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Prediction of human drug-induced liver injury (DILI) in relation to oral doses and blood concentrations

Dissertation zur Erlangung des akademischen Grades
des Doktors der Naturwissenschaften (Dr. rer. nat.)

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“To strive, to seek, to find and not to yield.”

Alfred, Lord Tennyson

“All sorts of things can happen when you’re open to new ideas and playing with things.”

Stephanie Kwolek

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Summary

Drug-induced liver injury (DILI) is the leading cause for acute liver failure in the USA and in Germany and one of the most common reasons for withdrawal of drugs from the market or failure of a drug candidate during development. Since DILI cannot be accurately predicted by animal models, a reliable *in vitro* test system for the prediction of human hepatotoxicity would be a valuable asset for drug development as well as for regulatory purposes.

In this thesis an *in vitro/in silico* approach for the prediction of human hepatotoxicity in relation to blood concentrations and oral doses was established. This approach combines *in vitro* effective concentrations derived from a cytotoxicity assay, *in vivo* concentrations obtained by physiologically based pharmacokinetic (PBPK) modelling and a support vector machine (SVM) classifier based on these concentration pairs to predict the risk for hepatotoxicity for specific exposure scenarios. For systematic test system evaluation and optimization two novel performance metrics, the Toxicity Separation Index (TSI) and Toxicity Estimation Index (TEI), were utilized. These indices eliminate the need for *a priori* defined *in vitro* and *in vivo* concentrations and foster the systematic evaluation of the benefit of additional readouts.

As a first step the feasibility of the *in vitro/in silico* approach was tested for primary human hepatocytes (PHH) and a training set of 28 compounds with in total 30 different *in vitro/in vivo* concentration vectors, yielding a sensitivity of 100%, a specificity of 88% and an accuracy of 93% in the leave-one-out classification with the SVM based classifier. A SVM based classifier utilizing all vectors was then applied to derive in combination with reverse PBPK modelling an acceptable daily intake (ADI) for the hepatotoxicant pulegone. The derived ADI was comparable to published ADIs based on two rodent studies.

Next, the compound set was extended to a total of 80 compounds with 82 distinct *in vitro/in vivo* concentration pairs. The SVM leave-one-out classification resulted in a sensitivity of 77.8%, a specificity of 59.4% and an accuracy of 70.1%.

Furthermore, the feasibility of the approach substituting HepG2 cells for the PHH and a combination of both cell culture systems for the extended compound set was evaluated. The obtained sensitivity was 88.9% and 86.7% and the specificity 62.5% and 65.6%, respectively. The accuracy was in both cases 77.9%.

Zusammenfassung

Arzneimittel bedingte Leberschäden (DILI) sind in den USA und Deutschland die häufigste Ursache für das akute Leberversagen. Zudem sind sie einer der häufigsten Gründe dafür, bereits zugelassene Medikamente vom Markt zu nehmen, beziehungsweise die Entwicklung von Wirkstoffkandidaten abzubrechen. Da eine zuverlässige Erkennung von potenzieller Hepatotoxizität im Tierversuch sehr schwierig ist, wäre ein präzises *in vitro* Testsystem für humane Lebertoxizität ein wichtiger Fortschritt, sowohl für die Entwicklung neuer Wirkstoffe als auch für die Risikoabschätzung und Regulierung von Chemikalien.

Im Rahmen dieser Arbeit wird ein *in vitro/in silico* Ansatz zur Vorhersage von humaner Hepatotoxizität in Bezug zu Blutkonzentrationen und oralen Dosen vorgestellt. Bei diesem Ansatz werden durch einen Zytotoxizitätstest ermittelte effektive *in vitro* Konzentrationen mit durch physiologisch basierter pharmakokinetischer (PBPK) Modellierung errechneten *in vivo* Konzentrationen verglichen. Die Vorhersage der Hepatotoxizität erfolgt durch einen „support vector machine“ (SVM) Klassifikator basierend auf diesen Konzentrationspaaren. Für die systematische Beurteilung und Optimierung des Testsystems wurden zwei neuartige Maßzahlen, der „ Toxicity Separation Index“ (TSI) und der „ Toxicity Estimation Index“ (TEI) angewendet. Durch diese Indices entfällt die Notwendigkeit sich *a priori* auf bestimmte *in vitro* und *in vivo* Konzentrationen festzulegen. Zusätzlich erleichtern sie die systematische Beurteilung, ob zusätzliche *in vitro* Parameter die Testsystemperformanz verbessern.

In einem ersten Schritt wurde die Realisierbarkeit des Ansatzes anhand eines Trainingssatzes von 28 Substanzen in primären menschlichen Hepatozyten (PHH) mit insgesamt 30 *in vitro/in vivo* Konzentrationspaaren überprüft. Die ,,Leave-One-Out“ Kreuzvalidierung mit dem SVM Klassifikator hat eine Sensitivität von 100%, eine Spezifizität von 88% und eine Korrekt-Klassifikationsrate von 93% ergeben. Zusätzlich wurde ein auf allen 30 Konzentrationsvektoren basierender SVM Klassifikator angewendet, um in Kombination mit reverser PBPK Modellierung eine akzeptable tägliche Aufnahme (ADI) für die hepatotoxische Substanz Pulegone abzuleiten. Der erhaltene Wert ist ähnlich zu den abgeleiteten ADI Werten aus zwei Studien in Nagern.

Im nächsten Schritt wurde das Substanzset auf insgesamt 80 Substanzen mit 82 verschiedenen *in vitro/in vivo* Konzentrationsvektoren erweitert. Die Kreuzvalidierung des Klassifikators resultierte in einer Sensitivität von 77.8%, einer Spezifizität von 59.4% und einer Korrekt-Klassifikationsrate von 70.1%.

Zusätzlich wurde die Realisierbarkeit des Ansatzes mit HepG2 Zellen anstatt primärer humaner Hepatozyten sowie einer Kombination der beiden Zellkultursysteme untersucht. Die Kreuzvalidierung für das komplette Substanzset führte zu Sensitivitäten von 88.9% beziehungsweise 86.7% und Spezifitäten von 62.5% beziehungsweise 65.6%. Die Korrekt-Klassifikationsrate betrug in beiden Fällen 77.9%.

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Abbreviations

%	Percent
°C	Degree Celsius
[HD]	High doses
4pLL model	Four-parameter log-logistic model
ADI	Acceptable daily intake
ADME	Absorption, distribution, metabolism, elimination
Allo	Allopurinol
AMIO	Amiodarone
APAP	Acetaminophen
ASP	Aspirin
ATP	Adenosine triphosphate
ATRO	Atropine
AUC	Area under the curve
AVS	Atorvastatin
BOS	Bosentan
BPR	Buspirone
BSO	DL-buthionine-sulfoximine
BUSF	Busulfan
BZB	Benzbromarone
BZT	Benztropine
CBZ	Carbamazepine
CEFS	Committee of Experts on Flavouring Substances of the Council of Europe
CHL	Chlorpheniramine
CIPRO	Ciprofloxacin
CL	Left cursor
CLFI	Clofibrate
CLON	Clonidine
CMZ	Chlorpromazine
CZP	Clozapine
cm	Centimeter
C_{av}	Average concentration
C_{max}	Peak concentration
CO_2	Carbon dioxide
COD	Codeine
CsA	Cyclosporin A
CTB	Cell Titer Blue assay
CYP1B1	Cytochrome P450 1B1
CYP3A7	Cytochrome P450 3A7
CZP	Clozapine
DBB	Dibrombimane
DFN	Diclofenac
Digi	Digoxin
DILI	Drug-induced liver injury
DIPY	Dipyridamole

DMEM	Dulbecco's modified eagles medium
DMSO	Dimethyl sulfoxide
DPH	Diphenhydramine
DRESS	Drug reaction with eosinophilia and systemic symptoms
EC	Effective concentration
EC_{10}	Concentration at which a 10% decrease occurs
EC_{20}	Concentration at which a 20% decrease occurs
EC_{50}	Concentration at which a 50% decrease occurs
EC_{80}	Concentration at which a 80% decrease occurs
EC_k	Concentration at which an effect size decrease of k% occurs
EDTA	Ethylene diamine tetra acetic acid
EMA	European Medicines Agency
Ery	Erythromycine
EtOH	Ethanol
FAM	Famotidine
FBS	Fetal bovine serum
FCA	Fluconazole
FDA	Food & Drug Administration (in the United States)
FFD	Fexofenadine
FLT	Fluoxetine
FN	False negative
FP	False positive
g	Gram
G6PD	Glucose-6-phosphate dehydrogenase
GLC	Glucose
GSH	Glutathione
h	Hour
HIV	Human immunodeficiency virus
HD	High dose
HepG2	Hepatoma G2 cell line
hLiMT	Human liver microtissue
HMPC	Committee on Herbal Medicinal Products of the EMA
HOECHST 33342	2'-(4-Ethoxyphenyl)-6-(4-methyl-1-piperazinyl)-1*H*,3'*H*-2,5'-bibenzimidazole
HuMPCC	Human micropatterned coculture
HYZ	Hydroxyzine
IBU	Ibuprofen
IC	Inhibitory concentration
IMP	Imipramine
INAH	Isoniazid
Indo	Indomethacine
ISS	Isosorbide dinitrate
ITS	Insulin transferrin selenite supplement
KC	Ketoconazole
KCl	Potassium chloride
kg	Kilogram

KH_2PO_4	Potassium dihydrogen phosphate
l	Liter
LAB	Labetalol
LEV	Levofloxacin
LFM	Leflunomide
LOEC	Lowest observed effective concentration
log	Logarithm
mM	Millimol per liter
MEL	Melatonin
MePa	Methylparaben
mg	Milligram
mg/l	Milligram per liter
mg/ml	Milligram per milliliter
ml	Milliliter
mm	Millimeter
MOS	Margin of safety
MTX	Methotrexate
MW	Molecular weight
N/A	Not applicable
NAC	N-acetylcysteine
NaCl	Sodium chloride
NaOH	Sodium hydroxide
Na_2HPO_4	Disodium hydrogen phosphate
NDP	Nifedipine
NFT	Nitrofurantoin
ng	Nanogram
ng/ml	Nanogram per milliliter
NIM	Nimesulide
NL	Left normalizing cursor
nM	Nanomol per liter
nm	Nanometer
NOAEL	No observed adverse effect level
NPV	Negative predictive value
NVP	Nevirapine
OXC	Oxycodone
OXM	Oxymorphone
p	Probability
PBS	Phosphate buffered saline
PBPK	Physiologically based pharmacokinetic modeling
PDX	Pyridoxine
PhB	Phenylbutazone
PHH	Primary human hepatocytes
PHNA	Phenacetin
pH	Potentia hydrogenii
PIN	Pindolol
PIO	Pioglitazone
PM	Poor metabolizer

PMZ	Promethazine
PPL	Propranolol
PPV	Positive predictive value
PRI	Primidone
Prima	Primaquine
PTN	Phenytoin
PUL	Pulegone
PV	Portal vein
PXT	Paroxetin
RFU	Relative fluorescence unit
RGZ	Rosiglitazone
RIF	Rifampicin
ROC	Receiver operating characteristic
ROS	Rosuvastatin
rpm	Rounds per minute
SIM	Simvastatin
SPB	Sodium phenylbutyrate
ss	Steady state
STS	Sitaxentan sodium
SVM	Support vector machine
t	Threshold
TC	Toxic concentration
t-BHQ	Tert-butylhydroquinone
TEI	Toxicity estimation index
Terbi	Terbinafine
THE	Theophylline
TN	True negative
TOL	Tolbutamide
Tolc	Tolcapone
TP	True positive
TROG	Troglitazone
TSI	Toxicity separation index
TSN	Triclosan
TTD	Tolterodine
U	Units
U/ml	Units per milliliter
VANC	Vancomycin
VERA	Verapamil
VitC	Vitamin C
VPA	Valproic acid
WB	Whole blood
x	Fold
ZAL	Zaleplon
µg/ml	Microgram per milliliter
µl	Microliter
µM	Micromol/l
µm	Micrometer

1 Introduction

1.1 The liver and drug-induced liver injury (DILI)

The liver is the largest internal organ of the human body with a weight between 1200 and 1800 g in adults. Functionally, it is a major site of detoxification and elimination of endogenous and exogenous substances. Furthermore, it is involved in glucose homeostasis, crucial for lipid and cholesterol metabolism and site of synthesis for clotting factors, albumin, transport proteins, acute phase proteins and growth factors (Drenckhahn and Fahimi 2008; Jaeschke 2013; Trefts et al. 2017; Qin and Crawford 2018).
Drug-induced liver injury (DILI) represents a major problem for the pharmaceutical industry and clinicians as it is the most common cause of acute liver failure in Germany and the US (Ostapowicz 2002; Hadem et al. 2012) in addition of being the most common reason for failure to get approval as drug or post-marketing withdrawal of approved drugs (Tran and Lee 2013; Onakpoya et al. 2016).

DILI arises due to a wide variety of different proposed mechanisms (Kaplowitz 2001; Jaeschke et al. 2002; Holt and Ju 2006; Abboud and Kaplowitz 2007; Gomez-Lechon et al. 2010; Jaeschke 2013) and manifests with a plethora of phenotypes including for example acute hepatitis, chronic hepatitis, acute cholestasis, chronic cholestasis, mixed hepatitis/cholestasis, atypical hepatitis, nonalcoholic steatohepatitis, fibrosis/cirrhosis, peliosis hepatis, nodular regenerative hyperplasia, neoplasms of the liver, microvesicular steatosis, granulomas, phospholipidosis, veno-occlusive disease and Budd-Chiari syndrome (Abboud and Kaplowitz 2007; Kaplowitz 2013; Andrade et al. 2019). Despite continuing efforts an *in vitro* prediction of DILI remains challenging as published test systems show limitations in sensitivity and accuracy and thus in predictive performance (Kaplowitz 2005; Xu et al. 2008; Gomez-Lechon et al. 2010; Khetani et al. 2013; Proctor et al. 2017; Vorrink et al. 2018).

1.2 Publicly available databases on hepatotoxicity

For the selection of training set and test set compounds for test system development curated databases are a useful tool. During preparation of this thesis three data bases were utilized: the National Institute of Diabetes and Digestive and Kidney Diseases' LiverTox database, the U.S. Food & Drug Administration (FDA)'s DILIrank dataset and the DILIst dataset.

The LiverTox data base (Clinical and Research Information on Drug-Induced Liver Injury: https://www.ncbi.nlm.nih.gov/books/NBK547852) provides drug dossiers with information about indication and dose of the drug, frequency, phenotype and proposed mechanisms of liver injury caused by the drug and case reports of liver injury due to the drug. For a range of compounds a likelihood score based on the number and credibility according to experts of case reports is provided.

Introduction

The drugs with likelihood scores are classified as described in **Table 1.1.** Drugs, which cause DILI only when used at high doses or at over doses are marked by an additional [HD].

Table 1.1: Likelihood score classification utilized by the LiverTox database
As given in (Categorization of the likelihood of drug induced liver injury).

https://www.ncbi.nlm.nih.gov/books/NBK548392/

Category	Description	Requirements
A	Well known cause of liver injury	Well known, well described and well reported cause of direct or idiosyncratic DILI with a characteristic signature - more than 50 cases including case series have been described
B	Known or highly likely cause of liver injury	Reported cause of idiosyncratic DILI with a characteristic signature- 12-50 described cases including small case series
C	Probable cause of liver injury	Probably linked to idiosyncratic liver injury but uncommonly reported with no characteristic signature - less than 12 identified cases without significant case series
D	Possible cause of liver injury	Single reports implicating the drug - less than 3 cases have been reported, case reports may be unconvincing, no characteristic signature
E	Not believed or unlikely cause of liver injury	No evidence of cause of DILI despite extensive use - single reports may have been published but are largely unconvincing
E*	Unproven but suspected cause of liver injury	Suspicion of capability of causing liver injury without convincing cases in medical literature - possible reports of acute liver injury to regulatory agencies or reported in large clinical studies without available specifics supporting the causality
X	Unknown	Recently introduced or rarely used drugs with inadequate information on the risk for developing liver injury

The FDA's DILIrank dataset (https://www.fda.gov/science-research/liver-toxicity-knowledge-base-ltkb/drug-induced-liver-injury-rank-dilirank-dataset) assigns one out of four DILI risk categories based on the FDA label and verification of causality found in literature. The classification scheme is detailed in **Table 1.2** (Chen et al. 2016).

Table 1.2: DILI concern categories in the DILIrank dataset according to Chen et al. 2016
For classification only one of the two conditions has to be fulfilled.

Category	Condition 1	Condition 2
Verified Most- DILI concern	Withdrawn due to DILI or DILI related black box labeling	Warnings and precautions with severe DILI indication with verified causality assessment
Verified Less-DILI concern	Mild DILI indication labeling in warnings and precautions or DILI labeling in adverse reactions with verified causality	No DILI indication in labeling but verified DILI causality
Verified No-DILI concern	No DILI indication in labeling without verified DILI causality	Not applicable
Ambiguous DILI concern	Warnings and precautions with severe DILI indication without verified causality assessment	Mild DILI indication labeling in warnings and precautions or DILI labeling in adverse reactions without verified causality

The DILIst dataset (Thakkar et al. 2020) is the DILIrank dataset augmented with data from the LiverTox database and three published dataset classifying compounds binary into DILI positive and DILI negative compounds based on concordance analysis.

1.3 Aim of this work

The aim of this work was to develop, evaluate and systematically optimize an *in vitro/in silico* approach for classification of drugs according to their risk for causing human DILI at therapeutic blood concentrations. Such a test system could be applied in drug development to detect possible DILI risks already prior to *in vivo* studies to reduce the number of animals used in testing as well as improve volunteer and patient safety. A further application could be risk assessment in deriving acceptable daily intakes for humans for food additives.

Assuming a relationship between effective *in vitro* concentrations and *in vivo* concentrations following therapeutic exposure scenarios, an *in vitro* cytotoxicity assay in primary human hepatocytes (PHH) as well as physiologically based pharmacokinetic (PBPK) modelling were performed for a set of 28 compounds.

Two novel performance metrics were introduced to allow the systematic comparison of different *in vitro* readouts (*e.g.* EC_{10} versus EC_{50}) and modelled *in vivo* concentrations (*e.g.* systemic concentrations versus portal vein, free versus total concentration) and thus systematic optimization of the test system (Albrecht et al. 2019). In addition, the performance of the optimized *in vitro/in silico* test system performance was evaluated utilizing a leave-one-out cross validation with a support vector machine (SVM) based classifier. As a first

applicability test of the *in vitro/in silico* approach for risk evaluation an acceptable daily intake (ADI) for pulegone was derived by combining the cytotoxicity test with reverse PBPK modelling.

The second part of the thesis focusses on the extension of the compound library with further 47 compounds. For five further compounds with ambiguous human hepatotoxicity status a classification with a SVM based classifier based on 77 *in vitro/in vivo* concentration vectors with defined human hepatotoxicity status was performed. Additionally, the inclusion of glutathione depletion as a further *in vitro* readout for the improvement of the test system performance was evaluated. Lastly, the utilization of the hepatocellular carcinoma cell line HepG2 in the *in vitro/in silico* test system is was evaluated including a comparison and the combination of both of the two cell systems.

2 Material and Methods

2.1 Material

2.1.1 Technical equipment

Table 2.1: Technical equipment in the laboratory

Equipment	Company
Autoclave Systec VX-150	Systec
Autoclave 5075 ELV	Tuttenauer
Balance	EW, Kern
Bright Field Microscope	Primovert, Zeiss, Software ZEN from Zeiss
Bunsen Burner	IBS Fireboy, Integra Bioscences
Casy®	Innovatis
Centrifuge	Megafuge 1.0R, Thermo Scientific
Centrifuge	Centrifuge 5415R, Eppendorf
Centrifuge with cooling function	5424R, Eppendorf
Centrifuge with cooling function	Biofuge Fresco, Heraeus
Fumehood	Electronics FAZ 2, Waldner
Fumehood	Köttermann
Incubators	CO_2 Incubator C150 R Hinge 230, Binder
Infinite M200 Pro Plate reader	Tecan
Laminar Flow Hood	CLEAN AIR SYSTEMS
Laminar Flow Hood	Heraeus HERASAFE
Laminar Flow Hood	Heraeus LaminAir HBB 2472
Magnetic stirrer	IKAMAG RCT, IKA
Microcentrifuge	Mini Spin Plus, Eppendorf
Multichannel pipette	Discovery, Abimed
Multichannel pipette	Research, Eppendorf
Multichannel pipette	Research Plus, Eppendorf
Multichannel pipette	Research Pro, Eppendorf
Multichannel pipette	XPlorer, Eppendorf
pH Meter	CG 842, Schott
Pipetteboy	Integra
Pipettes	ErgoOne, Starlab
Pipettes	Pipetman, Gibson
Pipettes	Research, Eppendorf
Pipettes	Research Plus, Eppendorf
Pipettes	Reference, Eppendorf
Precision balance	ALJ, 200-5DA Kern
Precision balance	EW150-3M, Kern
Precision balance	AE 240, Mettler
Precision balance	ME235P, Sartorius
Reagent reservoir	Dual solution, Heathrow Scientific

Equipment	Company
Reagent reservoir	StarTub PP Reagent Reservoir for Multichannel pipettes
Sonification bath	Labson 200 Bender& Hobein
Spectrometer	NanoDrop 2000, Thermo Scientific
Vacuum pump	Diaphragm Vacuum Pump, Vacuumbrand
Vortex	Vortex-genie 2, Bender& Hobein
Water purification system	Maxima Ultra-Pure Water, ELGA
Water purification system	Milli- Q® Integral 15 System, Merck
Waterbath	GFL 1083, Gesellschaft für Labortechnik
Waterbath	Precision GP28 Thermo Scientific

2.1.2 Chemicals and kits

Table 2.2: Commercial chemicals and kits

Compound	Company	Catalog number
Acetaminophen	Sigma-Aldrich Corp, St. Louis, MO, USA	A7085
Acetic acid	Carl-Roth, Karlsruhe, Germany	3738.5
Allopurinol	Cayman Chemicals, Ann Arbor, MI, USA	10012597
Amiodarone hydrochloride	Sigma-Aldrich Corp, St. Louis, MO, USA	A8423
Aspirin	Sigma-Aldrich Corp, St. Louis, MO, USA	A5376
Atorvastatin calcium salt	Cayman Chemicals, Ann Arbor, MI, USA	10493
Atropine sulfate monohydrate	Santa Cruz, Dallas TX, USA	sc-203322
Benztropine mesylate	Cayman Chemicals, Ann Arbor, MI, USA	16214
Bosentan hydrate	Sigma-Al Sigma-Aldrich Corp, St. Louis, MO, USA	SML 1265
Buspirone hydrochloride	Sigma-Al Sigma-Aldrich Corp, St. Louis, MO, USA	B7148
Busulfan	Sigma-Al Sigma-Aldrich Corp, St. Louis, MO, USA	B2635
Carbamazepine	Sigma-Aldrich Corp, St. Louis, MO, USA	C4024
Cell Titer Blue Assay	Promega	G8081
Chlorpheniramine maleate	Sigma-Aldrich Corp, St. Louis, MO, USA	C3025

Compound	Company	Catalog number
Chlorpromazine hydrochloride	Sigma-Aldrich Corp, St. Louis, MO, USA	C8138
Ciprofloxacin hydrochloride	Cayman Chemicals, Ann Arbor, MI, USA	14286
Clofibrate	Sigma-Aldrich Corp, St. Louis, MO, USA	C6643
Clonidine hydrochloride	Sigma-Aldrich Corp, St. Louis, MO, USA	C7897
Clozapine	Cayman Chemicals, Ann Arbor, MI, USA	12059
Codeine anhydrate	LGC Standards	MM0004.10
Cyclosporin A	Sigma-Aldrich Corp, St. Louis, MO, USA	30024
Dibrombimane	Sigma-Aldrich Corp, St. Louis, MO, USA	34025
Diclofenac sodium	Sigma-Aldrich Corp, St. Louis, MO, USA	D6899
Digoxin	Cayman Chemicals, Ann Arbor, MI, USA	22266
Dimethyl sulfoxide	Sigma-Aldrich Corp, St. Louis, MO, USA	34869-M
Diphenhydramine hydrochloride	Santa Cruz, Dallas TX, USA	sc-204729
Dimethyl sulfoxide	PanReac Applichem	A36720050
Dipyridamole	Sigma-Aldrich Corp, St. Louis, MO, USA	D9766
Disodium hydrogen phosphate	Carl-Roth, Karlsruhe, Germany	T876.2
DL-buthionine-sulfoximine	Sigma-Aldrich Corp, St. Louis, MO, USA	19176
Erythromycin ethylsuccinate	Santa Cruz, Dallas TX, USA	sc-204743
Ethanol	VWR Chemicals, Germany	20821.2
Famotidine	Sigma-Aldrich Corp, St. Louis, MO, USA	F6889
Fexofenadine hydrochloride	Cayman Chemicals, Ann Arbor, MI, USA	18191
Fluoxetine hydrochloride	Cayman Chemicals, Ann Arbor, MI, USA	14418
Glucose monohydrate	Sigma-Aldrich Corp, St. Louis, MO, USA	49159
HOECHST 33342	ThermoFisher Scientific	H1399
Hydroxyzine hydrochloride	Sigma-Aldrich Corp, St. Louis, MO, USA	H8885

Compound	Company	Catalog number
Ibuprofen	Sigma-Aldrich Corp, St. Louis, MO, USA	I7905
Imipramine hydrochloride	Cayman Chemicals, Ann Arbor, MI, USA	15890
Indomethacine	Santa Cruz, Dallas TX, USA	Sc200503
Isoniazid	Sigma-Aldrich Corp, St. Louis, MO, USA	I3377
Isosorbide dinitrate	Cayman Chemicals, Ann Arbor, MI, USA	23990
Ketoconazole	Sigma-Aldrich Corp, St. Louis, MO, USA	K1003
Labetalol hydrochloride	Sigma-Aldrich Corp, St. Louis, MO, USA	L1011
Leflunomide	Cayman Chemicals, Ann Arbor, MI, USA	14860
Levofloxacin	Sigma-Aldrich Corp, St. Louis, MO, USA	40922
Melatonin	Sigma-Aldrich Corp, St. Louis, MO, USA	M5250
Methotrexate	Sigma-Aldrich Corp, St. Louis, MO, USA	PHR1396
Methylparaben	Sigma-Aldrich Corp, St. Louis, MO, USA	PHR1012
N-acetylcysteine	Sigma-Aldrich Corp, St. Louis, MO, USA	A9165
Nevirapine	Cayman Chemicals, Ann Arbor, MI, USA	15117
Nifedipine	Cayman Chemicals, Ann Arbor, MI, USA	11106
Nimesulide	Sigma-Aldrich Corp, St. Louis, MO, USA	N1016
Nitrofurantoin	Sigma-Aldrich Corp, St. Louis, MO, USA	N7878
Oxycodone hydrochloride	Sigma-Aldrich Corp, St. Louis, MO, USA	01378
Oxymorphone hydrochloride monohydrate	LGC Standards	MM0673.00
Phenacetin	Sigma-Aldrich Corp, St. Louis, MO, USA	77440
Phenylbutazone	Sigma-Aldrich Corp, St. Louis, MO, USA	P8386
Phenytoin	Sigma-Aldrich Corp, St. Louis, MO, USA	PHR1139
Pindolol	Sigma-Aldrich Corp, St. Louis, MO, USA	P0778

