system used in for CALUX® assay

Compound	Cytotoxicity (-log conc.)
Difenoconazole (TC)	-4.339
Cyproconazole	-3.955
Epoxiconazole	NA
Fenbuconazole	NA
Itraconazole	NA
Ketoconazole	NA
Propiconazole	NA
Prothioconazole	-4.234
Tebuconazole	NA
1,2,4-triazole	NA
Hydroxyitraconazole	-5.002
Rotenone	-5.915
Tunicamycin (from Streptomyces)	-4.481
2-Phenylphenol	-NA

Note: NA: No cytotoxic effects observed

Cytotoxicity in the parental HepG2 cells in the ToxProfiler assays was assessed by propidium iodide (PI) staining in both glucose- and galactose-containing media after 24 h exposure. The positive control rotenone induced more than a two-fold higher level of cytotoxicity in galactose medium compared to glucose medium. A similar trend in cytotoxicity induction was observed for several conazole compounds including cyproconazole, difenoconazole, epoxiconazole, fenbuconazole, ketoconazole, propiconazole, and tebuconazole. In contrast, itraconazole as well as the metabolites 1,2,4-triazole and hydroxyitraconazole exhibited minimal to no cytotoxicity across the tested concentration range (**Figure A3.4.**).

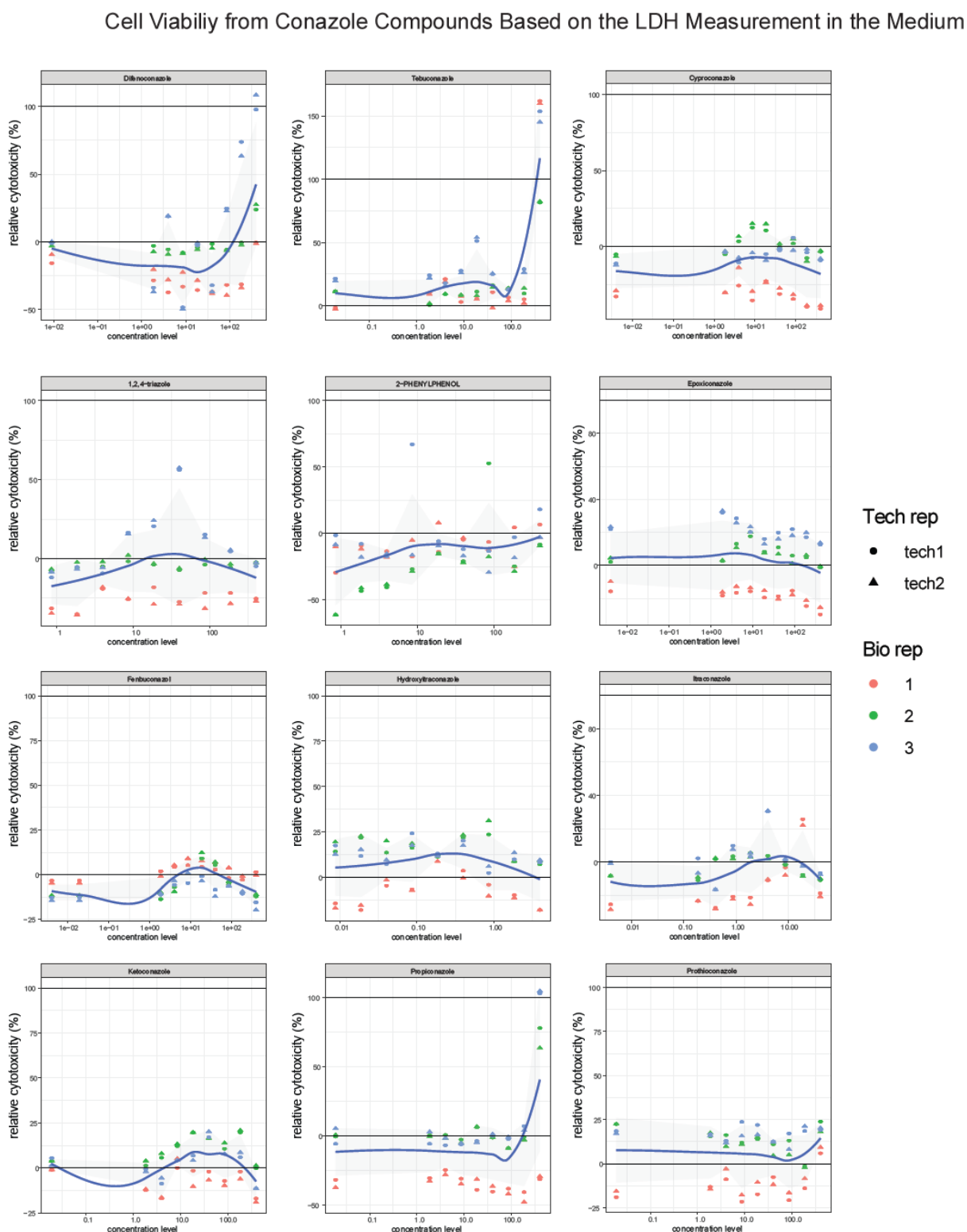
Figure A3.4. Cytotoxicity concentration-response curves of conazole compounds in parental HepG2 cells after 24 h exposure



Note: In the ToxProfiler assay, cytotoxicity was assessed using propidium iodide (PI) staining. Data are expressed as mean $\pm$ standard deviation from three biological replicates. Colors indicate the different biological replicates and the two medium conditions (glucose- or galactose-containing medium).

Cytotoxicity measurement of the PHHs exposed to conazole compounds were based on the LDH release, corresponding to 0% - vehicle control (minimal LDH release) to 100% cell death – 2% triton exposed cells (maximum LDH release). Difenoconazole, tebuconazole and propiconazole showed at higher concentration levels (>100) increasing trends of cytotoxic effects with some replicates reaching up to 100%. The remaining compounds exposed to the PHH were mostly below or slightly at the baseline level of 0% cytotoxicity (**Figure A3.5**).

Figure A3.5. Cytotoxicity measurement of the PHHs exposed to conazole compounds based on the LDH release



Note: The shape of the point indicates the technical replicates, and the color of the points indicates the biological replicate. The cytotoxicity values were normalized to the medium as the 0% cell death (minimum LDH release – 0%) and triton as the 100% cell death (maximum LDH release – 100%).

CALUX® assay

The effect of conazoles in the case study, hydroxyitraconazole, 1,2,4-triazole, positive controls-tunicamycin, rotenone and negative control, 2-phenylphenol for different receptors was measured using CALUX® assays (Table A3.4). Biokinetics analysis was carried out using VIVD model to predict free media concentration which was regarded as LEC of the assay (Table A3.4c). As the static version of VIVD model was used (Fisher, Simeon, Jamei, Gardner, & Bois, 2019), the ratio between nominal to free media concentration is constant for all the concentrations. This ratio was used to predict LEC showing effect in the assays (Table A3.4b). All the conazoles were active in the androgen receptor antagonistic (AR-) CALUX® assay, displaying range of potencies. All conazoles except itraconazole showed antagonistic effect on progesterin receptor (PR-). Cyproconazole, difenoconazole, epoxiconazole, itraconazole, ketoconazole and propiconazole showed potent effect on glucocorticoid receptor antagonism (GR-). All conazoles except itraconazole and ketoconazole showed response in PXR CALUX® assay with varying potency. However, itraconazole and ketoconazole showed response in PPARγ antagonistic assay along with difenoconazole, epoxiconazole and prothioconazole. Ketoconazole, prothioconazole and tebuconazole also showed effect in NRF2 response assay. Additional individual responses were observed in the CALUX® test battery, highlighting that, while some common biological effects were shared, differences in activity also emerged, likely due to the structural variations among the conazoles.

Among the metabolites, hydroxyitraconazole showed response in the cytotoxicity assessment by CALUX® assay but 1,2,4-triazole did not show effect in any of the CALUX® assays. The positive controls rotenone and tunicamycin also showed potent response in AR-, GR- and PR- assays which are consistent with the known biology of these compounds. The negative control 2-phenylphenol also showed expected effects such as AR agonism.

In summary, the TC showed similar biological activity to SCs cyproconazole, epoxiconazole, propiconazole, fenbuconazole and tebuconazole but showed dissimilar biological activity to SCs itraconazole, ketoconazole and prothioconazole as all these compounds showed thyroid hormone receptor beta antagonistic (TRβ-) activity.

Table A3.4. Biological activity of conazoles, metabolites, positive controls and negative controls in CALUX® assays

a)

Original readouts

Compound	Cytotox	API	AR+	AR-	ER+	ER-	ESRE	GR+	GR-	NRF2	P21	PR+	PR-	ppara+	ppara-	ppard+	ppard-	pparg+	pparg-	PXR	RAR	TRb+	TRb-
Cyproconazol	-4.0	-3.0	-3.0	-5.4	-3.0	-3.0	-3.0	-3.0	-5.5	-3.0	-3.0	-3.0	-5.1	-3.0	-3.0	-3.0	-3.0	-3.0	-3.0	-4.9	-3.0	-3.0	-3.0
Difenoconazole	-4.3	-3.0	-3.0	-5.8	-3.0	-3.0	-3.0	-3.0	-7.1	-3.0	-3.0	-3.0	-5.6	-3.0	-5.4	-6.0	-3.0	-3.0	-5.1	-5.4	-3.0	-3.0	-3.0
Epoxiconazole	-3.0	-3.0	-3.0	-6.7	-3.0	-3.0	-3.0	-3.0	-8.0	-3.0	-3.0	-3.0	-5.9	-3.0	-3.0	-3.0	-3.0	-3.0	-4.8	-5.5	-3.0	-3.0	-3.0
Fenbuconazol	-3.0	-3.0	-3.0	-5.7	-3.0	-3.0	-3.0	-3.0	-3.0	-3.0	-3.0	-3.0	-7.0	-3.0	-3.0	-3.0	-3.0	-3.0	-3.0	-5.2	-3.0	-3.0	-3.0
Hydroxyitraconazole	-5.0	-3.0	-3.0	-3.0	-3.0	-3.0	-3.0	-3.0	-3.0	-3.0	-3.0	-3.0	-3.0	-3.0	-3.0	-3.0	-3.0	-3.0	-3.0	-3.0	-3.0	-3.0	-3.0
Itraconazole	-3.0	-3.0	-3.0	-6.0	-3.0	-3.0	-3.0	-3.0	-7.2	-3.0	-3.0	-3.0	-3.0	-3.0	-5.9	-3.0	-3.0	-3.0	-5.8	-3.0	-3.0	-3.0	-6.5
Ketoconazole	-3.0	-3.0	-3.0	-5.6	-3.0	-3.0	-3.0	-3.0	-6.4	-4.9	-3.0	-3.0	-5.7	-3.0	-3.0	-3.0	-3.0	-3.0	-4.3	-3.0	-3.0	-3.0	-5.8
Propiconazole	-3.0	-3.0	-3.0	-6.6	-3.0	-3.0	-3.0	-3.0	-6.2	-3.0	-3.0	-3.0	-6.4	-3.0	-3.0	-3.0	-3.0	-3.0	-3.0	-5.6	-3.0	-3.0	-3.0
Prothioconazole	-4.2	-3.0	-3.0	-5.1	-5.0	-3.0	-3.5	-3.0	-3.0	-3.6	-3.0	-3.0	-5.4	-3.0	-5.0	-3.0	-5.0	-3.0	-4.7	-5.2	-3.0	-3.0	-4.4
Tebuconazole	-3.0	-3.0	-3.0	-6.6	-3.0	-3.0	-3.0	-3.0	-3.0	-5.0	-3.0	-3.0	-5.3	-3.0	-3.0	-3.0	-3.0	-3.0	-3.0	-5.8	-3.0	-3.0	-3.0
1,2,4-triazole	-3.0	-3.0	-3.0	-3.0	-3.0	-3.0	-3.0	-3.0	-3.0	-3.0	-3.0	-3.0	-3.0	-3.0	-3.0	-3.0	-3.0	-3.0	-3.0	-3.0	-3.0	-3.0	-3.0
Rotenone	-5.9	-3.0	-3.0	-9.3	-3.0	-3.0	-3.0	-3.0	-9.6	-3.0	-3.0	-3.0	-8.9	-3.0	-6.5	-3.0	-3.0	-3.0	-6.8	-3.0	-3.0	-3.0	-6.6
Tunicamycin	-4.5	-3.0	-3.0	-8.2	-3.0	-7.3	-8.6	-3.0	-7.7	-3.0	-3.0	-3.0	-8.4	-3.0	-6.5	-3.0	-7.5	-3.0	-6.5	-3.0	-3.0	-3.0	-7.8
2-Phenylphenol	-3.0	-3.0	-6.7	-3.0	-4.4	-3.0	-3.0	-3.0	-5.5	-3.0	-8.7	-3.0	-3.0	-3.0	-3.0	-7.4	-3.0	-8.2	-3.0	-3.9	-4.6	-3.0	-4.4

Concentration

highest

lowest

b)

LEC

Compound	Cytotox	API	AR+	AR-	ER+	ER-	ESRE	GR+	GR-	NRF2	P21	PR+	PR-	ppara+	ppara-	ppard+	ppard-	pparg+	pparg-	PXR	RAR	TRb+	TRb-
Difenoconazole	3.148			0.099					0.005				0.164		0.249	0.069			0.576	0.256			
Cyproconazole	76.91			2.472					2.338				4.988							8.308			
Epoxiconazole				0.084					0.004				0.512						5.369	1.256			
Fenbuconazole				1.015									0.051							3.041			
Hydroxyitraconazole	0.461																						
Itraconazole				0.051					0.003						0.053				0.071				0.016
Ketoconazole				0.143					0.022	0.693			0.119						3.414				0.103
Propiconazole				0.063					0.146				0.094							0.553			
Prothioconazole	52.04	282.3		6.472	8.563		256.4			234			3.284	9.744		9.732		18.3	5.348			31.73	
Tebuconazole				0.064						2.66			1.276							0.432			
1,2,4-triazole																							
Rotenone	0.131			6E-05		129			3E-05				1E-04		0.033				0.018				0.029
Tunicamycin	33			0.007		0.047	0.003		0.021				0.004		0.302		0.034		0.321				0.016
2-Phenylphenol			0.083		16.54				1.526		0.001					0.017		0.003		58.35	10.72		17.49

Agonistic assay
Antagonistic assay
Cell viability assay

Unclassified

c) Biokinetics analysis for CALUX® assays

Compound	Media Conc. (mol L ⁻¹)	Media Mass Balance (%)	Air Conc. (mol L ⁻¹)	Air Mass Balance (%)	Plastic Conc. (mol L ⁻¹)	Plastic Mass Balance (%)	Cell Conc. (mol L ⁻¹)	Cell Mass Balance (%)	Lyso Conc. (mol L ⁻¹)	Lyso Mass Balance (%)	Mito Conc. (mol L ⁻¹)	Mito Mass Balance (%)	Total Mass Balance (%)	Nominal Conc (M)	Ratio (Nominal/ Media Conc.)
Cyproconazole	6.94E-07	9.42E+01	5.13E-14	6.16E-07	3.83E-08	5.73E+00	3.43E-06	4.27E-02	3.43E-06	4.79E-04	3.43E-06	4.79E-03	1.00E+02	1.00E-06	1.44
Difenoconazole	6.87E-08	8.69E+01	6.38E-17	7.66E-10	8.63E-08	1.29E+01	1.11E-05	1.38E-01	1.11E-05	1.55E-03	1.11E-05	1.55E-02	1.00E+02	1.00E-06	14.6
Epoxiconazole	3.77E-07	8.95E+01	1.56E-14	1.88E-07	6.95E-08	1.04E+01	6.98E-06	8.68E-02	6.98E-06	9.75E-04	6.98E-06	9.75E-03	1.00E+02	1.00E-06	2.65
Fenbuconazole	5.03E-07	9.12E+01	4.36E-12	5.23E-05	5.80E-08	8.68E+00	5.53E-06	6.88E-02	5.53E-06	7.73E-04	5.53E-06	7.73E-03	1.00E+02	1.00E-06	1.99
hydroxyitraconazole	4.63E-08	8.71E+01	2.23E-32	2.67E-25	8.52E-08	1.28E+01	1.15E-05	1.43E-01	1.14E-05	1.60E-03	1.15E-05	1.61E-02	1.00E+02	1.00E-06	21.6
Itraconazole	4.63E-08	8.71E+01	6.09E-28	7.31E-21	8.52E-08	1.28E+01	1.15E-05	1.43E-01	1.14E-05	1.60E-03	1.15E-05	1.61E-02	1.00E+02	1.00E-06	21.6
Ketoconazole	6.13E-08	8.69E+01	2.70E-25	3.24E-18	8.62E-08	1.29E+01	9.99E-06	1.24E-01	5.44E-06	7.59E-04	1.00E-05	1.40E-02	1.00E+02	1.00E-06	16.3
Propioconazole	2.34E-07	8.78E+01	4.19E-14	5.03E-07	8.05E-08	1.21E+01	8.73E-06	1.09E-01	8.73E-06	1.22E-03	8.73E-06	1.22E-02	1.00E+02	1.00E-06	4.28
Prothioconazole	8.92E-07	9.77E+01	8.81E-17	1.06E-09	1.51E-08	2.26E+00	1.36E-06	1.69E-02	1.36E-06	1.90E-04	1.36E-06	1.90E-03	1.00E+02	1.00E-06	1.12
Tebuconazole	2.43E-07	8.79E+01	3.67E-15	4.41E-08	7.99E-08	1.20E+01	8.62E-06	1.07E-01	8.62E-06	1.20E-03	8.62E-06	1.20E-02	1.00E+02	1.00E-06	4.12
1,2,4-triazole	1.00E-06	1.00E+02	1.59E-10	1.91E-03	2.32E-11	3.48E-03	3.15E-07	3.91E-03	3.15E-07	4.39E-05	3.15E-07	4.39E-04	1.00E+02	1.00E-06	1
Rotenone	1.07E-07	8.69E+01	3.86E-15	4.63E-08	8.64E-08	1.29E+01	1.05E-05	1.30E-01	1.05E-05	1.46E-03	1.05E-05	1.46E-02	1.00E+02	1.00E-06	9.31
Tunicamycin	1.00E-06	1.00E+02	6.02E-47	7.22E-40	4.34E-11	6.50E-03	3.15E-07	3.92E-03	3.15E-07	4.40E-05	3.15E-07	4.40E-04	1.00E+02	1.00E-06	1
2-phenylphenol	4.48E-07	9.05E+01	2.01E-12	2.41E-05	6.31E-08	9.46E+00	6.16E-06	7.65E-02	6.16E-06	8.60E-04	6.16E-06	8.60E-03	1.00E+02	1.00E-06	2.23

Note: (a) Lowest concentrations in molar showing activity are shown as log values and (b) LEC (µM) obtained from using (c) biokinetics analysis using VIVD model (Fisher et al., 2019). Panels (a) and (b) use a red-to-green color scale to indicate increasing concentration values, with red representing the lowest and green the highest.

ToxProfiler assay

ToxProfiler assay was used to evaluate the response of compounds to different stress assays (Table A3.5). The nominal concentrations associated with toxicity were converted to free media concentrations which was regarded as LEC (Table A3.5b) using the biokinetics analysis of the compounds (Table A3.5c). All compounds showed effect in cell cycle stress assessed with p21-GFP assay. Hydroxyitraconazole, itraconazole and positive control rotenone showed the most potent effect in cell-cycle stress while the negative control 2-phenylphenol and cyproconazole showed least potency. All conazoles except fenbuconazole, hydroxyitraconazole and itraconazole showed an increase in oxidative stress measured by SRXN1-GFP signal. Effects of conazole on autophagy and mitochondrial toxicity measured by glucose and galactose ratio were also observed. The glucose/galactose cytotoxicity ratio of ketoconazole and difenoconazole is 2.86 and 4.44 suggesting that these compounds can cause mitochondrial toxicity. The positive control rotenone which is a known agent of oxidative stress also showed high glucose/galactose cytotoxicity ratio. None of the tested compounds showed any effect on inflammation marker ICAM1-GFP. Protein stress was observed by only prothioconazole while effect on ion stress was observed by fenbuconazole, ketoconazole, prothioconazole and cyproconazole. All conazoles except itraconazole and hydroxyitraconazole show increase in ER-Stress measured by quantifying the levels of nuclear CHOP protein. ER stress is usually associated with liver toxicity and the development of various liver diseases.

The TC difenoconazole exhibited biological responses strongly similar to the SCs propiconazole and epoxiconazole, and moderately similar to cyproconazole and tebuconazole—consistent with patterns observed in the CALUX® assays. However, in contrast to the CALUX® results, fenbuconazole displayed dissimilar responses compared to difenoconazole in the ToxProfiler assays. The similarity between TC difenoconazole and SCs propiconazole, epoxiconazole, cyproconazole and tebuconazole were also observed in structure-based clustering (Figure A3.2).