Compound	Company	Catalog number
Pioglitazone hydrochloride	Sigma-Aldrich Corp, St. Louis, MO, USA	PHR1632
Potassium chloride	Fluka Chemie AG, Switzerland	60129
Potassium dihydrogen phosphate	Merck, Darmstadt, Germany	1.04873.1000
Primaquine phosphate	Santa Cruz, Dallas TX, USA	sc-205817
Primidone	Santa Cruz, Dallas TX, USA	Sc-204861
Promethazine hydrochloride	Sigma-Aldrich Corp, St. Louis, MO, USA	P4651
Propranolol hydrochloride	Sigma-Aldrich Corp, St. Louis, MO, USA	P08884
Pulegone	Sigma-Aldrich Corp, St. Louis, MO, USA	376388
Pyridoxine hydrochloride	Sigma-Aldrich Corp, St. Louis, MO, USA	P9755
Rifampicin	Sigma-Aldrich Corp, St. Louis, MO, USA	R3501
Rosiglitazone maleate	Toronto Research Chemical, North York, ON, Canada	R693500
Rosuvastatin calcium salt	Cayman Chemicals, Ann Arbor, MI, USA	18813
Simvastatin	Sigma-Aldrich Corp, St. Louis, MO, USA	S6196
Sitaxentan sodium	BIOZOL TGM	T6672
Sodium chloride	Carl-Roth, Karlsruhe, Germany	3957.2
Sodium hydroxzide	Merck, Darmstadt, Germany	1.06482
Sodium phenylbutyrate	Cayman Chemicals, Ann Arbor, MI, USA	11323
Terbinafine hydrochloride	Santa Cruz, Dallas TX, USA	sc-200751
tert-Butylhydroquinone	Sigma-Aldrich Corp, St. Louis, MO, USA	112941
Theophylline	Cayman Chemicals, Ann Arbor, MI, USA	23760
Tolbutamide	Sigma-Aldrich Corp, St. Louis, MO, USA	T0891
Tolcapone	Sigma-Aldrich Corp, St. Louis, MO, USA	1670207
Tolterodine tartrate	TargetMol, Boston, MA, USA	TGM-T0099

Compound	Company	Catalog number
Triclosan	Sigma-Aldrich Corp, St. Louis, MO, USA	PHR1338
Troglitazone	Cayman Chemicals, Ann Arbor, MI, USA	71750
Valproic acid	Sigma-Aldrich Corp, St. Louis, MO, USA	PHR1061
Vancomycin hydrochloride	Sigma-Aldrich Corp, St. Louis, MO, USA	PHR1732
Verapamil hydrochloride	Sigma-Aldrich Corp, St. Louis, MO, USA	V4629
Vitamin C	Sigma-Aldrich Corp, St. Louis, MO, USA	A0278
Zaleplon	Mikromol Luckenwalde, Germany	MM1322.00

Table 2.3: Chemicals provided by industrial cooperation partners

Compound	Company
Benzbromarone	Astra Zeneca
Fluconazole	Astra Zeneca
Paroxetine hydrochloride	Astra Zeneca

2.1.3 Consumables

Table 2.4: Consumables

Consumable	Company	Catalog number
Cell culture microtiter plate black, 96 well	Greiner Bio-One, Kremsmünster, Austria	655986
Cell culture microtiter plate black, 96 well	Sigma-Aldrich Corp, St. Louis, MO, USA	M0562
Centrifugation tube, 15 ml	Sarstedt, Numbrecht, Germany	62.554.512
Centrifugation tube, 50 ml	Sarstedt, Numbrecht, Germany	62.547.254
Filtropur S 0.2 syringe filter	Sarstedt, Numbrecht, Germany	83.1826.001
Omnifix syringe, 10ml	B. Braun, Melsungen, Germany	1616103V
Parafilm Wrap	Cole-Parmer, Kehl/Rhein, Germany	PM-992
Pasteur pipette, glass, 150 mm	Carl Roth, Karlsruhe, Germany	4518.1
Pasteur pipette, glass, 230 mm	Carl Roth, Karlsruhe, Germany	4522.1

Consumable	Company	Catalog number
Pipette Tips, 5000 µl	Eppendorf, Hamburg, Germany	022492080
Pipette Tips 1250 µl, long	Sarstedt, Numbrecht, Germany	70.1186
Pipette Tips, 1000 µl	Sarstedt, Numbrecht, Germany	70.762
Pipette Tips, 200 µl	Sarstedt, Numbrecht, Germany	70.760.002
Pipette Tips, 20 µl	Sarstedt, Numbrecht, Germany	70.1116
SafeSeal 5 ml microtube	Sarstedt, Numbrecht, Germany	72.701
SafeSeal 2 ml microtube	Sarstedt, Numbrecht, Germany	72.695.500
SafeSeal 1.5 ml microtube	Sarstedt, Numbrecht, Germany	72.706
SafeSeal 0.5 ml microtube	Sarstedt, Numbrecht, Germany	72.699
Serological Pipette, 50 ml	Sarstedt, Numbrecht, Germany	86.1256.001
Serological Pipette, 25 ml	Sarstedt, Numbrecht, Germany	86.1685.001
Serological Pipette, 10 ml	Sarstedt, Numbrecht, Germany	86.1254.001
Serological Pipette, 5 ml	Sarstedt, Numbrecht, Germany	86.1253.001
Tissue Culture Plate Flat-Bottom 24 Well Plate	Sarstedt, Numbrecht, Germany	83.1836
Tissue Culture Plate Flat-Bottom 96 Well Plate	Sarstedt, Numbrecht, Germany	83.3924
Tissue Culture Flask T25	Sarstedt, Numbrecht, Germany	83.3910.002
Tissue Culture Flask T75	Sarstedt, Numbrecht, Germany	83.3911.002
Tissue Culture Flask T175	Sarstedt, Numbrecht, Germany	83.3912.002
Vacuum Filtration Unit, 0.22 µm, 250 ml	Sarstedt, Numbrecht, Germany	83.1822.001
Weighing tray	Sarstedt, Numbrecht, Germany	719923211
Weighing tray	Sarstedt, Numbrecht, Germany	719923212

2.1.4 Cell culture material, media and buffers

Table 2.5: Cell culture supplies

Supply	Company	Catalog number
CASY cups	OMNI Life Science, Bremen, Germany	OLS5651794
Casyton solution	Roche Diagnostics GmbH, Mannheim, Germany	5651808001
Collagen lyophyilized (rat tail), 10 mg	Roche Diagnostics GmbH, Mannheim, Germany	11171179001
CryoPure cryovials 1ml	Sarstedt, Numbrecht, Germany	72377992
Dexamethason	Sigma-Aldrich Corp, St. Louis, MO, USA	D4902
Dulbecco's modified eagles medium (DMEM)	PAN Biotech GmbH, Aidenbach, Germany	P04-04500
Freezing container (Mr. Frosty)	Thermo Fisher Scientific, Waltham, MA, USA	
Gentamicin	PAN Biotech GmbH, Aidenbach, Germany	P06-13001
Hemocytometer cover glasses	Marienfeld Superior, Germany	
Hemocytometer Neubauer improved (depth 0.1 mm, 0.0025 mm^2)	Marienfeld Superior, Germany	
Insulin transferrin selenite supplement (ITS)	Sigma-Aldrich Corp, St. Louis, MO, USA	13146
Penicillin/Streptomycin	PAN Biotech GmbH, Aidenbach, Germany	P06-07100
Sera Plus (Special Processed FBS)	PAN Biotech GmbH, Aidenbach, Germany	3702-P103009
Stable – L Glutamine	PAN Biotech GmbH, Aidenbach, Germany	P04-82100
Trypan blue solution	Sigma-Aldrich Corp, St. Louis, MO, USA	T8154
Trypsin/EDTA	Sigma-Aldrich Corp, St. Louis, MO, USA	P10-023100
William's E medium	PAN Biotech GmbH, Aidenbach, Germany	P04-29510

2.1.4.1 Phosphate buffered saline (PBS) buffer for cell culture

Table 2.6: Recipe for 5 l 10x PBS for cell culture

Compound	Amount [g]
KCl	10
KH_2PO_4	10
NaCl	400
Na_2HPO_4	46

After the reagents in **Table 2.6** were dissolved completely in double distilled water the pH was adjusted to 7.4 and autoclaved. Prior to usage in cell culture the 10x PBs was diluted in sterile filtered double distilled water and autoclaved.

2.1.4.2 Cell culture media

Table 2.7: PHH plating medium

Component	Volume [ml]
William's E medium	500
Sera Plus	50
Penicillin/Streptomycin	5
Gentamicin	0.5
Dexamethasone	0.02
ITS supplement	0.005

Table 2.8: PHH culture medium

Component	Volume [ml]
William's E medium	500
Penicillin/Streptomycin	5
Gentamicin	0.5
Dexamethasone	0.02
ITS supplement	0.005

Table 2.9: HepG2 culture medium

Component	Volume [ml]
DMEM	500
Penicillin/Streptomycin	5
Heat inactivated Sera Plus	50

2.1.5 Cell lines and cryopreserved primary human hepatocytes

2.1.5.1 Cryopreserved primary human hepatocytes

Cryopreserved re-plateable human hepatocytes were purchased from BioIVT (product numbers M00995-P, F00995-T-CERT and F00995-P) and Lonza (catalog number HUCPI). Prior to purchase the plateability was checked in our laboratory. Detailed information about the donors is given in **Supplement 2.**

2.1.5.2 HepG2 cell line

The HepG2 cell line is an adherent human hepatocellular carcinoma cell line derived from cells of a 15 year old male Caucasian donor. The HepG2 cells were purchased from ATCC LGC Standards (product number HB-8065).

2.2 Methods

2.2.1 Heat inactivation of FBS

For heat inactivation the thawed FBS was placed in a water bath at 56°C for 30 minutes and rotated every 10 minutes. The heat inactivated FBS was aliquoted and immediately stored at - 20°C.

2.2.2 Collagen coating of 96-well cell culture plates

For collagen coating of cell culture plates a 0.25 mg/l rat tail collagen solution was prepared by dissolving the rat tail collagen for at least 4 hours at 4°C in sterile 0.2% acetic acid. For a 96-well plate 100 µl collagen were pipetted in each well and removed after 1-2 minutes. The plates were left drying for at least 2 hours at room temperature. Prior to cell plating the collagen coated plates were washed three times with 1x PBS.

2.2.3 Cell culture of primary human hepatocytes (PHH)

2.2.3.1 Thawing of primary human hepatocytes (PHH)

On the day of plating the vials were taken out of the liquid nitrogen and were quickly defrosted in a 37°C water bath. The cells were immediately transferred into 5 ml/vial of PHH plating medium (**Table 2.7**) and re-suspended by gently inverting the tube. To ensure complete transfer of the cells, the empty vial was rinsed with PHH plating medium. In preparation of the yield and viability determination by the Trypan blue exclusion method, 50 µl of the cell suspension was gently mixed with 350 µl plating medium and 100 µl 0.4% sterile Trypan blue.

The cell viability and cell yield were determined by the following equations:

I) $$Average\ vital\ cells\ = \frac{counted\ vital\ cells}{number\ of\ counted\ counting\ chamber\ quadrants}$$

II) $$Average\ total\ cells\ = \frac{counted\ vital\ \ cells + counted\ dead\ cells}{number\ of\ counted\ counting\ chamber\ quadrants}$$

III) $$Viability = \frac{Average\ vital\ cells}{Average\ total\ cells}\ x\ 100\%$$

To adjust for further cell loss during plating and attachment, the cell number was corrected for vitality.

IV) $$\frac{Viable\ cells}{ml} = Average\ vital\ cells\ x\ viability\ x\ 10^4\ x\ 10$$

Cell suspensions with a viability of less than 60% were not used. For pipetting of the cells only cut pipette tips were used.

2.2.3.2 Cultivation of cryopreserved primary human hepatocytes on collagen monolayer coated 96-well plates

After determination of the yield and viability the cryopreserved PHH were plated on collagen monolayer coated 96-well plates (**2.2.2**). 50,000 viable cells in 200 µl PHH plating medium (**Table 2.7**) were plated per well and left for attachment for 3-4 hours at 37°C. Afterwards the cells were washed 3 times with 1x PBS to remove non-attached cells and cell debris and wash out collagen bound FBS and 200 µl PHH cultivation medium per well was added (**Table 2.8**)**.** The medium change was carried out by turning the plates upside down on a paper towel. This reduces the risk of the cells drying out as well as the risk of accidently aspirating the cells. The cells were cultivated at 37°C and 5% CO_2 for 16 – 20 hours prior to treatment.

2.2.3.3 Cytotoxicity testing in cryopreserved PHH with the CellTiter-Blue® viability assay (CTB)

As a read out for the cell viability the CellTiter-Blue® Cell Viability Assay (CTB) from Promega was utilized. The supplied reagent contains the dark blue dye resazurin, which can be reduced to the highly fluorescent dye resorufin (Promega). Due to the metabolization of resazurin in viable cells a color change of the medium from dark blue to pink can be observed, while in dead cells no color change occurs. The fluorescence intensity serves as a measure for metabolic capacity and cell viability.

For cytotoxicity testing cryopreserved primary human hepatocytes from at least three different donors were used. 50,000 viable cells were seeded (**2.2.3.2** and **2.2.3.3**) into each well of a collagen monolayer coated 96-well plate (**2.2.2**)**.** Preferably, the cells are plated into

black 96-well plates and the outer wells are not seeded with cells and instead filled with PBS. A general overview of the time frame is given in **Figure 2.1.**

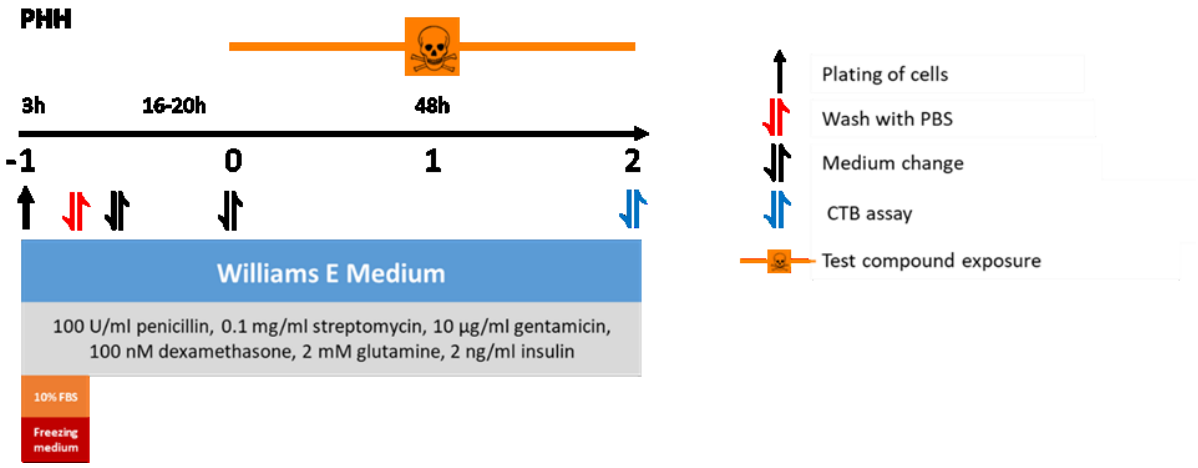

Figure 2.1: Exposure scheme for cytotoxicity testing in PHH

A general overview over the time schedule from begin of cultivation until end of compound exposure. Freezing medium refers to the medium the cryopreserved cells are provided in (Reference: Supplement 3A Albrecht et al. 2019).

On the next morning treatment solutions of the test compounds were prepared. Medium soluble compounds were directly dissolved in PHH cultivation medium (**Table 2.8**) and sterile filtered with a 10 ml syringe with a syringe filter. The further concentrations were prepared by serial dilution. For non-soluble compounds stock solutions in DMSO or ethanol were prepared. The final solvent concentration should not exceed 0.5%. At least five concentrations with a dilution factor of 3.16 and solvent controls were prepared for each compound. For each concentration 200 µl/well of treatment solution was pipetted into three wells per donor. The cells were incubated with the compounds for 48 hours at 37°C and 5% CO_2. Shortly before the end of the incubation period the morphology of the cells was checked under the microscope.

After incubation with the compounds the cells were washed 3 times with sterile warm 1x PBS to remove any remaining compound. This is necessary to avoid interference of the test compounds with the indicator dyes resazurin and resorufin. The CTB reagent was diluted 1:5 in PHH cultivation medium and 100 µl/well was added. The PHH were incubated with the CTB mixture for 2-4 hours until a color change from blue to pink was observed in the control indicating metabolization of resazurin and resorufin (Supplement 3A Albrecht et al. 2019).

When the PHH were plated on conventional cell culture plates, the supernatant was quickly transferred into new black 96-well plates. Otherwise, the black plate with the cells was directly transferred into a Tecan Infinite M200 Pro plate reader (i-control software version 1.7.1.12) and read out with the excitation set at 540 nm and emission set at 594 nm.

On each plate 100 µl of the CTB mixture was pipetted into some empty wells to serve as background control. During each medium change or washing step the supernatant was removed by tipping the plate on a paper towel. The average fluorescence of the CTB background control was subtracted from the fluorescence of the other wells. Since the vehicle

controls and compound-treated cells underwent the same procedure, the viability of the untreated cells was set to 100%.

All tested concentrations and donors are given in **Supplement 1** and **Supplement 2,** while all obtained raw data are tabulated in **Supplement 4.**

2.2.3.4 Glutathione depletion assay in primary human hepatocytes

For quantification of glutathione levels dibrombimane was used. Dibrombimane (DBB) reacts with the thiol group of glutathione becoming fluorescent in the process (Kosower and Kosower 1987, Cox and Cardozo-Pelaez 2007). 50,000 cells were plated in 200 µl medium per well of a collagen monolayer coated black 96-well plate and incubated for 16-20 hours at 37°C and 5% CO_2. The general time frame was identical to **Figure 2.1**. On the next day treatment solutions of the compounds were prepared freshly in PHH culture medium. For medium soluble compounds, these were dissolved in medium, sterile filtered and diluted via serial dilution. For non-medium soluble compounds, stock solutions in DMSO were prepared and serially diluted. The final DMSO concentration should not exceed 0.5%. For each compound 5 concentrations with a dilution factor of 3.16 and a solvent control were prepared. Per concentration three cell culture replicates were used. In addition as positive and negative controls t-BHQ and BSO were used, respectively. The cells were exposed to the compounds for 48 hours at 37°C and 5% CO_2.

The staining procedure consisted of two steps. A 0.5 mg/ml HOECHST 33342 stock solution in PBS was diluted in HepG2 medium to a final concentration of 0.5 µg/ml. The nuclei of the cells were stained with 100 µl of the HOECHST medium for 30 minutes at 37°C and 5% CO_2. The plates were quickly transferred into the plate reader and the fluorescence was read out at the excitation wavelength of 340 nm and the emission wavelength of 450 nm.

In preparation of the second staining step a 40 mM DBB stock solution in DMSO was diluted to a final concentration of 40 µM in PBS and the cells were washed once with 1x PBS. The cells were incubated with 100 µl of the 40 µM DBB solution/well for 30 minutes at 37°C and 5% CO_2. The plates were quickly transferred into the plate reader and the fluorescence was measured at the excitation wavelength of 393 nm and emission wavelength of 477 nm.
For data analysis, first the background fluorescence from an empty well was subtracted from the DBB measurements of all treated wells. This value was normalized by division of the HOECHST fluorescence in the same well. The normalized value of the vehicle controls were set to 100% GSH content.

All relevant information about tested concentration ranges, solvents and donors are summarized in **Supplement 1** and **Supplement 2.** The obtained raw data is provided in **Supplement 4**.

2.2.4 Cell culture of HepG2 cells

2.2.4.1 Thawing and freezing HepG2 cells

The cells were thawed quickly in a water bath at 37°C, and were immediately transferred into a 50 ml centrifugation tube containing 9 ml of cold HepG2 medium (**Table 2.9**), centrifuged for 5 minutes at 600 rpm at room temperature and the supernatant was discarded. In order to remove any remaining DMSO from the freezing medium, 10 ml of cold 1x PBS were added, the cells were centrifuged again at 600 rpm for 5 minutes at room temperature and the supernatant was discarded. Afterwards the pellet was re-suspended in 10 ml of warm HepG2 medium, transferred into conventional T75 cell culture flasks and cultured at 37°C and 5% CO_2. For cell stocks with low yield the pellet was re-suspended in 5 ml of HepG2 medium and transferred into a T25 flask.

For preparation of cell stocks usually cells with 80-90 % confluency were used. In preparation for trypsinization the medium was aspirated and the cells were washed one time with 1x PBS. For a T75 flask 2 ml of trypsin were added and the cells were trypsinized for 7-8 minutes at 37°C before 8 ml of HepG2 medium were added to prevent digestion of the cells. The cell suspension was transferred into a centrifugation tube and centrifuged at 800 rpm for 5 minutes at 4°C. The supernatant was removed and the cells were re-suspended in either 3 ml freezing medium (90 % HepG2 medium + 10 % DMSO) or preferably in 3 ml 90 % heat inactivated FCS + 90 % DMSO. The cell suspension was split into three cryovials and slowly frozen using a freezing container filled with isopropanol at -80°C. For long time storage the cell stocks were kept in liquid nitrogen.

2.2.4.2 Passaging and maintenance of HepG2 cells

For maintenance of the cell culture the cells were sub-cultured when 80-90 % confluence was reached. The medium was aspirated and the cells were washed once with 10 ml 1x PBS to remove remaining FBS. For a T75 flask the cells were trypsinized with 2 ml of trypsin for 7-8 minutes at 37°C and 5% CO_2. The trypsinization was stopped by adding 8 ml of HepG2 culture medium (**Table 2.9**), the cell suspension was transferred into a centrifugation tube and then centrifuged for 5 minutes at 600 rpm at room temperature. Afterwards the supernatant was discarded and the cell pellet was re-suspended in 1ml of medium by pipetting repeatedly up and down with a 1 ml pipette tip. The cell suspension was diluted with warm medium and re-suspended again by pipetting repeatedly up and down with a serological pipette prior to distribution into new T75 flasks with 10ml medium. The cells were cultivated further at 37°C and 5% CO_2 with medium changes every 2-3 days.

2.2.4.3 Plating of HepG2 cells

On the afternoon before treatment day the HepG2 cells were plated. For the experiments usually cells were used which reached 70-90% confluency and were sub-cultured maximally 23 times after purchase. The medium was aspirated, the cells were washed once with warm,

sterile 1x PBS and 2ml of trypsin was added per T75 flask. The cells were trypsinized for 7-8 minutes at 37°C and 5%CO_2. 8ml of medium (**Table 2.9**) was added and the cell suspension was transferred into a 50ml centrifugation tube. Following centrifugation for 5 minutes at 600 rpm and room temperature the supernatant was discarded and the pellet was re-suspended in 1ml of medium by repeatedly pipetting up and down with a 1000µl pipette tip. The cell suspension was filled up to 12-15ml total volume with medium and well mixed by pipetting up and down with a serological pipette. For counting of the cells and vitality assessment 100µl of the cell suspension were transferred into a CASY cup containing 10ml of Casyton and counted using the Casy® (setting for dead cells: NL= 7.13; CL = 9.5). Cells with a viability of less than 75% were not used for experiments. The remaining cell suspension was well re-suspended and cell suspensions with the appropriate cell number and volumes were prepared by diluting the original cell suspension with medium. Until treatment started the cells were cultivated at 37°C and 5% CO_2. Superfluous cell suspension was used for continuation of the cell culture.

2.2.4.4 Cytotoxicity testing in HepG2 cells with the CellTiter-Blue® viability assay (CTB)

A general overview of time frame for the cytotoxicity assay in HepG2 cells is given in figure **2.2**, while the principle of the assay is described in section **2.2.3.3**.

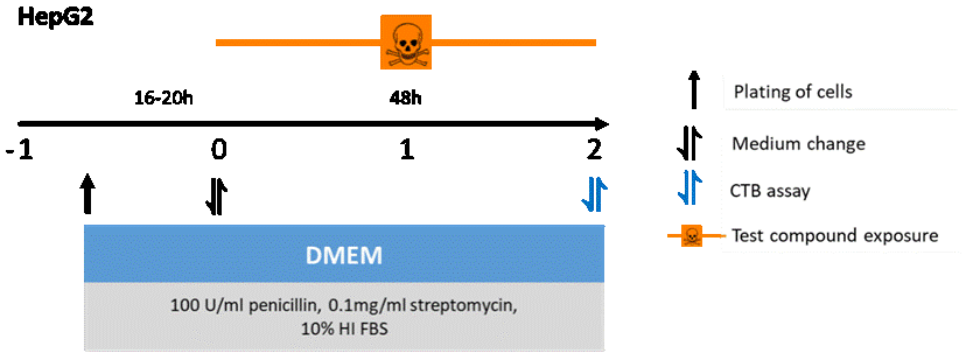

Figure 2.2: Exposure scheme for the cytotoxicity assay in HepG2 cells
A general overview of the time frame from plating to incubation with the CTB reagent (Modified from Supplement 3A Albrecht et al. 2019).

The HepG2 were plated either on conventional 24-well plates with 62,500 viable cells/well or on black 96-well plates with 15,625 viable cells/well. During preparation of this thesis it proofed beneficial to plate the HepG2 cells on collagen monolayer coated black 96-well plates. After over-night culture at 37°C and 5% CO_2 the cells were treated with the compounds. The treatment solutions were prepared freshly on treatment day. Medium soluble compounds were directly dissolved in HepG2 medium (**Table 2.9**) and sterile filtered. Further concentrations were obtained by serial dilution. For non-medium soluble compounds stock solutions in DMSO or ethanol were prepared. The final solvent concentration should not exceed 0.5%. For each compound at least five concentrations with a dilution factor of 3.16 and solvent controls were prepared. For a 24-well plate 0.5 ml/well and for a 96-well plate 0.2

ml/well of treatment were used. Three cell culture replicates were prepared for each concentration.

Shortly before the 48 hour compound exposure at 37°C and 5% CO_2 was completed the cells were checked for their morphology under the bright field microscope as an internal quality control measure. After the compound exposure was completed the cells were washed three times with sterile 1x PBS. For 24-well plates 600 µl of the CTB mixture (100 µl CTB reagent + 500µl HepG2 medium) were used for each well, while for 96-well plates 200 µl of the CTB mixture (40 µl CTB reagent + 160µl HepG2 medium) were used/well. The cells were incubated with the CTB mixture at 37°C and 5% CO_2 for 1-2 hours until a color change was observed. When 24 well plates were used 100 µl of the supernatant from each well were transferred into three wells of a black 96-well plate. For each plate some empty wells with CTB mixture were used as a background control. The fluorescence was read out in the plate reader at wavelength 540 nm and emission wavelength 594 nm.

The average fluorescence of the CTB background control was subtracted from the fluorescence of the other wells. Since the vehicle controls and compound treated cells underwent the same procedure the viability of the untreated cells was set to 100%.

All relevant information concerning tested concentrations, solvents and obtained raw data is summarized in **Supplement 1** and **Supplement 4.**

2.2.4.5 Glutathione depletion assay in HepG2 cells

15,625 viable cells were plated in 200 µl HepG2 medium (**Table 2.9**) per well of a black 96-well plate and incubated for 16-20 hours at 37°C and 5% CO_2. The general time frame was identical to **Figure 2.2**. On the next day treatment solution of the compounds were prepared freshly in HepG2 medium. The further steps were identical to **Section 2.2.3.4.**

Detailed information about the tested concentration ranges, solvents and all obtained raw data is given in **Supplement 1** and **Supplement 4.**

2.2.5 Physiologically based pharmacokinetic modeling (PBPK) with the Simcyp simulator

In order to obtain information about human blood concentrations following exposure to therapeutic doses of the test drugs physiologically based pharmacokinetic modeling was utilized. The simulations were done in close cooperation with Dr. Iain Gardner and Dr. Mian Zhang from the Simcyp Division of Certara UL Ltd (Sheffield, UK). The following method description and the obtained data correspond in large parts to the publication Albrecht et al. 2019. For model construction and PBPK simulation the Simcyp Simulator (Version 15, Version 18 and Version 19; Simcyp, Sheffield UK) was used.