Table A3. 5. Biological effects of the conazoles, positive controls and negative controls evaluated using ToxProfiler Cell stress assays

a)

Original readouts

Compounds	Oxidative stress SRXN1-GFP	ER stress CHOP-GFP	Cell cycle stress p21-GFP	Ion stress MT1X-GFP	Protein Stress HSPA1B-GFP	Autophagy LC3-GFP	Inflammation ICAM1-GFP	Cytotoxicity Glucose	Cytotoxicity Galactose
Cyproconazole	120	259	234	271		120			242
Difenoconazole	9	14	8			59		40	9
Epoxiconazole	86	86	60			86		251	100
Fenbuconazole		60	35	60				128	60
Hydroxyitraconazole			1			0.4			
Itraconazole			2			0.4			
Ketoconazole	9	13	9	86		3		86	30
Propiconazole	40	30	35			141		126	60
Prothioconazole	86	120	69	120	120	295		230	120
Tebuconazole	60	111	50			102			105
1,2,4-triazole									
Rotenone		<0.18	1					1	<0.18
Tunicamycin	2	0.4				4			
2-Phenylphenol	86	240	185					244	236



b)

LEC

Compounds	Oxidative stress SRXN1-GFP	ER stress CHOP-GFP	Cell cycle stress p21-GFP	Ion stress MT1X-GFP	Protein Stress HSPA1B-GFP	Autophagy LC3-GFP	Inflammation ICAM1-GFP	Cytotoxicity Glucose	Cytotoxicity Galactose
Difenoconazole	0.63	0.99	0.56			4.15		2.82	0.63
Cyproconazole	83.86	180.99	163.52	189.38		83.86			169.11
Epoxiconazole	32.97	32.97	23.00			32.97		96.22	38.34
Fenbuconazole		29.94	17.47	29.94				63.88	29.94
Hydroxyitraconazole			0.05			0.02			
Itraconazole			0.10			0.02			
Ketoconazole	0.57	0.82	0.57	5.43		0.19		5.43	1.90
Propiconazole	9.55	7.16	8.35			33.65		30.07	14.32
Prothioconazole	76.86	107.25	61.67	107.25	107.25	263.66		205.57	107.25
Tebuconazole	14.86	27.49	12.38			25.26			26.01
1,2,4-triazole									
Rotenone		0.02	0.04					0.11	0.02
Tunicamycin	2	0.4				4			
2-Phenylphenol	39.09	109.09	84.09					110.91	107.27

c) Biokinetics analysis for ToxProfiler assay

Compound	Media Conc. (mol L ⁻¹)	Media Mass Balance (%)	Air Conc. (mol L ⁻¹)	Air Mass Balance (%)	Plastic Conc. (mol L ⁻¹)	Plastic Mass Balance (%)	Cell Conc. (mol L ⁻¹)	Cell Mass Balance (%)	Lyso Conc. (mol L ⁻¹)	Lyso Mass Balance (%)	Mito Conc. (mol L ⁻¹)	Mito Mass Balance (%)	Total Mass Balance (%)	Nominal Conc (M)	Ratio (Nominal/Media Conc.)
Cyproconazole	6.99E-07	9.38E+01	5.17E-14	9.83E-06	3.85E-08	4.73E+00	1.20E-05	1.35E+00	1.20E-05	1.52E-02	1.20E-05	1.52E-01	1.00E+02	1.00E-06	1.43
Difenoconazole	7.04E-08	8.52E+01	6.54E-17	1.24E-08	8.85E-08	1.09E+01	3.08E-05	3.48E+00	3.08E-05	3.91E-02	3.08E-05	3.91E-01	1.00E+02	1.00E-06	14.2
Epoxiconazole	3.83E-07	8.85E+01	1.59E-14	3.02E-06	7.06E-08	8.67E+00	2.26E-05	2.56E+00	2.26E-05	2.87E-02	2.26E-05	2.87E-01	1.00E+02	1.00E-06	2.61
Fenbuconazole	5.09E-07	9.04E+01	4.41E-12	8.39E-04	5.87E-08	7.20E+00	1.85E-05	2.09E+00	1.85E-05	2.35E-02	1.85E-05	2.35E-01	1.00E+02	1.00E-06	1.96
Hydroxyitraconazole	4.75E-08	8.53E+01	2.29E-32	4.34E-24	8.74E-08	1.07E+01	3.09E-05	3.50E+00	3.06E-05	3.90E-02	3.09E-05	3.93E-01	1.00E+02	1.00E-06	21.03
Itraconazole	4.75E-08	8.53E+01	6.25E-28	1.19E-19	8.74E-08	1.07E+01	3.09E-05	3.50E+00	3.06E-05	3.90E-02	3.09E-05	3.93E-01	1.00E+02	1.00E-06	21.03
Ketoconazole	6.32E-08	8.56E+01	2.78E-25	5.28E-17	8.89E-08	1.09E+01	2.75E-05	3.12E+00	1.32E-05	1.68E-02	2.77E-05	3.53E-01	1.00E+02	1.00E-06	15.82
Propioconazole	2.39E-07	8.65E+01	4.27E-14	8.12E-06	8.22E-08	1.01E+01	2.69E-05	3.05E+00	2.69E-05	3.43E-02	2.69E-05	3.43E-01	1.00E+02	1.00E-06	4.19
Prothioconazole	8.94E-07	9.75E+01	8.83E-17	1.68E-08	1.51E-08	1.85E+00	4.80E-06	5.44E-01	4.80E-06	6.11E-03	4.80E-06	6.11E-02	1.00E+02	1.00E-06	1.12
Tebuconazole	2.48E-07	8.66E+01	3.75E-15	7.12E-07	8.15E-08	1.00E+01	2.67E-05	3.02E+00	2.67E-05	3.39E-02	2.67E-05	3.39E-01	1.00E+02	1.00E-06	4.04
1,2,4-triazole	9.99E-07	9.99E+01	1.59E-10	3.03E-02	2.32E-11	2.85E-03	5.72E-07	6.48E-02	5.72E-07	7.28E-04	5.72E-07	7.28E-03	1.00E+02	1.00E-06	1
Rotenone	1.10E-07	8.53E+01	3.95E-15	7.51E-07	8.85E-08	1.09E+01	3.01E-05	3.41E+00	3.01E-05	3.83E-02	3.01E-05	3.83E-01	1.00E+02	1.00E-06	9.09
Tunicamycin	9.99E-07	9.99E+01	6.01E-47	1.14E-38	4.34E-11	5.32E-03	5.77E-07	6.54E-02	5.77E-07	7.34E-04	5.77E-07	7.34E-03	1.00E+02	1.00E-06	1
2-phenylphenol	4.55E-07	8.96E+01	2.04E-12	3.88E-04	6.41E-08	7.86E+00	2.03E-05	2.30E+00	2.03E-05	2.58E-02	2.03E-05	2.58E-01	1.00E+02	1.00E-06	2.2

Note: Lowest (a) total concentrations (μM) showing response in cell stress assays converted to (b) LEC (μM) obtained using (c) biokinetics analysis of the compounds using VIVD model (Fisher et al., 2019).

Cell Painting PLUS (CPP)

The Cell Painting PLUS (CPP) assay was used to evaluate the impact of the compounds on various cellular components, including actin filaments, DNA, the endoplasmic reticulum, Golgi apparatus, lysosomes, mitochondria, RNA, and general cell morphology. To assess the cellular impact of conazoles, the median benchmark concentration (BMC) was calculated for each organelle-specific feature and used to compare compound-induced effects across cellular structures. The nominal median BMC was converted to free media concentration (**Table A3.6b**) by biokinetics analysis (**Table A3.6c**). This free media concentration was regarded as LEC.

In the CPP assay, itraconazole, difenoconazole and ketoconazole were more potent than the other conazoles (**Table A3.6**). Cyproconazole had the highest median BMCs for all organelles and was the least potent of the conazoles. All the conazoles showed strong effects on mitochondria which may be one mechanism and morphological change of liver toxicity by this class of compounds. Hydroxyitraconazole and itraconazole showed comparable potency on actin and RNA. Hydroxyitraconazole was three times more potent than itraconazole on DNA and ER, as well as seven times more potent on golgi apparatus and lysosomes. Hydroxyitraconazole was six times more potent than itraconazole for the effect on mitochondria.

In summary, the TC difenoconazole showed similar response to all SCs, except cyproconazole which showed lower potency than other conazoles. This differs from the responses observed in the CALUX® and ToxProfiler assays, which may be attributed to the distinct biological targets and mechanisms assessed by each assay type. The overall potency of TC (showed as low median BMC) is high in the CPP assay.

Table A3. 6. BMCs of the conazoles, positive controls and negative controls for CPP assay

a)

Original readouts

Compounds	Actin	DNA	ER	Generic	Golgi	Lyso	Mito	RNA
Cyproconazole	96.25	84.15	101.60	83.50	186.43	147.83	8.29	162.11
Difenoconazole	4.35	1.79	4.23	2.74	3.38	2.39	2.75	3.41
Epoxiconazole	67.08	26.36	37.78	17.08	40.11	55.82	10.11	31.75
Fenbuconazole	20.56	21.66	24.62	17.96	21.40	26.17	2.64	32.38
Hydroxyitraconazole	3.19	1.94	3.19	3.32	0.48	0.50	2.76	3.59
Itraconazole	3.42	19.70	8.82	4.34	3.61	3.88	3.70	1.15
Ketoconazole	16.73	26.34	15.60	3.11	4.69	4.58	0.21	12.83
Propiconazole	28.39	20.45	40.05	17.95	29.21	44.68	1.97	38.94
Prothioconazole	36.40	36.96	39.62	36.40	34.58	36.56	6.84	36.38
Tebuconazole	43.65	63.47	46.88	32.82	41.12	60.88	24.04	55.98
1,2,4-triazole								
Rotenone	0.00318	0.00318	0.00318	0.00318	0.00318	0.00318	0.00318	0.00318
Tunicamycin	8.31	5.10	1.56	0.59	1.22	1.33	0.76	1.32
2-Phenylphenol	87.47	15.78	68.82	63.54	83.54	80.45	44.41	70.23

b)

LEC

Compound	Actin	DNA	ER	Generic	Golgi	Lyso	Mito	RNA
Difenoconazole	0.50	0.20	0.51	0.32	0.41	0.29	0.33	0.37
Cyproconazole	76.51	52.07	81.11	64.74	135.31	109.62	6.13	119.21
Epoxiconazole	30.60	13.84	19.83	9.11	21.42	28.92	5.31	16.77
Fenbuconazole	13.45	14.02	15.16	10.75	13.40	15.56	1.72	19.32
Hydroxyitraconazole	0.26	0.13	0.26	0.18	0.04	0.04	0.20	0.29
Itraconazole	0.43	0.35	0.83	0.55	0.30	0.32	1.06	0.35
Ketoconazole	1.76	1.32	1.68	0.33	0.51	0.50	0.02	1.32
Propiconazole	10.53	7.02	14.38	6.29	10.28	15.87	0.70	12.28
Prothioconazole	29.49	34.22	34.73	34.07	21.12	34.67	6.14	34.33
Tebuconazole	19.84	22.19	17.23	12.98	14.46	21.12	8.88	21.66
1,2,4-triazole								
Rotenone	0.00058	0.00058	0.00058	0.00058	0.00058	0.00208	0.00058	0.00058
Tunicamycin	4.88	3.92	1.37	0.61	1.21	1.32	0.7	1.22
2-Phenylphenol	31.87	39.17	37.48	37.89	41.13	42.68	25.43	40.59

Concentration

highest

lowest

c) Biokinetics analysis for Cell Painting analysis

Compound	Media Conc. (mol L ⁻¹)	Media Mass Balance (%)	Air Conc. (mol L ⁻¹)	Air Mass Balance (%)	Plastic Conc. (mol L ⁻¹)	Plastic Mass Balance (%)	Cell Conc. (mol L ⁻¹)	Cell Mass Balance (%)	Lyso Conc. (mol L ⁻¹)	Lyso Mass Balance (%)	Mito Conc. (mol L ⁻¹)	Mito Mass Balance (%)	Total Mass Balance (%)	Nominal Conc.	Ratio (Nominal/Media Conc.)
Cyproconazole	8.03E-07	9.40E+01	5.94E-14	2.67E-06	4.43E-08	5.43E+00	1.37E-05	5.19E-01	1.37E-05	5.83E-03	1.37E-05	5.83E-02	1.00E+02	1.00E-06	1.25
Difenoconazole	1.21E-07	7.91E+01	1.12E-16	5.05E-09	1.52E-07	1.86E+01	5.28E-05	1.99E+00	5.28E-05	2.24E-02	5.28E-05	2.24E-01	1.00E+02	1.00E-06	8.28
Epoxiconazole	5.25E-07	8.68E+01	2.18E-14	9.80E-07	9.67E-08	1.19E+01	3.09E-05	1.17E+00	3.09E-05	1.31E-02	3.09E-05	1.31E-01	1.00E+02	1.00E-06	1.9
Fenbuconazole	6.47E-07	8.98E+01	5.61E-12	2.52E-04	7.46E-08	9.15E+00	2.35E-05	8.87E-01	2.35E-05	9.97E-03	2.35E-05	9.97E-02	1.00E+02	1.00E-06	1.54
hydroxyitracon	8.33E-08	7.89E+01	4.00E-32	1.80E-24	1.53E-07	1.88E+01	5.42E-05	2.04E+00	5.36E-05	2.27E-02	5.42E-05	2.30E-01	1.00E+02	1.00E-06	12.01
Itraconazole	8.33E-08	7.89E+01	1.10E-27	4.93E-20	1.53E-07	1.88E+01	5.42E-05	2.04E+00	5.36E-05	2.27E-02	5.42E-05	2.30E-01	1.00E+02	1.00E-06	12.01
Ketoconazole	1.09E-07	7.92E+01	4.79E-25	2.16E-17	1.53E-07	1.88E+01	4.74E-05	1.79E+00	2.29E-05	9.71E-03	4.78E-05	2.03E-01	1.00E+02	1.00E-06	9.18
Propioconazole	3.59E-07	8.31E+01	6.44E-14	2.90E-06	1.24E-07	1.52E+01	4.05E-05	1.53E+00	4.05E-05	1.72E-02	4.05E-05	1.72E-01	1.00E+02	1.00E-06	2.78
Prothioconazole	9.36E-07	9.78E+01	9.24E-17	4.16E-09	1.58E-08	1.94E+00	5.05E-06	1.90E-01	5.05E-06	2.14E-03	5.05E-06	2.14E-02	1.00E+02	1.00E-06	1.07
tebuconazole	3.71E-07	8.33E+01	5.61E-15	2.52E-07	1.22E-07	1.50E+01	3.99E-05	1.51E+00	3.99E-05	1.69E-02	3.99E-05	1.69E-01	1.00E+02	1.00E-06	2.7
Rotenone	1.83E-07	7.99E+01	6.56E-15	2.95E-07	1.47E-07	1.80E+01	5.00E-05	1.89E+00	5.00E-05	2.12E-02	5.00E-05	2.12E-01	1.00E+02	1.00E-06	5.48
Tunicamycin	1.00E-06	1.00E+02	6.02E-47	2.71E-39	4.34E-11	5.32E-03	5.96E-07	2.25E-02	5.96E-07	2.53E-04	5.96E-07	2.53E-03	1.00E+02	1.00E-06	1
2-phenylphenol	5.96E-07	8.86E+01	2.68E-12	1.20E-04	8.40E-08	1.03E+01	2.66E-05	1.00E+00	2.66E-05	1.13E-02	2.66E-05	1.13E-01	1.00E+02	1.00E-06	1.68
1,2,4-triazole	1.00E-06	1.00E+02	1.59E-10	7.16E-03	2.32E-11	2.85E-03	5.92E-07	2.23E-02	1.69E-06	7.15E-04	5.91E-07	2.50E-03	1.00E+02	1.00E-06	1

Note: Median (a) BMCs (µM) showing response in CPP assay converted to (b) LEC (µM) obtained using (c) biokinetics analysis of the compounds using VIVD model (Fisher et al., 2019).

Combined analysis by all high-throughput assays

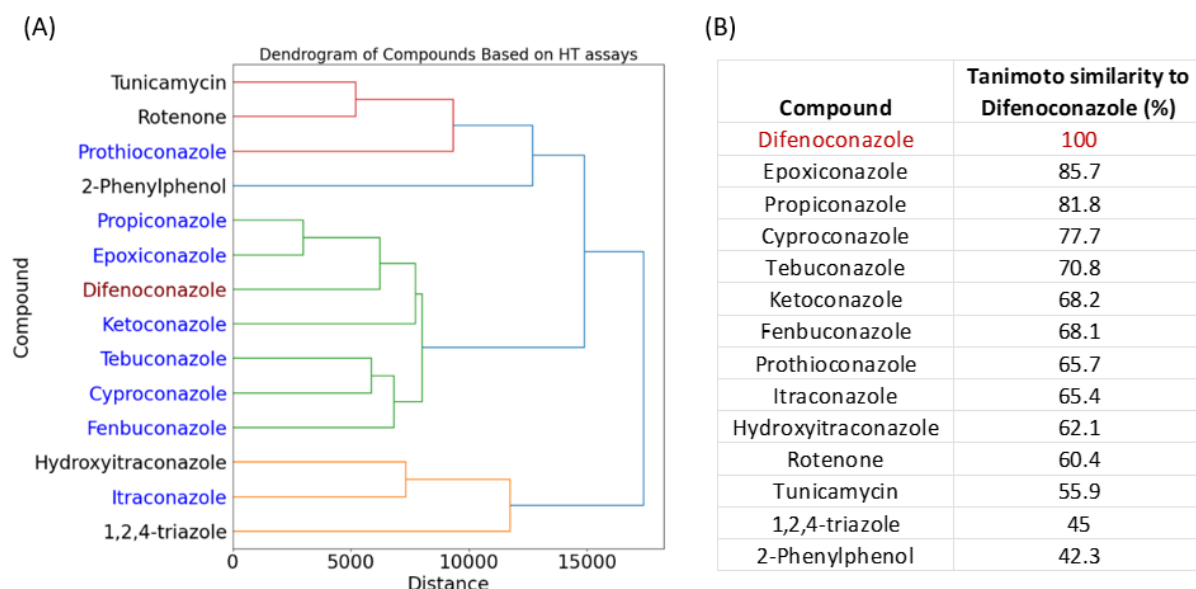
The response observed in CALUX®, ToxProfiler (Cell stress assays) and CPP assays was combined, and clustering was performed using the LEC at which compounds elicited biological responses to identify relationships between effect profiles and compound potency (**Figure A3.6.**). Hierarchical clustering was performed using Ward's method on the standardized dataset. The resulting linkage matrix was used to generate a dendrogram, which was visualized using the dendrogram function from the SciPy library (version 1.10.1).

The TC, difenoconazole was clustered with a group formed by propiconazole and epoxiconazole, with ketoconazole also joining this cluster. Additionally, the SCs, tebuconazole and cyproconazole were clustered together and fenbuconazole was grouped with this subcluster. Thus, the TC was clustered with the SCs, propiconazole, epoxiconazole, ketoconazole, tebuconazole, cyproconazole, and fenbuconazole.

The positive controls rotenone and tunicamycin are clustered together reflecting similar potency of these compounds (**Figure A3.6A.**). Prothioconazole and 2-phenylphenol are also clustered in this subcluster. Notably, prothioconazole is structurally distinct from other conazoles, featuring a triazolinethione ring in which a sulfur atom replaces the nitrogen typically found in the triazole ring of other compounds in this class. Prothioconazole is metabolized into desthio-prothioconazole which is considered to also contribute to its antifungal activity. The cell systems used in the high throughput assays are non-metabolizing cells. The distinct clustering of prothioconazole from other conazoles may be attributed to its unique chemical structure, specifically the presence of a triazolinethione ring. Hydroxyitraconazole and itraconazole clustered closely together, indicating that the parent compound and its hydroxylated metabolite exhibit largely similar biological effects and potencies. 1,2,4-triazole was clustered with this cluster.

In summary, the subcluster formed by propiconazole, epoxiconazole, and difenoconazole in the structural similarity analysis (**Figure A3.2.**) was largely preserved in the clustering based on high-throughput assay data (**Figure A3.6A.**). Additionally, ketoconazole, tebuconazole, cyproconazole, and fenbuconazole were grouped with this subcluster, suggesting that these conazoles share comparable biological activity profiles and are suitable for read-across analysis of difenoconazole. Additionally, the similarity calculation according to Tanimoto method was conducted using the LEC as input. Epoxiconazole, followed by propiconazole and cyproconazole were revealed as the top three of the most similar substances (**Figure A3.6B.**).

Figure A3.6. A) Hierarchical clustering and B) Tanimoto similarity of SCs, metabolites, positive controls and negative controls from the TC (difenoconazole) assessed by high throughput assays based on the LEC



6. PHH transcriptomics analysis

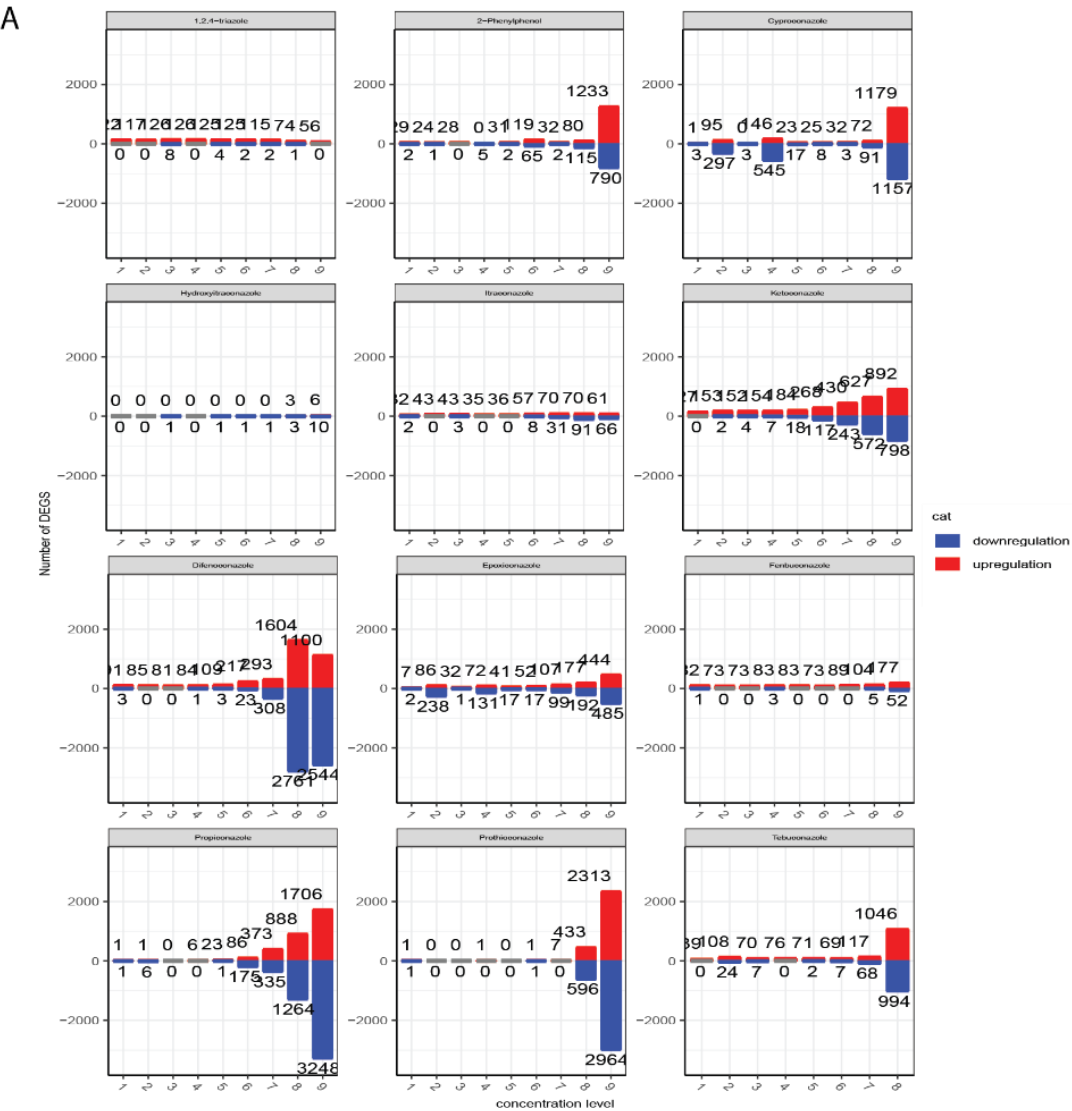
The effect of conazoles in PHHs was investigated using transcriptomics endpoints followed by evaluation of biological effect based on the PHH TXG-MAPr pathway enrichment analysis. The compounds show concentration dependent transcriptomic perturbation based on the number of differentially expressed genes (DEGs) in **Figure A3.7A**.

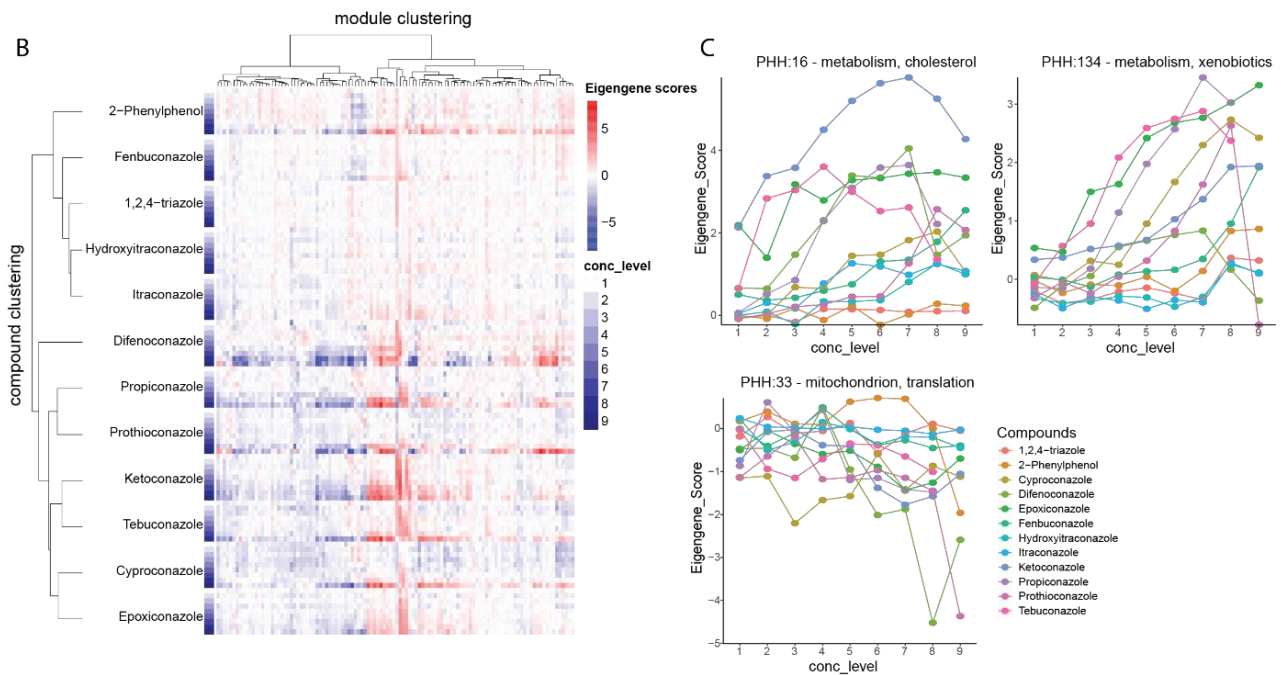
The pathways perturbed due to conazole exposure were identified based on the module-based enrichment from the PHH TXG-MAPr. This platform was used to investigate the mode of action of the different conazoles. The clustering of compounds using module-based analysis grouped epoxiconazole and cyproconazole, tebuconazole and ketoconazole, prothioconazole and propiconazole together (**Figure A3.7B**). Difenoconazole was clustered with these conazoles. Itraconazole and hydroxyitraconazole were grouped together and fenbuconazole was clustered with it (**Figure A3.7B**). Metabolism of cholesterol and metabolism of xenobiotics are the strongest activated pathways and show a clear concentration-response pattern upon the exposure towards conazole compounds (**Figure A3.7C**).

The conazoles and the metabolites showed different number of DEGs at the tested concentrations (**Figure A3.8A**). The strongly modulated responses included metabolism in cholesterol and metabolism of xenobiotics having the highest module scores (**Figure A3.8B**). We also noticed the activation of stress response pathways predominantly by endoplasmic reticulum stress and oxidative stress in agreement with the results from the high throughput test methods (**Figure A3.8B**). The normalized AUC (normAUC) derivatization from the absolute eigengene scores was used to aggregate the concentration–response effects for each compound. This approach is particularly relevant given that the concentration ranges tested differed across compounds. The normAUC values were used to evaluate the compound similarity by performing correlation analysis. The clustering analysis shows 3 distinct clusters (**Figure A3.8C**). Consistent with the observation from number of DEGs (**Figure A3.8A**) and module activity (**Figure A3.7B**), low perturbing conazoles (1,2,4-triazole, hydroxyitraconazole, and itraconazole) are grouped together in cluster 1. Moderate perturbing conazoles: tebuconazole, ketoconazole, and epoxiconazole are grouped in cluster 2.

The TC- difenoconazole and 2 SC, propiconazole and prothioconazole classified as the strong perturbing conazoles are clustered together in cluster 3. Overall, this clustering is in the agreement with the structure-based clustering (**Figure A3.2.**) as well as high-throughput *in vitro* assays based clustering (**Figure A3.6.**).

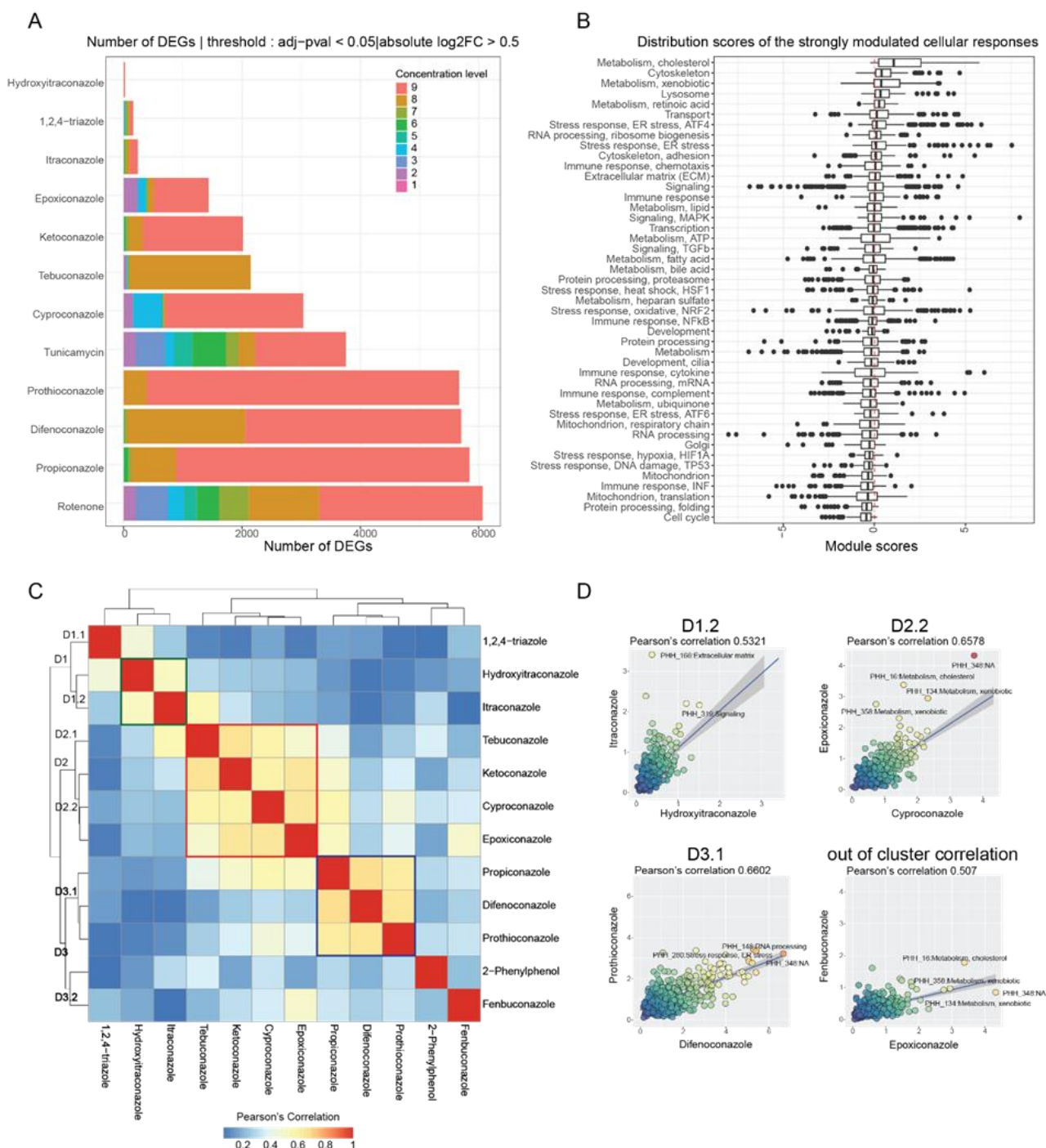
Figure A3.7. Transcriptomic perturbation upon conazole exposure





Note: (A) The number of differentially expressed genes upon the exposure of conazoles in every concentration level. The color of the bar plot indicates the direction of the perturbation. (B) A heatmap exhibiting the overall responses based on the significant annotated modules upon exposure to conazoles. The order of the compounds on the y-axis and the modules on the x-axis are arranged using hierarchical clustering based on the module responses. The color indicator assigned to each compound on the y-axis represent the concentration level. The color of the heatmap represent the scale of the module scores (red – activation, blue – deactivation). (C) dose-response plots of modules annotated with the highly perturbed pathways, from left to right: PHH16 – metabolism cholesterol, PHH134 – metabolism xenobiotics, PHH33 – mitochondrial translation. The color of the plots indicate the compounds.

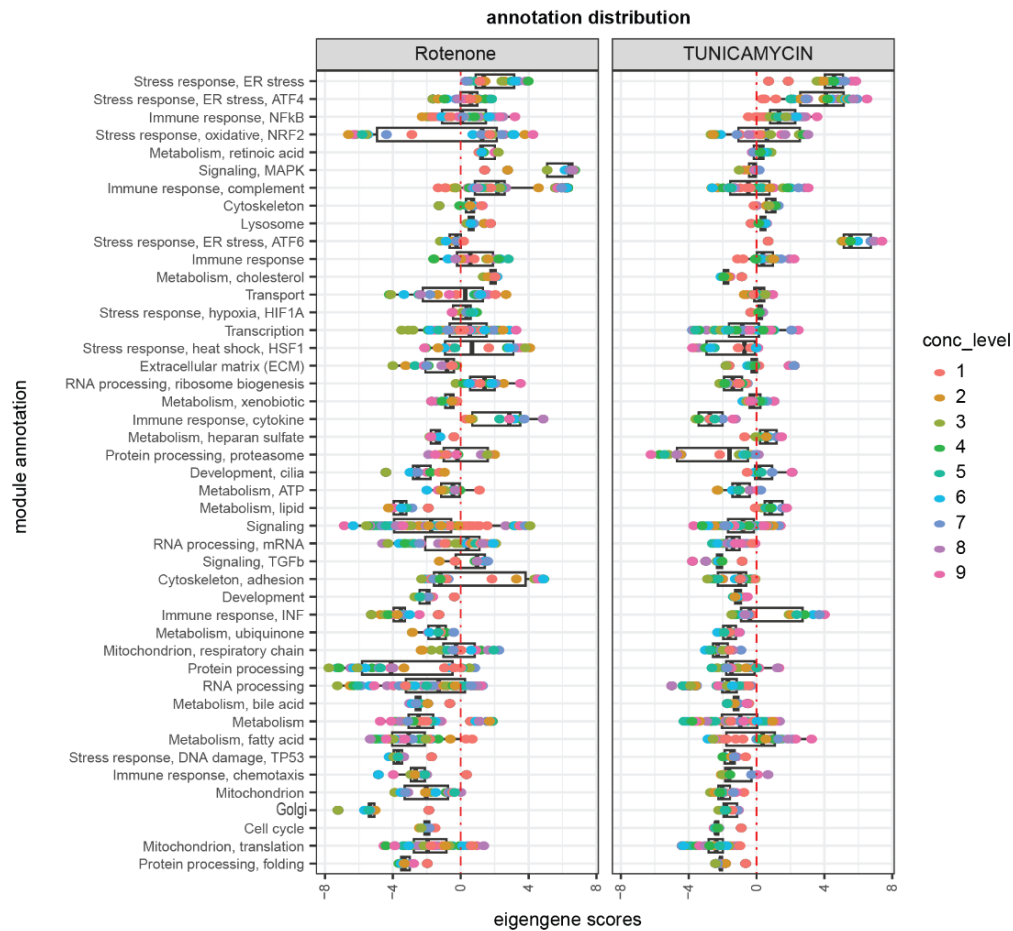
Figure A3.8. Transcriptomics outcomes of PHHs exposed to conazoles



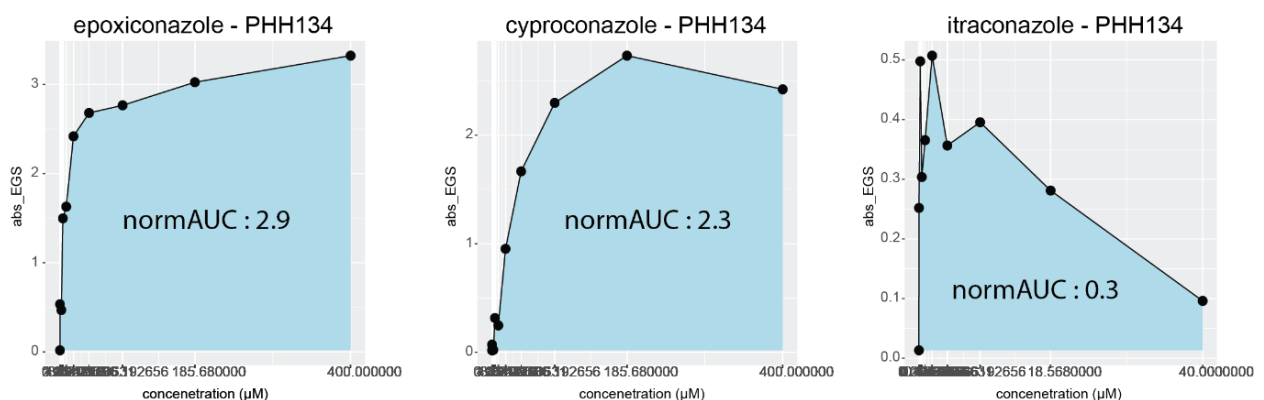
Note: (A) The number of differentially expressed genes (DEGs - threshold: FDR < 0.05 and absolute log2 fold change > 0.5) for each conazole. Colors of the bars indicate concentration level. (B) Distribution plots of modulated pathways based on the module scores upon conazole exposure. The bar plots consist of the data points of each module annotated with the entailed term from every exposure condition. (C) Correlation matrix scores based on the AUC of the module reveal similarities among conazoles. There are 3 clusters observed based on the dendrogram, suggesting 3 groups of conazoles with similarity of the activity towards PHH with each group. The color of the matrix indicates Pearson's correlation scores. (D) Individual correlation plots showing the similarities between conazoles for each group. The dots represent the modulation of the gene networks from two conazoles. The color of the dots represents the average scores of the indicated modules.

Figure A3.9. Outcomes from module-based analysis

A



B



Note: (A) The distribution of module scores for every annotation of the positive controls, rotenone and tunicamycin. The colors of the dots indicate concentration levels. (B) comparison of normAUC value from moderate, strong, and low perturbing conazole: epoxiconazole, cyproconazole, and itraconazole reflecting the magnitude of the modulation of PHH134 – metabolism xenobiotic.

As PHHs have higher metabolizing machinery, it could be that prothioconazole is more active in PHHs due to the formation of desthio metabolite. Upon further inspection on the individual correlation plots, we

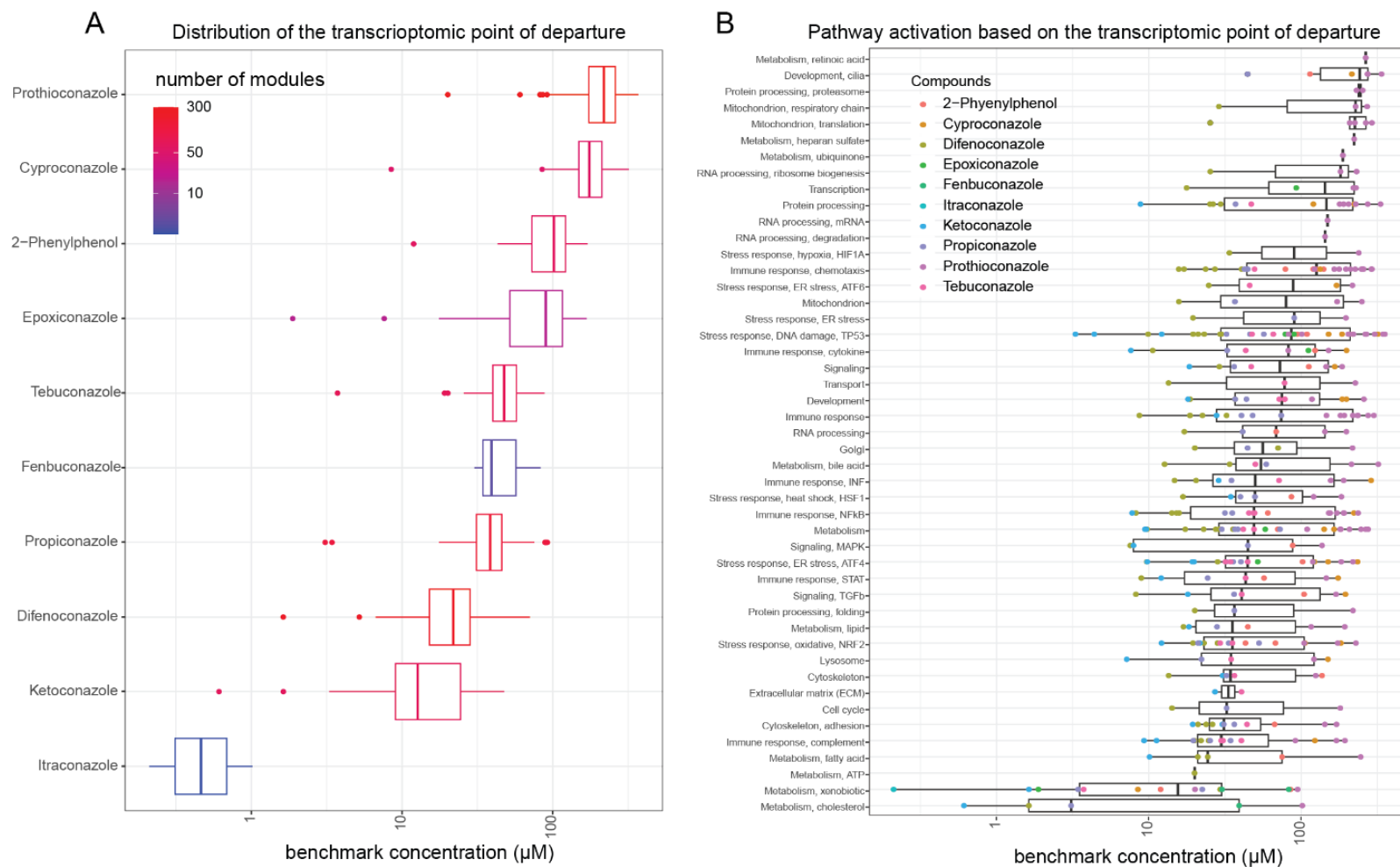
observed the correlation between itraconazole and hydroxyitraconazole (D1.2) showing mostly low magnitude of module activation. The correlation between epoxiconazole and cyproconazole (D2.2) and prothioconazole and difenoconazole (D3.1) highlight strong activation of PHH16 – metabolism cholesterol, PHH134 and PHH358 – metabolism xenobiotics, PHH280: stress response, ER stress, and PHH148: RNA processing. We also noticed out of cluster correlation between epoxiconazole and fenbuconazole with similar modules being highly activated (**Figure A3.8D**).

We evaluated the responses of the reference compounds, tunicamycin and rotenone, to ensure the accuracy of the transcriptomics endpoints. Based on our data, we captured the expected responses of the PHH towards these reference compounds (**Figure A3.9A**). The normAUC values are able to correctly reflect the overall magnitude of the module activation as it is showed from the dose-response of PHH134 where moderate and strong perturbing conazole, epoxiconazole and cyproconazole have higher normAUC values (2.9 and 2.3 respectively) compared to low perturbing conazole, itraconazole (0.4) (**Figure A3.9B**).

Upon quantifying the median BMD values leveraging the PHH TXG-MAPr gene networks, we noticed different potency amongst the conazole compounds. Interestingly, the sensitivity based on the BMD values does not reflect the magnitude of transcriptomic perturbation of the conazole compounds. Itraconazole was found to be the most sensitive compound showing the lowest BMD distribution despite having only less than 10 responsive modules. On the other hand, prothioconazole and cyproconazole were found to have rather high BMD values with more than 200 responsive modules. The TC – difenoconazole is showed to also have low BMD values along with other SCs including ketoconazole and propiconazole (**Figure A3.10A**) indicating similar potency. Focusing on the annotated responsive modules, metabolism cholesterol and metabolism xenobiotics are the most activated pathways by the most conazole compounds (**Figure A3.10B**). We found the most sensitive pathway of the SCs (ketoconazole, prothioconazole) and TC (difenoconazole) to be cholesterol metabolism (hPHH_16). While for fenbuconazole and prothioconazoles, this pathway was within top 20 most sensitive pathways (**Figure A3.11A**). The other most sensitive pathway was metabolism of xenobiotics consisting of 2 modules (PHH134 and PHH358). Cyproconazole, epoxiconazole, prothioconazole, and tebuconazole show the most sensitive activation on PHH134 (8.5, 1.4, 1.03, 1.7 μ M respectively) – metabolism xenobiotics with the CYP3A family to be the genes with low BMC values (**Figure A3.10B**). On the other hand, fenbuconazole and itraconazole exhibit most sensitive perturbation of PHH358 (1.3 and 5.4 μ M) – metabolism xenobiotics with low BMC values from CYP1A family (**Figure A3.10B**). Interestingly, for the TC-difenoconazole, PHH 134 was not within the top 20 most sensitive modules despite also being activated at relatively low concentration (24.4 μ M). Also, pathways related to stress responses are activated at the higher concentration indicating the stage of higher severity of the responses upon conazole exposure.