PBPK modeling requires information about the compound, the studied population and the treatment scenario. The compound information comprises physicochemical properties and ADME data of the compound (Absorption, Distribution, Metabolism, and Elimination). The ADME parameters can be experimental values from *in vitro* or *in vivo* studies or predicted from physicochemical properties of the compound (Jamei et al. 2009).

Information about demographic, genetic, anatomical and physiological factors of several populations were provided with the software. The simulator uses a correlated Monte Carlo approach to generate a population of virtual subjects (Jamei et al. 2009). The treatment scenarios were chosen according to information obtained from literature.

The constructed models were performance checked against clinical data and refined when necessary. The simulations were done with a virtual population of 100 healthy North European Caucasian subjects, half female, half male, aged 20-50 (Albrecht et al. 2019).

Input parameters for all models are given in **Supplement 3**. Certara UK (Simcyp Division) granted free access to the Simcyp Simulators through an academic licence (subject to conditions).

2.2.6 Statistical analysis

The statistical analysis was done in close cooperation with Franziska Kappenberg, Dr. Marianne Grinberg and Prof. Dr. Jörg Rahnenführer from the Department of Statistics at the TU Dortmund University. The following sections correspond closely to Albrecht et al. 2019. For all statistical analysis the statistical programming language R-version 3.5.1 was utilized.

2.2.6.1 Curve fitting and determination of EC values

The cytotoxicity and glutathione depletion assay data were analyzed using the R package *drc* version 3.0-1 (Ritz et al. 2015). A four-parameter log-logistic model (4pLL) was fitted using the least square method with the Gauss-Newton algorithm. The EC values (effective concentration) were defined as the concentration at which the viability or glutathione content was reduced by k % compared to the control (EC_k = (100-k) %).

The concentration-response curve is described by the equation:

$$f(x|b,c,d,e) = c + \frac{d-c}{1+\exp(b(\log(x)-\log(e)))}$$

Where *f* is the response, *x* is the concentration, *b* is the slope of the function, *c* is the lower horizontal asymptote corresponding to cells treated with a high concentration of the test compound, *d* is the upper horizontal asymptote corresponding to the control and *e* is the concentration at which 50 % of the overall effect is observed. For the specific case that *c* = 0 % and *d* = 100 % then *e* corresponds to our definition of an EC_{50} (Ritz 2010; Albrecht et al. 2019) .

In case that the fitting procedure lead to an $d \neq 100$ % a refitting was performed by dividing all values by d, multiplying with 100 % and fitting a curve to the new data (Albrecht et al. 2019). The EC values were obtained by the inverse function of the curve fit. The confidence intervals of the EC values were calculated by the delta method (Grinberg 2017).

As a quality control of the curve fit the goodness-of-fit (GOF) was determined by the equation:

$$GOF = 1 - \frac{Sum\ of\ squared\ differences\ between\ the\ data\ points\ and\ fitted\ curve}{Sum\ of\ squared\ differences\ between\ the\ data\ points\ and\ the\ mean\ response}$$

An optimal fit would yield a value of close to 1, while a poor fit yield values close to zero. For further analysis of the training set compounds, only curves with a GOF of at least 0.55 were used to calculate EC values (Albrecht et al. 2019).

For the compounds of the test set and for the glutathione depletion assay in PHH for curves with a GOF below 0.55 and a viability of at least 70% at the highest tested concentration, a curve fit using the Brian-Cousens function as described in Kappenberg et al. 2020 was performed. If the GOF was still under 0.55 the EC_{10} was set to > highest tested concentration. For curves with a viability less than 70 % at the highest tested concentration, the GOF was not utilized for the test set.

To avoid estimated EC values which lie too far outside from the limits of the tested concentration range only EC values were accepted which were in the range of 0.2 x the lowest tested concentration to 5 x the highest tested concentration. EC values below 0.2 x the lowest concentration were set to < the lowest tested concentration. EC values exceeding 5 x highest tested concentration were set to > the highest tested concentration range. If the respective EC value could not be determined because the curve never reached the respective value the EC value was set to > the highest tested concentration.

For further analysis, which depends on the availability of minimum, median and maximum EC values, EC values < lowest tested concentration were set to 0.2 x the lowest tested concentration and EC values > 5x highest tested concentration were set to 5 x the highest tested concentration (Albrecht et al. 2019). All obtained curves are given in **Supplement 5,** while all obtained EC values are summarized in **Supplement 6.**

2.2.6.2 Calculation of toxicity separation and toxicity estimation indices

For systematic test system evaluation and optimization two performance metrics were utilized as described in Albrecht et al. 2019. For calculation of these indices, *in vivo* concentrations for a given exposure scenario, effective *in vitro* concentrations, and information about the *in vivo* toxicity status are needed. It is important that both concentrations have the same unit.

The Toxicity Separation Index (TSI) is a quantitative measure of how well an *in vitro* test system differentiates between toxic and non-toxic compounds. A TSI of 1 represents optimal separation where as a random class assignment yields a TSI of 0.5.

In the first step of the algorithm the difference between the *in vivo* concentration and the effective *in vitro* concentration for each compound was calculated with the equation:

$$\Delta = \log_{10}(\frac{C\ in\ vivo}{c\ in\ vitro})$$

The compounds were sorted according to Δ in ascending order. A cutoff value (t) was chosen for each interval between two consecutive differences. Additionally, a cutoff value below the minimal distance and a cutoff value above the maximal distance were chosen.

Next, the compounds were classified for each cutoff value as either toxic (Δ > t) or non-toxic (Δ ≤ t). The assigned toxicity status was compared to the true toxicity status and for each t the sensitivity ($\frac{True\ positive\ (TP)}{True\ positive\ (TP)+false\ negative\ (FN)}$) and

specificity ($\frac{True\ negative(TN)}{True\ negative\ (TN)+false\ positive\ (FP)}$) were determined.

Since for a given interval between two differences all possible cutoff values yield an identical sensitivity and specificity, this approach represents all possible cutoff values in the observed range of differences (Albrecht et al. 2019 supplemental material 3 B).

The specificity was then used to calculate the false positive rate (1-specificity) and a receiver operating characteristic (ROC) curve was constructed. The TSI is defined as the AUC of the ROC curve. For the analysis the R package *pROC* version 1.13 was used (Robin et al. 2011; Albrecht et al. 2019). In case of missing alert concentrations in the gene expression data taken from Albrecht et al. 2019 concentrations the difference was set to a difference greater than the highest observed difference as described in Albrecht et al. 2019 supplement 3B.

The toxicity estimation index (TEI) quantifies how well toxic blood concentrations *in vivo* can be estimated by applying the *in vitro* test system. It cannot be excluded that at blood concentrations corresponding to therapeutic or expected doses non-toxic compounds, could become toxic at higher concentrations. Since information about the dose at, which would occur is often missing for the calculation of the TEI only compounds with known toxicity at therapeutic or expected were considered (Albrecht et al. 2019).

The TEI is calculated by the following equation:

$$\mathrm{TEI} = 1 - \frac{1}{5}\frac{\sum_{i=1}^{n} \mathbb{1}_{toxic}(i)\mathbb{1}_{x(i)>y(i)} \left|\log_{10}(\frac{y(i)}{x(i)})\right|}{\sum_{i=1}^{n} \mathbb{1}_{toxic}(i)}$$

in which i=1…n represents the individual compounds for a set of *n* compounds, $x(i)$ represents the effective concentration *in vitro* and $y(i)$ represents the *in vivo* concentration for a given exposure scenario. The terms $\mathbb{1}_{toxic}$ and $\mathbb{1}_{x(i)>y(i)}$ are indicator functions. For toxic compounds $\mathbb{1}_{toxic}$= 1 while for non-toxic compounds $\mathbb{1}_{toxic}$= 0. Therefore for all non-toxic compounds $\mathbb{1}_{toxic}\ \mathbb{1}_{x(i)>y(i)}$= 0 regardless of their respective *in vitro* and *in vivo* concentrations.

Ideally, an *in vitro* test system should yield an alert at an *in vitro* concentration ≤ the relevant *in vivo* concentration for toxic compounds in order to minimize risks of dangerously high exposures. For toxic compounds that fulfil this condition $\mathbb{I}_{toxic}\ \mathbb{I}_{x(i)>y(i)}$= 0. For compounds, with a higher effective *in vitro* concentration than *in vivo* concentration $\mathbb{I}_{toxic}\ \mathbb{I}_{x(i)>(y(i)}$= 1.

The algorithm is briefly described in the following. First, the subset of toxic compounds was identified for which the effective *in vitro* concentration is higher than the critical *in vivo* concentration. For each of these compounds the absolute value of the difference between the *in vitro* and *in vivo* concentration was obtained by $log_{10}\ (\frac{y(i)}{x(i)})$. The absolute differences were then summed up and divided by the total number of tested toxic compounds. Finally, the obtained ratio is scaled by multiplication with 0.2 and subtraction from one. This last step was performed to assign a TEI of 1 for an optimal estimation. In addition the multiplication with 0.2 leads to a scaling where a decrease of 0.2 corresponds to an increase of the mean distance of the toxic compounds to the iso-concentration line by the factor of 10. In case of missing alerts in the gene expression data taken from Albrecht et al. 2019, the *in vitro* concentration was set to 10 times the highest tested concentration.

2.2.6.3 Support vector machine (SVM) based prediction of hepatotoxicity

For classification of the compounds a support vector machine (SVM) was utilized. Each compound is represented by a vector with the effective *in vitro* concentration and the *in vivo* concentration as its coordinates. For hyperplane selection a maximal margin approach was applied. In order to allow for outliers and resulting misclassifications a 'soft margin' was introduced. The optimal hyperplane for separation between the two classes 'toxic' and 'non-toxic' was chosen by simultaneously maximizing the margin and minimizing the sum of errors by the slack constant 'C' (Noble 2006;). The SVM was built with the program R using the packages *mlr* version 2.13 (Bischl et al. 2016) and *kernlab* version 0.9-27 (Karatzoglou et al. 2004) with default settings for the hyperplane for training and evaluation of the classifier and SVM classification, respectively. Each compound was assigned a probability to belong to the 'toxic' class by using a fitted logistic regression model to a separating line between the two classes. This method allowed for the definition of lines corresponding to different probabilities of toxicity (Platt 2000;). For assessment of classification performance a leave one out approach was applied (Albrecht et al. 2019). For classification of the compounds with undefined toxicity status a SVM classifier was build utilizing all vectors with defined human hepatotoxicity status.

3 Results

Note*: Excerpts of this thesis have been published in Albrecht et al. 2019. The content of pages 25-90 corresponds largely to this publication.*

3.1 Test compounds and exposure scenarios

3.1.1 Hepatotoxicity of the selected test compounds

The compounds for the test and training set were selected based on the LiverTox database, the DILIrank data set, the DILIst dataset, literature search and suggestions from experts in the EuToxRisk and InnoSysTox research consortia. The DILIst data set became available after publication of Albrecht et al. 2019 and was therefore only considered for the toxicity assessment of the test set compounds. The toxicity status for the training set was taken from Albrecht et al. (2019). **Table 3.1** provides a brief overview over the toxicity data for the selected compounds of the test and training set. Detailed information on the toxicity is given in **Supplement 1.** For acetaminophen and ethanol exposure scenarios leading to *in vivo* concentrations with and without associated human hepatotoxicity are known. Therefore, these compounds are – depending on the exposure scenario – included in the toxic as well as the non-toxic subsets. The training set included 12 hepatotoxic, 14 non hepatotoxic compounds and 2 further compounds (acetaminophen and ethanol) with one *vivo* concentration associated with an increased risk of causing human hepatotoxicity and one *in vivo* concentration without an increased risk for causing human hepatotoxicity each. The test set included 31 hepatotoxic compounds and 16 non-hepatotoxic compounds. In addition, 5 compounds (clofibrate, dipyridamole, sodium phenylbutyrate, tolbutamide and vancomycin) with unclear hepatotoxicity status were tested. In total the extended compound set comprised 82 *in vitro/in vivo* concentration pairs (45 associated with human hepatotoxicity, 32 not associated with human hepatotoxicity and 5 with unclear human hepatotoxicity association).

Table 3.1 Overview of the selected compounds for the training and test set

The hepatotoxicity status refers to the toxicity status used in this work. Classification of the training and test compounds according to three different hepatotoxicity databases accessed on the 18.11.2020. The likelihood score refers to the LiverTox database, the DILIconcern to the DILIrank dataset and the DILIst classification to the DILIst dataset. The respective classifications are explained in section 1.2.

Compound	Abbreviation	Hepatotoxicity status	Compound set	LiverTox Likelihood score	DILI rank DILI concern	DILIst classification
Acetaminophen	APAP	Toxic/Non-toxic	Training	A [HD]	Verified Most-DILI concern	DILI positive
Allopurinol	Allo	Toxic	Test	A	Verified Most-DILI concern	DILI positive
Amiodarone	AMIO	Toxic	Test	A	Verified Most-DILI concern	DILI positive
Aspirin	ASP	Toxic	Training	A [HD]	Verified Less–DILI concern	DILI positive
Atorvastatin	AVS	Toxic	Test	A	Verified Most-DILI concern	DILI positive
Atropine	ATRO	Non-toxic	Test	E	Verified No-DILI concern	DILI negative
Benzbromarone	BZB	Toxic	Test	B	Verified Most-DILI concern	DILI positive
Benztropine	BZT	Non-toxic	Test	E	Verified No-DILI concern	DILI negative

Compound	Abbreviation	Hepatotoxicity status	Compound set	LiverTox Likelihood score	DILI rank DILI concern	DILIst classification
Bosentan	BOS	Toxic	Test	C	Verified Most-DILI concern	DILI positive
Buspirone	BPR	Non-toxic	Training	E	Ambiguous DILI concern	DILI negative
Busulfan	BUSF	Toxic	Training	A	Verified Most-DILI concern	DILI positive
Carbamazepine	CBZ	Toxic	Training	A	Verified Most-DILI concern	DILI positive
Chlorpheniramine	CHL	Non-toxic	Test	E	Verified No-DILI concern	DILI negative
Chlorpromazine	CMZ	Toxic	Test	A	Verified Less-DILI concern	DILI positive
Ciprofloxacin	CIPRO	Toxic	Test	B	Verified Most-DILI concern	DILI positive
Clofibrate	CLFI	Unclear	Test	D	Verified Less-DILI concern	DILI positive
Clonidine	CLON	Non-toxic	Training	E	Ambiguous DILI concern	DILI negative
Clozapine	CZP	Toxic	Test	B	Verified Most - DILI concern	DILI positive
Codeine	COD	Non-toxic	Test	E	Verified No-DILI concern	DILI negative
Cyclosporin A	CsA	Toxic	Test	C	Verified Most-DILI concern	DILI positive
Diclofenac	DFN	Toxic	Training	A	Verified Most-DILI concern	DILI positive

Compound	Abbreviation	Hepatotoxicity status	Compound set	LiverTox Likelihood score	DILI rank DILI concern	DILIst classification
Digoxin	Digi	Non-toxic	Test	E	Verified No-DILI concern	DILI negative
Dimethyl sulfoxide	DMSO	Non-toxic	Training	Not included	Not included	Not included
Diphenhydramine	DPH	Non-toxic	Test	E	Verified No-DILI concern	DILI negative
Dipyridamole	DIPY	Unclear	Test	E*	Ambiguous DILI concern	Not included
Erythromycin	Ery	Toxic	Test	A	Verified Most-DILI concern	DILI positive
Ethanol	EtOH	Toxic/Non-toxic	Training	Not included	Not included	DILI positive
Famotidine	FAM	Non-toxic	Training	C	Verified Less-DILI concern	DILI positive
Fexofenadine	FFD	Non-toxic	Test	E	Verified No-DILI concern	DILI negative
Fluconazole	FCA	Toxic	Test	B	Verified Most-DILI concern	DILI positive
Fluoxetine	FLT	Toxic	Test	C	Verified Less-DILI concern	DILI positive
Glucose	GLC	Non-toxic	Training	Not included	Not included	Not included
Hydroxyzine	HYZ	Non-toxic	Training	E	Verified No-DILI concern	Not included
Ibuprofen	IBU	Toxic	Test	A	Verified Less-DILI concern	DILI positive
Imipramine	IMP	Toxic	Test	B	Verified Less-DILI concern	DILI positive
Indomethacin	Indo	Toxic	Test	C	Verified Most-DILI concern	DILI positive

Compound	Abbreviation	Hepatotoxicity status	Compound set	LiverTox Likelihood score	DILI rank DILI concern	DILIst classification
Isoniazid	INAH	Toxic	Training	A	Verified Most-DILI concern	DILI positive
Isosorbide dinitrate	ISS	Non-toxic	Test	E	Verified No-DILI concern	DILI negative
Ketoconazole	KC	Toxic	Training	A	Verified Most-DILI concern	DILI positive
Labetalol	LAB	Toxic	Training	C	Verified Most-DILI concern	DILI positive
Leflunomide	LFM	Toxic	Test	B	Verified Most-DILI concern	DILI positive
Levofloxacin	LEV	Toxic	Training	A	Verified Most-DILI concern	DILI positive
Melatonin	MEL	Non-toxic	Training	E	Not included	DILI negative
Methotrexate	MTX	Toxic	Test	A	Verified Most-DILI concern	DILI positive
Methylparaben	MePa	Non-toxic	Training	Not included	Not included	Not included
N acetylcysteine	NAC	Non-toxic	Training	E	Verified No-DILI concern	DILI negative
Nevirapine	NVP	Toxic	Test	A	Verified Most-DILI concern	DILI positive
Nifedipine	NDP	Toxic	Test	B	Verified Less-DILI concern	DILI positive
Nimesulide	NIM	Toxic	Training	A	Verified Most-DILI concern	DILI positive
Nitrofurantoin	NFT	Toxic	Training	A	Verified Most-DILI concern	DILI positive

Compound	Abbreviation	Hepatotoxicity status	Compound set	LiverTox Likelihood score	DILI rank DILI concern	DILIst classification
Oxycodone	OXC	Non-toxic	Test	Not given	Verified No-DILI concern	DILI negative
Oxymorphone	OXM	Non-toxic	Test	Not given	Verified No-DILI concern	DILI negative
Paroxetine	PXT	Toxic	Test	B	Verified Less-DILI concern	DILI positive
Phenacetine	PHNA	Toxic	Test	Not included	Not included	DILI positive
Phenylbutazone	PhB	Toxic	Training	Not included	Not included	DILI positive
Phenytoin	PTN	Toxic	Test	A	Verified Most-DILI concern	DILI positive
Pindolol	PIN	Toxic	Test	E	Verified No-DILI concern	DILI negative
Pioglitazone	PIO	Toxic	Test	C	Verified Less-DILI concern	DILI positive
Primaquine	Prima	Non-toxic	Test	E	Verified No-DILI concern	DILI negative
Primidone	PRI	Non-toxic	Test	E*	Verified No-DILI concern	DILI negative
Promethazine	PMZ	Non-toxic	Training	E	Verified Less-DILI concern	DILI positive
Propranolol	PPL	Non-toxic	Training	E	Ambiguous DILI concern	DILI negative
Pyridoxine	PDX	Non-toxic	Test	E	Verified No-DILI concern	DILI negative
Rifampicin	RIF	Toxic	Training	A	Verified Most-DILI concern	DILI positive

Compound	Abbreviation	Hepatotoxicity status	Compound set	LiverTox Likelihood score	DILI rank DILI concern	DILIst classification
Rosiglitazone	RGZ	Toxic	Test	C	Verified Less-DILI concern	DILI positive
Rosuvastatin	ROS	Toxic	Test	B	Verified Less-DILI concern	DILI positive
Simvastatin	SIM	Toxic	Test	A	Verified Less-DILI concern	DILI positive
Sitaxentan	SXS	Toxic	Test	Not included	Verified Most-DILI concern	DILI positive
Sodium phenylbutyrate	SPB	Unclear	Test	E	Ambiguous DILI concern	Not included
Terbinafine	Terbi	Toxic	Test	B	Verified Most-DILI concern	DILI positive
Theophylline	THE	Non-toxic	Test	E	Verified No-DILI concern	DILI negative
Tolbutamide	TOL	Unclear	Test	B (class)	Ambiguous DILI concern	DILI negative
Tolcapone	Tolc	Toxic	Test	C	Verified Most-DILI concern	DILI positive
Tolterodine	TTD	Non-toxic	Test	Not given	Verified No-DILI concern	DILI negative
Triclosan	TSN	Non-toxic	Training	Not included	Not included	Not included
Valproic acid	VPA	Toxic	Training	A	Verified Most-DILI concern	DILI positive
Vancomycin	VANC	Unclear	Test	B (usually in association with DRESS)	Verified Less-DILI concern	DILI positive

Compound	Abbreviation	Hepatotoxicity status	Compound set	LiverTox Likelihood score	DILI rank DILI concern	DILIst classification
Verapamil	VERA	Toxic	Test	B	Verified Less-DILI concern	DILI positive
Vitamin C	VitC	Non-toxic	Training	E	Verified No-DILI concern	DILI negative
Zaleplon	ZAL	Non-toxic	Test	E	Verified No-DILI concern	DILI negative

3.1.2 Exposure scenarios for the test compounds.

For obtaining the *in vivo* concentrations the following exposure scenarios as given in **Table 3.2** were used. For the compounds of the training set the same exposure scenarios as in Albrecht et al. 2019 were used. A detailed summary of the obtained *in vivo* concentrations as well as the input parameters are given in **Supplement 3.**

Table 3.2: Overview of the used exposure scenarios for all compounds

Compound	Abbreviation	Indication/ Scenario	Dose [mg]	Dose intervall	Route	Duration	Reference
Acetaminophen (toxic)	APAP	Over dose	10000	Single dose	Oral	Single dose	Albrecht et al. 2019
Acetaminophen (non-toxic)	APAP	Pain and fever	1000	8 hours	Oral	1 week	Albrecht et al. 2019
Allopurinol	Allo	Gout	300	24 hours	Oral	7 days	LiverTox, e medicines
Amiodarone	AMIO	Arrhythmias	200	8 hours	Oral	143 days	LiverTox
Aspirin	ASP	Pain and fever	1000	8 hours	Oral	7 days	Albrecht et al. 2019
Atorvastatin	AVS	Hypercholesterolemia	20	24 hours	Oral	30 days	LiverTox, e medicines
Atropine	ATRO	Irritable bowel disease	0.6	24 hours	Oral	7 days	LiverTox, e medicines ISDB WHO Single medicines
Benzbromarone	BZB	Gout	100	24 hours	Oral	7 days	Benzbromarone product leaflet
Benztropine	BZT	Parkinsonism	2	24 hours	Oral	30 days	LiverTox, Rx list
Bosentan	BOS	Pulmonary hypertension	125	12 hours	Oral	13.5 days	LiverTox Medscape
Buspirone	BPR	Anxiety disorders	30	12 hours	Oral	6 months	Albrecht et al. 2019
Busulfan	BUSF	Chronic myelogenous leukemia	0.06/kg	24 hours	Oral	30 days	LiverTox, e medicines
Carbamazepine	CBZ	Epilepsy	200	6 hours	Oral	6 months	Albrecht et al. 2019
Chlorpheniramine	CHL	Allergy, rhinitis	4	6 hours	Oral	2 weeks	Albrecht et al. 2019
Chlorpromazine	CMZ	Psychosis	25	8 hours	Oral	30 days	LiverTox, Drugs. Com

Compound	Abbreviation	Indication/ Scenario	Dose [mg]	Dose intervall	Route	Duration	Reference
Ciprofloxacin	CIPRO	Bacterial infections	250	12 hours	Oral	14 days	LiverTox, e medicines
Clofibrate	CLFI	Hyperlipidemia	500	6 hours	Oral	7 days	LiverTox, Drugs. com
Clonidine	CLON	Restless leg syndrome	0.3	24 hours	Oral	6 months	Albrecht et al. 2019
Clozapine	CZP	Schizophrenia	150	12 hours	Oral	20 days for multiple dose: day 1 12.5 mg, day 2 25 mg followed by daily increases of 12.5 mg to a final dose of 150 mg	LiverTox, Drugs com, e medicines
Codeine	COD	Pain	30	6 hours	Oral	3 days	LiverTox, e medicines
Cyclosporin A	CsA	Solid organ transplantation (maintenance dose)	10/kg	24 hours	Oral	30 days	Drugs.com
Diclofenac	DFN	Rheumatoid arthritis	50	8 hours	Oral	6 months	Albrecht et al. 2019
Digoxin	Digi	Mild to moderate heart failure	0.25	24 hours	Oral	30 days	Digoxin product leaflet / maintenance dose slow digitalization
Dimethyl sulfoxide	DMSO	Osteo arthritis (as an ingredient of dermal formulations)	650.65	6 hours	Dermal	15 days	Albrecht et al. 2019
Diphenhydramine	DPH	Allergies	50	8 hours	Oral	30 days	LiverTox, e medicines

Compound	Abbreviation	Indication/ Scenario	Dose [mg]	Dose intervall	Route	Duration	Reference
Dipyridamole	DIPY	Thrombosis prophylaxis	100	8 hours	Oral	30 days	LiverTox, e medicines
Erythromycin	Ery	Bacterial infections	500	6 hours	Oral	7 days	LiverTox, e medicines
Ethanol (toxic)	EtOH	Alcoholic beverages	16000	24 hours	Oral	6 months	Albrecht et al. 2019
Ethanol (non-toxic)	EtOH	Alcohol containing sprays	9720	24 hours	Dermal	6 months	Albrecht et al. 2019
Famotidine	FAM	Peptic ulcer	40	24 hours	Oral	6 weeks	Albrecht et al. 2019
Fexofenadine	FFD	Chronic urticaria	180	24 hours	Oral	30 days	LiverTox
Fluconazole	FCA	Cryptococcal meningitis/ prevention of candida infections	400	24 hours	Oral	30 days	LiverTox, e medicines
Fluoxetine	FLT	Depression	20	24 hours	Oral	60 days	LiverTox
Glucose	GLC	Nutrition	84000	7:30 am, 12:30 pm, 6:00 pm	Oral	15 days	Albrecht et al. 2019
Hydroxyzine	HYZ	Anxiety and tension	100	8 hours	Oral	3 months	Albrecht et al. 2019
Ibuprofen	IBU	Rheumatoid arthritis	600	8 hours	Oral	30 days	LiverTox
Imipramine	IMP	Depression	44.26	12 hours	Oral	30 days	LiverTox, e medicines
Indomethacin	Indo	Rheumatoid disorders	25	8 hours	Oral	30 days	LiverTox, e medicines
Isoniazid	INAH	Tuberculosis	300	24 hours	Oral	9 months	Albrecht et al. 2019
Isosorbide dinitrate	ISS	Angina pectoris	30	12 hours	Oral	30 days	LiverTox, e medicines
Ketoconazole	KC	Fungal infections	400	24 hours	Oral	2 weeks	Albrecht et al. 2019
Labetalol	LAB	Hypertension	400	12 hours	Oral	2 weeks	Albrecht et al. 2019

Compound	Abbreviation	Indication/ Scenario	Dose [mg]	Dose intervall	Route	Duration	Reference
Leflunomide	LFM	Rheumatoid arthritis	Single dose: 100 multiple dose day 1-3 100 mg then 20 mg	24 hours	Oral	33 days	LiverTox
Melatonin	MEL	Insomnia, migraine	5	24 hours	Oral	4 weeks	Albrecht et al. 2019
Methotrexate	MTX	Rheumatoid arthritis	15	7 days	Oral	35 days	LiverTox
Methylparaben	MePa	Personal care products	10.2	24 hours	Dermal	6 months	Albrecht et al. 2019
N -acetylcysteine	NAC	Bronchitis	200	8 hours	Oral	1 week	Albrecht et al. 2019
Nevirapine	NVP	HIV	Single dose:400 multiple dose: day 1-14 200 mg then 400 mg	24 hours	Oral	44 days	LiverTox, e medicines
Nifedipine	NDP	Prophylaxis of angina pectoris	30	24 hours	Oral	30 days	LiverTox, e medicines
Nimesulide	NIM	Acute pain	100	12 hours	Oral	2 weeks	Albrecht et al. 2019
Nitrofurantoin	NFT	Urinary tract infections	100	6 hours	Oral	2 weeks	Albrecht et al. 2019
Oxycodone	OXC	Severe pain	5	6 hours	Oral	30 days	E medicines
Oxymorphone	OXM	Severe pain	10	6 hours	Oral	30 days	Drugs com

Compound	Abbreviation	Indication/ Scenario	Dose [mg]	Dose intervall	Route	Duration	Reference
Paroxetine	PXT	Depression	20	24 hours	Oral	30 days	LiverTox
Phenacetin	PHNA	Pain and fever	300	6 hours	Oral	7 days	Inxight drugs
Phenylbutazone	PhB	Inflammatory pain, arthritis	100	8 hours	Oral	1 week	Albrecht et al. 2019
Phenytoin	PTN	Epilepsy	100	8 hours	Oral	30 days	LiverTox
Pindolol	PIN	Hypertension/ angina pectoris	5	12 hours	Oral	30 days	LiverTox
Pioglitazone	PIO	Diabetes mellitus	30	24 hours	Oral	30 days	LiverTox, e medicines
Primaquine	Prima	Prevention of malaria	30	24 hours	Oral	30 days	LiverTox
Primidone	PRI	Epilepsy	Single dose:250 multiple dose day 1-9: 125mg day 10 onward 250 mg	Day 1-3 24h day 4 -6 12h day 7 onward 8 hours	Oral	39 days	LiverTox, Medscape
Promethazine	PMZ	Nausea, vomiting, motion sickness	25	6 hours	Oral	5 days	Albrecht et al. 2019
Pyridoxine	PDX	Nutritional supplement vegetarian/vegan diet	1.4	24 hours	Oral	7 days	Orthomol, LiverTox
Rifampicin	RIF	Tuberculosis	600	24 hours	Oral	6 months	Albrecht et al. 2019
Rosiglitazone	RGZ	Diabetes mellitus	4	12 hours	Oral	30 days	LiverTox
Rosuvastatin	ROS	Hypercholesterolemia	10	24 hours	Oral	30 days	LiverTox, e medicines
Simvastatin	SIM	Hypercholesterolemia	20	24 hours	Oral	30 days	LiverTox, e medicines

Compound	Abbreviation	Indication/ Scenario	Dose [mg]	Dose intervall	Route	Duration	Reference
Sitaxentan	SXS	Pulmonary hypertension	100	24 hours	Oral	30 days	EMA
Sodium phenylbutyrate	SPB	Urea cycle disorders	4000/m^2	8 hours	Oral	30 days	LiverTox, e medicines
Terbinafine	Terbi	Tinea ungium	250	24 hours	Oral	42 days	LiverTox
Theophylline	THE	Asthma	300	8 hours	Oral	30 days	LiverTox
Tolbutamide	TOL	Diabetes mellitus	1000	12 hours	Oral	30 days	LiverTox
Tolcapone	Tolc	Parkinsonism	100	8 hours	Oral	30 days	LiverTox, e medicines
Triclosan	TSN	Personal care products	4	8 hours	Oral	6 months	Albrecht et al. 2019
Valproic acid	VPA	Eepilepsy	15/kg	24 hours	Oral	6 months	Albrecht et al. 2019
Vancomycin	VANC	Bacterial infections	1000	12 hours	Intravenous infusion (10 mg/h)	7 days	LiverTox, e medicines
Vitamin C	VitC	Nutrition	82.5	24 hours	Oral	15 days	Albrecht et al. 2019
Zaleplon	ZAL	Insomnia	10	24 hours	Oral	30 days	LiverTox

3.2 Optimization of cytotoxicity assay based DILI prediction in PHH

3.2.1 Establishment of two novel performance metrics

For optimization of the cytotoxicity assay based prediction of human DILI two performance metrics were introduced, which allow to systematically compare different effective *in vitro* concentration thresholds *e.g.* EC_{10} or EC_{50}, different incubation times and different human *in vivo* concentrations *e.g.* total blood concentrations, unbound blood concentration, systemic blood concentrations and portal vein blood concentrations. The concept of these novel indices is based on the assumption that *in vivo* hepatotoxic compounds would show an *in vitro* toxicity at concentrations close to or below the *in vivo* relevant concentrations, whereas *in vivo* non-toxic compounds would show *in vitro* toxicity effects only at concentrations higher than the *in vivo* relevant concentrations.

As a measure for the capability of a combination of *in vitro* and *in vivo* concentrations to separate toxic from non-toxic compounds the Toxicity Separation Index (TSI) was utilized. An optimal separation of toxic and non-toxic on a diagram with the effective *in vitro* concentration on the abscissa and the relevant *in vivo* concentration on the ordinate would yield a TSI of 1 whereas a random distribution of toxic and non-toxic compounds on such a diagram would yield a TSI of 0.5. Thus the higher the TSI the better is the separation of toxic and non-toxic compounds using a specific pair of *in vitro* and *in vivo* concentrations.

The Toxicity Estimation Index (TEI) measures how close the effective *in vitro* concentration for a toxic compound is to the relevant *in vivo* concentration. *In vivo* hereby describes the concentration at therapeutic doses for pharmaceuticals or following common exposure scenarios in case of other chemicals. Since it has to be assumed that at these concentrations non-toxic compounds, could become hepatotoxic at unknown higher *in vivo* concentrations all non-toxic compounds were excluded for the calculation of the TEI.

A TEI of 1 indicates that all tested toxic compounds had effective *in vitro* concentrations at or below the *in vivo* relevant concentrations. The TEI is scaled in a way that a decrease of 0.2 corresponds to a tenfold increase of the ratio effective *in vitro* concentration to the relevant *in vivo* concentration.

An ideal *in vitro* test system for the classification of compounds into toxic and non-toxic compounds and estimation of toxic in *vivo* concentrations would be expected to have a TSI and TEI of 1. The principle of these metrics is illustrated for four hypothetical scenarios in **Figure 3.1** (taken from Albrecht et al 2019). Panels A and B show scenarios with good separation of toxic and non-toxic compounds while Panels C and D depict scenarios with poor separation of toxic and non-toxic compounds. Panels A and C illustrate scenarios with a good estimation of toxic *in vivo* concentrations by the effective *in vitro* concentrations, whereas panels B and D exemplify scenarios where the mean effective *in vitro* concentrations is higher than the toxic *in vivo* concentration.

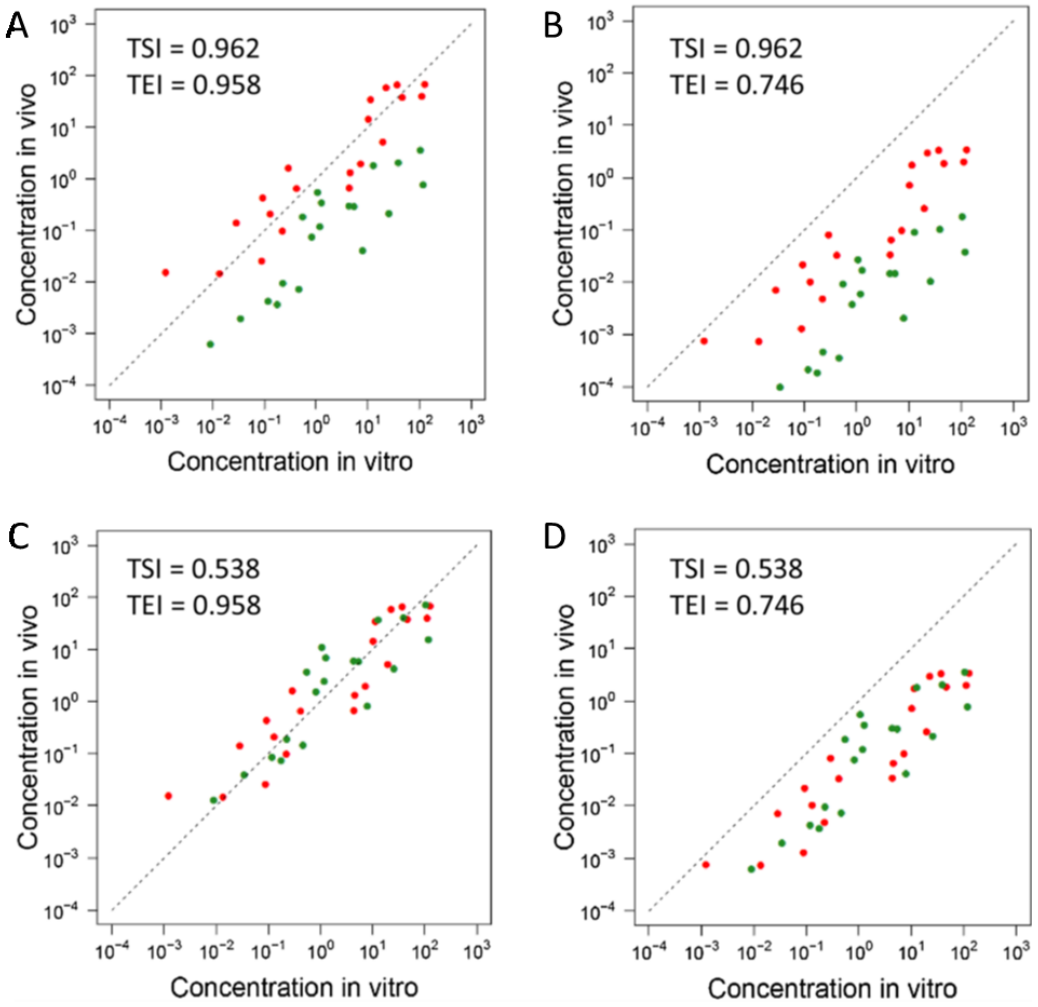

Figure 3.1: Concept of Toxicity Separation Index (TSI) and Toxicity Estimation Index (TEI)
The concept of the metrics is shown for four hypothetical scenarios. The effective in vitro and the relevant in vivo concentrations are given in the same units. Toxic compounds are shown in red and non-toxic compounds are depicted in green. The dotted line is the iso-concentration line. (A) A scenario with good separation of toxic and non-toxic compounds and good estimation of toxic in vivo concentrations. (B) A scenario with good separation of toxic and non-toxic compounds with worse estimation of toxic in vivo concentrations. (C) A scenario without separation of toxic and non-toxic compounds but good estimation of toxic blood concentrations for toxic compounds. (D) A scenario without separation of toxic and non-toxic compounds and worse estimation of toxic in vivo concentrations for toxic compounds (Figure is taken from Albrecht et al. 2019).

3.2.2 Selection of the optimal *in vitro* effective concentration and influence of donor variability and incubation time

The first questions of interest were which *in vitro* effective concentration and which donor should be considered. To answer these questions the TSI and TEI were calculated for a broad range of different effective *in vitro* concentrations obtained for all 28 compounds of the training set after 48 hours incubation with cells from three different donors using the 95% percentile of the total systemic blood concentration as *in vivo* concentration. **Figure 3.2 A** depicts the results of this exercise. The next question for optimizing the test system was the selection of the ideal incubation time. Therefore, the TSI and TEI were calculated for three selected different effective *in vitro* concentrations obtained for all 28 compounds of the training set after 1 day, 2 days and 7 days of incubation with cells from three different donors using the 95% percentile of the total systemic blood concentration as *in vivo* concentration. The results of this analysis are illustrated by **Figure 3.2 B.**

For test system optimization the TSI was prioritized over the TEI. All utilized *in vivo* and *in vitro* concentrations as well as the obtained indices are given in **Supplement 3**, **6** and **7.**

For these analysis the minimum was defined as the most sensitive donor and the maximum as the least sensitive donor **Figure 3.2 A** shows that the highest TSI value was obtained when using the median EC_{10} values as *in vitro* parameter. As shown in **Figure 3.2 B** the highest TSI and TEI were obtained after 48 hours of compound exposure. Therefore, the median EC_{10} value after 48 hours of compound exposure was selected for further analysis.

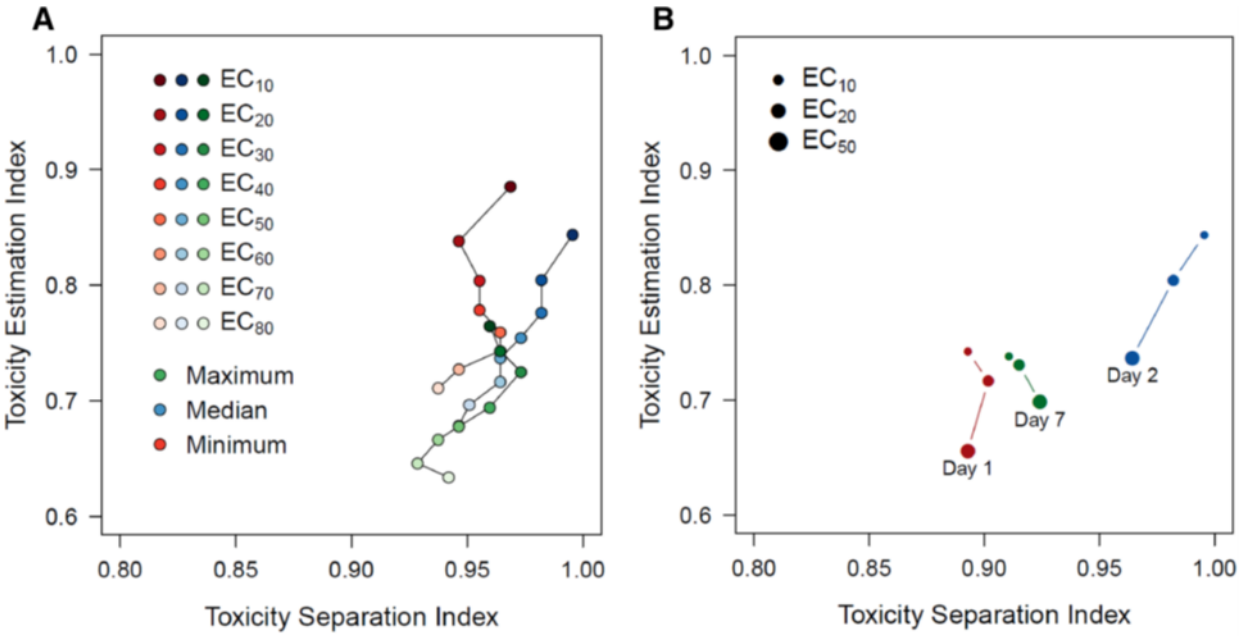

Figure 3.2: TSI and TEI for different effective *in vitro* concentrations, donors and incubation times

As in vivo concentration the 95% percentile of the total systemic blood concentration was used. (A) The TSIs and TEIs for different effective in vitro concentrations in three different donors after 48 hours of incubation with the 28 training set compounds. (B) The TSI and TEI for three different effective in vitro concentrations for three donors after three different incubation times with the compounds of the training set. The minimum refers to the most sensitive donor and the maximum to the least sensitive donor for each compound. The median designates the donor in-between these. (Figure is taken from Albrecht et al. 2019).

3.2.3 Selection of the optimal *in vivo* concentration

After establishing the best *in vitro* parameter for usage in the cytotoxicity assay based *in vitro in silico* test system the next question to be addressed was which *in vivo* concentration should be used. Therefore the TSI and TEI were calculated for a variety of different *in vivo* concentrations obtained by physiologically based pharmacokinetic (PBPK) modelling utilizing the median EC_{10} after 48 hours of the training set compounds as *in vitro* parameter. The results for this are shown in **Figure 3.3**.

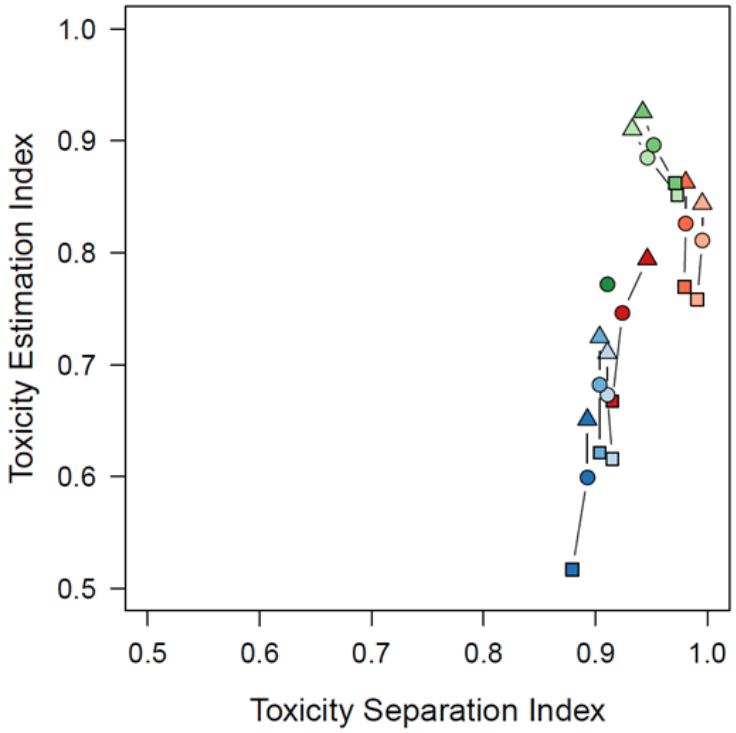

Figure 3.3: TSI and TEI for different *in vivo* concentrations from a population of 100 virtual subjects with the median EC_{10} after 48h exposure with the 28 training set compounds as *in vitro* parameter
WB = whole blood, Cmax = peak concentration after single dose, ss = steady state after multiple dose, PV = portal vein. All experiments were done with PHH from three different donors (Figure is taken from Albrecht et al. 2019).

As **Figure 3.3** illustrates the highest TSI was achieved when using the 95% percentile of the peak total systemic blood concentration after a single dose. Therefore, this was chosen as *in vivo* parameter of the test system for further analysis.

Figure 3.4 depicts the data situation for the 30 exposure scenarios for the training set compounds using the median EC_{10} after 48 hours of compound exposure as *in vitro* parameter and the 95% percentile of the peak total systemic whole blood concentration after single exposure as *in vivo* parameter. **Table 3.3** summarizes the optimized *in vitro* and *in vivo* parameters for the cytotoxicity assay in PHH with the training set compounds.

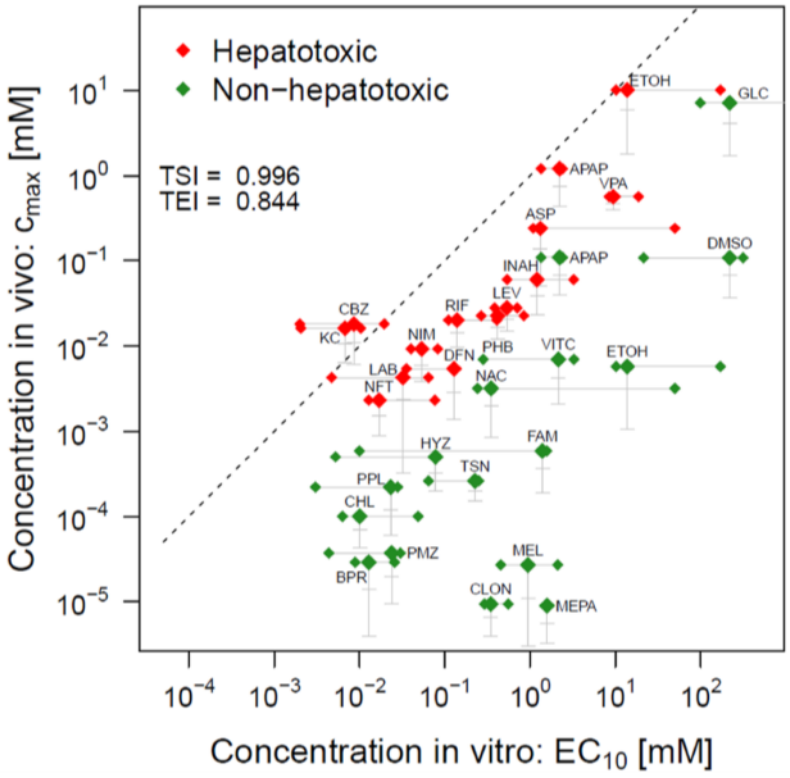

Figure 3.4: Data situation for the compounds of the 28 training set compounds
As *in vitro concentration the median EC_{10} using PHH of three different donors after 48 hours of compound exposure is chosen. As in vivo concentration the 95% percentile of peak total systemic whole blood concentration was used. Each compound is represented by a cross shape, the color indicating the toxicity status (green: non-hepatotoxic; red: hepatotoxic). The horizontal diamonds represent the minimal, median and maximal EC_{10} values for this compound while the vertical diamonds depict the 5% percentile, mean and 95% percentile of the blood concentration of the respective compound. The dashed line is the iso-concentration line (Figure is taken from Albrecht et al. 2019).*

As reflected by the very high TSI of 0.996 and **Figure 3.4** a nearly optimal separation of hepatotoxic and non-hepatotoxic compounds was observed for this combination of parameters. The TEI of 0.844 indicates that the observed median EC_{10} was on average within the 10 x range of the 95% percentile of the peak total systemic whole blood concentration after single exposure as obtained by PBPK modelling.

Table 3.3: Summary of the optimized *in vitro* and *in vivo* parameters for the cytotoxicity assay in PHH with the training set compounds

The EC_{10} is the median EC_{10} after 48 hours of compound exposure. As in vivo concentration the 95% percentile of the total systemic peak whole blood concentration obtained by PBPK modelling is given. (The values are taken from Albrecht et al. 2019).

Compound	Abbreviation	Toxicity	EC_{10} Median [mM]	Total systemic peak blood concentration, 95% CI [mM]
Acetaminophen	APAP	Hepatotoxic	2.221	$12.1*10^{-1}$
Acetaminophen	APAP	Non-hepatotoxic	2.221	$10.9*10^{-2}$
Aspirin	ASP	Hepatotoxic	1.321	$24.0*10^{-2}$
Buspirone	BPR	Non-hepatotoxic	0.013	$29.0*10^{-6}$
Carbamazepine	CBZ	Hepatotoxic	0.009	$18.1*10^{-3}$
Chlorpheniramine	CHL	Non-hepatotoxic	0.010	$10.0*10^{-5}$
Clonidine	CLON	Non-hepatotoxic	0.346	$94.0*10^{-7}$
Diclofenac	DFN	Hepatotoxic	0.129	$54.2*10^{-4}$
Dimethyl sulfoxide	DMSO	Non-hepatotoxic	219.581	$10.8*10^{-2}$
Ethanol	EtOH	Hepatotoxic	13.780	$10.1*10^{0}$
Ethanol	EtOH	Non-hepatotoxic	13.780	$57.6*10^{-4}$
Famotidine	FAM	Non-hepatotoxic	1.395	$59.0*10^{-5}$
Glucose	GLC	Non-hepatotoxic	219.428	$71.5*10^{-1}$
Hydroxyzine	HYZ	Non-hepatotoxic	0.078	$50.0*10^{-5}$
Isoniazid	INAH	Hepatotoxic	1.206	$60.0*10^{-3}$
Ketoconazole	KC	Hepatotoxic	0.007	$16.2*10^{-3}$
Labetalol	LAB	Hepatotoxic	0.032	$42.8*10^{-4}$
Levofloxacin	LEV	Hepatotoxic	0.534	$28.0*10^{-3}$
Melatonin	MEL	Non-hepatotoxic	0.946	$27.0*10^{-6}$
Methylparaben	MePa	Non-hepatotoxic	>0.316	$90.0*10^{-7}$
N-acetylcysteine	NAC	Non-hepatotoxic	0.350	$31.9*10^{-4}$
Nimesulide	NIM	Hepatotoxic	0.054	$92.6*10^{-4}$
Nitrofurantoin	NFT	Hepatotoxic	0.017	$23.2*10^{-4}$
Phenylbutazon	PhB	Hepatotoxic	0.418	$22.6*10^{-3}$
Promethazine	PMZ	Non-hepatotoxic	0.024	$37.2*10^{-6}$

Compound	Abbreviation	Toxicity	EC_{10} Median [mM]	Total systemic peak blood concentration, 95% CI [mM]
Propranolol	PPL	Non-hepatotoxic	0.023	$22.0*10^{-5}$
Rifampicin	RIF	Hepatotoxic	0.140	$20.1*10^{-3}$
Triclosan	TSN	Non-hepatotoxic	0.226	$26.0*10^{-5}$
Valproic acid	VPA	Hepatotoxic	9.461	$56.9*10^{-2}$
Vitamin C	VitC	Non-hepatotoxic	2.153	$59.9*10^{-4}$

3.2.4 SVM based classification of the compounds of the training set

With the selected *in vitro* and *in vivo* parameters a support vector machine based classifier was build and tested by leave-one-out cross validation with the training set compounds. As illustrated by **Figure 3.5** all *in vivo* hepatotoxic compounds were correctly classified as toxic by the classifier, whereas two *in vivo* non-hepatotoxic compounds were misclassified as toxic. This lead to a sensitivity of 100 %, a specificity of 87.5 %, an accuracy of 93.3 %, a positive predictive value of 87.5 % and a negative predictive value of 100 % for the compounds of the training set. The performance metrics are summarized in **Table 3.4.** All probabilities are given in **Supplement 8.**

Table 3.4: Summary of the performance metrics for the cytotoxicity assay based SVM based leave-one-out classifier for PHH and the training set compounds
(Data taken from Albrecht et al. 2019).