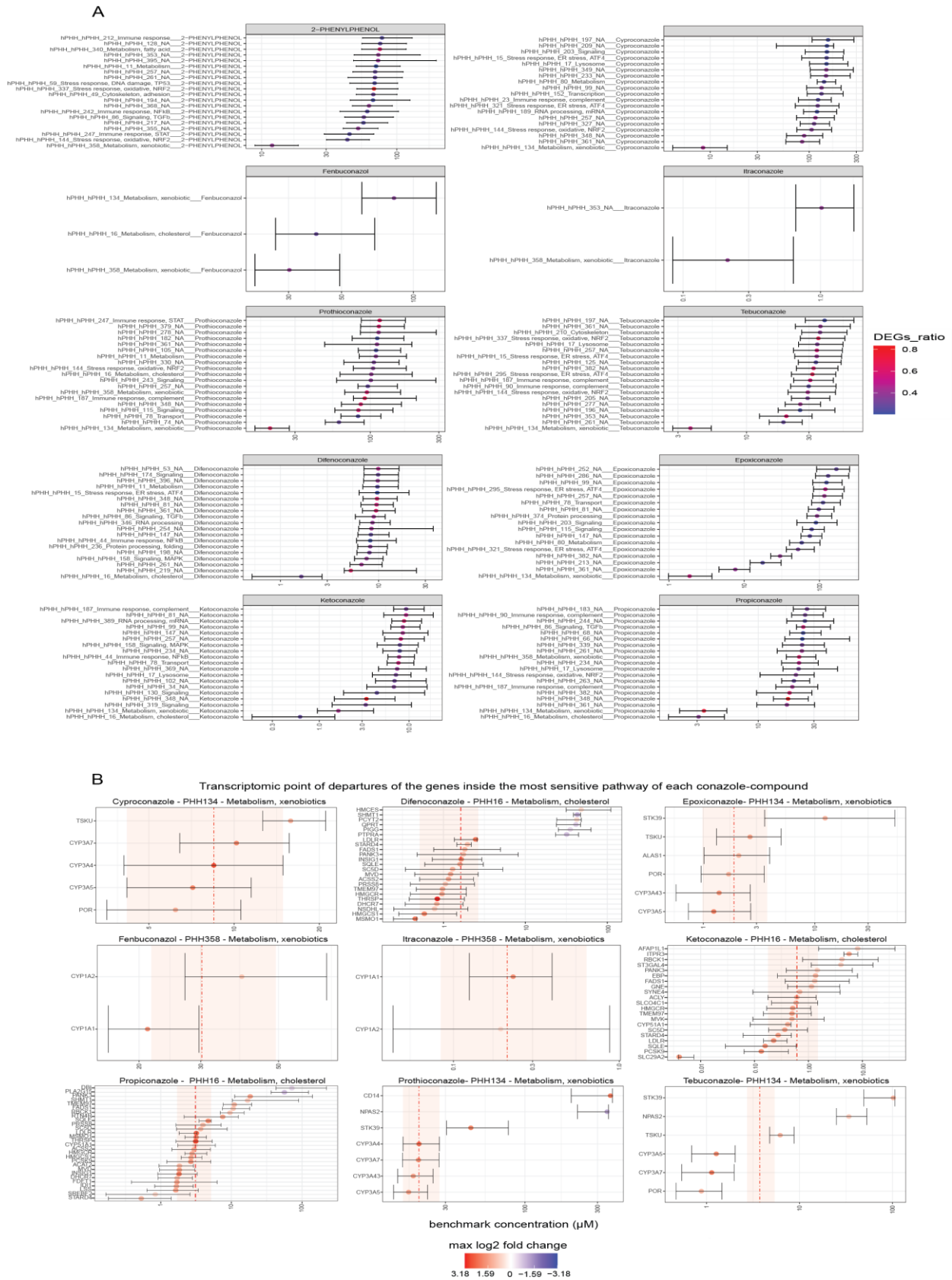
Based on these findings, the transcriptomics analysis could unravel responses similarity among conazoles. Our results showed that the conazoles activate common pathways including metabolism cholesterol and xenobiotic metabolism (**Figure A3.11A and A3.11B**). However, their potency to perturb the genes associated with these pathways differs. We also demonstrate the potential of transcriptomics endpoints combined with the gene network-based pathway enrichment approach to identify common modulated pathways of conazoles and point out the similarities between SCs and TC-difenoconazole supporting the concept of read-across.

Figure A3.10. Derivatization of transcriptomics point of departure (tPOD) based on the benchmark concentration values



Note: (A) Distribution of benchmark concentration (BMC) for each conazole. The x-axis represents the BMC in log10 scale bar. The color of the plot indicates the number of the modules consisting of the box plots. (B) Distribution of pathway activation based on the benchmark concentration of the modules annotated with the entailed terms. The dots indicate the benchmark concentration of every module annotated with the entailed term from every condition. The color of the dots indicates the compound which the BMC of the module is derived.

Figure A3.11. Outcomes from the BMD express



Note: (A) Top 20 most activated modules based on the BMD values of every conazole. (B) The transcriptomic point of departure for every gene in the most sensitive module for each conazole. The dots indicate BMC values, and the bars show the range of BMDL-BMDU values. The color of the dots shows the maximum log₂ fold change for the genes for each conazole. The red line shows the BMC of the indicated module (median BMC of the gene BMC) with the shadow represents the range of BMDL-BMDU of the module (median values of the gene BMDL-BMDU).

Comparison of transcriptomics response between PHH and RPTEC-TERT cells

The transcriptomic responses in PHHs and RPTEC-TERT cells were compared to assess the effects of conazoles across different cell types. This was achieved by analyzing pathway enrichment and module responses using PHH TXG-MAPr in both cell models (**Figures A3.12.** and **A3.13.**).

Pathway enrichment analysis revealed that cholesterol metabolism was the most perturbed pathway in both cell types, consistent with the known mode of action of conazoles. This indicates that the expected biological response to conazoles was captured in both PHH and RPTEC-TERT cells (**Figure A3.12.**). In PHH, xenobiotic metabolism was also among the top perturbed pathways, which was not observed in RPTEC-TERT cells. This difference is attributed to the liver-specific nature of xenobiotic metabolism, as the liver is the primary organ responsible for this function.

On the other hands, we observed different compound clustering patterns between the two cell types based on the module scores:

- In RPTEC-TERT cells, difenoconazole, cyproconazole, propiconazole, fenbuconazole, and prothioconazole clustered together. Tebuconazole and epoxiconazole clustered with 2-phenylphenol, while ketoconazole clustered with 1,2,4-triazole. Hydroxyitraconazole and itraconazole formed a separate cluster.
- In PHHs, difenoconazole clustered with propiconazole, prothioconazole, ketoconazole, tebuconazole, cyproconazole, and epoxiconazole. Hydroxyitraconazole and itraconazole also clustered together, with fenbuconazole joining this cluster.

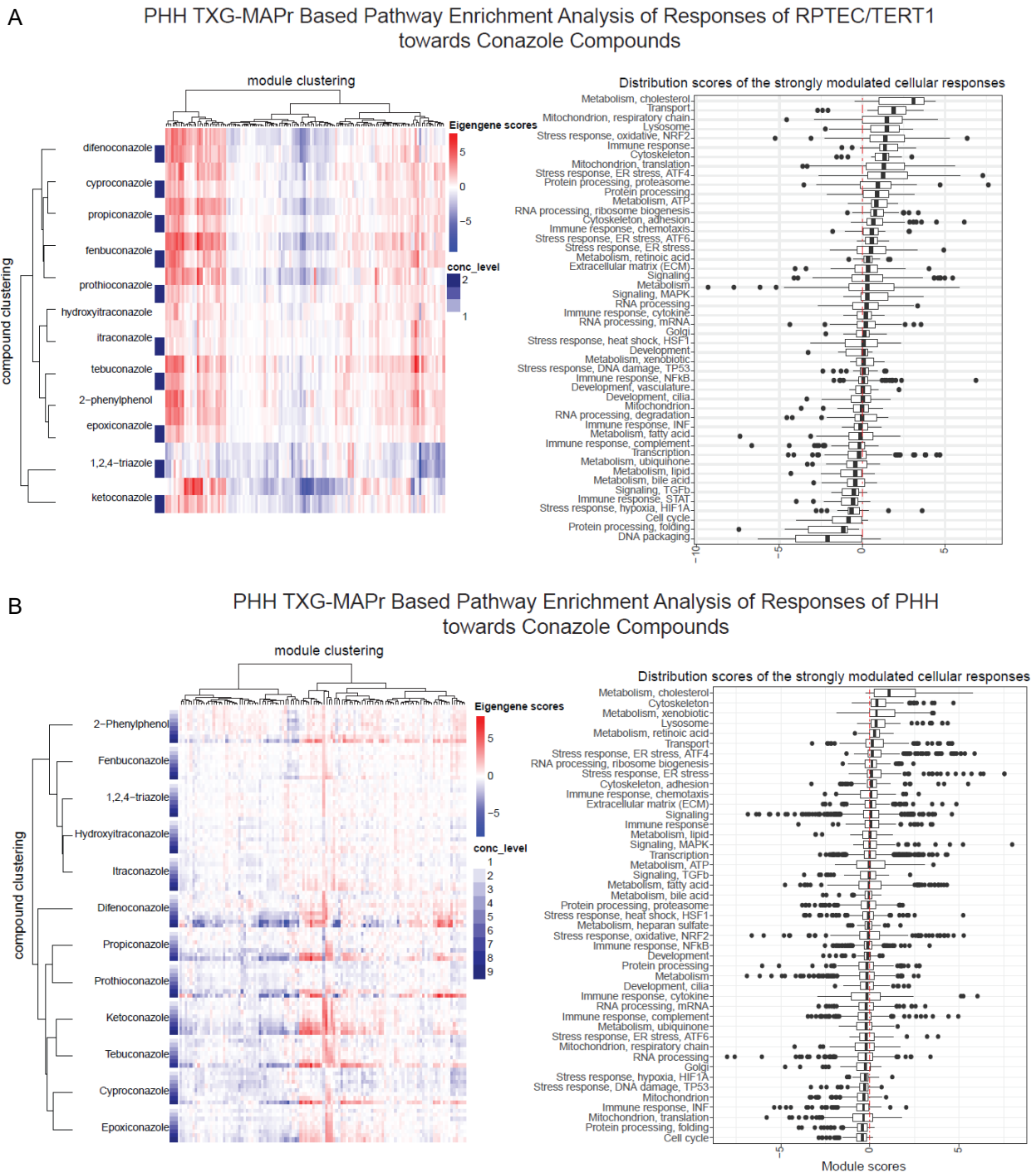
Notably, 1,2,4-triazole elicited a stronger transcriptomic response in RPTEC-TERT cells compared to PHH. To validate this observation, transcriptomic profiling in RPTEC-TERT cells is being repeated across the full concentration range.

Correlation analysis of transcriptomic responses at the module level revealed two distinct clusters in RPTEC-TERT cells and three clusters in PHH (**Figure A3.13.**).

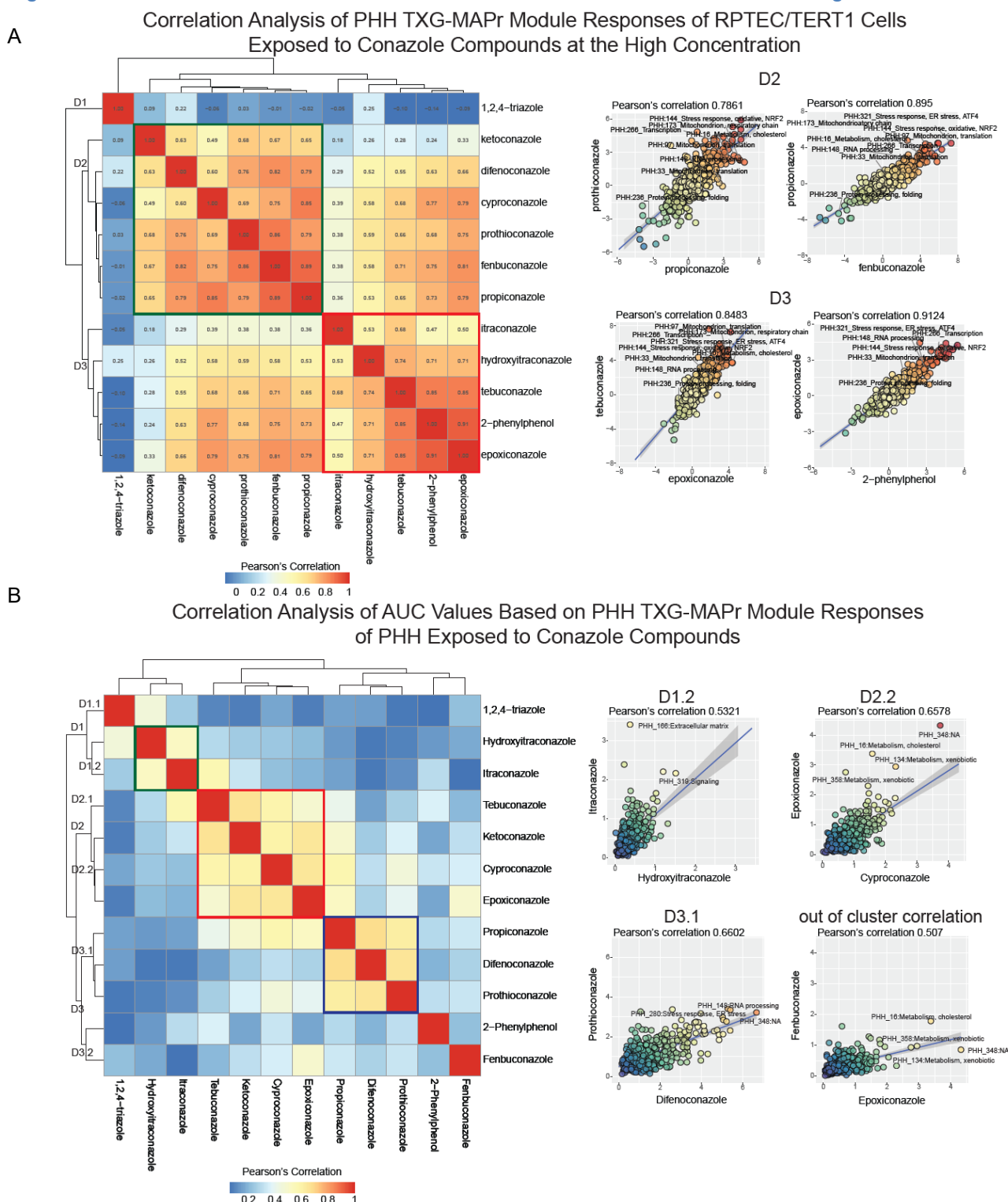
- In RPTEC-TERT cells (**Figure A3.13A**), the TC, difenoconazole, clustered with the SCs: cyproconazole, prothioconazole, fenbuconazole, and propiconazole. A second cluster included tebuconazole, epoxiconazole, and itraconazole, which grouped with hydroxyitraconazole and 2-phenylphenol. Interestingly, the responses of 1,2,4-triazole seems to be different from the rest of the conazole compounds as this compound is separately grouped.
- In PHH cells (**Figure A3.13B**), the TC, difenoconazole, clustered with propiconazole and prothioconazole. A separate cluster comprised SCs, tebuconazole, ketoconazole, cyproconazole, and epoxiconazole, while hydroxyitraconazole and itraconazole formed a third distinct cluster.

Despite the differences in clustering and module responses, difenoconazole and propiconazole consistently clustered together in both cell types, suggesting a shared transcriptomic response across hepatic and renal cell models.

Figure A3. 12. Heatmap showing the responses of the strong modulated modules upon conazole exposure to RPTEC/TERT1 cells

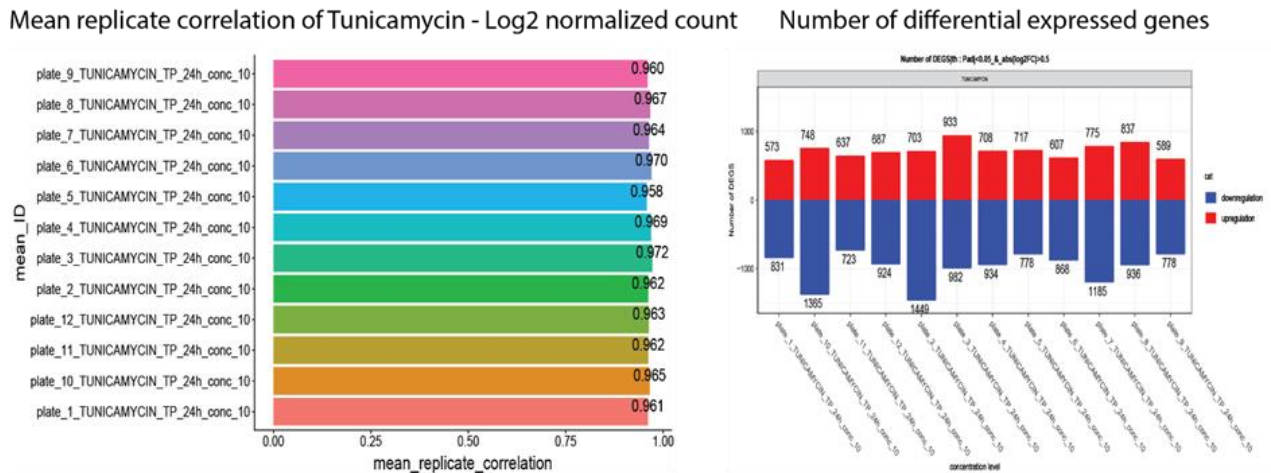


Note: (A – left) the color of the heatmap represents the direction of the modulation (red – activation, blue – de-activation) with the intensity indicating the magnitude of the eigengene scores. The clustering of the compounds and modules was done using ward.d2 method with Euclidian distant. The distribution of the eigengene scores of the activities of annotated modules upon the exposure of conazole compounds in RPTEC/TERT1 cells (A – right). The red line indicates the 0 eigengene score value. Heatmap showing the responses of the strong modulated modules upon conazole exposure to PHHs (B – left) the color of the heatmap represents the direction of the modulation (red – activation, blue – de-activation) with the intensity indicating the magnitude of the eigengene scores. The clustering of the compounds and modules was done using ward.d2 method with Euclidian distant. The distribution of the eigengene scores of the activities of annotated modules upon the exposure of conazole compounds in PHHs (AB– right). The red line indicates the 0 eigengene score value.

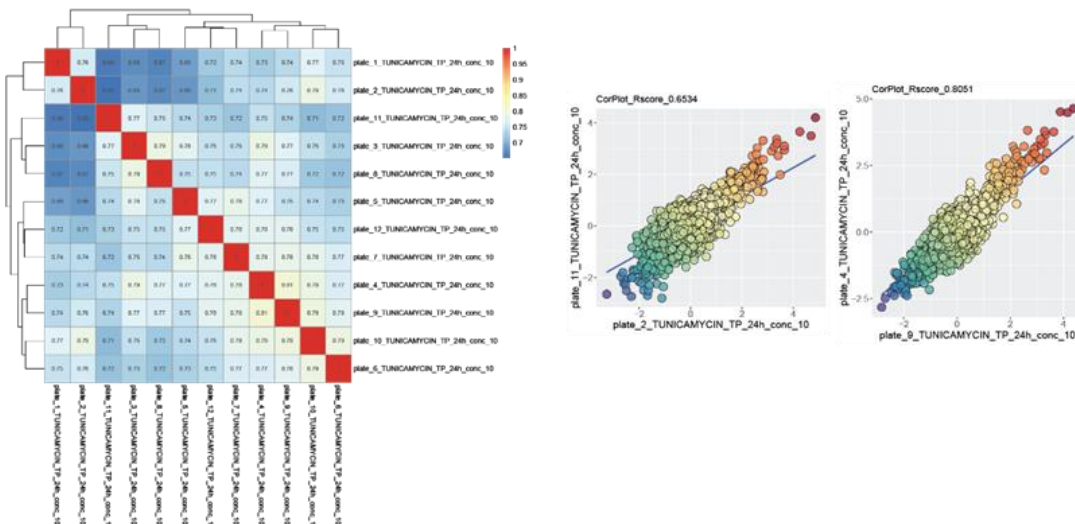
Figure A3. 13. Left: Correlation matrix scores of the module reveal similarities among conazoles

Note: The color of the matrix indicates Pearson's correlation scores. There are 3 clusters observed based on the dendrogram for A. RPTEC/TERT1 cells exposed to the highest concentration (based on the eigengene scores of the modules) and B. PHHs (based on the AUC values of the eigengene scores across concentration). Right: Individual correlation plots showing the similarities between conazoles for each group for A. RPTEC/TERT1 cells and B. PHH TXG-MAPr. The dots represent the modulation of the gene networks from two conazoles. The color of the dots represents the average scores of the indicated modules.

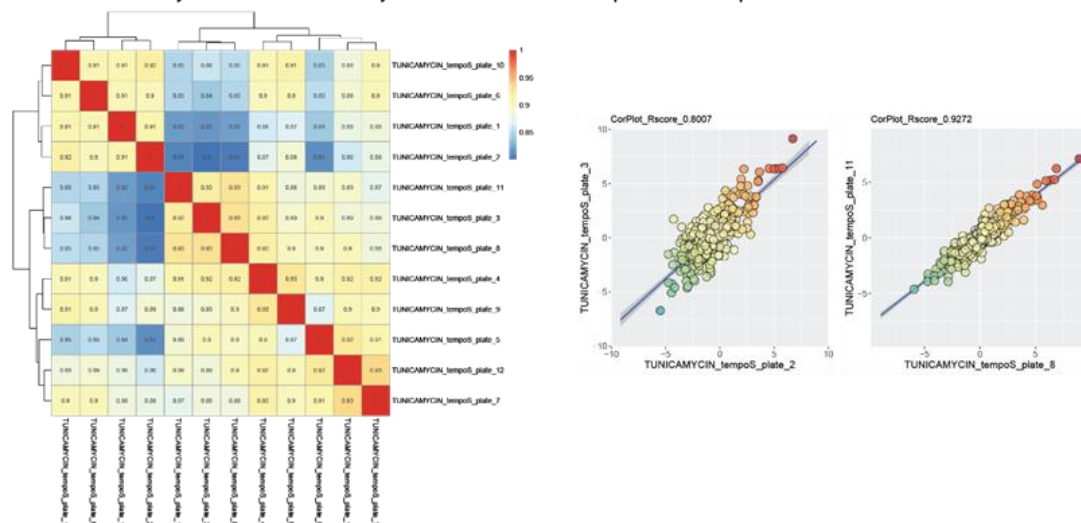
Figure A3. 14. Top: Correlation between three biological replicates of based on the normalized count (left) and number of differentially expressed genes – th: $\text{abs}(\log\text{FC}) > 0.5$ and $\text{FDR} < 0.05$ (right). Middle: Pearson correlation analysis of the positive control tunicamycin from different experimental plates based on the log2 fold change values as correlation matrix heatmap (left) and correlation plots showing the lowest and highest correlation the conditions (right). Bottom: Pearson correlation analysis of the positive control tunicamycin from different experimental plates based on the module scores as heatmap (left) and correlation plots showing the lowest and highest correlation from all the conditions(right).



Correlation analysis of the tunicamycin from different experimental plates based on the log2 fold change values



Correlation analysis of the tunicamycin from different experimental plates based on the module scores



For positive controls Tunicamycin 10 μ M, within one screen, the correlation between three biological replicates is around 0.96. Moreover, the cells also showed comparable magnitude of responses based on the number of differentially expressed genes. The response correlation at the log2 fold change level ranges from 0.6 to 0.7 and from the module level from 0.8 to 0.92 (**Figure A3.14.**). Based on these findings, we can demonstrate the strong robustness of the transcriptomic responses of the PHHs. Additionally, in multiple publications (Callegaro et al., 2021, Kunnen et al., 2024) the authors show that modules activation can be reproduced qualitatively across transcriptomics platform (Tunicamycin, microarray versus TempOSeq) and across different experimental setups (Cyclosporin A, PHH with or without sandwich culture).

7. H295R steroidogenesis assay

The endocrine-disrupting potential of conazoles and their metabolites was evaluated in this case study. All active compounds exhibited a downregulatory effect on androgen and estrogen pathways (**Table A3.7.**). The total lowest observed effect concentrations (LOEC) were converted to free media concentrations (**Table A3.7B**) using biokinetics analysis to compare the potency of the conazoles (**Table A3.7C**). The free media concentration was regarded as LEC.

For androgen disruption, the most potent compounds were ranked as follows: Itraconazole = Hydroxyitraconazole > Ketoconazole > Epoxiconazole > Fenbuconazole > Tebuconazole > Propiconazole > Prothioconazole > Cyproconazole. Notably, difenoconazole and 1,2,4-triazole did not show any androgen-disrupting activity.

For estrogen disruption, the potency ranking was: Hydroxyitraconazole > Ketoconazole > Difenoconazole > Itraconazole > Propiconazole > Fenbuconazole > Cyproconazole > Tebuconazole > Epoxiconazole. In this case, prothioconazole and 1,2,4-triazole did not exhibit any estrogen-disrupting activity.

Table A3. 7. Steroidogenesis potential of conazole compounds assessed using the H295R steroidogenesis assay.

A) Original read-outs

Compound	Highest non-cytotoxic concentration in H295R (μM)	Androgens			Estrogens		
		LEC (μM)	↑/↓ hormone levels	max fold (average 2)	LEC (μM)	↑/↓ hormone levels	max fold (average 2)
Difenoconazole	3.33	-	↘	0.75	1.00	↘	0.65
Cyproconazole	33.3	33.30	↘	0.07	1.83	↘	0.22
Epoxiconazole	33.3	1.83	↘	0.13	3.33	↘	0.65
Fenbuconazole	10	1.83	↘	0.15	1.83	↘	0.58
Hydroxyitraconazole	1	0.33	↘	0.17	0.33	↘	0.63
Itraconazole	10	0.33	↘	0.28	3.33	↘	0.44
Ketoconazole	3.33	0.58	↘	0.14	0.58	↘	0.15
Propiconazole	10	10.00	↘	0.31	1.83	↘	0.27
Prothioconazole	33.3	10.00	-	0.63	-	-	0.86
Tebuconazole	10	3.33	↘	0.08	3.16	↘	0.24
1,2,4-triazole	100	-	-	0.92	-	-	0.92

B) LEC

Compound	Highest non-cytotoxic concentration in H295R (μM)	Androgens			Estrogens		
		LEC (μM)	↑/↓ hormone levels	max fold (average 2)	LEC (μM)	↑/↓ hormone levels	max fold (average 2)
Difenoconazole	3.33	-	↘	0.75	0.24	↘	0.65
Cyproconazole	33.3	30.11	↘	0.07	1.65	↘	0.22
Epoxiconazole	33.3	1.31	↘	0.13	2.38	↘	0.65
Fenbuconazole	10	1.48	↘	0.15	1.48	↘	0.58
Hydroxyitraconazole	1	0.06	↘	0.17	0.06	↘	0.63
Itraconazole	10	0.06	↘	0.28	0.56	↘	0.44
Ketoconazole	3.33	0.12	↘	0.14	0.12	↘	0.15
Propiconazole	10	5.60	↘	0.31	1.02	↘	0.27
Prothioconazole	33.3	9.72	-	0.63	-	-	0.86
Tebuconazole	10	1.90	↘	0.08	1.81	↘	0.24
1,2,4-triazole	100	-	-	0.92	-	-	0.92

Concentration
highest
lowest

Note: The (A) original read-outs (LOECs) and (B) LEC are shown in different panels. The LOECs was defined as the lowest tested concentration at which hormone levels increased to ≥ 1.50 fold or decreased to ≤ 0.66 fold relative to the solvent control, which are also the interpretation criteria set in OECD TG456 (OECD, 2025). The direction of change (up ↑/ down ↓) is indicated in the hormone level trend column. For conazoles that did not meet the LOEC criteria but exhibited a consistent upward or downward trend exceeding thresholds used in earlier TG456 guideline versions (≥ 1.20 fold increase or ≤ 0.66 fold decrease), the trend in hormone level variation is also reported.

8. Developmental Neurotoxicity (DNT) assays

In this case study, three assays from the Developmental Neurotoxicity (DNT) test battery—UKN2, UKN4, and UKN5 were used to assess the effects of conazole compounds and five additional control compounds. Each assay targets a critical neurodevelopmental process:

UKN2 - The cMINC Neural Crest Cell Migration Assay: Assesses the impact of compounds on the migration of neural crest cells, which are essential for the development of various peripheral tissues and structures.

UKN4 - NeuriTox Neurite Outgrowth of CNS Neurons Test: Uses LUHMES (human mesencephalic cells) to evaluate compound effects on neurite outgrowth in central nervous system neurons.

UKN5 – PeriTox Neurite Outgrowth of PNS Neurons Test: Investigates how compounds influence neurite outgrowth in peripheral neurons, focusing on peripheral nervous system development.

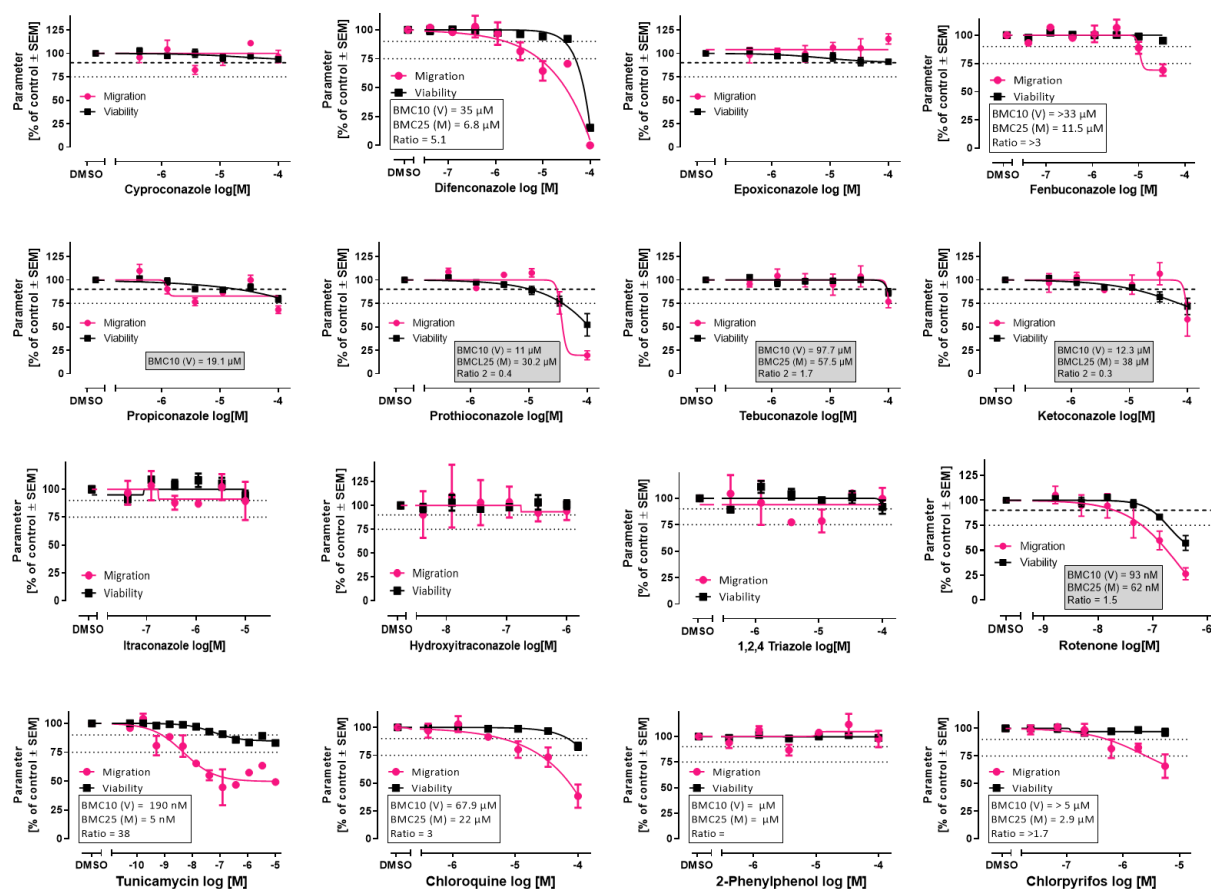
These assays collectively provide insight into the potential developmental neurotoxicity of the tested substances by targeting key cellular events involved in neurodevelopment.

UKN2 – The cMINC Neural Crest Cell Migration Assay

The conazoles were tested in a full concentration response curve up to a concentration of 100 µM (except from itraconazole (10µM and hydroxyitraconazole (1µM)). Additionally, the control compounds rotenone, chlorpyrifos, chloroquine, tunicamycin and 2-phenylphenol were tested. Neural crest cells are exposed to the test compounds for 24 hours and at the same time migrate into a cell free space. As a readout, the migration of neural crest cells as well as the viability of the cells is assessed by high content imaging. The BMC25 (migration) is used as a threshold concentration for migration impairment and the BMC10 (viability) is assumed as the lowest nontoxic concentration. These values were indicated for each compound (**Figure A3.15.**).

The compounds difenoconazole, prothioconazole, fenbuconazole, ketoconazole, rotenone, tunicamycin and chloroquine affected neural crest cell migration, some of the compounds also reduced the viability of the cells (**Figure A3.15.**). If the ratio of BMC25(migration)/BMC10(viability) ≥ 1.3 , compounds are defined as specific hit in the assay. If a compound did not lead to cytotoxicity in the tested range, the highest tested concentration was used to replace the BMC10 viability. The positive control compound tunicamycin showed a ratio of 38 and is therefore defined as a specific assay hit. Among the conazoles, difenoconazole (BMC(M)= 6.8 µM; BMC Ratio = 5.1) as well as fenbuconazole (BMC(M)=11.5µM; BMC ratio=3) were identified as DNT positives in the the cMINC Neural Crest Cell Migration Assay (UKN2 Test) (**Figure A3.15., Table A3.8.**).

Figure A3.15. Conazoles and case study control compounds were tested in the cMNC, which assessed migration (displayed in pink) as well as viability (displayed in black) as a readout.



Note: Concentration–response curves are given for every compound; $n \geq 3$. The data in the insert boxes are derived from curve fitting of the data. BMC25 (Migration) was considered as the relevant threshold concentration for migration impairment. BMC10 (Viability) was assumed to be the highest non-cytotoxic concentration. A ratio of BMC25 (M)/BMC10 (V) was calculated, and a specific assay hit was defined as ≥ 1.3 . If no effect could be observed even at the highest tested concentration (HTC), the HTC is used to calculate the ratio.

Table A3.8. Summary table of the testing in the cMINC

	UKN (cMINC)		
	BMC25(M) in μM	BMC10(V) in μM	Ratio(BMC25(M)/BMC10(V))
Cyproconazole	>200	>200	NE
Difenoconazole	6.8	35	5.1
Epoxiconazole	>200	>200	NE
Fenbuconazole	11.5	>33	>3
Propiconazole	>200	19.1	
Prothioconazole	30.2	11	0.4
Tebuconazole	57.5	97.7	1.7
Ketoconazole	12.3	38	0.3
Itraconazole	>20	>20	NE
Hydroxy-itraconazole	>2	>2	NE
1,2,4-triazole	>200	>200	NE
Rotenone	62	93	1.5
Chloroquine (bis)phosphate	22	68	3
Tunicamycin	0.005	0.19	38
2-Phenylphenol	>200	>200	NE
Chlorpyrifos	2.9	>5	>1.7

Note: BMC25 (Migration) was considered as the relevant threshold concentration for migration impairment. BMC10 (Viability) was assumed to be the highest non-cytotoxic concentration. A ratio of BMC25 (M)/BMC10 (V) was calculated, and a specific assay hit was defined as ≥ 1.3 . If no effect could be observed even at the highest tested concentration (HTC), to calculate the ratio, the HTC is used. Yellow marked compounds indicate the assay specific hits.

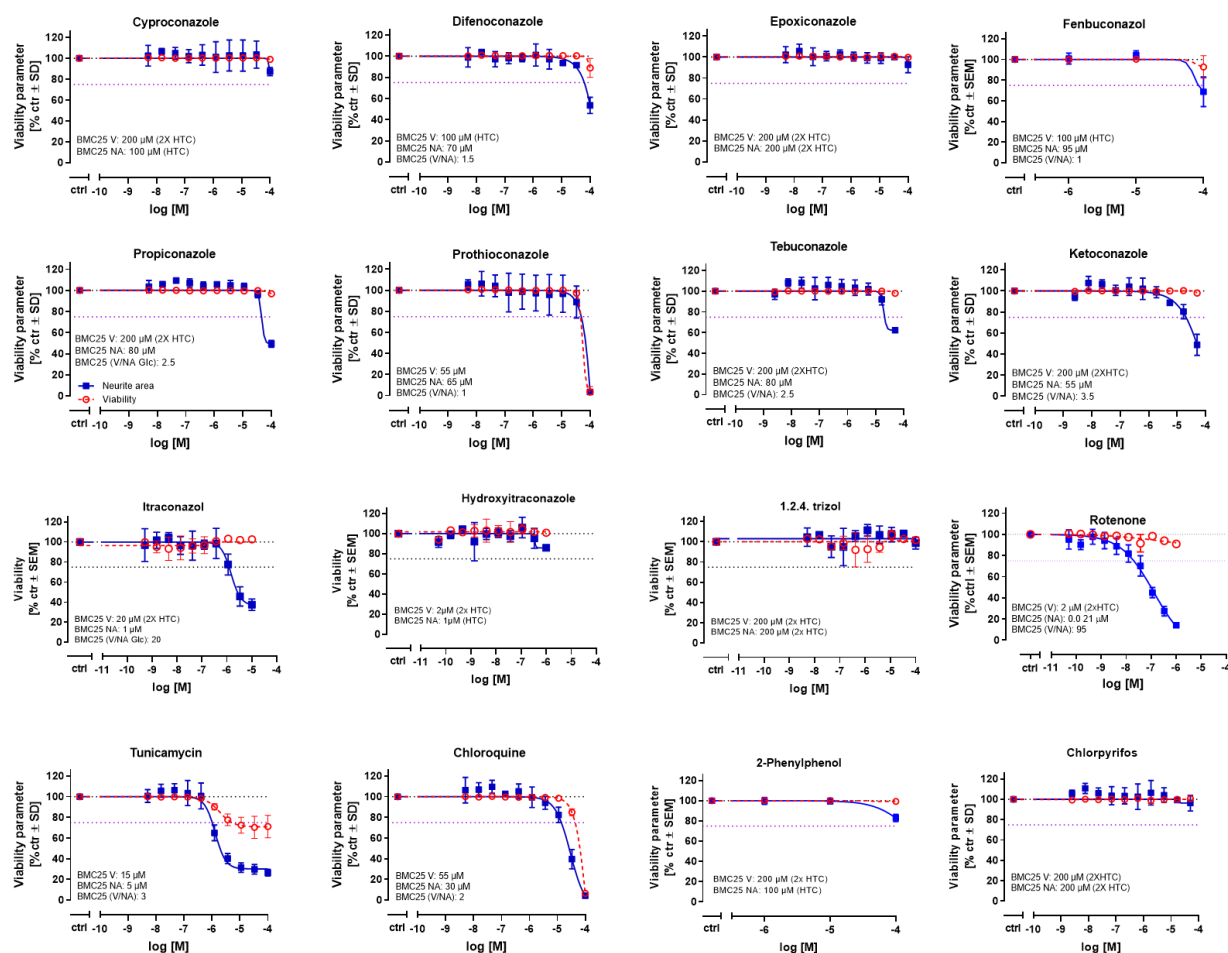
UKN4 – NeuroTox Neurite Outgrowth of CNS Neurons Test

The conazole compounds were tested up to a concentration of 100 μM (except from itraconazole (10 μM and hydroxyitraconazole (1 μM)). Additionally, the control compounds rotenone, chlorpyrifos, chloroquine, tunicamycin and 2-phenylphenol were tested.

LUHMES cells were exposed to the compounds for 24 hours, then the cells were stained with Hoechst and calcein and imaged for total neurite area with an automated microscope. The viable cells and the neurite area were determined by double positivity and measurement of calcein-positive pixels by the software of the microscope. The BMC25(neurite outgrowth) as well as the BMC25(viability) is indicated for each compound (**Figure A3.16.**).

The compounds difenoconazole, propiconazole, prothioconazole, ketoconazole, tebuconazole, itraconazole, rotenone, tunicamycin and chloroquine did affect the neurite outgrowth. Some of the compounds also reduced the viability of the cells (**Figure A3.16.**). If the ratio of BMC25 (neurite outgrowth)/BMC25(Viability) ≥ 4 , compounds are defined as specific hits in the assay. If a compound did not lead to cytotoxicity in the tested range, the 2x the highest tested concentration was used to calculate the BMC ratio. The positive control compound rotenone showed a ratio of 95 and is therefore defined as a specific hit in the UKN4 assay. Among the conazoles itraconazole (BMC(NA)= 1 μM ; BMC Ratio = >20) was identified as a specific assay hit in the NeuroTox. Since the Metabolite hydroxyitraconazole could only be tested in a low concentration, the specific effect could not be confirmed. However, it showed a neurite impairing effect with 1 μM . Ketoconazole showed an borderline effect in high concentrations (BMC(NA)=55 μM ; Ratio >3.5) (**Figure A3.16., Table A3.9.**).

Figure A3.16. Conazoles and case study control compounds were tested in the NeuroTox, which assessed neurite outgrowth (displayed in blue) as well as viability (displayed in red) as a readout.



Note: Concentration-response curves are given for every compound; $n \geq 3$. The data in the insert boxes are derived from curve fitting of the data. BMC25 (Neurite outgrowth) was considered as the relevant threshold concentration for developmental impairment. BMC25 (Viability) was assumed to be the highest non-cytotoxic concentration. A ratio of BMC25 (V)/BMC25 (NA) was calculated, and a specific assay hit was defined as ≥ 4 . If no effect could be observed even at the highest tested concentration (HTC), to calculate the ratio, the 2 x HTC is used.