Parameter	Value
True positives	14
True negatives	14
False negatives	0
False positives	2
Sensitivity	100 %
Specificity	87.5 %
Accuracy	93.3 %
Positive predictive value	87.5 %
Negative predictive value	100 %

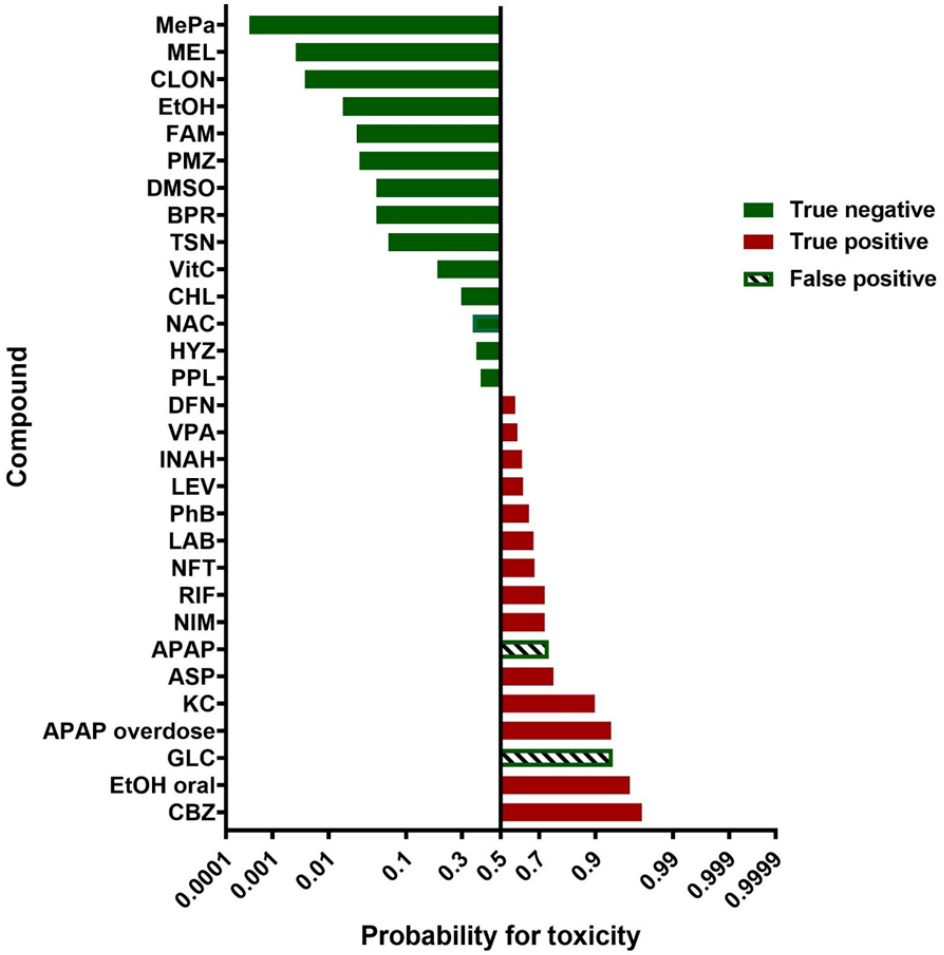

Figure 3.5: SVM based leave-one-out classification of the 28 training set compounds
As in vitro concentration the median EC_{10} using PHH of three different donors after 48 hours of compound exposure is chosen. As in vivo concentration the 95% percentile of peak total systemic whole blood concentration was used. Compounds exceeding a probability for toxicity of 0.5 were predicted to be toxic (Data taken from Albrecht et al. 2019).

3.3 *In vitro in silico* based prediction of an ADI for pulegone

As a first test of applicability of the *in vitro/in silico* test system for estimation of an acceptable daily intake (ADI), pulegone was investigated. Pulegone is a natural occurring minty flavoring agent and fragrance constituent and a known hepatotoxicant at high doses (Chen et al. 2011; Khojasteh et al. 2012). First, the cytotoxicity assay was performed with PHH from three donors and 48 hours incubation time. The resulting curves are shown in **Figure 3.6.**

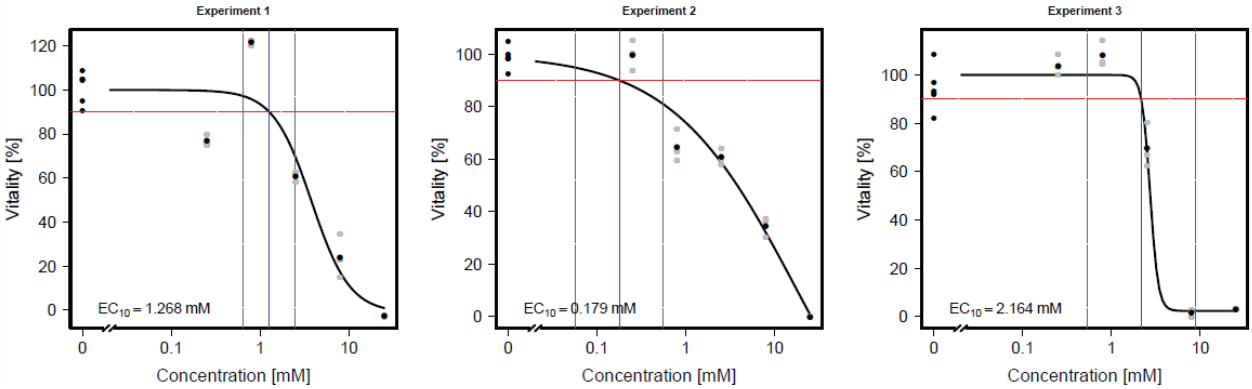

Figure 3.6: Concentration-response curves for the cytotoxicity assay in PHH from three different donors after 48 hours of exposure to pulegone
The red and blue lines indicate the EC_{10}. The blue and grey lines show the confidence interval of the EC_{10}. (Figure is taken from Albrecht et al. 2019).

As **Figure 3.6** illustrates cytotoxicity was observed in PHH from all three donors after 48 hours of compound exposure with a rounded median EC_{10} of 1.27 mM. In the next step the *in vivo* concentrations corresponding to different probabilities of human hepatotoxicity were calculated by means of the SVM classifier build with all 30 *in vitro/in vivo* concentration pairs of the training set compounds. The results are given in **Table 3.5** and **Figure 3.7.**

Table 3.5: *In vivo* concentrations of pulegone corresponding to different probabilities of hepatotoxicity derived by the SVM classifier
The probability of hepatotoxicity indicates the probability of the compound having an increased risk of causing human hepatotoxicity. In the linear equation y denotes the log_{10} of the in vivo concentration, x indicates the log_{10} of the median EC_{10} of the cytotoxicity assay in PHH after 48 hours of incubation. The probability influences the y- intercept. The Cmax denotes the 95% percentile of the peak blood concentration (Data taken from Albrecht et al. 2019).

Probability of hepatotoxicity	Linear equation	Cmax [µM]
0.5	y= -1.600+0.796x	30.34
0.1	y= -2.559+0.796x	3.33
0.05	y= -2.885+0.796x	1.57
0.01	y= -3.606+0.796x	0.299
0.005	y= -3.910+0.796x	0.148
0.001	y= -4.615+0.796x	0.029

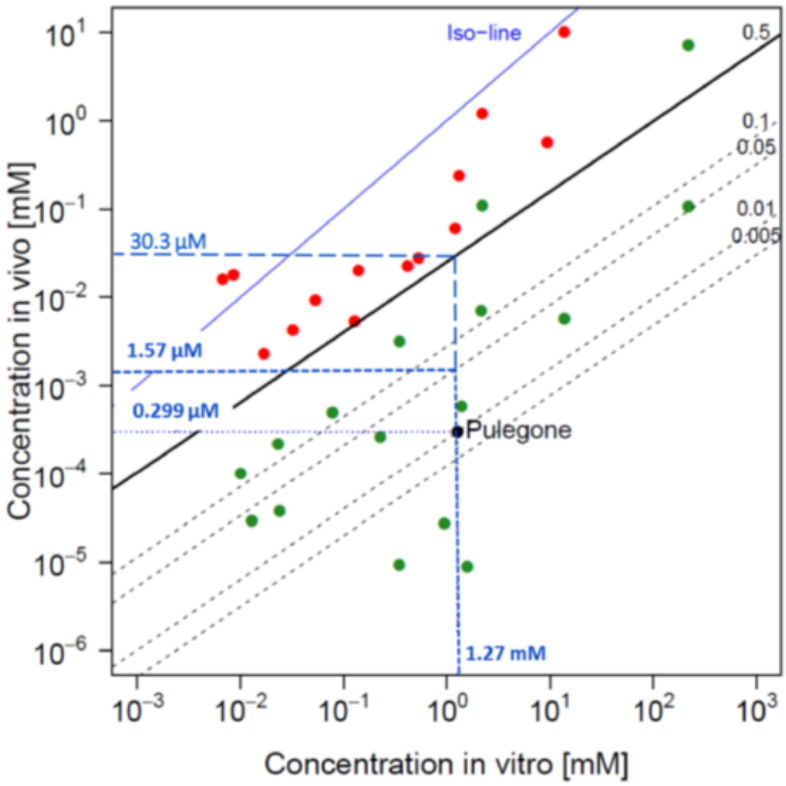

Figure 3.7: *In vivo* concentrations for pulegone associated with different probabilities of an increased risk of causing human hepatotoxicity as derived by the SVM classifier
The green circles symbolize the non-hepatotoxic compounds of the training set while the red circles depict the hepatotoxic compounds of the training set. The blue line denotes the iso-concentration line. The different probabilities of an association with an increased risk of causing human hepatotoxicity are represented by the black lines. All experiments were done with PHH from three different donors (Figure is taken from Albrecht et al. 2019).

The final step consisted of reverse PBPK modelling to obtain the doses which would lead to the derived *in vivo* concentrations after single or repeated oral exposure. The input parameters are given in **Supplement 3**. The modelled dosages are summarized in **Table 3.6.** For this case study a probability of 5% of association with an increased risk of causing human hepatotoxicity was considered appropriate for ADI determination. This leads to a maximal acceptable blood concentration of 1.57 µM and a derived ADI of 268 µg/kg/day for a human. The derived ADI was in good agreement with established ADIs derived from rodent *in vivo* studies of 375-750 µg/kg/day (HMPC) and 100 µg kg/day (CEFS).

Table 3.6: Oral doses of pulegone associated with different probabilities of increased risk of hepatotoxicity derived by the SVM classifier and reverse PBPK modelling
The probability of hepatotoxicity indicates the probability of the compound having an increased risk of causing human hepatotoxicity. The repeated oral dose is given for an average body weight of 70.8 kg (Walpole et al. 2012; data taken from Albrecht et al. 2019).

Probability of hepatotoxicity	Single oral dose [mg]	Repeated oral dose [mg]	Repeated oral dose [mg/kg]
0.5	4000	350	4.944
0.1	86	40	0.565
0.05	41	19	0.268
0.01	7.5	3.6	0.051
0.005	3.8	1.8	0.025
0.001	0.72	0.35	0.005

3.4 Expansion of the compound set for the cytotoxicity assay based classification of compounds in PHH

3.4.1 TSI and TEI for the extended compound set

In the next step the compound set was extended to include 47 additional compounds with established toxicity status and 5 compounds with unclear human DILI risk. These additional compounds are referred to as test set. The toxicity status of the entire set of compounds (extended compound set) and the exposure scenarios considered are given in **Section 3.1**.

Detailed TSI and TEI analysis were first performed for all compounds with established human hepatotoxicity status. Hence 77 *in vitro/in vivo* data pairs for 75 compounds were utilized. All obtained indices are given in **Supplement 7.**

Figure 3.8 depicts the comparison of different effective concentrations obtained from the cytotoxicity assay in PHH of three different donors after 48 hours of compound exposure. As *in vivo* concentration the 95% percentile of the peak total systemic blood concentration was utilized. As shown in this graph the optimal TSI was obtained for the maximal EC_{20}, referring to the EC_{20} for the least sensitive donor.

Next, the influence of numerous modelled *in vivo* concentrations on the TSI and TEI using the maximal EC_{20} was analyzed. The results are visualized in **Figure 3.9.** It shows that the highest TSI was obtained for the 95% percentile of the peak total systemic blood concentration yielding a TSI of 0.804 and a TEI of 0.605.

The corresponding data situation is illustrated in **Figure 3.10**. In general a trend for the separation of hepatotoxic and non-hepatotoxic compounds was observed. The TEI in **Figure 3.10** shows that the mean effective *in vitro* concentration for the hepatotoxic compounds was in the 100-fold range of the relevant *in vivo* concentration. **Table 3.7** provides an overview of the optimized *in vitro* and *in vivo* concentrations for PHH for the extended compound set.

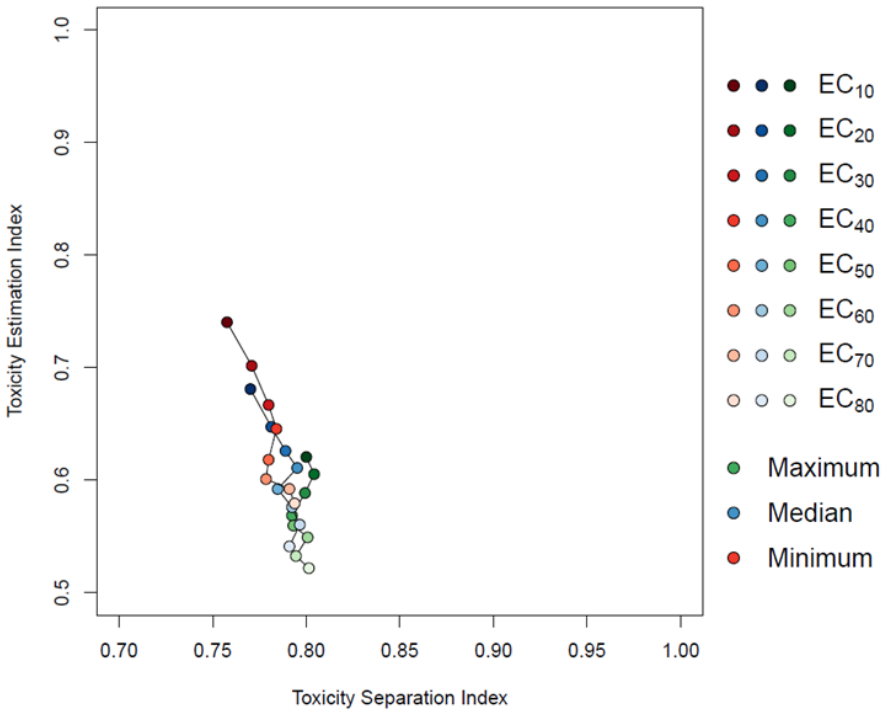

Figure 3.8: Toxicity Separation and Toxicity Estimation Indices for different effective concentrations from the cytotoxicity assay for the extended compound set obtained in PHH from at least three different donors after 48 hours of incubation

As in vivo concentration the 95% percentile of the peak total systemic blood concentration was utilized.

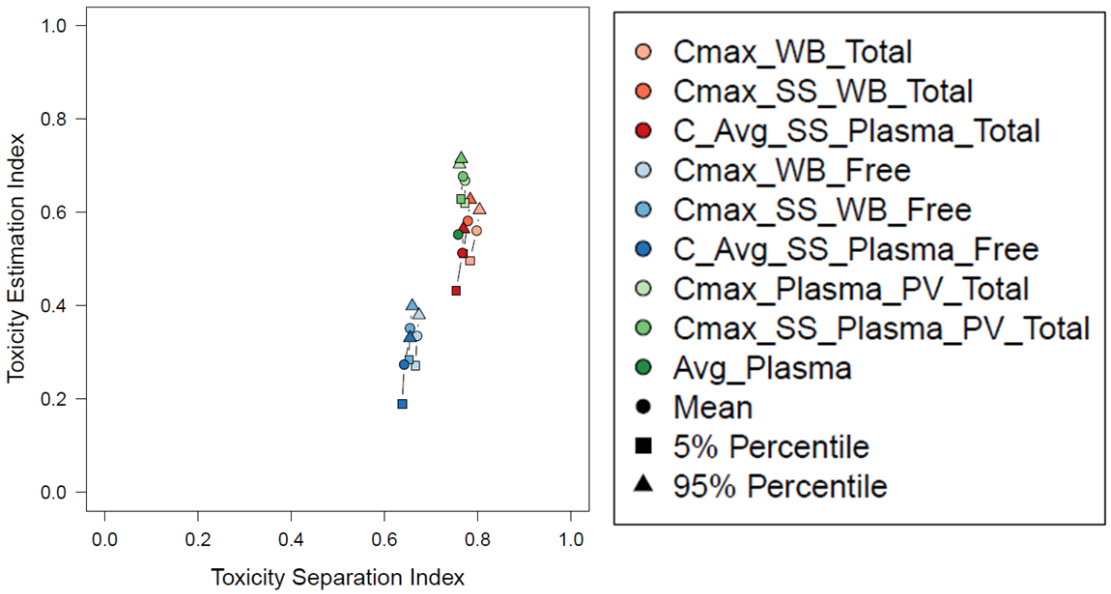

Figure 3.9: Toxicity Separation and Toxicity Estimation Indices for different modelled *in vivo* concentrations for the extended compound set

As in vitro concentration the maximum EC_{20} obtained from the cytotoxicity assay in PHH from at least three different donors after 48 hours of incubation was used. WB= whole blood, Cmax = peak concentration after single dose, ss = steady state after multiple dose, PV = portal vein.

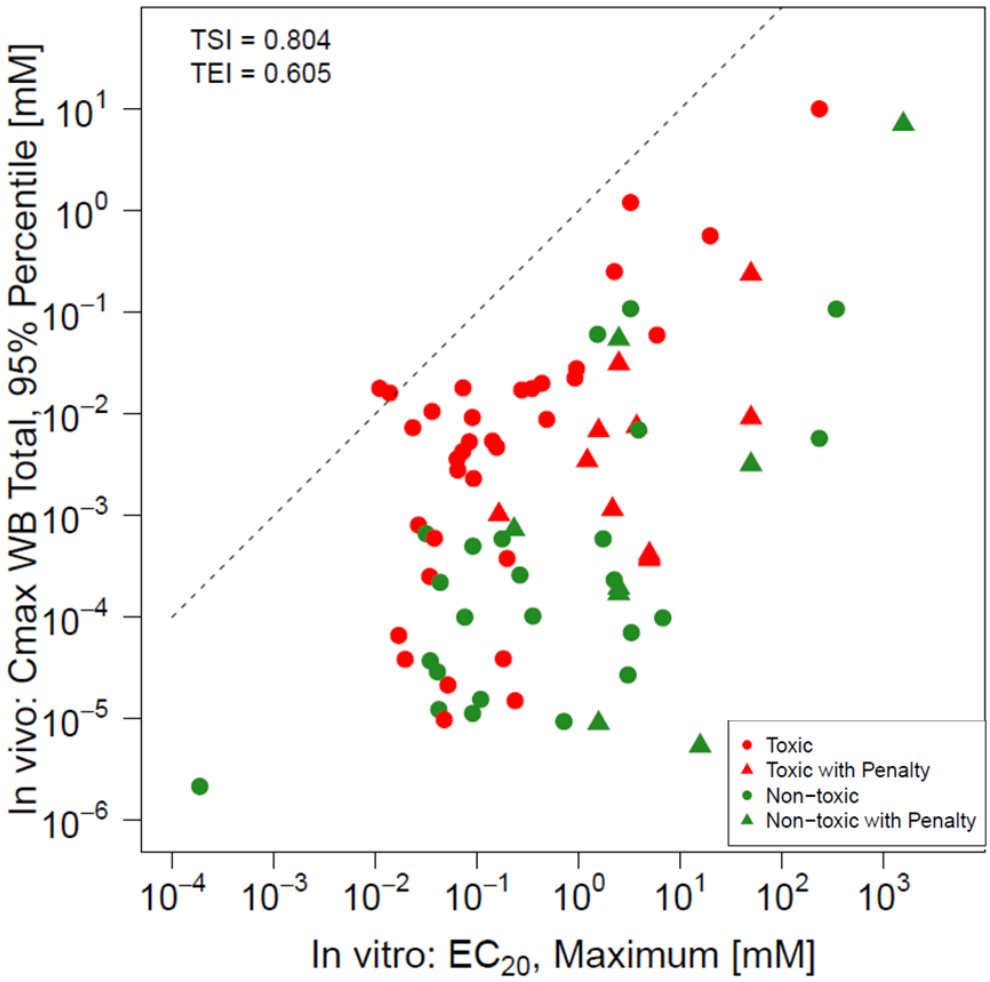

Figure 3.10: Data situation for the cytotoxicity assay of the extended compound set in PHH
The maximal EC_{20} after 48 hours of compound exposure was utilized as in vitro concentration and the 95% percentile of the peak total systemic blood concentration was used as in vivo concentration. Red symbols depict hepatotoxic compounds, while green symbols represent non-hepatotoxic compounds. Circles symbolize compounds for which the EC_{20} was observed whereas triangles illustrate compounds for which no EC_{20} was observed. In this case the highest tested concentration was multiplied by five (referred to as "Penalty"). The dotted line is the iso-concentration line. PHH from at least three different donors were used.

Table 3.7: Summary of the optimized *in vitro* and *in vivo* parameters for the cytotoxicity assay in PHH with the extended set of compounds

The EC_{20} is the maximal EC_{20} after 48 hours of compound exposure. As in vivo concentration the 95% percentile of the total systemic peak whole blood concentration obtained by PBPK modelling is given (The values for the training set compounds are taken from Albrecht et al. 2019).

Compound	Abbreviation	Toxicity	EC_{20} Maximum [mM]	Total systemic peak blood concentration, 95% CI [mM]
Acetaminophen	APAP	Hepatotoxic	3.263	$12.1*10^{-1}$
Acetaminophen	APAP	Non-hepatotoxic	3.263	$10.9*10^{-2}$
Allopurinol	Allo	Hepatotoxic	> 0.75	$7.5*10^{-3}$
Amiodarone	AMIO	Hepatotoxic	> 1	$3.7*10^{-4}$
Aspirin	ASP	Hepatotoxic	> 10	$24.0*10^{-2}$
Atorvastatine	AVS	Hepatotoxic	0.238	$1.5*10^{-5}$
Atropine	ATRO	Non-hepatotoxic	> 3.16	$53.8*10^{-7}$
Benzbromarone	BZB	Hepatotoxic	$1.104*10^{-2}$	$17.9*10^{-3}$
Benztropine	BZT	Non-hepatotoxic	$42.288*10^{-3}$	$1.2*10^{-5}$
Bosentan	BOS	Hepatotoxic	$6.496*10^{-2}$	$2.8*10^{-3}$
Buspirone	BPR	Non-hepatotoxic	$4.087*10^{-2}$	$29.0*10^{-6}$
Busulfan	BUSF	Hepatotoxic	> 1	$3.9*10^{-4}$
Carbamazepine	CBZ	Hepatotoxic	$7.313*10^{-2}$	$18.1*10^{-3}$
Chlorpheniramine	CHL	Non-hepatotoxic	$7.607*10^{-2}$	$10.0*10^{-5}$
Chlorpromazine	CMZ	Hepatotoxic	0.182	$38.9*10^{-6}$
Ciprofloxacin	CIPRO	Hepatotoxic	0.157	$4.7*10^{-3}$
Clofibrate	CLFI	Unclear	> 1	$14.7*10^{-6}$
Clonidine	CLON	Non-hepatotoxic	0.720	$94.0*10^{-7}$
Clozapine	CZP	Hepatotoxic	$6.387*10^{-2}$	$3.6*10^{-3}$
Codeine	COD	Non-hepatotoxic	2.253	$2.3*10^{-4}$
Cyclosporin A	CsA	Hepatotoxic	$2.341*10^{-2}$	$7.3*10^{-3}$
Diclofenac	DFN	Hepatotoxic	$1.427*10^{-1}$	$54.2*10^{-4}$
Digoxin	Digi	Non-hepatotoxic	$0.187*10^{-3}$	$21.5*10^{-7}$
Dimethyl sulfoxide	DMSO	Non-hepatotoxic	346.033	$10.8*10^{-2}$
Diphenhydramine	DPH	Non-hepatotoxic	0.177	$5.9*10^{-4}$
Dipyridamole	DIPY	Unclear	> 0.313	$2.7*10^{-3}$
Erythromycin	Ery	Hepatotoxic	0.487	$88.6*10^{-4}$

Compound	Abbreviation	Toxicity	EC_{20} Maximum [mM]	Total systemic peak blood concentration, 95% CI [mM]
Ethanol	EtOH	Hepatotoxic	235.657	$10.1*10^{0}$
Ethanol	EtOH	Non-hepatotoxic	235.657	$57.6*10^{-4}$
Famotidine	FAM	Non-hepatotoxic	1.750	$59.0*10^{-5}$
Fexofenadine	FFD	Non-hepatotoxic	$> 4.646*10^{-2}$	$7.3*10^{-4}$
Fluconazole	FCA	Hepatotoxic	> 0.5	$31.4*10^{-3}$
Fluoxetine	FLT	Hepatotoxic	$1.968*10^{-2}$	$3.8*10^{-5}$
Glucose	GLC	Non-hepatotoxic	>316	$71.5*10^{-1}$
Hydroxyzine	HYZ	Non-hepatotoxic	$91.398*10^{-3}$	$50.0*10^{-5}$
Ibuprofen	IBU	Hepatotoxic	2.263	$252.6*10^{-3}$
Imipramine	IMP	Hepatotoxic	$3.437*10^{-2}$	$2.5*10^{-4}$
Indomethacine	Indo	Hepatotoxic	$> 2.445*10^{-1}$	$34.8*10^{-4}$
Isoniazid	INAH	Hepatotoxic	5.9410	$60.0*10^{-3}$
Isosorbide dinitrate	ISS	Non-hepatotoxic	0.356	$10.3*10^{-5}$
Ketoconazole	KC	Hepatotoxic	$1.385*10^{-2}$	$16.2*10^{-3}$
Labetalol	LAB	Hepatotoxic	$7.253*10^{-2}$	$42.8*10^{-4}$
Leflunomide	LFM	Hepatotoxic	$2.664*10^{-2}$	$80.9*10^{-5}$
Levofloxacin	LEV	Hepatotoxic	$9.589*10^{-2}$	$28.0*10^{-3}$
Melatonin	MEL	Non-hepatotoxic	3.075	$27.0*10^{-6}$
Methotrexate	MTX	Hepatotoxic	> 0.033	$10.3*10^{-4}$
Methylparaben	MePa	Non-hepatotoxic	> 0.316	$90.0*10^{-7}$
N-acetylcysteine	NAC	Non-hepatotoxic	> 10	$31.9*10^{-4}$
Nevirapine	NVP	Hepatotoxic	$3.486*10^{-1}$	$17.8*10^{-3}$
Nifedipine	NDP	Hepatotoxic	$> 43.282*10^{-2}$	$11.6*10^{-4}$
Nimesulide	NIM	Hepatotoxic	$9.036*10^{-2}$	$92.6*10^{-4}$
Nitrofurantoin	NFT	Hepatotoxic	$9.262*10^{-2}$	$23.2*10^{-4}$
Oxycodone	OXC	Non-hepatotoxic	6.775	$9.8*10^{-5}$
Oxymorphone	OXM	Non-hepatotoxic	$9.089*10^{-2}$	$11.3*10^{-6}$
Paroxetine	PXT	Hepatotoxic	$17.008*10^{-3}$	$6.6*10^{-5}$
Phenacetine	PHNA	Hepatotoxic	> 10	$91.9*10^{-4}$
Phenylbutazon	PhB	Hepatotoxic	$9.225*10^{-2}$	$22.6*10^{-3}$
Phenytoin	PTN	Hepatotoxic	> 0.316	$68.7*10^{-4}$