Table A3.9. Summary table of the testing in the NeuroTox

	UKN4		
	BMC25(NA) in μM	BMC25(V) in μM	Ratio(BMC25(NA)/BMC25(V))
Cyproconazole	>200	>200	NE
Difenoconazole	70	100	1.5
Epoxiconazole	>200	>200	NE
Fenbuconazole	95	100	1
Propiconazole	80	200	2.5
Prothioconazole	65	55	1
Tebuconazole	80	200	2.5
Ketoconazole	55	>200	>3.5 (borderline)
Itraconazole	1	>20	>20
Hydroxy-itraconazole	1	>2	NE (tested too low)
1,2,4-triazole	>200	>200	NE
Rotenone	0.021	>2	>95
Chloroquine (bis)phosphate	30	55	2
Tunicamycin	5	15	3
2-Phenylphenol	>100	>200	NE
Chlorpyrifos	>200	>200	NE

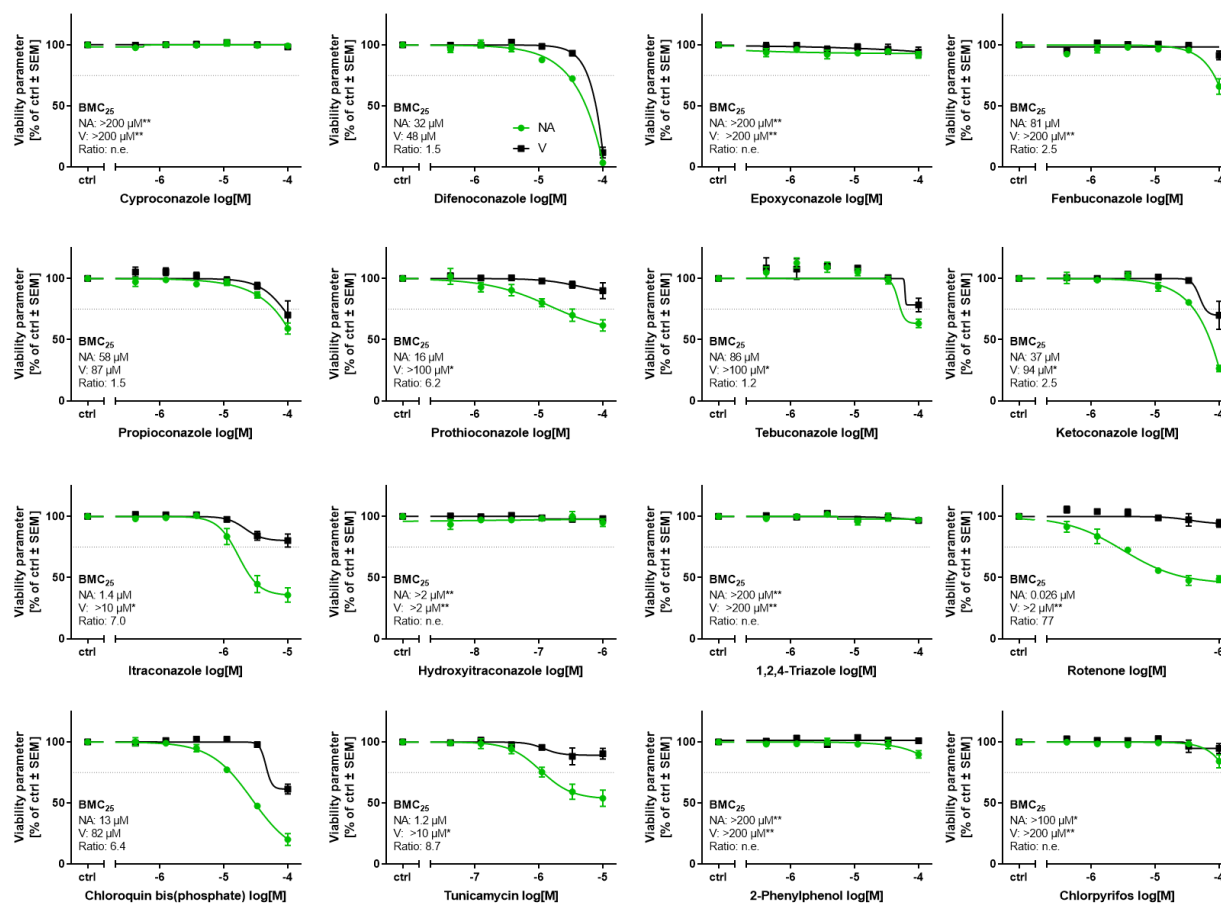
Note: BMC25 (Neurite outgrowth) was considered as the relevant threshold concentration for developmental impairment. BMC25 (Viability) was assumed to be the highest non-cytotoxic concentration. A ratio of BMC25 (M)/BMC25 (V) was calculated, and a specific assay hit was defined as ≥ 4 . If no effect could be observed even at the highest tested concentration (HTC), to calculate the ratio, 2x HTC is used. Yellow marked compounds indicate assay specific hits.

UKN5 – PeriTox Neurite Outgrowth of PNS Neurons Test

The compounds were tested up to a concentration of 100 μM (except from itraconazole (10 μM and hydroxyitraconazole (1 μM)). Additionally, the control compounds rotenone, chlorpyrifos, chloroquine, tunicamycin and 2-phenylphenol were tested (**Figure A3.17.**).

IPS derived peripheral neurons were treated for 24 hours with the test chemicals and stained with Hoechst and calcein. For imaging and quantification of viable cells and neurite area as well as the viability of the cells was assessed. The BMC25(neurite outgrowth) as well as the BMC25(viability) is indicated for each compound. The compounds difenoconazole, fenbuconazole, propiconazole, prothioconazole, ketoconazole, tebuconazoles, itraconazole rotenone, tunicamycin and chloroquine did affect the neurite outgrowth (**Figure A3.17.**). Some of the compounds also reduced the viability of the cells. If the ratio of BMC25 (neurite outgrowth)/BMC25(Viability) ≥ 3 , compounds are defined as specific hit in the assay. If a compound did not lead to cytotoxicity in the tested range, the 2x the highest tested concentration was used as a reference to calculate the BMC ratio. The positive control compound rotenone showed a ratio of 77, chloroquine a ratio of 6.4 and tunicamycin a ratio of 8.7. They are defined as a specific hit in the UKN5 assay. Among the conazoles, prothioconazole (BMC(NA)= 16 μM ; BMC Ratio = >6.2) and itraconazole (BMC(NA)=1.4 μM , BMC Ratio= 7) were identified as specific assay hits in the PeriTox (**Figure A3.17.**, **Table A3.10.**).

Figure A3.17. Conazoles and case study control compounds were tested in the PeriTox, which assessed neurite outgrowth (displayed in green) as well as viability (displayed in black) as a readout. Concentration–response curves are given for every compound; $n \geq 3$.



Note: The data in the insert boxes are derived from curve fitting of the data. BMC25 (Neurite outgrowth) was considered as the relevant threshold concentration for developmental impairment. BMC25 (Viability) was assumed to be the highest non-cytotoxic concentration. A ratio of BMC25(NA)/BMC25(V) was calculated, and a specific assay hit was defined as ≥ 3 . If no effect could be observed even at the highest tested concentration (HTC), $2 \times \text{HTC}$ is used to calculate the ratio.

Table A3.10. Summary table of the testing in the PeriTox

	UKN5		
	BMC25(NA) in μM	BMC25(V) in μM	Ratio(BMC25(NA)/BMC25(V))
Cyproconazole	>200	>200	NE
Difenoconazole	32	48	1.5
Epoxiconazole	>200	>200	NE
Fenbuconazole	81	>200	2.5
Propiconazole	58	87	1.5
Prothioconazole	16	>100	6.2
Tebuconazole	86	>100	1.2
Ketoconazole	37	94	2.5
Itraconazole	1.4	10	7
Hydroxy-itraconazole	>2	>2	NE
1,2,4-triazole	>200	>200	NE
Rotenone	0.026	>2	77
Chloroquine (bis)phosphate	13	82	6.4
Tunicamycin	1.2	>10	8.7
2-Phenylphenol	>200	>200	NE
Chlorpyrifos	>200	>200	NE

Note: BMC25 (Neurite outgrowth) was considered as the relevant threshold concentration for developmental impairment. BMC25 (Viability) was assumed to be the highest non-cytotoxic concentration. A ratio of BMC25 (M)/BMC25 (V) was calculated, and a specific assay hit was defined as ≥ 3 . If no effect could be observed even at the highest tested concentration (HTC), 2 x HTC is used to calculate the ratio. Yellow marked compounds indicate the assay specific hits.

Biokinetics analysis to predict LEC was carried out for compounds that tested DNT positive in the UKN2, UKN4 and UKN5 assays.

Table A3. 11. LEC of the conazole compounds tested for the developmental neurotoxicity (DNT) using A) UKN2, B) UKN4 and C) UKN5 assays

A) UKN2 assays			
UKN2 assays	Total	Biokinetics	Free
Compounds	BMC25 (M) (μM)	Ratio (Nominal/Free media)	BMC25 (LEC) (M) (μM)
Difenoconazole	6.8	1.85	3.67
Fenbuconazole	11.5	1.08	10.67
Tebuconazole	57.5	1.22	47.04
B) UKN4 assays			
UKN4 assays	Total	Biokinetics	Free
Compounds	BMC25(NA) (μM)	Ratio (Nominal/Free media)	BMC25 (LEC) (NA) (μM)
Itraconazole	1	19.9	0.05
Ketoconazole	55	1.99	27.58
C) UKN5 assays			
UKN5 assays	Total	Biokinetics	Free
Compounds	BMC25(NA) (μM)	Ratio (Nominal/Free media)	BMC25 (LEC) (NA) (μM)
Itraconazole	1.4	21.67	0.06
Prothioconazole	16	1.01	15.78

Note: The nominal BMC25 for DNT assays converted to free media concentrations using biokinetics analysis were considered as the LEC for a measurable effect. Abbreviations: NA: Neurite outgrowth, M: Migration.

9. Physiologically based Kinetic (PBK) modelling

For the kinetics assessment of the compounds, published ADME parameters were supplemented with the data generated in the project. The permeability of all the compounds in the Caco-2 cells was experimentally determined in the project. The experimentally determined fraction unbound in plasma was available in literature for all the compounds except epoxiconazole and prothioconazole. These were determined in the project using equilibrium dialysis method described in **Annex I**. Clearance in PHHs was sourced from literature for cyproconazole, difenoconazole and fenbuconazole. Clearance in PHHs were measured experimentally in the project by substrate depletion method for epoxiconazole, propiconazole, prothioconazole and tebuconazole. For ketoconazole, both fractions unbound in plasma and clearance in PHHs measured in the H2020 EU-project EUTOXRISK were used. All the input parameters used in the PBK model development, along with their sources, are described in **Annex I**. LC-MS was used to detect the hydroxy-metabolite and 1,2,4-triazole in the supernatant of primary human hepatocytes incubated with conazoles. While the hydroxy metabolites of conazoles could be detected, its quantification was not possible due to the lack of reference standards. 1,2,4-triazole was not detected for any of the conazoles incubated with PHHs.

Simulations for all the conazoles were run in healthy volunteers in Simcyp V24 for repeat dosing every 24 hours for 15 days at a dose of 10 mg/kg bw/day (**Figure A3.18.** to **Figure A3.27.**). The predicted total plasma C_{max} at steady state for TC was within 3-fold of the predicted total plasma C_{max} at steady state of SCs itraconazole, propiconazole, fenbuconazole, tebuconazole and ketoconazole while it was higher than 3-fold for SCs epoxiconazole, cyproconazole and prothioconazole (**Table A3.12.**). However, the unbound C_{max} for prothioconazole was ~3-fold of the SC due to low fraction unbound value. These differences in predicted unbound C_{max} were considered when comparing relative toxicity of the SCs and carrying out reverse dosimetry and comparing NAM-based PoDs into human oral equivalent (hOED) dose. The TC had both total and unbound plasma C_{max} within 4-fold for propiconazole and fenbuconazole. It is noteworthy that the SC propiconazole clustered with the TC difenoconazole in structural-based clustering (**Figure A3.2.**), high-throughput *in vitro* assay-based clustering (**Figure A3.6.**) and AUC-correlation based clustering in PHH transcriptomics analysis as well (**Figure A3.8.**).

Table A3.12. The total and unbound maximal plasma concentration (μM) at steady state for all SCs and TC used in the case study

Compound	Dose (mg/kg bw/day)	Plasma C_{max} (μM)	Unbound plasma C_{max} (μM)	Allometric LOAELs (mg/kg bw/day)	Unbound plasma C_{max} at allometric LOAEL (μM)
Difenoconazole	10	12.65	0.12	NA*	NA*
Cyproconazole	10	78.81	8.44	5.95	5.02
Epoxiconazole	10	62.30	1.83	2.71	0.50
Fenbuconazole	10	20.00	0.37	6.32	0.24
Itraconazole	10	8.30	0.13	2.50	0.01
Ketoconazole	10	29.33	0.50	2.50	0.12
Propiconazole	10	9.47	0.47	10.14	0.47
Prothioconazole	10	114.02	0.38	14.29	0.54
Tebuconazole	10	23.05	1.59	8.70	1.38

Note: A red/yellow/green color scale has been applied to indicate plasma concentrations at toxic doses, with red representing the lowest concentrations, yellow indicating intermediate levels, and green the highest.

Figure A3.18. Concentration time profile for (a) total plasma and (b) unbound concentration for repeat dose exposure of 10 mg/kg bw/day of cyproconazole predicted using PBK modelling

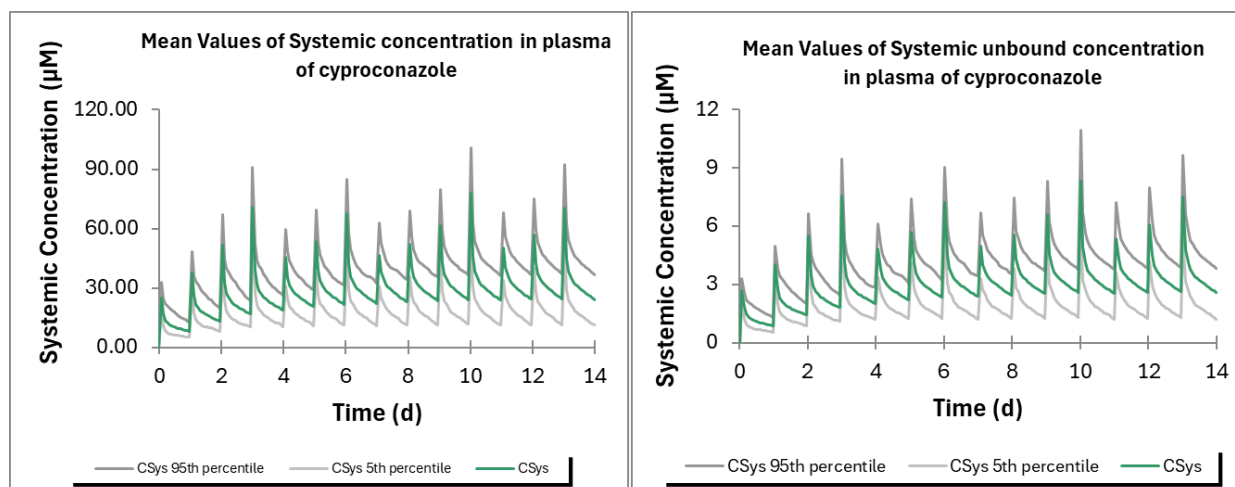


Figure A3.19. Concentration time profile for (a) total plasma and (b) unbound concentration for repeat dose exposure of 10 mg/kg bw/day of difenoconazole predicted using PBK modelling

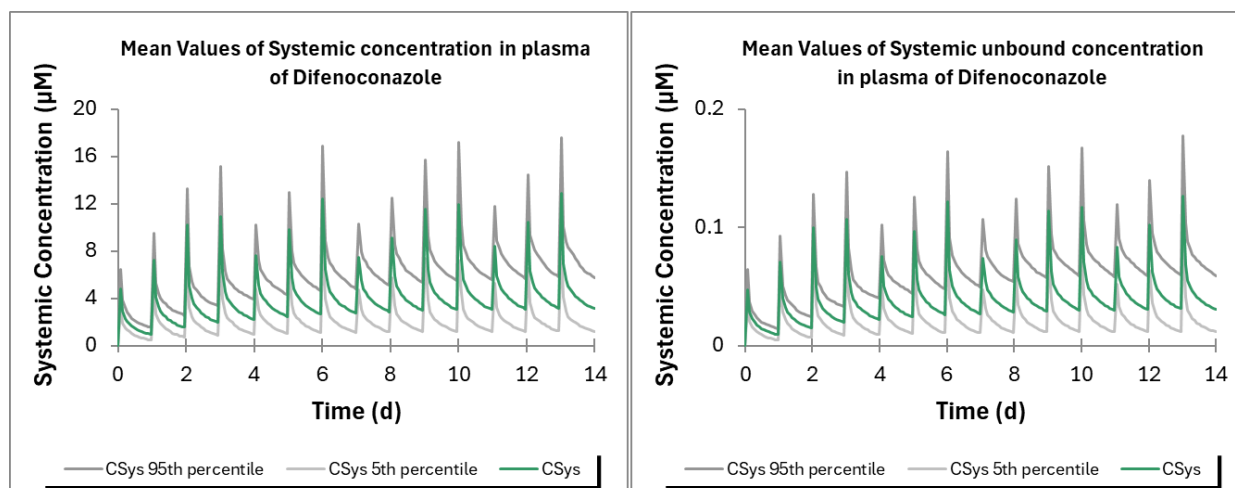


Figure A3.20. Concentration time profile for (a) total plasma and (b) unbound concentration for repeat dose exposure of 10 mg/kg bw/day of epoxiconazole predicted using PBK modelling

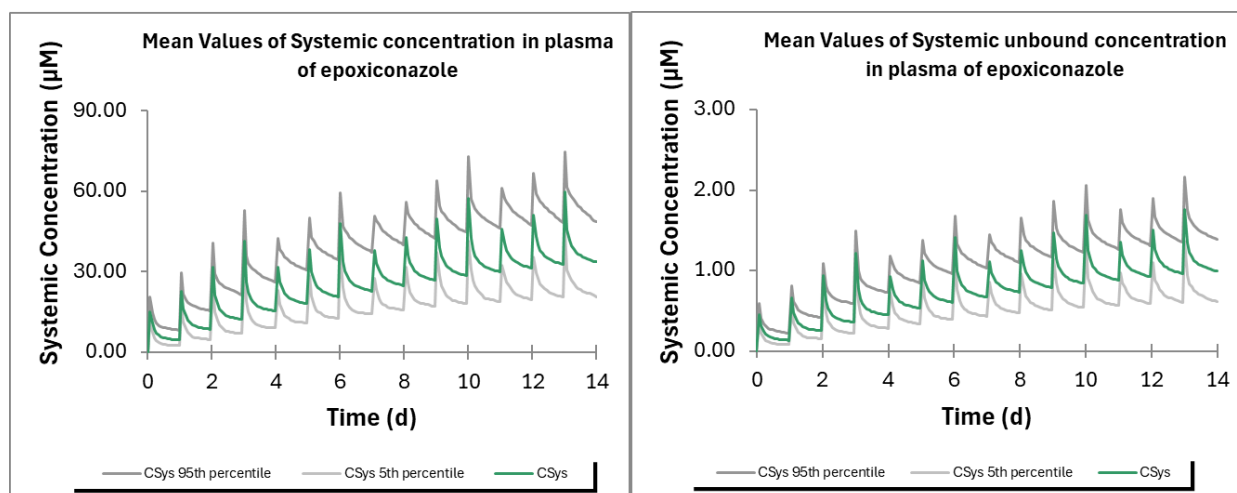


Figure A3.21. Concentration time profile for (a) total plasma and (b) unbound concentration for repeat dose exposure of 10 mg/kg bw/day of fenbuconazole predicted using PBK modelling

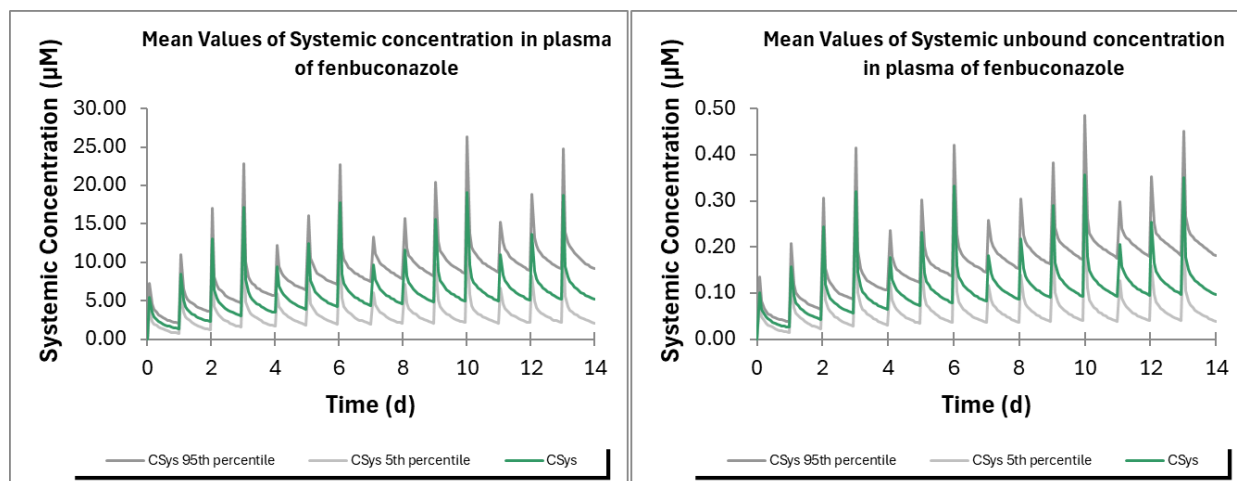


Figure A3.22. Concentration time profile for (a) total plasma and (b) unbound concentration for repeat dose exposure of 10 mg/kg bw/day of itraconazole predicted using PBK modelling

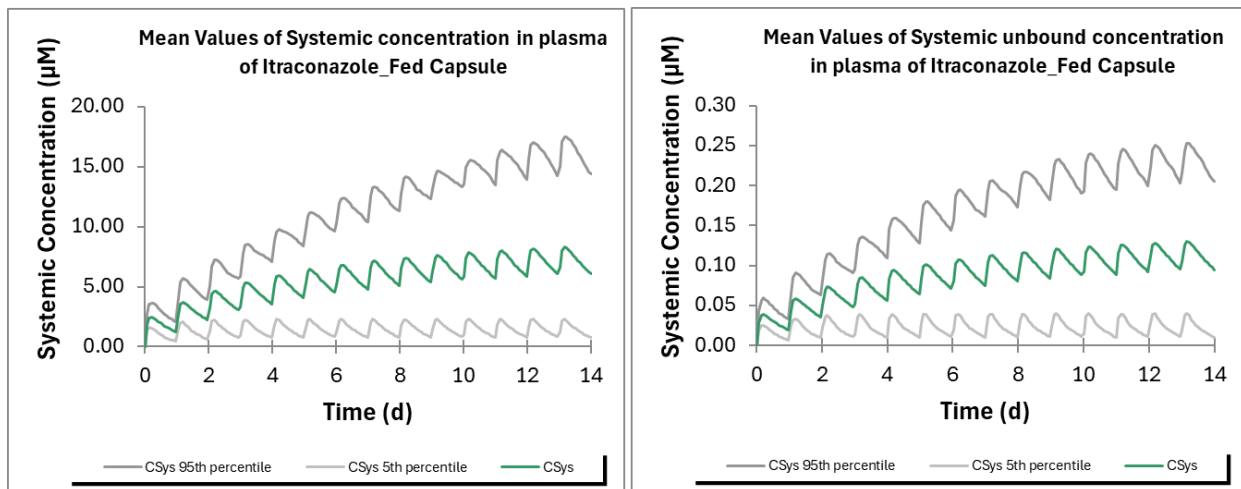


Figure A3.23. Concentration time profile for (a) total plasma and (b) unbound concentration of hydroxyitraconazole predicted after repeat dose exposure of 10 mg/kg bw/day of parent itraconazole predicted using PBK modelling