Compound	Abbreviation	Toxicity	EC_{20} Maximum [mM]	Total systemic peak blood concentration, 95% CI [mM]
Pindolol	PIN	Non-hepatotoxic	> 0.5	$17.1*10^{-5}$
Pioglitazone	PIO	Hepatotoxic	$8.431*10^{-2}$	$5.3*10^{-3}$
Primaquine	Prima	Non-hepatotoxic	$3.171*10^{-2}$	$6.6*10^{-4}$
Primidone	PRI	Non-hepatotoxic	> $49.966*10^{-2}$	$54.8*10^{-3}$
Promethazine	PMZ	Non-hepatotoxic	$3.478*10^{-2}$	$37.2*10^{-6}$
Propranolol	PPL	Non-hepatotoxic	$4.361*10^{-2}$	$22.0*10^{-5}$
Pyridoxine	PDX	Non-hepatotoxic	$33.195*10^{-1}$	$7.0*10^{-5}$
Rifampicin	RIF	Hepatotoxic	0.437	$20.1*10^{-3}$
Rosiglitazone	RGZ	Hepatotoxic	$38.197*10^{-3}$	$59.9*10^{-5}$
Rosuvastatin	ROS	Hepatotoxic	$47.775*10^{-3}$	$97.8*10^{-7}$
Simvastatin	SIM	Hepatotoxic	$5.184*10^{-2}$	$2.1*10^{-5}$
Sitaxentan sodium	SXS	Hepatotoxic	0.276	$17.3*10^{-3}$
Sodium phenylbutyrate	SPB	Unclear	> 10	$911.1*10^{-3}$
Terbinafine	Terbi	Hepatotoxic	> 1	$41.9*10^{-5}$
Theophylline	THE	Non-hepatotoxic	1.542	$609.6*10^{-4}$
Tolbutamide	TOL	Unclear	> 3.16	$313.6*10^{-3}$
Tolcapone	Tolc	Hepatotoxic	$3.621*10^{-2}$	$10.6*10^{-3}$
Tolterodine	TTD	Non-hepatotoxic	0.109	$15.5*10^{-6}$
Triclosan	TSN	Non-hepatotoxic	$2.657*10^{-1}$	$26.0*10^{-5}$
Valproic acid	VPA	Hepatotoxic	19.811	$56.9*10^{-2}$
Vancomycin	VANC	Unclear	> 1	$14.9*10^{-3}$
Verapamil	VERA	Hepatotoxic	$1.987*10^{-1}$	$37.8*10^{-5}$
Vitamin C	VitC	Non-hepatotoxic	3.915	$59.9*10^{-4}$
Zaleplon	ZAL	Non-hepatotoxic	> 0.5	$1.9*10^{-4}$

3.4.2 SVM based classification based on the cytotoxicity assay data in PHH for the extended compound set

For compounds of the extended set with a clear toxicity status a SVM based leave one out classification was performed utilizing the maximal EC_{20} after 48 hours of compound exposure as *in vitro* concentration and the 95% confidence interval of the total, systemic peak blood concentration as *in vivo* concentration. This led to a sensitivity of 77.8%, a specificity of 59.4%, an accuracy of 70.1%, a positive predictive value of 72.9% and a negative predictive value of 65.6%. The obtained results are depicted in **Figure 3.11**, while an overview of the classifier performance metrics is provided by **Table 3.8**. In addition, a classifier utilizing all 77 *in vitro/in vivo* concentration pairs was built and used for classification of the five compounds with unclear toxicity status. The results for these compounds are summarized in **Table 3.9.** All probabilities for toxicity are given in **Supplement 8.**

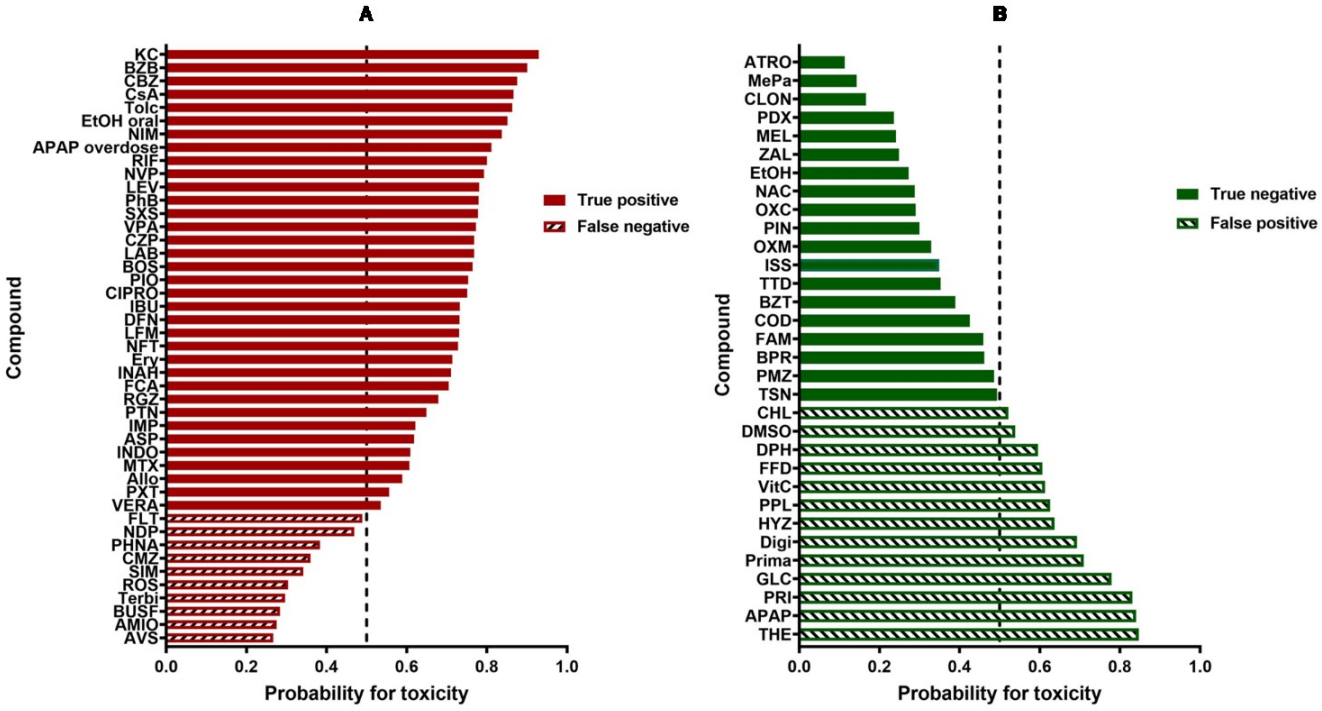

Figure 3.11: SVM based leave one out classification of the 77 extended set compounds with defined toxicity status in PHH

As in vitro concentration the maximal EC $_{20}$ using PHH of three different donors after 48 hours of compound exposure is chosen. As in vivo concentration the 95% percentile of peak total systemic whole blood concentration was used. Compounds exceeding a probability for toxicity of 0.5 were predicted to be toxic. (A) toxic compounds. (B) non-toxic compounds.

Table 3.8: Summary of the performance metrics for the cytotoxicity assay based SVM based classifier for PHH and the extended set compounds

Parameter	Value
True positives	35
True negatives	19
False negatives	10
False positives	13
Sensitivity	77.8%
Specificity	59.4%
Accuracy	70.1%
Positive predictive value (PPV)	72.9%
Negative predictive value (NPV)	65.6%

Table 3.9: SVM based classification of compounds with unclear toxicity status based on the cytotoxicity assay in PHH after 48 hours of compound exposure and comparison to literature data

Compound	Abbreviation	Classifier result	LIverTox Likelihood score	DILI rank DILI concern	DILIlist classification
Clofibrate	CLFI	Non-toxic	D	Verified Less-DILI concern	DILI positive
Dipyridamole	DIPY	Toxic	E*	Ambiguous DILI concern	Not included
Sodium phenylbutyrate	SPB	Toxic	E	Ambiguous DILI concern	Not included
Tolbutamide	TOL	Toxic	B (class)	Ambiguous DILI concern	DILI negative
Vancomycin	VANC	Toxic	B (usually in association with DRESS)	Verified Less-DILI concern	DILI positive

3.5 Glutathione depletion assay in PHH

Glutathione (GSH) depletion as indicator of oxidative stress was considered as an additional readout. The GDH depletion assay was performed **for the 28 compounds** of the training set in PHH from three donors. Analysis after curve fitting revealed that the best TSI was obtained if the minimal EC_{80} was selected as *in vitro* concentration. Combining this with the 95% percentile for the total systemic peak plasma concentration following a single exposure as *in vivo* concentration a TSI of 0.955 and a TEI of 0.706 were obtained. **Figure 3.12** summarizes the performance metrics for different thresholds and donors of the glutathione depletion assay in PHH, while **Figure 3.13** illustrates the data situation for the highest scoring threshold. Matching the TSI value a high degree of compound separation is displayed.

Following this optimization step the glutathione depletion assay was combined with the cytotoxicity assay as well as the previously published gene expression data for *CYP1B1*, *CYP3A7* and *G6PD* after 24 hours of compound exposure. As shown in **Table 3.10** the obtained TSI of the cytotoxicity assay could not be improved by the addition of the GSH depletion assay, the gene expression analysis or both. Moreover, inclusion of the GSH depletion data did – contrary to the gene expression analysis data – not improve the TEI. Therefore, inclusion of the glutathione depletion assay provided no added value for the test system.

Table 3.10: TSI and TEI for the combination of different *in vitro* assays in PHH with training set compounds

As in vivo in vivo concentration the 95% percentile of the total systemic peak blood concentration following a single exposure was utilized. For the combinations the LOEC was used as in vitro concentration (The gene expression, cytotoxicity and in vivo data was obtained from Albrecht et al. 2019).

***In vitro* assays**	**TSI**	**TEI**
Median EC_{10} cytotoxicity	0.996	0.844
Median alert concentration gene expression	0.739	0.770
Minimum EC_{80} GSH depletion	0.955	0.706
Cytotoxicity + gene expression	0.996	0.887
Cytotoxicity+ GSH depletion	0.996	0.844
Gene expression + GSH depletion	0.982	0.818
All assays	0.996	0.887

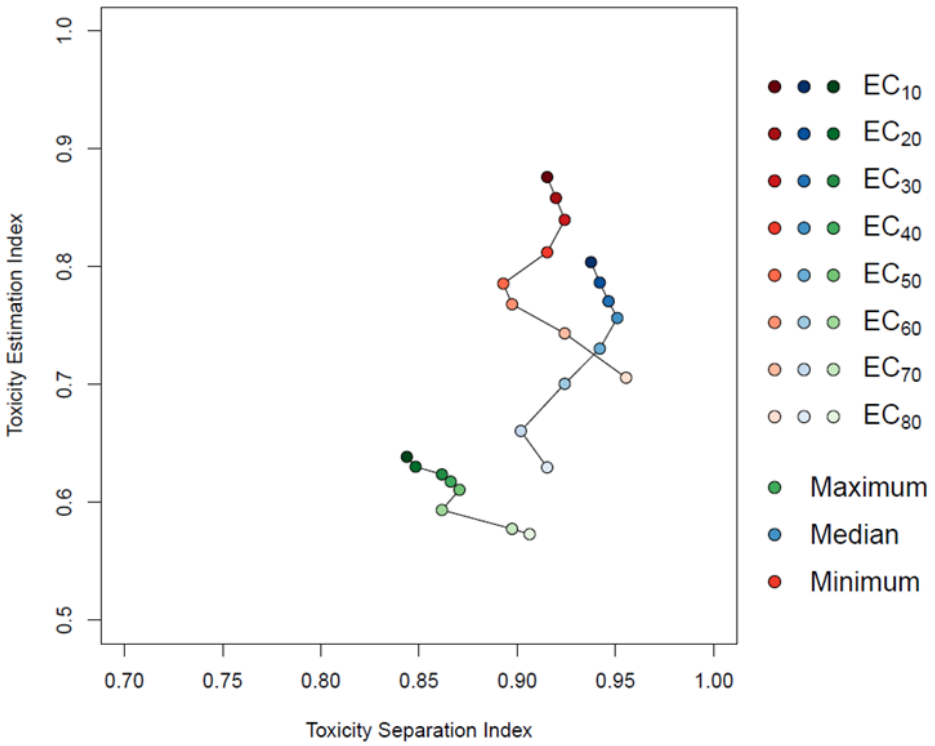

Figure 3.12: TSI and TEI for the GSH depletion assay in PHH after 48 hour incubation with the 28 training set compounds

The different colors depict the three independent donors while the shade of the color symbolizes the increasing effective concentrations with the EC_{10} being the darkest shade and the EC_{80} the lightest shade. As in vivo concentration the 95% percentile of the total systemic peak blood concentration following a single exposure was utilized. The experiments were done with cells from three different donors.

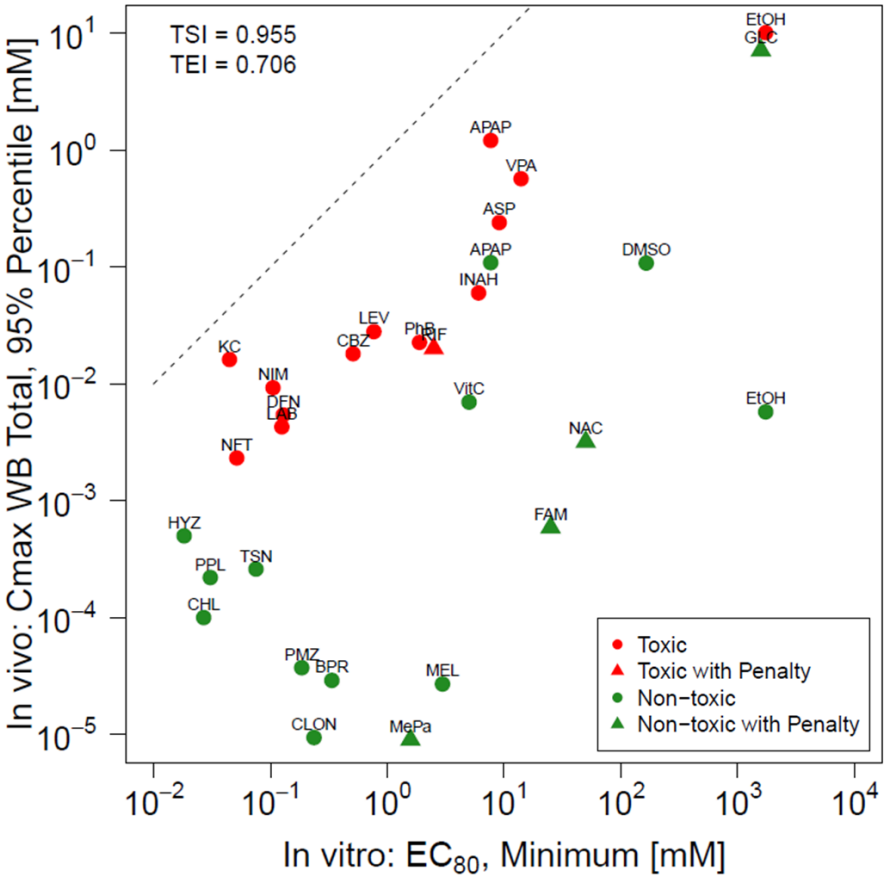

Figure 3.13: Data situation for the minimum EC_{80} of the GSH depletion assay in PHH following 48 hour exposure to the 28 training set compounds

Green shapes depict non-hepatotoxic compounds, while red shapes symbolize hepatotoxic compounds. The dashed line is the iso-concentration line. In case the EC80 was not observed the highest tested concentration was multiplied by 5 ("Penalty"). The minimum refers to the most sensitive tested donor. These compounds are represented by triangles. As in vivo concentration the 95% percentile of the total systemic peak blood concentration following a single exposure was utilized. The experiments were performed with cells from three different donors. Cmax: Peak concentration; WB: whole blood.

3.6 Performance of the *in vitro* cytotoxicity test system in HepG2 cells

3.6.1 Toxicity Separation Index and Toxicity Estimation Index for the training set compounds

In addition to the PHH the same *in vitro* assays were performed with the HepG2 cell line. As a first step the optimal *in vitro* effective concentration was determined by systematic evaluation of different effective concentrations for the training set compounds after 48 hours of compound exposure in three replicates utilizing the 95% percentile of the total systemic peak blood concentration after a single exposure as *in vivo* concentration. This analysis revealed

that the optimal TSI and TEI obtained were 0.951 and 0.880 achieved by the minimal EC_{10} (**Figure 3.14**).

The TSI was worse compared with the TSI of the cytotoxicity assay with the same compound set (TSI = 0.996) but the TEI was better compared with the TEI for the PHH (TEI = 0.844). **Figure 3.15** illustrates the data situation for this combination, while the resulting *in vitro* and *in vivo* concentration pairs are tabulated in **Table 3.11.** All indices are summarized in **Supplement 7.**

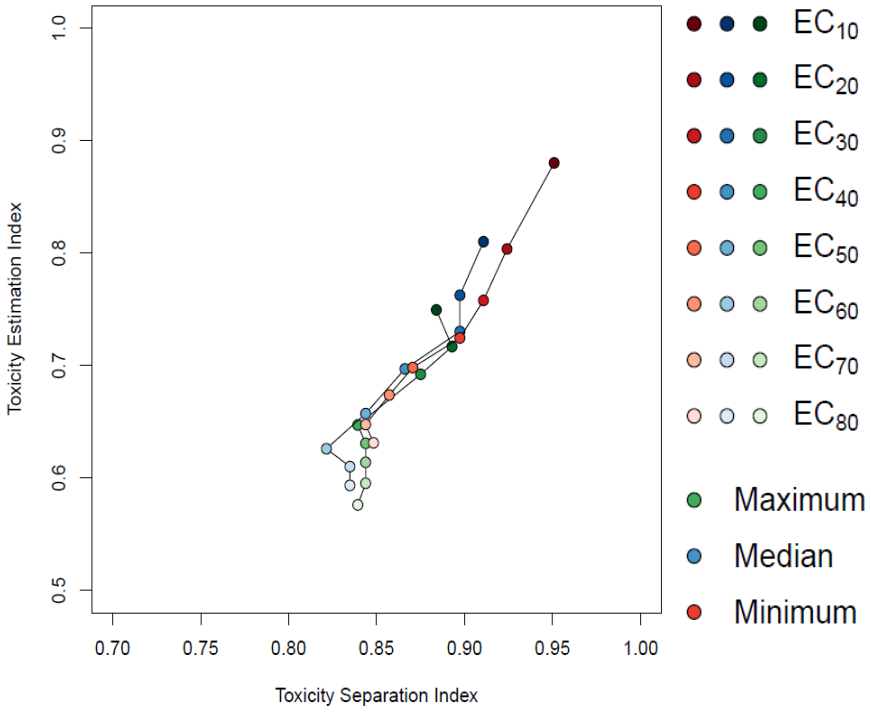

Figure 3.14: TSI and TEI after 48 hours of exposure with the training set compounds in HepG2
As in vitro concentration and the 95% percentile of the total, systemic blood concentration was used as in vivo concentration. All experiments were carried out in three replicates.

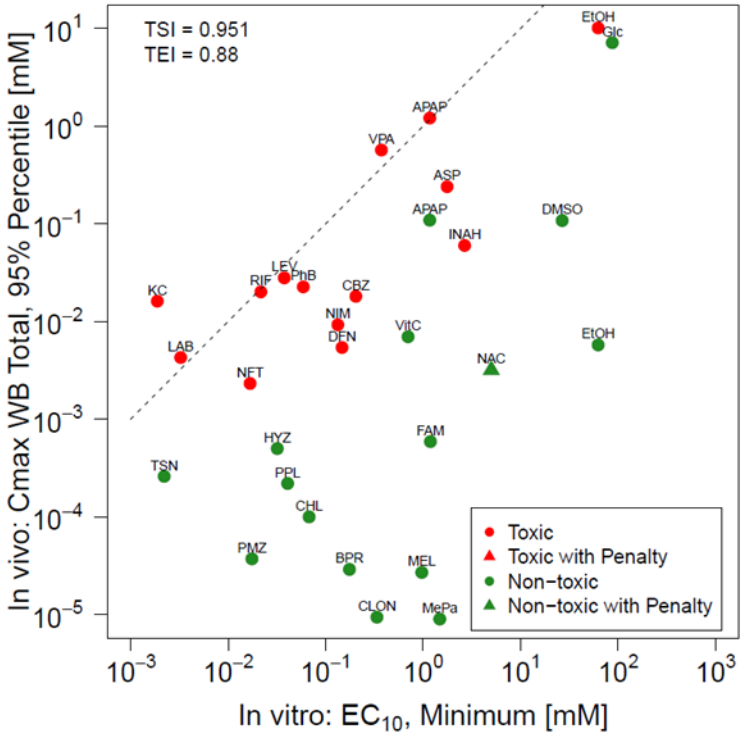

Figure 3.15: Data situation for the cytotoxicity asay after 48 hours compound exposure with the training set compounds in HepG2

As in vitro concentration the minimal EC_{10} was utilized, while the 95% percentile of the total, systemic blood concentration was used as in vivo concentration. The dashed line represents the iso-concentration line. In case the EC10 was not observed the highest tested concentration was multiplied by 5 ("Penalty"). The minimum is the most sensitive of all replicates. All experiments were carried out in three replicates (Data taken from Albrecht et al.2019

Table 3.11: Summary of the optimized *in vitro* and *in vivo* parameters for the cytotoxicity assay in HepG2 with the training set compounds

The EC_{10} is the minimal EC_{10} after 48 hours of compound exposure. As in vivo concentration the 95% percentile of the total systemic peak whole blood concentration obtained by PBPK modelling is given. The values are taken from Albrecht et al. 2019).

Compound	Abbreviation	Toxicity	EC10 Minimum [mM]	Total systemic peak blood concentration, 95% CI [mM]
Acetaminophen	APAP	Hepatotoxic	1.169	$12.1*10^{-1}$
Acetaminophen	APAP	Non-hepatotoxic	1.169	$10.9*10^{-2}$
Aspirin	ASP	Hepatotoxic	1.761	$24.0*10^{-2}$
Buspirone	BPR	Non-hepatotoxic	0.176	$29.0*10^{-6}$
Carbamazepine	CBZ	Hepatotoxic	0.024	$18.1*10^{-3}$

Compound	Abbreviation	Toxicity	EC10 Minimum [mM]	Total systemic peak blood concentration, 95% CI [mM]
Chlorpheniramine	CHL	Non-hepatotoxic	0.068	$10.0*10^{-5}$
Clonidine	CLON	Non-hepatotoxic	0.336	$94.0*10^{-7}$
Diclofenac	DFN	Hepatotoxic	0.147	$54.2*10^{-4}$
Dimethyl sulfoxide	DMSO	Non-hepatotoxic	26.670	$10.8*10^{-2}$
Ethanol	EtOH	Hepatotoxic	62.399	$10.1*10^{0}$
Ethanol	EtOH	Non-hepatotoxic	62.399	$57.6*10^{-4}$
Famotidine	FAM	Non-hepatotoxic	1.189	$59.0*10^{-5}$
Glucose	GLC	Non-hepatotoxic	87.153	$71.5*10^{-1}$
Hydroxyzine	HYZ	Non-hepatotoxic	0.032	$50.0*10^{-5}$
Isoniazid	INAH	Hepatotoxic	2.665	$60.0*10^{-3}$
Ketoconazole	KC	Hepatotoxic	0.002	$16.2*10^{-3}$
Labetalol	LAB	Hepatotoxic	0.003	$42.8*10^{-4}$
Levofloxacin	LEV	Hepatotoxic	0.038	$28.0*10^{-3}$
Melatonin	MEL	Non-hepatotoxic	0.968	$27.0*10^{-6}$
Methylparaben	MePa	Non-hepatotoxic	1.481	$90.0*10^{-7}$
N-acetylcysteine	NAC	Non-hepatotoxic	>10	$31.9*10^{-4}$
Nimesulide	NIM	Hepatotoxic	0.133	$92.6*10^{-4}$
Nitrofurantoin	NFT	Hepatotoxic	0.017	$23.2*10^{-4}$
Phenylbutazone	PhB	Hepatotoxic	0.059	$22.6*10^{-3}$
Promethazine	PMZ	Non-hepatotoxic	0.018	$37.2*10^{-6}$
Propranolol	PPL	Non-hepatotoxic	0.041	$22.0*10^{-5}$
Rifampicin	RIF	Hepatotoxic	0.022	$20.1*10^{-3}$
Triclosan	TSN	Non-hepatotoxic	0.002	$26.0*10^{-5}$
Valproic acid	VPA	Hepatotoxic	0.373	$56.9*10^{-2}$
Vitamin C	VitC	Non-hepatotoxic	0.701	$59.9*10^{-4}$

3.6.2 Toxicity separation index and toxicity estimation index for the extended compound set in HepG2 cells

For the 75 compounds with clear human hepatotoxicity status the TSI and TEI for a variety of different effective concentrations in the cytotoxicity after 48 hours of compound exposure in HepG2 were analyzed. As *in vivo* concentration the 95% percentile of the total, systemic peak blood concentration was used. This analysis revealed that the minimal EC_{10} provided the highest TSI of 0.843 as well as the highest TEI of 0.765. The obtained indices are illustrated in **Figure 3.16.** All indices are summarized in **Supplement 7.**

Figure 3.17 visualizes the data situation for the extended compound set in HepG2 utilizing the minimal EC_{10} as *in vitro* parameter and the 95% percentile of the total systemic peak blood concentration as *in vivo* parameter. As shown in the figure and indicated by the TSI a trend for separation of the hepatotoxic and non-hepatotoxic compounds was observed. The TEI indicates that the mean effective *in vitro* concentration for the hepatotoxic compounds was in the 31.6-fold range of the relevant *in vivo* concentrations. **Table 3.12** summarizes the optimized *in vitro* and *in vivo* concentrations.

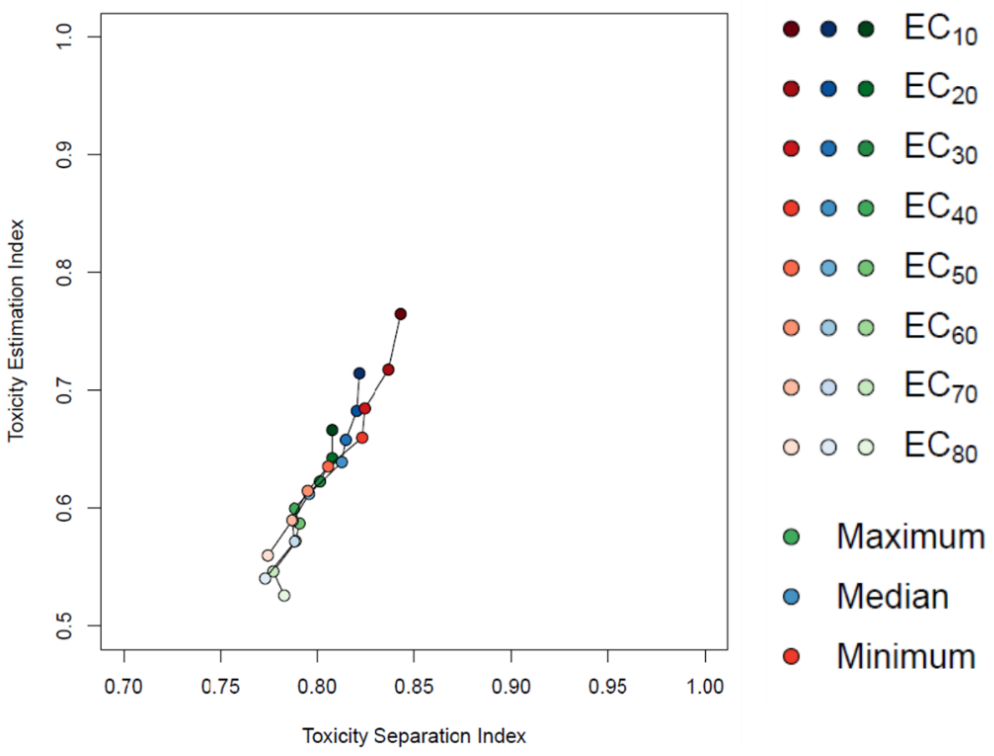

Figure 3.16: Toxicity Separation and Toxicity Estimation Indices for the cytotoxicity in HepG2
95% percentile of the peak total, systemic blood concentration was used. All experiments were done in at least three replicates.

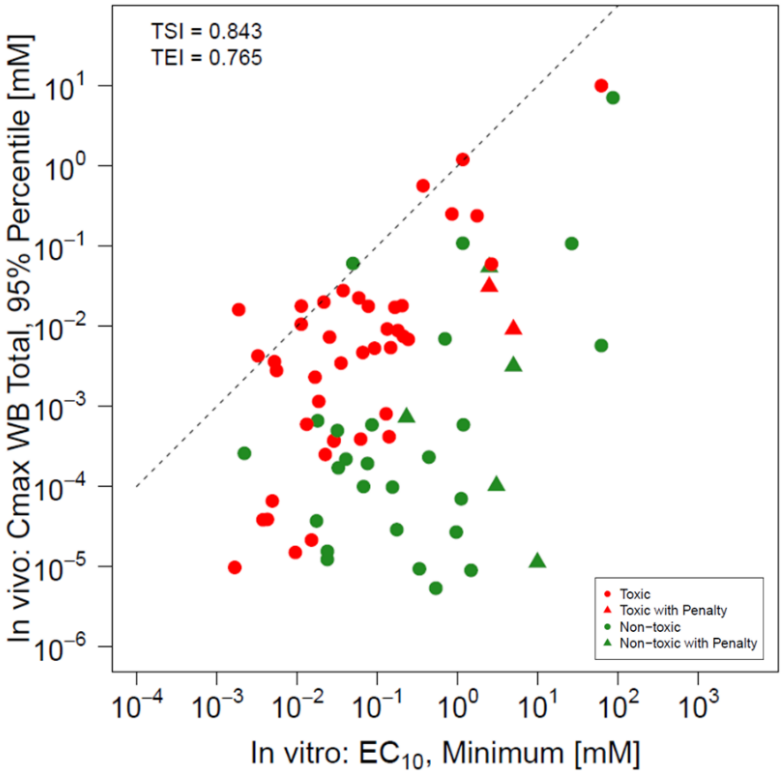

Figure 3.17: Data situation for the cytotoxicity assay for extended compound set in HepG2
The minimal EC_{10} after 48 hours of compound exposure was used as in vitro concentration and the 95% percentile of the peak total systemic blood concentration was used as in vivo concentration. Red symbols depict hepatotoxic compounds, while green symbols represent non-hepatotoxic compounds. Circles symbolize compounds for which the EC_{10} was observed whereas triangles illustrate compounds for which no EC_{10} was overserved. In the latter case the highest tested concentration was multiplied by five (penalty). The dotted line is the iso-concentration line. All experiments were done in at least three replicates.

Table 3.12: Summary of the optimized *in vitro* and *in vivo* parameters for the cytotoxicity assay in HepG2 cells with the extended set of compounds
The EC_{10} is the minimal EC_{10} after 48 hours of compound exposure. As in vivo concentration the 95% percentile of the total systemic peak whole blood concentration obtained by PBPK modelling is given (The values for the training set compounds are taken from Albrecht et al. 2019).

Compound	Abbreviation	Toxicity	EC_{10} Minimum[mM]	Total systemic peak blood concentration, 95% CI [mM]
Acetaminophen	APAP	Hepatotoxic	1.169	$12.1*10^{-1}$
Acetaminophen	APAP	Non-hepatotoxic	1.169	$10.9*10^{-2}$

Compound	Abbreviation	Toxicity	EC_{10} Minimum[mM]	Total systemic peak blood concentration, 95% CI [mM]
Allopurinol	Allo	Hepatotoxic	$2.119*10^{-1}$	$7.5*10^{-3}$
Amiodarone	AMIO	Hepatotoxic	$2.880*10^{-2}$	$3.7*10^{-4}$
Aspirin	ASP	Hepatotoxic	1.761	$24.0*10^{-2}$
Atorvastatine	AVS	Hepatotoxic	9.542*10-3	$1.5*10^{-5}$
Atropine	ATRO	Non-hepatotoxic	0.539	$53.8*10^{-7}$
Benzbromarone	BZB	Hepatotoxic	$1.133*10^{-2}$	$17.9*10^{-3}$
Benztropine	BZT	Non-hepatotoxic	$2.389*10^{-2}$	$1.2*10^{-5}$
Bosentan	BOS	Hepatotoxic	$5.569*10^{-3}$	$2.8*10^{-3}$
Buspirone	BPR	Non- -hepatotoxic	$1.756*10^{-1}$	$29.0*10^{-6}$
Busulfan	BUSF	Hepatotoxic	$6.226*10^{-2}$	$3.9*10^{-4}$
Carbamazepine	CBZ	Hepatotoxic	0.204	$18.1*10^{-3}$
Chlorpheniramine	CHL	Non-hepatotoxic	$6.766*10^{-2}$	$10.0*10^{-5}$
Chlorpromazine	CMZ	Hepatotoxic	$4.277*10^{-3}$	$38.9*10^{-6}$
Ciprofloxacin	CIPRO	Hepatotoxic	$6.617*10^{-2}$	$4.7*10^{-3}$
Clofibrate	CLFI	Unclear	>1	$14.7*10^{-6}$
Clonidine	CLON	Non-hepatotoxic	$3.359*10^{-1}$	$94.0*10^{-7}$
Clozapine	CZP	Hepatotoxic	$5.242*10^{-3}$	$3.6*10^{-3}$
Codeine	COD	Non-hepatotoxic	$4.417*10^{-1}$	$2.3*10^{-4}$
Cyclosporin A	CsA	Hepatotoxic	$25.499*10^{-3}$	$7.3*10^{-3}$
Diclofenac	DFN	Hepatotoxic	0.1473	$54.2*10^{-4}$
Digoxin	Digi	Non-hepatotoxic	$1.6*10^{-5}$	$21.5*10^{-7}$
Dimethyl sulfoxide	DMSO	Non-hepatotoxic	26.670	$10.8*10^{-2}$
Diphenhydramine	DPH	Non-hepatotoxic	$8.604*10^{-2}$	$5.9*10^{-4}$
Dipyridamole	DIPY	Unclear	$3.708*10^{-3}$	$2.7*10^{-3}$
Erythromycin	Ery	Hepatotoxic	0.181	$88.6*10^{-4}$
Ethanol	EtOH	Hepatotoxic	62.399	$10.1*10^{0}$
Ethanol	EtOH	Non-hepatotoxic	62.399	$57.6*10^{-4}$
Famotidine	FAM	Non-hepatotoxic	1.189	$59.0*10^{-5}$
Fexofenadine	FFD	Non-hepatotoxic	$>4.646*10^{-2}$	$7.3*10^{-4}$
Fluconazole	FCA	Hepatotoxic	>0.5	$31.4*10^{-3}$
Fluoxetine	FLT	Hepatotoxic	$3.764*10^{-3}$	$3.8*10^{-5}$

Compound	Abbreviation	Toxicity	EC_{10} Minimum[mM]	Total systemic peak blood concentration, 95% CI [mM]
Glucose	GLC	Non-hepatotoxic	87.153	$71.5*10^{-1}$
Hydroxyzine	HYZ	Non-hepatotoxic	$3.191*10^{-2}$	$50.0*10^{-5}$
Ibuprofen	IBU	Hepatotoxic	0.856	$252.6*10^{-3}$
Hydroxyzine	HYZ	Non-hepatotoxic	$3.191*10^{-2}$	$50.0*10^{-5}$
Ibuprofen	IBU	Hepatotoxic	0.856	$252.6*10^{-3}$
Imipramine	IMP	Hepatotoxic	$2.255*10^{-2}$	$2.5*10^{-4}$
Indomethacine	Indo	Hepatotoxic	$3.542*10^{-2}$	$34.8*10^{-4}$
Isoniazide	INAH	Hepatotoxic	$26.645*10^{-1}$	$60.0*10^{-3}$
Isosorbide dinitrate	ISS	Non-hepatotoxic	>0.614	$10.3*10^{-5}$
Ketoconazole	KC	Hepatotoxic	$14.488*10^{-3}$	$16.2*10^{-3}$
Labetalol	LAB	Hepatotoxic	$32.618*10^{-4}$	$42.8*10^{-4}$
Leflunomide	LFM	Hepatotoxic	0.129	$80.9*10^{-5}$
Levofloxacin	LEV	Hepatotoxic	$3.766*10^{-2}$	$28.0*10^{-3}$
Melatonin	MEL	Non-hepatotoxic	0.968	$27.0*10^{-6}$
Methotrexate	MTX	Hepatotoxic	$4*10^{-6}$	$10.3*10^{-4}$
Methylparaben	MePa	Non-hepatotoxic	1.481	$90.0*10^{-7}$
N-acetylcysteine	NAC	Non-hepatotoxic	>10	$31.9*10^{-4}$
Nevirapine	NVP	Hepatotoxic	$7.750*10^{-2}$	$17.8*10^{-3}$
Nifedipine	NDP	Hepatotoxic	$1.872*10^{-2}$	$11.6*10^{-4}$
Nimesulide	NIM	Hepatotoxic	0.133	$92.6*10^{-4}$
Nitrofurantoin	NFT	Hepatotoxic	$1.680*10^{-2}$	$23.2*10^{-4}$
Oxycodone	OXC	Non-hepatotoxic	0.155	$9.8*10^{-5}$
Oxymorphone	OXM	Non-hepatotoxic	>1.991	$11.3*10^{-6}$
Paroxetine	PXT	Hepatotoxic	4.9267	$6.6*10^{-5}$
Phenacetine	PHNA	Hepatotoxic	>1	$91.9*10^{-4}$
Phenylbutazon	PhB	Hepatotoxic	$5.901*10^{-2}$	$22.6*10^{-3}$
Phenytoin	PTN	Hepatotoxic	0.244	$68.7*10^{-4}$
Pindolol	PIN	Non-hepatotoxic	$3.277*10^{-2}$	$17.1*10^{-5}$
Pioglitazone	PIO	Hepatotoxic	$9.270*10^{-2}$	$5.3*10^{-3}$
Primaquine	Prima	Non-hepatotoxic	$1.811*10^{-2}$	$6.6*10^{-4}$

Compound	Abbreviation	Toxicity	EC_{10} Minimum[mM]	Total systemic peak blood concentration, 95% CI [mM]
Primidone	PRI	Non-hepatotoxic	>49.966*10^{-2}	54.8*10^{-3}
Promethazine	PMZ	Non-hepatotoxic	1.755*10^{-2}	37.2*10^{-6}
Propranolol	PPL	Non-hepatotoxic	4.078*10^{-2}	22.0*10^{-5}
Pyridoxine	PDX	Non-hepatotoxic	1.117	7.0*10^{-5}
Rifampicin	RIF	Hepatotoxic	2.164*10^{-2}	20.1*10^{-3}
Rosiglitazone	RGZ	Hepatotoxic	13.205*10^{-2}	59.9*10^{-5}
Rosuvastatin	ROS	Hepatotoxic	1.676*10^{-3}	97.8*10^{-7}
Simvastatin	SIM	Hepatotoxic	1.519*10^{-2}	2.1*10^{-5}
Sitaxentan sodium	SXS	Hepatotoxic	0.166	17.3*10^{-3}
Sodium phenylbutyrate	SPB	Unclear	3.125	911.1*10^{-3}
Terbinafine	Terbi	Hepatotoxic	1.409*10-1	41.9*10^{-5}
Theophylline	THE	Non-hepatotoxic	4.982*10^{-2}	609.6*10^{-4}
Tolbutamide	TOL	Unclear	0.908	313.6*10^{-3}
Tolcapone	Tolc	Hepatotoxic	1.131*10^{-2}	10.6*10^{-3}
Tolterodine	TTD	Non-hepatotoxic	2.395*10^{-2}	15.5*10^{-6}
Triclosan	TSN	Non-hepatotoxic	22.099*10^{-4}	26.0*10^{-5}
Valproic acid	VPA	Hepatotoxic	0.373	56.9*10^{-2}
Vancomycin	VANC	Unclear	>1	14.9*10^{-3}
Verapamil	VERA	Hepatotoxic	2.898*10^{-2}	37.8*10^{-5}
Vitamin C	VitC	Non-hepatotoxic	7.006*10^{-1}	59.9*10-^{4}
Zaleplon	ZAL	Non-hepatotoxic	7.576*10^{-2}	1.9*10^{-4}

3.6.3 SVM based classification based on the cytotoxicity assay data in HepG2 for the extended compound set

Analogous to the analysis in PHH, a SVM based leave-one-out classification for HepG2 cells was performed utilizing the minimal EC_{10} after 48 hours of compound exposure as *in vitro* concentration and the 95% confidence interval of the total, systemic peak blood concentration as *in vivo* concentration for the compounds of the extended set with a clear toxicity status. This yielded a sensitivity of 88.9 %, a specificity of 62.5%, an accuracy of 77.9 %, a positive predictive value of 76.9% and a negative predictive value of 80%. The obtained results are

depicted in **Figure 3.18,** while an overview of the classifier performance metrics is provided by **Table 3.13**. In addition, a classifier utilizing all 77 *in vitro/in vivo* concentration pairs was built and used for classification of the five compounds with unclear toxicity status. The results are summarized in **Table 3.14.** All probabilities are given in **Supplement 8**.

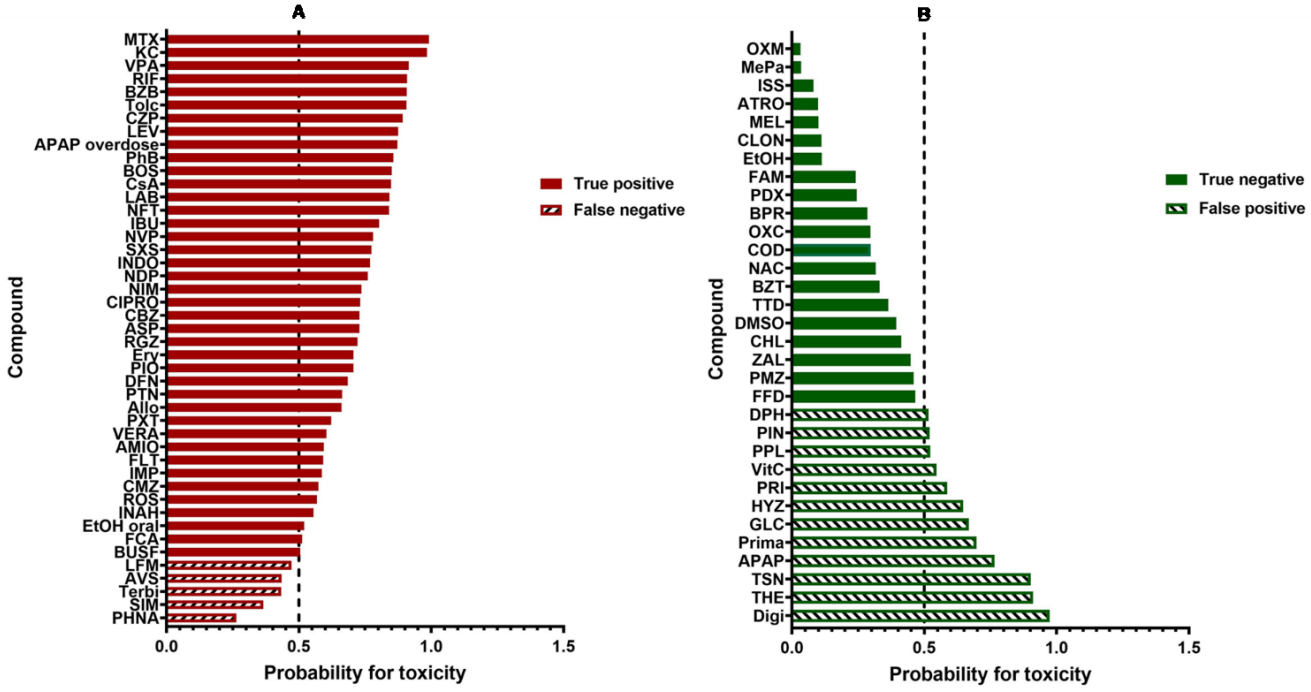

Figure 3.18: SVM based leave-one-out classification of the 75 extended set compounds with defined toxicity status utilizing HepG2 cells

As in vitro concentration the minimal EC_{10} using HepG2 cells of after 48 hours of compound exposure is chosen. As in vivo concentration the 95% percentile of peak total systemic whole blood concentration was used. Compounds exceeding a probability for toxicity of 0.5 were predicted to be toxic. (A) toxic compounds. (B) non-toxic compounds.

Table 3.13: Summary of the performance metrics for the cytotoxicity assay based SVM based classifier for HepG2 and the extended set compounds

Parameter	Value
True positives	40
True negatives	20
False negatives	5
False positives	12
Sensitivity	88.9%
Specificity	62.5%
Accuracy	77.9%
Positive predictive value (PPV)	76.9%
Negative predictive value (NPV)	80%

Table 3.14: SVM based classification of compounds with unclear toxicity status based on the cytotoxicity assay in HepG2 after 48 hours of compound exposure and comparison to literature data

Compound	Abbreviation	Classifier result	LiverTox Likelihood score	DILI rank DILI concern	DILIlist classification
Clofibrate	CLFI	Non-toxic	D	Verified Less-DILI concern	DILI positive
Dipyridamole	DIPY	Toxic	E*	Ambiguous DILI concern	Not included
Sodium phenylbutyrate	SPB	Toxic	E	Ambiguous DILI concern	Not included
Tolbutamide	TOL	Toxic	B (class)	Ambiguous DILI concern	DILI negative
Vancomycin	VANC	Non-toxic	B (usually in association with DRESS)	verified Less-DILI concern	DILI positive

3.6.4 Glutathione depletion assay in HepG2 cells

For the training set compounds the GSH depletion assay was carried out with the HepG2 cell line. **Figure 3.19** summarizes the obtained TSI and TEI for a multitude of different effective concentrations after 48 hours of compound exposure. Utilizing the 95% percentile of the total, systemic, peak blood concentration as *in vivo* parameter the maximal EC_{10}, the maximal EC_{20}, the maximal EC_{30} and the minimal EC_{50} all yielded the optimal TSI of 0.960. Since the maximal EC_{10} provided the highest TEI of 0.773 out of these options it was chosen as the optimal *in vitro* parameter. All indices are given in **Supplement 7**.

Figure 3.20 illustrates the data situation for the optimal combination demonstrating an almost complete separation of the toxic and non-toxic compounds as reflected in the high TSI. To

evaluate a possible improvement by inclusion of the GSH depletion assay into the test battery for HepG2, the data obtained from Albrecht et al. 2019 for cytotoxicity, gene expression analysis and glutathione depletion assay were combined and the TSI and TEI calculated.

The results are summarized in **Table 3.15**. Interestingly, the GSH assay alone yielded already a better TSI than the cytotoxicity assay alone. The combination of the cytotoxicity and the GSH depletion assay yielded the optimal TSI and TEI of 0.969 and 0.892 respectively, showing that the GSH depletion assay may be useful to improve the HepG2 *in vitro* test system. Further inclusion of the gene expression analysis data did not improve the test system in HepG2.

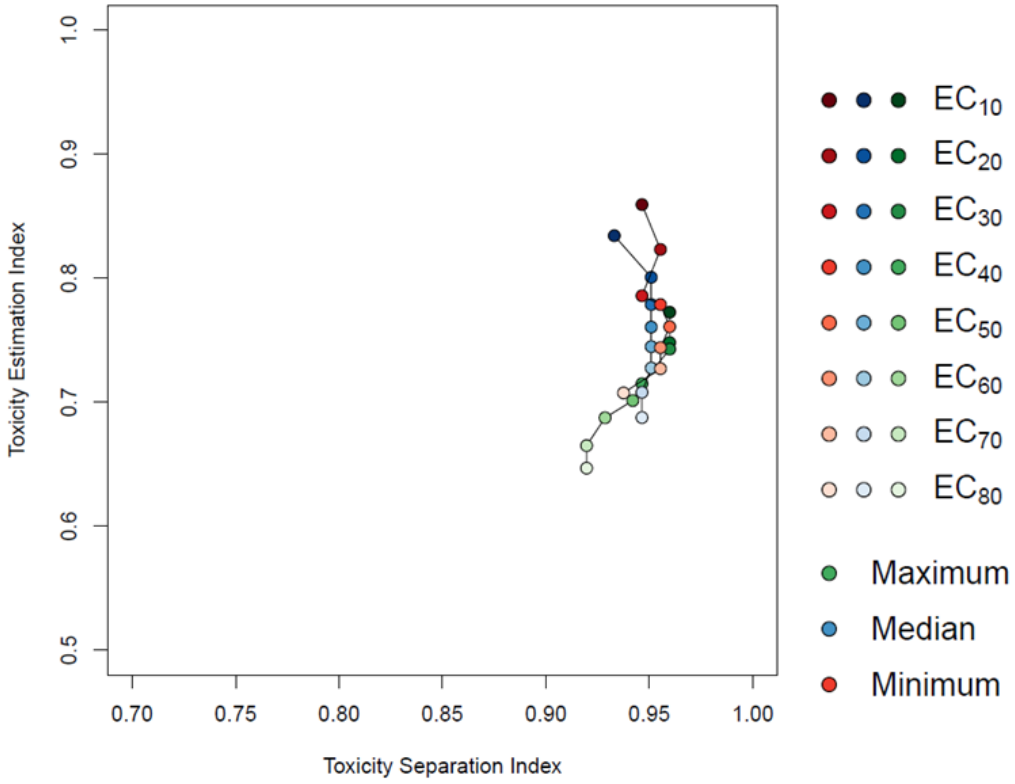

Figure 3.19: Indices for different effective concentrations for the GSH depletion assay in HepG2 following 48 hours of exposure to the 28 training set compounds
As in vivo concentration the 95% percentile of the total systemic peak blood concentration following a single exposure was utilized. The experiments were done with cells from three different passages (Data taken from Albrecht et al. 2019).

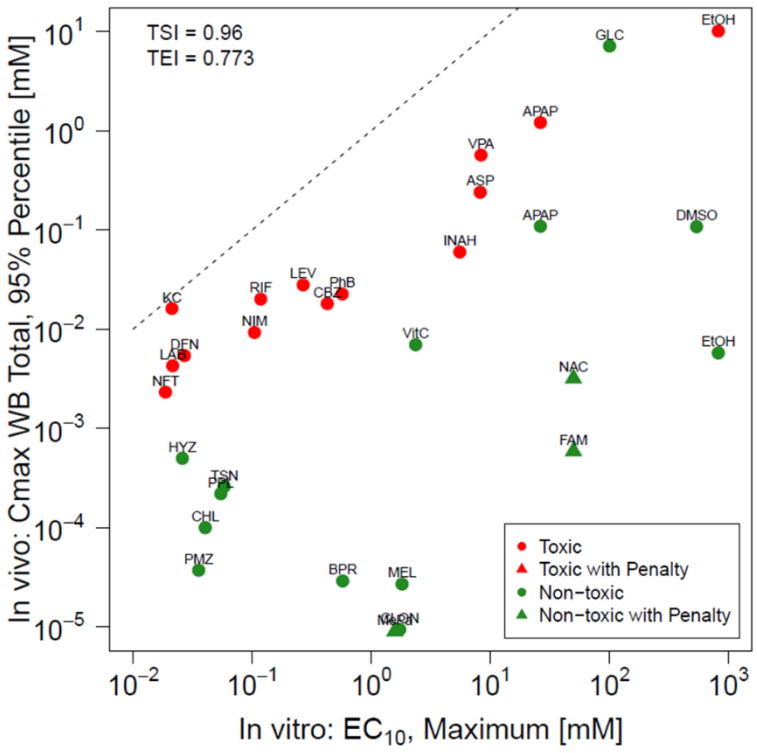

Figure 3.20: Data situation for the GSH depletion assay in HepG2 following 48 hour exposure to the 28 training set compounds

As in vitro concentration the maximal EC_{10} was utilized while as in vivo concentration the 95% percentile of the total systemic peak blood concentration following a single exposure was used. The experiments were done with cells from three different passages. In case the EC10 was not observed the highest tested concentration was multiplied by 5 (penalty). These compounds are represented by triangles. The maximum refers to the least sensitive replicate. Cmax: peak concentration; WB: Whole blood (Data taken from Albrecht et al. 2019).

Table 3.15: TSI and TEI for the combination of different *in vitro* assays in HepG2 with compounds from the training set

The lowest tested concentration leading to an at least 2.5 fold induction of at least one of the genes CYP1B1, CYP3A7 or G6PD was defined as alert concentration (All data was obtained from Albrecht et al. 2019). As in vivo in vivo concentration the 95% percentile of the total systemic peak blood concentration following a single exposure was utilized. For the combinations the LOEC was used as in vitro concentration.

In vitro assays	TSI	TEI
Minimal EC10 cytotoxicity	0.951	0.880
Median alert concentration gene expression	0.513	0.595
Maximal EC10 GSH depletion	0.960	0.772
Cytotoxicity + gene expression	0.951	0.880
Cytotoxicity+ GSH depletion	0.969	0.892
Gene expression + GSH depletion	0.960	0.786
All assays	0.969	0.892

3.7 Combination of the cytotoxicity assay data from PHH and HepG2 to predict human hepatotoxicity

3.7.1 Toxicity Separation Index and Toxicity Estimation Index for the extended compound set utilizing the combined cytotoxicity data of PHH and HepG2 cells.

Next, the cytotoxicity assay data for the extended compound set obtained from PHH and HepG2 were combined to investigate whether these improves the performance metrics. As *in vitro* concentration the lower of the maximal EC_{20} for PHH or the minimal EC_{10} for HepG2 was utilized while the 95% percentile of the total peak systemic blood concentration was used as *in vivo* parameter. For this combination a TSI of 0.849 and a TEI of 0.771 were obtained representing slight improvements compared to the usage of each individual cell system alone. The data situation for the extended data set is illustrated in **Figure 3.21** and the relevant *in vitro* and *in vivo* values are summarized in **Table 3.16.**

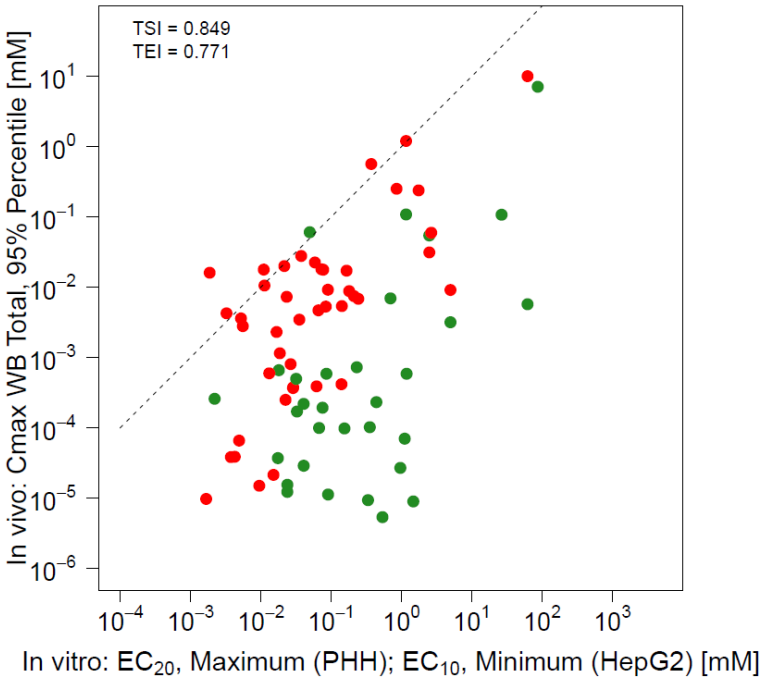

Figure 3.21: Data situation for the lowest observed effect concentration of the cytotoxicity assay after 48 hours of compound exposure for extended compound set

As in vitro parameter the lower of the maximal EC_{20} for PHH or the minimal EC_{10} for HepG2 were used. As in vivo the 95% percentile of the peak total systemic blood concentration was utilized. Red symbols depict hepatotoxic compounds, while green symbols represent non-hepatotoxic compounds. The dotted line is the iso-concentration line. All experiments were done with PHH from at least three different donors and in at least three replicates for the HepG2 cells.