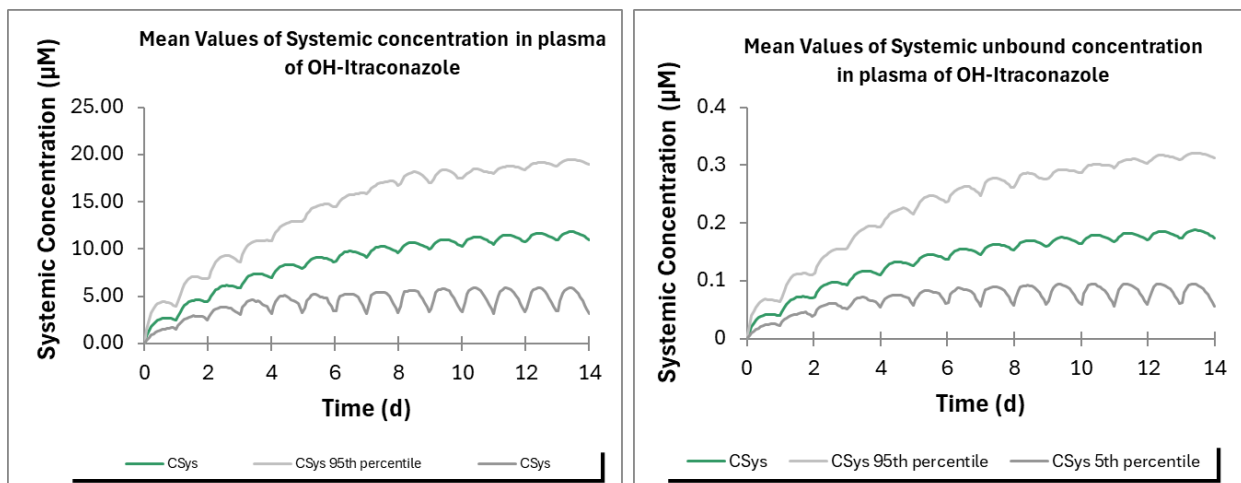


Figure A3.24. Concentration time profile for (a) total plasma and (b) unbound concentration for repeat dose exposure of 10 mg/kg bw/day of ketoconazole predicted using PBK modelling

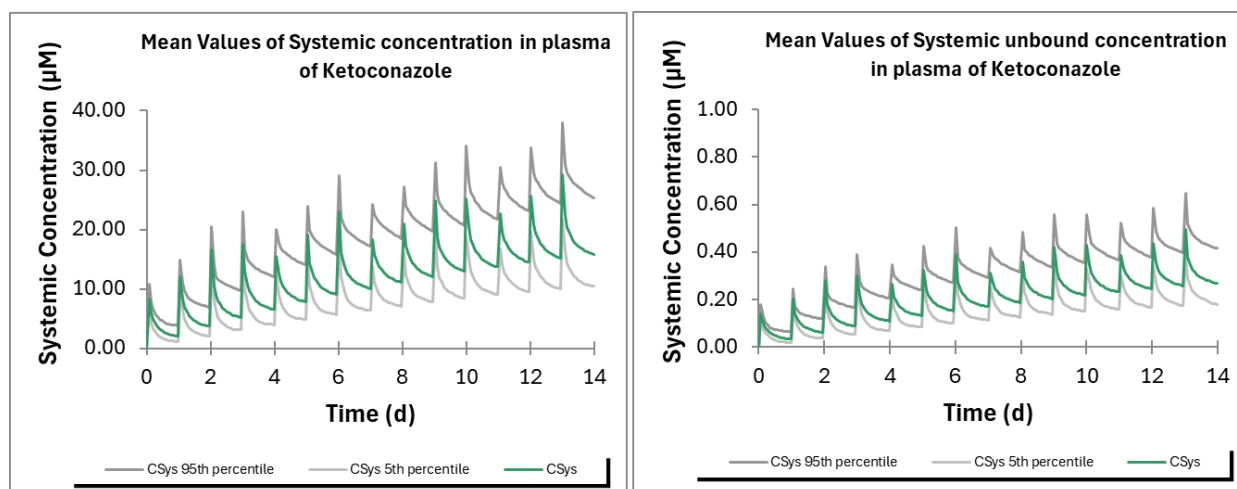


Figure A3.25. Concentration time profile for (a) total plasma and (b) unbound concentration for repeat dose exposure of 10 mg/kg bw/day of propiconazole predicted using PBK modelling

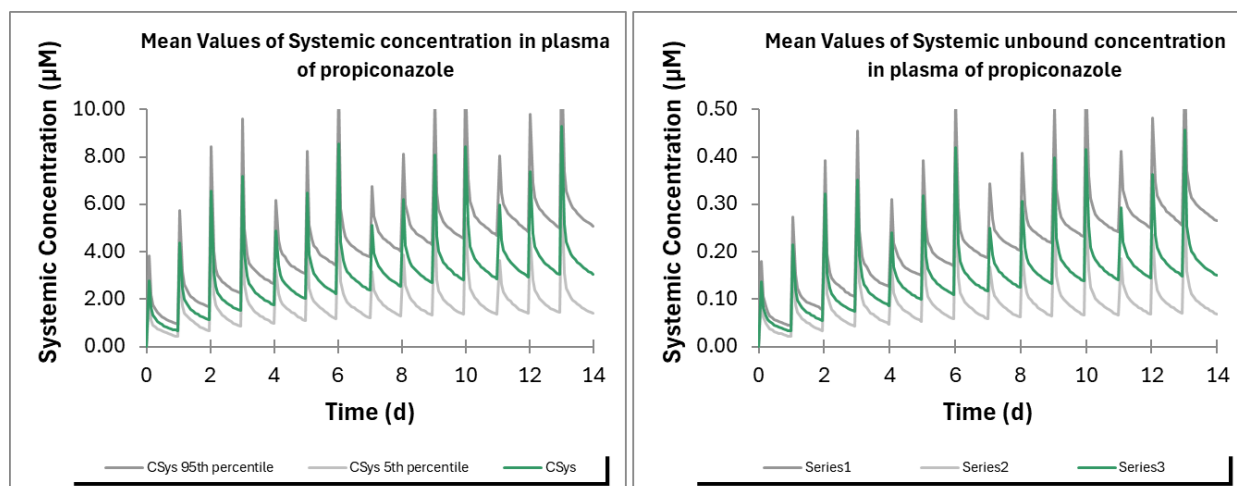


Figure A3.26. Concentration time profile for (a) total plasma and (b) unbound concentration for repeat dose exposure of 10 mg/kg bw/day of prothioconazole predicted using PBK modelling

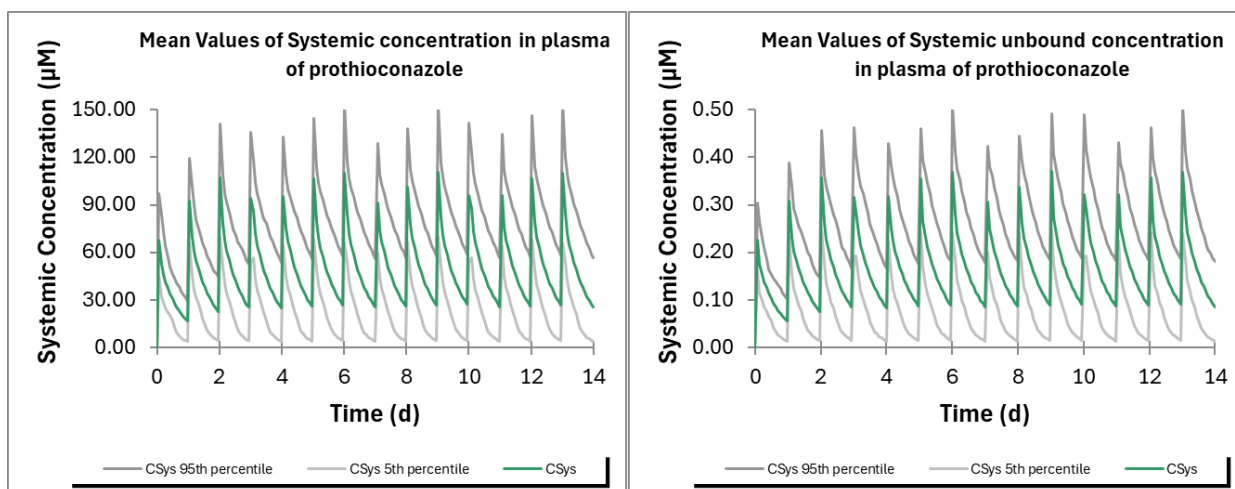
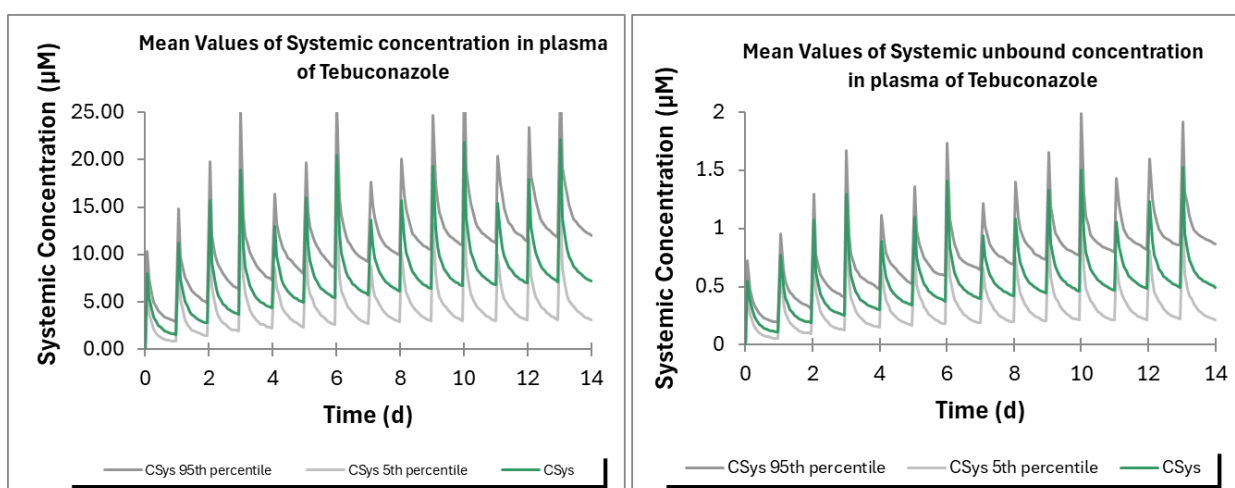
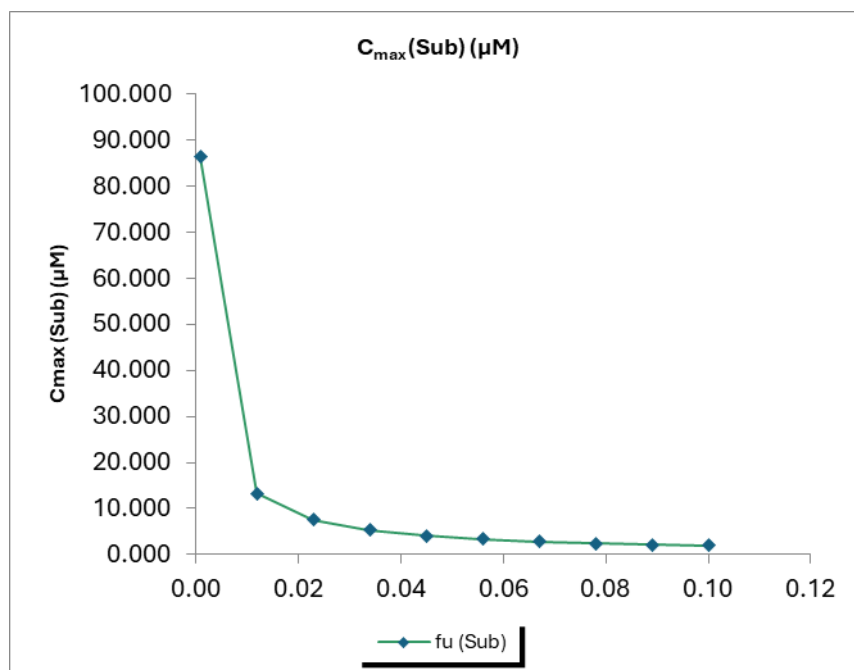


Figure A3.27. Concentration time profile for (a) total plasma and (b) unbound concentration for repeat dose exposure of 10 mg/kg bw/day of tebuconazole predicted using PBK modelling



The fraction unbound of conazoles is less than 0.10 which could be difficult to accurately measure in the *in vitro* assays. The sensitivity analysis was carried out to see the effect of fraction unbound in plasma on the C_{max} of the TC, difenoconazole. The sensitivity analysis showed that the C_{max} is highly sensitive to fraction unbound in the region lower than 0.01 but is less sensitive to values between 0.01 -0.10 (**Figure A3.28.**). Note, the experimentally determined fraction unbound (fu) in plasma for all conazole compounds, except prothioconazole, was either 0.01 or more than 0.01 (**Annex I**). This indicates that, for most conazoles, small changes in fu above this threshold have a relatively minor impact on pharmacokinetic sensitivity. In contrast, when fu falls below 0.01, as observed with prothioconazole, the system becomes more sensitive to changes in binding, potentially leading to more pronounced effects on free drug concentrations.

Figure A3.28. Sensitivity analysis to show relationship between fraction unbound in plasma and $C_{\max}$ for the TC, difenoconazole



10. Expert judgement

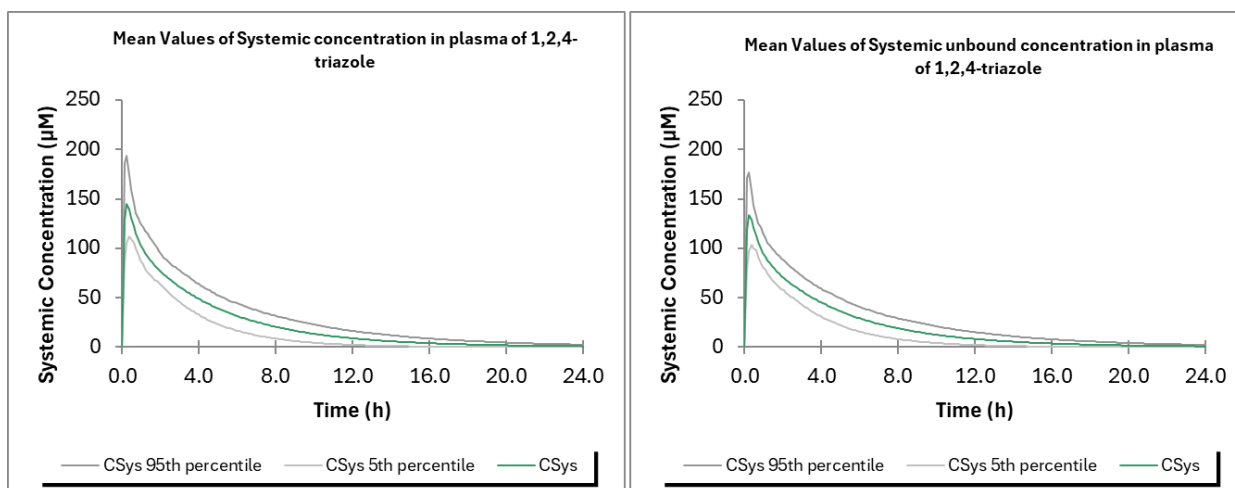
The TC, difenoconazole, showed toxic activity in high throughput *in vitro* assays, transcriptomics in PHHs and H295R steroidogenesis assays. The clustering based on molecular structure (**Figure A3.2.**) and *in vitro* high throughput assays (**Figure A3.6.**) showed that difenoconazole is related to propiconazole and epoxiconazole. In the AUC-correlation analysis from high throughput transcriptomics as well propiconazole, difenoconazole and prothioconazole cluster together (**Figure A3.13**). However, in the clustering using just annotated modules from transcriptomics analysis difenoconazole is not clustered while propiconazole and prothioconazole are clustered together (**Figure A3.12**). The toxicokinetics analysis shows that both total and unbound plasma $C_{\max}$ of difenoconazole is within 3-fold for fenbuconazole and propiconazole (**Table A3.12.**). Thus, the SC, propiconazole, showed similarity to the TC, difenoconazole, in all structural, toxicokinetics and toxicodynamics analyses.

The LC-MS analysis for metabolite detection showed that the conazoles form hydroxy metabolites after incubation in primary human hepatocytes and can also form it in the liver (**Appendix II**). These metabolites could not be quantified due to the absence of reference standards of hydroxy metabolites of each conazoles. Simcyp simulations of itraconazole and hydroxyitraconazole showed that at a daily dose of 200 mg, hydroxyitraconazole can be cleared within 24 - 30 hours. Note, the 10 mg/kg dose used to compare $C_{\max}$ of all conazoles is at least 4 times higher than 200 mg daily dose. The high-throughput *in vitro* data, transcriptomics in PHHs and existing data indicate that hydroxy itraconazole has comparable toxicity as the parent compound. The high throughput assays and plated PHHs have limited metabolic capacity and may overpredict the toxicity of the parent compound. Therefore, we assume that PoDs predicted by the NAMs in this case study capture toxicity of both parent and hydroxy metabolite of the conazoles.

Another metabolite of concern is 1,2,4-triazole, which is known to be associated with craniofacial alterations. Given the high toxicity associated with this metabolite, its potential contribution to the overall risk posed by parent conazoles is of particular concern. Therefore, as part of the risk assessment for conazoles, we also aimed to evaluate the potential toxicity arising from the formation of 1,2,4-triazole. No toxicity of 1,2,4-triazole

was observed in any of the *in vitro* assay used in this case study. 1,2,4-triazole showed some effect in PHH transcriptomics assay but did not pass the filtering criteria. The PBK modelling of 1,2,4-triazole at 4 mg/kg dose only with renal excretion accounted by mechanistic kidney (MechKim) model in Simcyp (Neuhoff et al., 2013), shows that 1,2,4-triazole will be completely excreted from the body within 24 hours (**Figure A3.29**).

Figure A3.29. Concentration time profile for (a) total and (b) unbound plasma concentration for repeat dose exposure of 4 mg/kg bw/day of 1,2,4-triazole predicted using PBK modelling



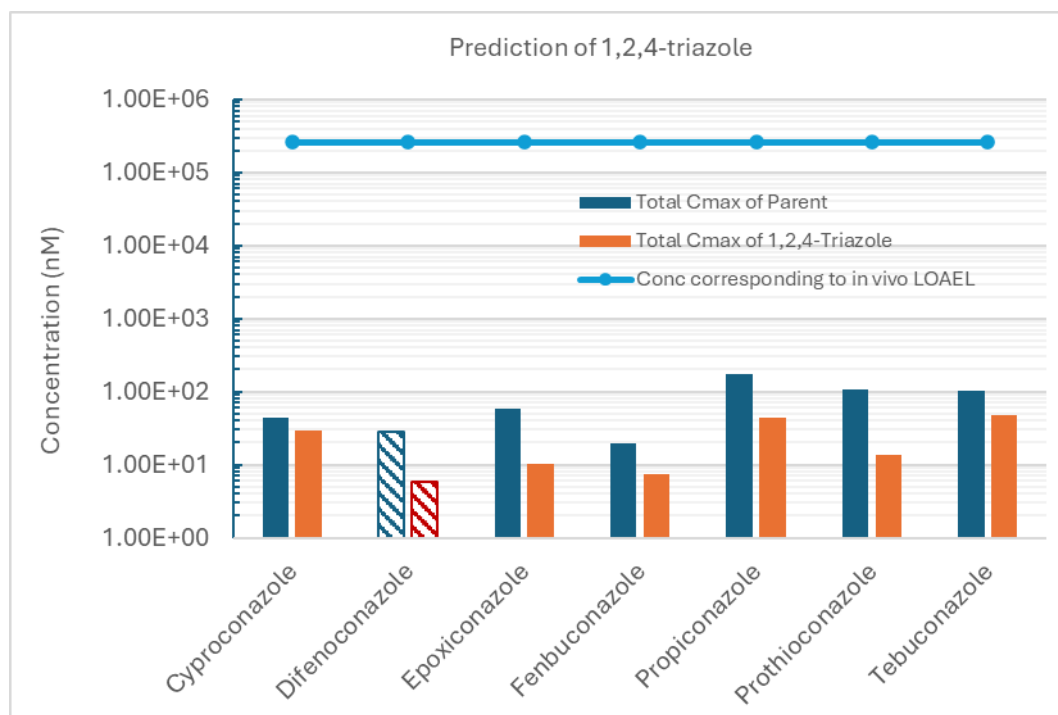
Evidence for the *in vivo* formation of 1,2,4-triazole from conazoles is limited, and the quantity formed, if any, has not been well characterized. 1,2,4-triazole could not be detected in LC-MS analysis of supernatant of conazoles incubated with PHH. To predict how much 1,2,4-triazole can theoretically be formed from parent conazoles, simulations were carried out assuming 100% conversion of the parent conazole to its metabolite incorporated using human liver microsome based clearance predicted from ADMET predictor. The ADI of each conazoles from EFSA were used as the dose for conazoles. This is a highly conservative estimate as:

- (a) Only renal excretion of 1,2,4-triazole is considered
- (b) 100 % conversion of conazole to 1,2,4-triazole is assumed
- (c) ADI of conazoles is considered as the input dose. In real situation, the intake of conazole will most likely be lower than ADI.

The analysis showed that even with this very conservative estimate, the unbound concentration corresponding to allometrically-scaled *in vivo* LOAEL (Agency, 2006)⁷¹ was predicted as 6000 times higher than the highest concentration of 1,2,4-triazole that can be formed by these set of conazoles (**Figure A3.30**).

⁷¹ HED Records Center Series 361 Science Reviews - File R124628, US EPA, 2006 https://www3.epa.gov/pesticides/chem_search/cleared_reviews/csr_PC-600011_7-Feb-06_a.pdf

Figure A3.30. Maximal concentration of parent compound and 1,2,4-triazole at ADI, repeat exposure of parent conazoles when 100% metabolite formation was assumed.



Note: The blue line corresponds to concentrations corresponding to in vivo LOAEL of 1,2,4-triazole.

Uncertainty analysis

The estimated means of replicate variability on micro molar (μM) on the log10 scale range from range from 0.17 to 0.93 for CALUX® bioassays, is 0.37 for CPP and is 0.61 for transcriptomics modules with three replicates (Table A3.13). The reliability estimates for CALUX® are based on replicates of reference compounds which are a mixture of biological and technical replicates. For CPP, there was only replicates for one compound, rotenone. For transcriptomics the between replicate variability were estimated based on modules where all three replicates showed an effect, excluding modules where effects were seen in some but not all modules.

The between replicate variability can justify uncertainty factors added to individual BMDs or the derived overall PoD to set the LEC.

Table A3. 13. Estimated between replicate variability for log10 benchmark concentrations from in vitro NAMs and for the overall PoD (LEC) derived over multiple bioassays/Channels or Modules.

NAM	Feature category	Fixed effects	Estimate	Est. Error	l.95..CI	u.95..CI	calc sd	n_fix	n_random
CALUX®	DR-CALUX agonism	ref_2,3,7,8-Tetrachlorodibenzo-p-dioxin	0.17	0.24	0.01	0.86	0.05	1	3
CALUX®	HepG2-AhR agonism	ref_2,3,7,8-Tetrachlorodibenzo-p-dioxin	0.26	0.06	0.17	0.40	0.24	1	15
CALUX®	TRb-CALUX agonism	ref_3,3',5-triiodo-L-thyronine	0.44	0.11	0.28	0.71	0.38	1	12
CALUX®	AR-CALUX agonism	ref_5a-androstan-17b-ol-3-one	0.42	0.09	0.29	0.62	0.38	1	15
CALUX®	HepG2-p53 agonism	ref_Actinomycin D (from streptomyces species)					0.68	1	24
CALUX®	p21-CALUX agonism	ref_Actinomycin D (from streptomyces species)	0.44	0.10	0.30	0.68	0.40	1	15
CALUX®	p53-CALUX agonism	ref_Actinomycin D (from streptomyces species)	0.53	0.08	0.39	0.72	0.50	1	24
CALUX®	Hif1a-CALUX agonism	ref_Cobalt(II)chloride	0.46	0.09	0.31	0.68	0.41	1	15
CALUX®	Nrf2-CALUX agonism	ref_Curcumin					0.75	1	15
CALUX®	GR-CALUX agonism	ref_Dexamethasone	0.53	0.12	0.36	0.81	0.48	1	15
CALUX®	TRb-CALUX antagonism	ref_Diclozauril	0.60	0.13	0.41	0.92	0.57	1	15
CALUX®	ERa-CALUX agonism	ref_Estradiol	0.57	0.13	0.38	0.86	0.51	1	15
CALUX®	AR-CALUX antagonism	ref_Flutamide	0.27	0.06	0.18	0.42	0.27	1	15
CALUX®	PPARd-CALUX antagonism	ref_GSK3787	0.47	0.10	0.32	0.72	0.44	1	15
CALUX®	LXR-CALUX agonism	ref_GW3965 hydrochloride	0.78	0.19	0.50	1.24	1.23	1	15
CALUX®	PPARa-CALUX antagonism	ref_GW6471	0.70	0.15	0.47	1.05	0.65	1	15
CALUX®	PPARa-CALUX agonism	ref_GW7647	0.62	0.14	0.42	0.94	0.58	1	15
CALUX®	PPARg-CALUX antagonism	ref_GW9662	0.26	0.05	0.18	0.38	0.24	1	15
CALUX®	PPARd-CALUX agonism	ref_L-165,041					0.41	1	15
CALUX®	TCF-CALUX agonism	ref_Lithium chloride (anhydrous)	0.51	0.11	0.35	0.77	0.48	1	15
CALUX®	PR-CALUX agonism	ref_Medroxyprogesterone 17-acetate	0.45	0.10	0.30	0.70	0.39	1	15
CALUX®	GR-CALUX antagonism	ref_Mifepristone					0.32	1	15
CALUX®	PR-CALUX antagonism	ref_Mifepristone					0.19	1	15
CALUX®	PXR-6128-CALUX agonism	ref_Nicardipine hydrochloride	0.40	0.09	0.27	0.60	0.36	1	15
CALUX®	AP1-CALUX agonism	ref_Phorbol 12-myristate 13-acetate					0.43	1	15
CALUX®	RAR-CALUX agonism	ref_Retinoic acid	0.56	0.18	0.33	1.02	0.47	1	9
CALUX®	PPARg-CALUX agonism	ref_Rosiglitazone	0.72	0.15	0.50	1.07	0.66	1	15

CALUX®	ERa-CALUX antagonism	ref_Tamoxifen	0.49	0.11	0.32	0.76	0.47	1	15
CALUX®	Cytotox-CALUX antagonism	ref_Tributyltin acetate	0.93	0.58	0.32	2.45	0.62	1	4
CALUX®	ESRE-CALUX agonism	ref_Tunicamycin (from Streptomyces sp.)					0.45	1	16
CALUX®	Overall pPoD over bioassays	no replicates							
Cellpainting	Channels	Rotenone	0.37	0.43	0.06	1.55	0.36	8	3
Cellpainting	Overall pPoD over Channels	Rotenone	0.11	0.22	0.00	0.65	0.13	1	3
Transcriptomics	Modules	Ketoconazole	0.61	0.63	0.14	2.49	0.32	28	3
Transcriptomics	Overall pPoD over Modules	Ketoconazole	0.82	1.31	0.03	3.18	0.45	1	3

Note: One estimate per feature category, with n_fix fixed effects and n_random groups for random effects. The estimate, estimation error, 95% probability interval and in the case when the random effects model could not be fitted, a calculated standard deviation based on point estimates.

11. Outcomes and Conclusions

In vitro toxicologically relevant concentrations were compared to corresponding in vivo exposure levels. For conazoles in the case study, the lowest free (unbound) media concentration associated with a biological effect in each in vitro assay was identified. These concentrations were then compared to estimated free in vivo concentrations associated with toxicity. For conazole pesticides, this comparison was based on allometrically scaled LOAELs, while for pharmaceutical compounds (ketoconazole and itraconazole), it was based on clinically administered human doses.

To derive a final PoD for each compound, the lowest LEC (free media concentrations) associated with biological activity across all in vitro assays were calculated. This represents a conservative estimate of the concentrations eliciting effects.

Table A3.14. Comparison of the lowest LEC associated with biological activity across all in vitro assays for TC and SCs

Compounds	CALUX®		ToxProfiler		CPP		Transcriptomics		H295R assay		DNT assays	
	LEC	Biology	LEC	Biology	LEC	Biology	LEC	Biology	LEC	Biology*	LEC	Biology
Difenoconazole	0.005	GR	0.563	Cell cycle stress	0.202	DNA	1.630	Metabolism, cholesterol	0.24	Estrogen ↘	3.67	UKN2
Cyproconazole	2.338	GR	83.857	Oxidative Stress	6.132	Mitochondria	8.477	Metabolism, xenobiotic	1.65	Estrogen ↘	NA	Negative
Epoxiconazole	0.004	GR	23.001	Cell cycle stress	5.307	Mitochondria	1.883	Metabolism, xenobiotic	1.31	Androgen ↘	NA	Negative
Fenbuconazole	0.051	PR	17.467	Cell cycle stress	1.715	Mitochondria	30.216	Metabolism, xenobiotic	1.48	Androgen ↘ Estrogen ↘	10.67	UKN2
Itraconazole	0.003	GR	0.019	Autophagy	0.299	Golgi	0.211	Metabolism, xenobiotic	0.06	Androgen ↘	0.05	UKN4
Ketoconazole	0.022	GR	0.190	Autophagy	0.023	Mitochondria	0.612	Metabolism, cholesterol	0.12	Androgen ↘ Estrogen ↘	27.58	UKN4
Propiconazole	0.063	AR	7.159	ER stress	0.701	Mitochondria	3.089	Metabolism, cholesterol	1.02	Estrogen ↘	NA	Negative
Prothioconazole	3.284	PR	61.670	Cell cycle stress	6.137	Mitochondria	20.105	Metabolism, xenobiotic	9.72	Androgen ↘	15.78	UKN5
Tebuconazole	0.064	AR	12.385	Cell cycle stress	8.884	Mitochondria	3.734	Metabolism, xenobiotic	1.81	Estrogen ↘	47.04	UKN2

Note: The lowest effect concentration (LEC) was consistently observed in the CALUX®.

For all the SCs and TC, the minimum LECs from all assays was consistently observed in the CALUX® assays. These values were considered representative of the unbound concentrations potentially associated with *in vivo* toxicity and were designated as NAM-derived LOAELs. These concentrations were even lower than the LECs for H295R assays for all the compounds (**Table A3.14.**).

Assuming this unbound concentration in plasma will result in systemic toxicity, reverse dosimetry was used to derive hOED for all the compounds. This was compared to the *in vivo* LOAELs from rats and mice 90 days studies, which allometrically scaled to human dose (mg/kg bw/day) for all the SCs. The lowest allometrically scaled *in vivo* LOAELs from each SC were selected as representative for *in vivo* LOAELs. The NAM-based LOAELs were either lower or similar to the *in vivo* LOAELs for all compounds (**Table A3.15.**).

Table A3.15. Comparison of NAM derived hOED with allometrically scaled in vivo LOAEL

Compound	Minimum LEC from all <i>in vitro</i> assays (µM)	Unbound C _{max} at 10 mg/kg/day (µM)	NAM-derived LOAELs (mg/kg bw/day)	<i>In vivo</i> allometric LOAELs (mg/kg bw/day)
Difenoconazole	0.005	0.12	0.43	4.88
Cyproconazole	2.338	8.44	2.77	5.95
Epoxiconazole	0.004	1.83	0.02	2.71
Fenbuconazole	0.051	0.37	1.37	6.32
Itraconazole	0.003	0.13	0.21	2.5
Ketoconazole	0.022	0.5	0.44	2.5
Propiconazole	0.063	0.47	1.33	10.14
Prothioconazole	3.284	0.38	86.41	14.29
Tebuconazole	0.064	1.59	0.4	8.7

Note: The TC difenoconazole is marked in bold. A green–yellow–red colour scale highlights LOAEL values, where red indicates lower LOAELs and therefore higher potency.

The comparison between NAM-derived and *in vivo* LOAELs for the SCs supports confidence in the read-across approach. For most SCs, the NAM-derived LOAELs were lower than their respective *in vivo* LOAELs, indicating a conservative nature of the NAM-based assessments.

Epoxiconazole had the lowest NAM-derived LOAEL, consistent with its low *in vivo* LOAEL, alongside itraconazole and ketoconazole. However, the NAM-based LOAEL for epoxiconazole was 14 times lower than the *in vivo* value, reinforcing the conservative tendency of NAMs. Similarly, for propiconazole—clustered with the target compound (TC) across all assessments—the NAM-derived LOAEL was 6.5 times lower than the *in vivo* LOAEL.

Notably, prothioconazole was the only SC for which the NAM-based LOAEL exceeded the *in vivo* LOAEL, being predicted at 10 times higher. This discrepancy may be attributed to structural differences between prothioconazole and the other SCs. Additionally, it is also to be noted, that the *in vivo* studies used for the LOAELs comparison may have difference in/between species/studies. For instance, the *in vivo* LOAEL for the prothioconazole was allometrically scaled from the mice study, which showed effects on liver at study LOAEL (allom. LOAEL: 14.29 mg/kg bw/d). In rat studies, the lowest dose causing effects were seen at much higher dose (allom. LOAEL: 125 mg/kg bw/d) with notably as kidney one of the prominent the target organs in rats. Moreover, it is also known that prothioconazole metabolizes into prothioconazole-desthio, a more toxic metabolite. However, it remains unclear whether this metabolic conversion occurred in the cellular systems used in this study, as it was not specifically characterized. Interestingly, prothioconazole also exhibited the highest LOAEL among the SCs in both NAM-based and *in vivo* assessments.

A two-step approach was used for read-across analysis. In first step, hierarchical clustering of SCs and TCs based on structural information was employed as a screening tool to identify and prioritize potential read-across candidates for the TC, difenoconazole. This approach serves primarily as a preliminary screening method to highlight substances which require further consideration. Propiconazole and epoxiconazole were shown to be structurally similar to difenoconazole based on the MACCS fingerprints and Tanimoto distance (**Figure A3.2.**). However, structural similarity alone is not sufficient to conclude on the read-across candidate (Ball et al., 2016; Escher et al., 2019). A next step of integrating SC evaluation is essential to identify (dis)similarity, therefore refining the candidate selection. In this study, we integrated NAM data to evaluate the mechanistic aspect of the compounds and support read-across as a second step. The clustering based on the high-throughput NAM results refined the initial hypothesis by providing more information that the SCs propiconazole and epoxiconazole are not only structurally but also mechanistically similar to the TC (**Figure A3.6.**). The next step, which is data-gap filling (here LOAEL of TC) can be conducted using regular pattern, nearest neighbour or worst-case approach (ECHA, 2017; Escher et al., 2019). In this CS, we considered both the nearest neighbour and the worst-case approach to derive the LOAEL of the TC. These are discussed below:

- (a) The LOAEL value for TC was predicted using a *k*-nearest neighbour (kNN) model based on a Tanimoto similarity matrix derived from high-throughput assay response profiles. We used three compounds with the highest similarity scores and available LOAEL values, which included epoxiconazole, propiconazole and cyproconazole (**Figure A3.6B.**). The predicted LOAEL was calculated as a similarity-weighted average of the neighbours' LOAEL values:

$$LOAEL_{pred} = (\text{sum}(S_i * LOAEL_i)) / (\text{sum}(S_i))$$

where S_i represents the Tanimoto similarity between the TC and neighbour i , calculated from the high-throughput assay response profiles, and $LOAEL_i$ is the allometrically scaled LOAEL for neighbour i (**Table A3.15.**). The allometric LOAEL from this approach was predicted to be **6.21 mg/kg/day** which is 1.3 times of the actual allometric LOAEL for difenoconazole (4.88 mg/kg bw/day) (**Main document, Table 2**).

- (b) An alternative interpretation would be to use a worst-case approach to predict the LOAEL of TC. Both structural similarity and high-throughput NAM assessments indicated that propiconazole and

epoxiconazole were the closest analogues to the TC. However, as epoxiconazole exhibited lower NAM-derived and *in vivo* LOAEL of the two SCs, the *in vivo* LOAEL for TC difenoconazole was inferred using epoxiconazole. Consequently, using this approach, an allometric LOAEL of **2.71 mg/kg bw/day** was established for the TC difenoconazole, matching that of epoxiconazole. When compared with the actual *in vivo* allometric LOAEL for difenoconazole (4.88 mg/kg bw/day) (**Main document, Table 2**), the predicted value of 2.71 mg/kg/day is lower but still within a two-fold range, supporting the conservative yet reliable nature of the read-across estimate.

Another alternative and scientifically justified approach would be to adopt the NAM-derived LOAEL for the TC as the final PoD. Despite the qualitative alignment in NAM-derived LOAELs among the SCs, there was no consistent fold-difference when compared to their respective *in vivo* LOAELs. Therefore, the use of a read-across approach based on NAM-derived similarity represents a pragmatic strategy for estimating missing toxicity value. The analysis presented above supports this conclusion, as both read-across approaches applied in this study predicted the LOAEL for TC within a two-fold range of its experimentally derived, allometrically scaled LOAEL. This level of agreement indicates that similarity in NAM bioactivity profiles can provide a reasonable basis for estimating *in vivo* toxicity thresholds within this chemical space

12. Uncertainties in the specific workflow followed

Uncertainty is described in a semiquantitative way using the terms:

Low – absence of conflicting evidence

Moderate – good evidence from primary line of data, few data gaps

High – some evidence, doubts can be raised about data gaps and relevance of approaches/data

Uncertainties specific to	Description	Weight (High/Medium/Low)
Problem formulation and regulatory context	The conazole case study aims to explore the applicability of NAM data in read-across scenario to predict the systemic toxicity and LOAEL of target compound difenoconazole. Source compounds (SC) belong to same category due to the <i>in vivo</i> study indicating liver as main target organ. Studies were extracted from DAR/RAR from Rapporteur Member States (RMS) available at EFSA Public consultation website. This is considered to be reliable.	Low
<i>in vivo</i> data	Studies were extracted from DAR/RAR from Rapporteur Member States (RMS) available at EFSA Public consultation website. There is small variation regarding the guideline for the studies, which is all indicated in the conclusion and executive summaries in the dossiers. Some studies were conducted according to guidelines or methods considered similar or equivalent to OECD guidelines. Some studies lack defined guidelines, or the guidelines are not specified. This served as supporting evidence. Hydroxy itraconazole and ketoconazole have few to no available <i>in vivo</i> rodent studies. Some ketoconazole studies are limited to 28 days, which may be insufficient for predicting longer-term effects. Our case study aims to assess toxicity at a subchronic level (90 days) for a better understanding of potential risks. Nevertheless, the results can still serve as supportive evidence.	Low
Biological coverage of <i>in vitro</i> screening battery	The case study explores the applicability of a NAMs, which cover a large variety of biological processes that might lead to adverse outcomes after repeated exposure. The Read-across hypothesis is that all compounds induce liver toxicity, which is covered by a specific assay the transcriptomics derived from PHHs. Although some MoA might be underrepresented e.g. compounds effecting endocrine endpoints this is of low relevance for the read-across assessment. Variability may exist among samples, batches, and laboratories where the experiments were conducted. The experimental results obtained from these tests do not directly predict adverse outcomes. However, they can provide insights into potential key events and modes of action. The <i>in vitro</i> assays in this case study used mostly the liver cells. However, it is important to consider a wider array of <i>in vitro</i> model to cover broader toxicological effects. Other models are being considered for the future of this case study, such as kidney, lung, DNT (developmental neurotoxicity). Further, due to the limited metabolic competence of <i>in vitro</i> test battery, metabolites were directly tested in the assays. It is noteworthy that extending this approach to a different set of compounds would require ensuring that the NAMs and <i>in vitro</i> screening battery used are relevant to those compounds and adequately capture their biological activity.	Low
Relevant <i>in vitro</i> testing range	The <i>in vitro</i> testing range must cover a large enough concentration range to assure that the biological activity is not missed. Some compounds behave with different concentration range. We expect that a retesting at higher concentrations for less sensitive assays will increase the confidence of the results.	Moderate
Kinetic models –PBK models	The PBK models provide an estimate of the bioavailable concentration using forward dosimetry. It is expected that this model overestimates the bioavailable concentration. As the models have been parametrized using <i>in vitro</i> clearance data, the uncertainty associated with the prediction is expected to be low.	Low

Kinetic models – VIVD	<i>In vitro</i> kinetic modelling was performed for all compounds to assess <i>in vitro</i> conversion factors. These are needed to compare benchmark concentration from different <i>in vitro</i> assays. The conazoles are within the applicability domain of the model so the uncertainty associated with the prediction is expected to be low.	Low
<i>In silico</i> data	<i>In silico</i> models rely on detailed input data and high-quality databases, and their applicability may be restricted to specific chemical classes. Ensuring accuracy requires cross-validation, but the available data is often limited, especially on human studies.	Low
Clustering analysis based on NAM data	For the compounds where no responses were observed, the highest tested concentration were used as input. The results were combined and for similarity and clustering analysis. The outcomes of the assays serve as fingerprint to calculate the similarity. However, the results of the similarity could vary based on the choice of fingerprint and algorithm. This uncertainty arises from potential limitations in representing the molecular feature captured by the fingerprints. Morgan fingerprints which are used in this case study use a circular method to cover atom environment for a specific radius. While this fingerprint could capture the local environment of the atom, this also dependent on the selected radius. The same scenario applies for the similarity calculation and hierarchical clustering, which could vary between algorithms and parameter used. While these considerations are important, the most significant aspect of using these approaches is to guide or provide initial indications about which substances might be potentially relevant to look at. The clustering analysis was also conducted for the NAM <i>in vitro</i> test readouts (CALUX®, ToxProfiler and CPP). Each assay readouts serve as feature to construct the biological fingerprints, which later used for the clustering. However, given the varying number of features for each assay outcomes, some of the assays could be overrepresented. Therefore, it is crucial to interpret the clustering with caution, by considering other data available in this case study, e.g. with <i>in vivo</i> data and transcriptomics.	Moderate
Uncertainties specific to the chemical assessed	Compounds with low solubility or high plastic binding properties can contribute to the uncertainty of the results. Most of the conazole compounds have logPow >3 which tend to be more hydrophobic and may bind to plastic materials. Therefore, the concentration was corrected using VIVD model. Compounds could interfere with the luminescence readouts and could produce false positives. Some chemicals are pH dependent, e.g. Prothioconazole. The water solubility of prothioconazole differ according to the condition regarding the pH based on the experimental data available. Water solubility varies among the compounds, with values ranging from 0.0005 mg/L for itraconazole to 300 mg/L for prothioconazole. It is important to note that the water solubility of prothioconazole is influenced by the pH of the testing conditions. Specifically, prothioconazole solubility can range from 5 mg/L at pH 4, to 300 mg/L at pH 8, and up to 2000 mg/L at pH 9 (RMS, 2005). For this analysis, we assume neutral conditions and have used the value for pH 8. This highlights the significant impact that minor changes in pH can have on prothioconazole's water solubility. Structurally, prothioconazole also differs from other triazole pesticides, due to triazolinone ring present. <i>In vitro</i> assays based clustering analysis also shows that prothioconazole is clustered differently from other conazoles. <i>In vivo</i> data shows higher LOAEL for prothioconazole. Further, the hydroxy metabolites of all compounds could not be quantified in the LC-MS analysis due to the absence of reference standards. However, as the toxicity of hydroxy itraconazole is similar to itraconazole, we assume it will be the same for the TC and its hydroxy metabolite. <i>In vivo</i> studies on 1,2,4-triazole indicate reproductive or developmental toxicity in male mice, including epididymis germ cell degeneration and testes atrophy at higher doses compared to parent compounds. This serves as indication of reproductive or developmental toxicity at higher doses. 1,2,4-triazole can be cleaved during the metabolism step of the triazole conazoles, which makes it relevant for testing. However, when tested in the <i>in vitro</i> metabolic profiling assays in hepatocytes and quantified using the LC-MS, 1,2,4-triazole was not present. This could be due to that 1,2,4-triazole was cleaved at the later stage of metabolism round which. 1,2,4-triazole also has more hydrophilic properties in comparison to other parent compounds. This could be due to the concentration not being high enough, or the 1,2,4-triazole has different metabolism pathways. When tested in assay panels such as CALUX®, ToxProfiler and CPP, 1,2,4-triazole did not show any results, despite evidence from <i>in vivo</i> study.	Moderate

13. Discussion of other interpretations (if applicable)

Not needed

14. References

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Supporting Information

- [Annex I CALUX concentrations.xlsx](#)
- [Annex I CPP concentrations.xlsx](#)
- [Annex I ToxProfiler concentrations.xlsx](#)
- [Annex I CALUX method.pdf](#)
- [Annex I CPP method.xlsx](#)
- [Annex I Toxprofiler method.pdf](#)
- [Appendix I Danish QSAR Toolbox.pdf](#)
- [Appendix II BMD transcriptomics.xlsx](#)
- [Appendix III LCMS metabolites.xlsx](#)
- [PBPK_workspaces_IATA.zip](#)
- [Data for uncertainty analysis.zip](#)