Table 3.16: Overview of the *in vitro* and *in vivo* values for the combination of the cytotoxicity assays in PHH and HepG2 cells for the extended compound set after 48 hours of compound exposure

Compound	Abbreviation	Toxicity	LOEC [mM]	Total systemic peak blood concentration, 95% CI [mM]	Cell system
Acetaminophen	APAP	Hepatotoxic	1.169	$12.1*10^{-1}$	HepG2
Acetaminophen	APAP	Non-hepatotoxic	1.169	$10.9*10^{-2}$	HepG2
Allopurinol	Allo	Hepatotoxic	$2.119*10^{-1}$	$7.5*10^{-3}$	HepG2
Amiodarone	AMIO	Hepatotoxic	$2.880*10^{-2}$	$3.7*10^{-4}$	HepG2
Aspirin	ASP	Hepatotoxic	1.761	$24.0*10^{-2}$	HepG2
Atorvastatine	AVS	Hepatotoxic	9.542*10-3	$1.5*10^{-5}$	HepG2
Atropine	ATRO	Non-hepatotoxic	0.539	$53.8*10^{-7}$	HepG2
Benzbromarone	BZB	Hepatotoxic	$1.104*10^{-2}$	$17.9*10^{-3}$	PHH
Benztropine	BZT	Non-hepatotoxic	$2.389*10^{-2}$	$1.2*10^{-5}$	HepG2
Bosentan	BOS	Hepatotoxic	$5.569*10^{-3}$	$2.8*10^{-3}$	HepG2
Buspirone	BPR	Non-hepatotoxic	$4.087*10^{-2}$	$29.0*10^{-6}$	PHH
Busulfan	BUSF	Hepatotoxic	$6.226*10^{-2}$	$3.9*10^{-4}$	HepG2
Carbamazepine	CBZ	Hepatotoxic	$7.313*10^{-2}$	$18.1*10^{-3}$	PHH
Chlorpheniramine	CHL	Non-hepatotoxic	$6.766*10^{-2}$	$10.0*10^{-5}$	HepG2
Chlorpromazine	CMZ	Hepatotoxic	$4.277*10^{-3}$	$38.9*10^{-6}$	HepG2
Ciprofloxacin	CIPRO	Hepatotoxic	$6.617*10^{-2}$	$4.7*10^{-3}$	HepG2
Clofibrate	CLFI	Unclear	> 1	$14.7*10^{-6}$	No difference
Clonidine	CLON	Non-hepatotoxic	$3.359*10^{-1}$	$94.0*10^{-7}$	HepG2
Clozapine	CZP	Hepatotoxic	$5.242*10^{-3}$	$3.6*10^{-3}$	HepG2
Codeine	COD	Non-hepatotoxic	$4.417*10^{-1}$	$2.3*10^{-4}$	HepG2

Compound	Abbreviation	Toxicity	LOEC [mM]	Total systemic peak blood concentration, 95% CI [mM]	Cell system
Cyclosporin A	CsA	Hepatotoxic	$2.341*10^{-2}$	$7.3*10^{-3}$	PHH
Diclofenac	DFN	Hepatotoxic	$1.427*10^{-1}$	$54.2*10^{-4}$	PHH
Digoxin	Digi	Non-hepatotoxic	$1.6*10^{-5}$	$21.\ 5*10^{-7}$	HepG2
Dimethyl sulfoxide	DMSO	Non-hepatotoxic	26.670	$10.8*10^{-2}$	HepG2
Diphenhydramine	DPH	Non-hepatotoxic	$8.604*10^{-2}$	$5.9*10^{-4}$	HepG2
Dipyridamole	DIPY	Unclear	$3.708*10^{-3}$	$2.7*10^{-3}$	HepG2
Erythromycin	Ery	Hepatotoxic	0.181	$88.6*10^{-4}$	HepG2
Ethanol	EtOH	Hepatotoxic	62.399	$10.1*10^{0}$	HepG2
Ethanol	EtOH	Non-hepatotoxic	62.399	$57.6\ *10^{-4}$	HepG2
Famotidine	FAM	Non-hepatotoxic	1.189	$59.0*10^{-5}$	HepG2
Fexofenadine	FFD	Non-hepatotoxic	$> 4.646\ *10^{-2}$	$7.3*10^{-4}$	No difference
Fluconazole	FCA	Hepatotoxic	> 0.5	$31.4*10^{-3}$	No difference
Fluoxetine	FLT	Hepatotoxic	$3.764*10^{-3}$	$3.8*10^{-5}$	HepG2
Glucose	GLC	Non-hepatotoxic	87.153	$71.5*10^{-1}$	HepG2
Hydroxyzine	HYZ	Non-hepatotoxic	$3.191*10^{-2}$	$50.0*10^{-5}$	HepG2
Ibuprofen	IBU	Hepatotoxic	0.856	$252.6*10^{-3}$	HepG2
Imipramine	IMP	Hepatotoxic	$2.255*10^{-2}$	$2.5*10^{-4}$	HepG2
Indomethacine	Indo	Hepatotoxic	$3.542*10^{-2}$	$34.8*10^{-4}$	HepG2
Isoniazid	INAH	Hepatotoxic	$26.645*10^{-1}$	$60.0*10^{-3}$	HepG2
Isosorbide dinitrate	ISS	Non-hepatotoxic	0.356	$10.3*10^{-5}$	PHH
Ketoconazole	KC	Hepatotoxic	$1.385*10^{-2}$	$16.2*10^{-3}$	PHH

Compound	Abbreviation	Toxicity	LOEC [mM]	Total systemic peak blood concentration, 95% CI [mM]	Cell system
Labetalol	LAB	Hepatotoxic	$32.618*10^{-4}$	$42.8*10^{-4}$	HepG2
Leflunomide	LFM	Hepatotoxic	$2.664*10^{-2}$	$80.9*10^{-5}$	PHH
Levofloxacin	LEV	Hepatotoxic	$3.766*10^{-2}$	$28.0*10^{-3}$	HepG2
Melatonin	MEL	Non-hepatotoxic	0.968	$27.0*10^{-6}$	HepG2
Methylparaben	MePa	Non-hepatotoxic	1.481	$90.0*10^{-7}$	HepG2
Methotrexate	MTX	Hepatotoxic	$4*10^{-6}$	$10.3*10^{-4}$	HepG2
N-acetylcysteine	NAC	Non-hepatotoxic	> 10	$31.9*10^{-4}$	No difference
Nevirapine	NVP	Hepatotoxic	$7.750*10^{-2}$	$17.8*10^{-3}$	HepG2
Nifedipine	NDP	Hepatotoxic	$1.872*10^{-2}$	$11.6*10^{-4}$	HepG2
Nimesulide	NIM	Hepatotoxic	$9.036*10^{-2}$	$92.6*10^{-4}$	PHH
Nitrofurantoin	NFT	Hepatotoxic	$1.680*10^{-2}$	$23.2*10^{-4}$	HepG2
Oxycodone	OXC	Non-hepatotoxic	0.155	$9.8*10^{-5}$	HepG2
Oxymorphone	OXM	Non-hepatotoxic	$9.089*10^{-2}$	$11.3*10^{-6}$	PHH
Paroxetine	PXT	Hepatotoxic	$17.008*10^{-3}$	$6.6*10^{-5}$	PHH
Phenacetine	PHNA	Hepatotoxic	> 1	$91.9*10^{-4}$	PHH
Phenylbutazon	PhB	Hepatotoxic	$5.901*10^{-2}$	$22.6*10^{-3}$	HepG2
Phenytoin	PTN	Hepatotoxic	0.244	$68.7*10^{-4}$	HepG2
Pindolol	PIN	Non-hepatotoxic	$3.277*10^{-2}$	$17.1*10^{-5}$	HepG2
Pioglitazone	PIO	Hepatotoxic	$8.431*10^{-2}$	$5.3*10^{-3}$	PHH
Primaquine	Prima	Non-hepatotoxic	$1.811*10^{-2}$	$6.6*10^{-4}$	HepG2
Primidone	PRI	Non-hepatotoxic	$> 49.966*10^{-2}$	$54.8*10^{-3}$	HepG2
Promethazine	PMZ	Non-hepatotoxic	$1.755*10^{-2}$	$37.2*10^{-6}$	HepG2

Compound	Abbreviation	Toxicity	LOEC [mM]	Total systemic peak blood concentration, 95% CI [mM]	Cell system
Propranolol	PPL	Non-hepatotoxic	$4.078*10^{-2}$	$22.0*10^{-5}$	HepG2
Pyridoxine	PDX	Non-hepatotoxic	1.117	$7.0*10^{-5}$	HepG2
Rifampicin	RIF	Hepatotoxic	$2.164*10^{-2}$	$20.1*10^{-3}$	HepG2
Rosiglitazone	RGZ	Hepatotoxic	$38.197*10^{-3}$	$59.9*10^{-5}$	PHH
Rosuvastatin	ROS	Hepatotoxic	$1.676*10^{-3}$	$97.8*10^{-7}$	HepG2
Simvastatin	SIM	Hepatotoxic	$1.519*10^{-2}$	$2.1*10^{-5}$	HepG2
Sitaxentan sodium	SXS	Hepatotoxic	0.166	$17.3*10^{-3}$	HepG2
Sodium phenylbutyrate	SPB	Unclear	3.125	$911.1*10^{-3}$	HepG2
Terbinafine	Terbi	Hepatotoxic	1.409*10-1	$41.9*10^{-5}$	HepG2
Theophylline	THE	Non-hepatotoxic	$4.982*10^{-2}$	$609.6*10^{-4}$	HepG2
Tolbutamide	TOL	Unclear	0.908	$313.6*10^{-3}$	HepG2
Tolcapone	Tolc	Hepatotoxic	$1.131*10^{-2}$	$10.6*10^{-3}$	HepG2
Tolterodine	TTD	Non-hepatotoxic	$2.395*10^{-2}$	$15.5*10^{-6}$	HepG2
Triclosan	TSN	Non-hepatotoxic	$22.099*10^{-4}$	$26.0*10^{-5}$	HepG2
Valproic acid	VPA	Hepatotoxic	0.373	$56.9*10^{-2}$	HepG2
Vancomycin	VANC	Unclear	> 1	$14.9*10^{-3}$	No difference
Verapamil	VERA	Hepatotoxic	$2.898*10^{-2}$	$37.8*10^{-5}$	HepG2
Vitamin C	VitC	Non-hepatotoxic	$7.006*10^{-1}$	59.9*10-4	HepG2
Zaleplon	ZAL	Non-hepatotoxic	$7.576*10^{-2}$	$1.9*10^{-4}$	HepG2

3.7.2 Support vector machine based classification for the extended compound set utilizing the combined cytotoxicity data of PHH and HepG2 cells

Since the combination of the cytotoxicity assay data of PHH and HepG2 slightly improved the test system performance a SVM based leave-one-out classification was carried out using the LOEC of the maximal EC_{20} in PHH or the minimal EC_{10} in HepG2 after 48 hours of compound exposure as *in vitro* concentration and the 95% confidence interval of the total, systemic peak blood concentration as *in vivo* concentration for the compounds of the extended set with a clear toxicity status. A sensitivity of 86.7 %, a specificity of 65.6%, an accuracy of 77.9 %, a positive predictive value of 78% and a negative predictive value of 77.8% were obtained. The observed results are illustrated in **Figure 3.22** while an overview of the classifier performance metrics is given in **Table3.17**. In addition, a classifier utilizing all 77 *in vitro/in vivo* concentration pairs was built and used for classification of the five compounds with unclear toxicity status. The results are summarized in **Table 3.18.** All probabilities are given in **Supplement 8.**

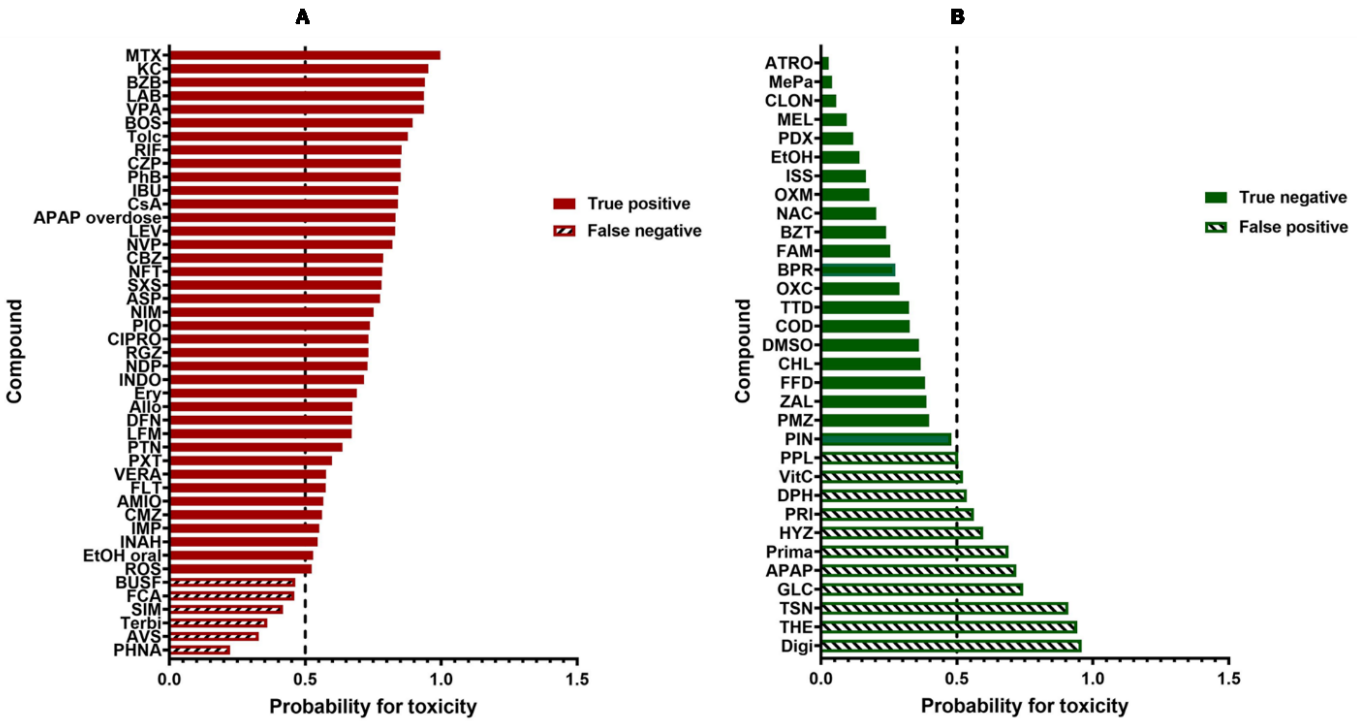

Figure 3.22: SVM based classification of the 77 extended set compounds with defined toxicity status using the combination of PHH and HepG2 cells

As in vitro concentration the LOEC of the maximal EC_{20} for PHH and the minimal EC_{10} using HepG2 cells of after 48 hours of compound exposure is chosen. As in vivo concentration the 95% percentile of peak total systemic whole blood concentration was used. Compounds exceeding a probability for toxicity of 0.5 were predicted to be toxic. (A) toxic compounds. (B) non-toxic compounds.

Table 3.17: Summary of the performance metrics for the cytotoxicity assay based SVM based leave-one-out classifier for the combination of PHH and HepG2 and the extended set compounds

Parameter	Value
True positives	39
True negatives	21
False negatives	6
False positives	11
Sensitivity	86.7%
Specificity	65.6%
Accuracy	77.9%
Positive predictive value (PPV)	78%
Negative predictive value (NPV)	77.8%

Table 3.18: SVM based classification of compounds with unclear toxicity status based on the combination of the cytotoxicity assay in PHH and HepG2 after 48 hours of compound exposure and comparison to literature data

Compound	Abbreviation	Classifier result	LIverTox Likelihood score	DILI rank DILI concern	DILIlist classification
Clofibrate	CLFI	Non-toxic	D	Verified Less-DILI concern	DILI positive
Dipyridamole	DIPY	Toxic	E*	Ambiguous DILI concern	Not included
Sodium phenylbutyrate	SPB	Toxic	E	Ambiguous DILI concern	Not included
Tolbutamide	TOL	Toxic	B (class)	Ambiguous DILI concern	DILI negative
Vancomycin	VANC	Non-toxic	B (usually in association with DRESS)	Verified Less-DILI concern	DILI positive

3.8 Comparison of the cytotoxicity and classification data obtained from PHH and HepG2 for the extended compound set

A comparison of three *in vitro/in* vivo test systems and the corresponding SVM based classifiers for the extended compound set revealed that the usage of the maximal EC_{20} of the cytotoxicity assay in PHH as *in vitro* parameter led to the worst performance metrics both for the *in vitro/in vivo* test system as well as the leave-one-out classifier. The usage of the lowest observed effective concentration (LOEC) of the combination of the cytotoxicity assays performed in PHH and HepG2 yielded the best TSI and TEI as well as the best specificity and positive predictive value (PPV) for the classifier. Utilization of the minimal EC_{10} for the cytotoxicity assay in HepG2 provided the best sensitivity as well as the best negative predictive value (NPV), while the obtained accuracy was equal to the accuracy of the classifier built with the LOEC.

The different performance metrics for the classifiers are given in **Table 3.19.** 50 out of 77 (64.9%) *in vitro/in vivo* concentration pairs were classified correctly by all three classifiers, two of the three classifiers led to correct classifications for 11 concentration vectors (14.3%), 2 compounds (2.6%) were predicted correctly by only one of the three classifiers and 14 concentration pairs (18.2%) were misclassified by all classifiers. Out of the five compounds with unclear toxicity only vancomycin was classified divergently by the different classifiers. All compounds with divergent classification results are summarized in **Table 3.20.** A more detailed comparison of compounds with divergent classifications based on the *in vitro* data in PHH or HepG2 is provided in **Table 3.2**. A closer analysis of the divergently classified compounds did not reveal a specific pattern regarding enzyme elevations, symptoms or proposed mechanism of toxicity (**Supplement 1**).

Table 3.19: Performance metrics of the *in vitro/in vivo* test systems and SVM based leave one out classifiers for the extended compound set based on the cytotoxicity assay in PHH, HepG2 or the combination thereof

The TSI and TEI are for the combination of the in vitro and in vivo parameter for all 77 in vitro/in vivo vectors with defined hepatotoxicity status. The performance metrics are for the leave one out classifications for the set of 77 in vitro/in vivo vectors with defined hepatotoxicity status. Uniquely correct classified compounds are compound, which were classified correctly solely by that particular classifier. Uniquely wrong classified compounds are compounds, which were wrongly classified only by that particular classifier. The linear equations refer to the classifiers used for the classification of compounds with uncertain toxicity status utilizing all 77 defined vectors. Y= log_{10} (in vivo concentration), x = log_{10} (in vitro concentration).

Parameter	PHH	HepG2	Combination
In vitro parameter	Maximal EC_{20}	Minimal EC_{10}	LOEC
In vivo parameter	95% CI total, systemic Cmax whole blood	95% CI total, systemic Cmax whole blood	95% CI total, systemic Cmax whole blood
TSI	0.804	0.843	0.849
TEI	0.605	0.765	0.771
Sensitivity	77.8%	88.9%	86.7%
Specificity	59.4%	62.5%	65.6%
Accuracy	70.1%	77.9%	77.9%
PPV	72.9%	76.9%	78.0%
NPV	65.6%	80.0%	77.8%
Uniquely correct classified compounds	1 (1.3%)	1 (1.3%)	0 (0.0%)
Uniquely wrong classified compounds	8 (10.4%)	2 (2.6%)	1 (1.3%)
Linear equation for a probability of 0.5 for toxicity utilizing all defined vectors	Y = -3.0453 + 0.820206*x	Y = -2.07331 + 1.150456*x	Y = -2.04486 + 1.14252*x

Table 3.20: Prediction results for the divergently classified compounds of the extended compound set across the SVM based classifiers
FN: false negative, FP: false positive, TN: true negative, TP: true positive.

Compound	Abbreviation	PHH	HepG2	Combination
Amiodarone	AMIO	FN	TP	TP
Busulfan	BUSF	FN	TP	FN
Chlorpheniramine	CHL	FP	TN	TN
Chlorpromazine	CMZ	FN	TP	TP
Dimethyl sulfoxide	DMSO	FP	TN	TN
Fexofenadine	FFD	FP	TN	TN
Fluconazole	FCA	TP	TP	FN
Fluoxetine	FLT	FN	TP	TP
Leflunomide	LFM	TP	FN	TP
Nifedipine	NDP	FN	TP	TP
Pindolol	PIN	TN	FP	TN
Rosuvastatin	ROS	FN	TP	TP
Triclosan	TSN	TN	FP	FP
Vancomycin	VANC	Toxic	Non-toxic	Non-toxic

Table 3.21: Probabilities for the divergently classified compounds of the extended compound set in SVM based leave-one-out classifiers built with in *vitro* data either from PHH or HepG2
A probability for toxicity greater than 0.5 leads to a classification as toxic, while a probability less than 0.5 leads to a classification as non-toxic. In case the relevant effective in vitro concentration was not reached a value of 5x the highest tested concentration was utilized.

Compound	Abbreviation	Toxicity status	Probability for toxicity PHH	Probability for toxicity HepG2	Correct classifier
Amiodarone	AMIO	Toxic	0.275	0.594	HepG2
Busulfan	BUSF	Toxic	0.284	0.504	HepG2
Chlorpheniramine	CHL	Non-toxic	0.522	0.413	HepG2
Chlorpromazine	CMZ	Toxic	0.360	0.575	HepG2
Dimethyl sulfoxide	DMSO	Non-toxic	0.538	0.395	HepG2
Fexofenadine	FFD	Non-toxic	0.606	0.466	HepG2
Fluoxetine	FLT	Toxic	0.489	0.591	HepG2
Leflunomide	LFM	Toxic	0.730	0.472	PHH
Nifedipine	NDP	Toxic	0.469	0.760	HepG2
Pindolol	PIN	Non-toxic	0.299	0.520	PHH
Rosuvastatin	ROS	Toxic	0.304	0.568	HepG2
Triclosan	TSN	Non-toxic	0.494	0.920	PHH

Out of the 14 *in vitro/in vivo* concentration pairs, which were always misclassified, four compounds (28.6%) were *in vivo* hepatotoxic. Out of these more detailed toxicity information was available for three compounds (atorvastatin, simvastatin and terbinafine) (**Supplement 1)**. For all three compounds an immune component was mentioned as either autoimmunity hepatitis like symptoms, Stevens-Johnson syndrome, a hypersensitivity mediated mechanism of toxicity or a combination of these (Devarbhavi et al. 2018, LiverTox). **Table 3.22** summarizes the unambiguously misclassified compounds. A comparison of the classification of the five compounds with uncertain toxicity by the classifiers utilizing all 77 in *vitro/in vivo* vectors with defined toxicity status is provided in **Table 3.23**.

Table 3.22: Probability of toxicity for the unambiguously misclassified compounds of the extended compound set in SVM based leave-one-out classifiers built with either in vitro data from PHH or HepG2 or the LOEC

A probability for toxicity greater than 0.5 leads to a classification as toxic, while a probability less than 0.5 leads to a classification as non-toxic. In case the relevant effective in vitro concentration was bot reached a value of 5x the highest tested concentration was utilized. FN: false negative, FP: false positive, LOEC: lowest observed effective concentration.

Compound	Abbreviation	PHH	HepG2	LOEC	Result
Acetaminophen	APAP	0.840	0.765	0.712	FP
Atorvastatin	AVS	0.267	0.434	0.329	FN
Digoxin	Digi	0.693	0.974	0.958	FP
Diphenhydramine	DPH	0.596	0.516	0.536	FP
Glucose	GLC	0.779	0.668	0.774	FP
Hydroxyzine	HYZ	0.637	0.647	0.596	FP
Phenacetine	PHNA	0.384	0.263	0.223	FN
Primaquine	Prima	0.709	0.697	0.690	FP
Primidone	PRI	0.831	0.587	0.562	FP
Propranolol	PPL	0.626	0.522	0.505	FP
Simvastatin	SIM	0.342	0.366	0.418	FN
Terbinafine	Terbi	0.296	0.432	0.360	FN
Theophylline	THE	0.847	0.911	0.942	FP
VitaminC	VitC	0.613	0.546	0.523	FP

Table 3.23: Probability of toxicity for the compounds with unclear toxicity status
Compounds with a probability for toxicity exceeding 0.5 were classified as toxic, while compounds with a probability lower than 0.5 were classified as non-toxic. In case the relevant effective in vitro concentration was not reached, the value was set to 5 x the highest tested concentration. LOEC: Lowest observed effective concentration.

Compound	Abbreviation	PHH	HepG2	LOEC
Clofibrate	CLFI	0.159	0.281	0.264
Dipyridamole	DIPY	0.556	0.924	0.927
Sodium phenylbutyrate	SPB	0.757	0.830	0.835
Tolbutamide	TOL	0.750	0.894	0.899
Vancomycin	VANC	0.612	0.354	0.342

3.9 Comparison with published data sets

Comparison of the 75 compounds with defined toxicity status of the extended compound set with three published data sets of in *vitro* test system for the prediction of human hepatotoxicity revealed that for 55 compounds (73.3% of compounds) data was available in at least one of these publications. 33 of the test compounds from Proctor et al. (Proctor et al. 2017) were included in the extended data set. For acetaminophen at therapeutic blood concentrations and chlorpheniramine, the true toxicity status used in the data set varied from the one in this work. Both were considered hepatotoxic by Proctor and colleagues. 40 compounds of the data set from Xu and colleagues (Xu et al. 2008) were matched by the extended compound set with 11 compounds having a divergent true toxicity status. These compounds were aspirin, erythromycin, fluoxetine, levofloxacin, paroxetine, pioglitazone, rosiglitazone and simvastatin described as non-hepatotoxic by Xu et al., while acetaminophen at therapeutic blood concentrations, chlorpheniramine and clonidine were considered hepatotoxic. 21 compounds of the extended compound overlapped with the data set of Khetani et al. (Khetani et al. 2013) with 2 compounds with contradictory DILI status, namely aspirin and fluoxetine being declared non-hepatotoxic. **Table 3.24** provides an overview over the different classification systems, while **Table 3.25** summarizes the classification results.

Table 3.24: Comparison of different *in vitro* test systems for human hepatotoxicity
PHH: primary human hepatotoxicity, HepG2: HepG2 cell line LOEC: lowest observed effective concentration, SVM: support vector machine, hLiMT: human liver microtissues, ATP: adenosine triphosphate, MOS = margin of safety, IC_{50} or TC50: concentration at which a change in effect of 50% was observed, Cmax: peak concentration, Hu:-MPCC human micropatterned coculture, GSH: glutathione.

Parameter	IfADo	Proctor (2018)	Xu (2008)	Khetani (2013)
Cell culture system	PHH and HepG2 collagen monolayer; LOEC out of these two	PHH monolayer; hLiMT spheroids	PHH collagen monolayer with matrigel overlay	Hu-MPCC
Duration of compound exposure [days]	2	hLiMT:14 PHH: 2	1	9
Number of treatments	1	hLiMT:3 PHH:1	1	4
In vitro read outs	Resazurin reduction (CTB assay)	ATP content	Imaging based calculation of 8 factors	Albumin secretion, urea secretion, ATP content, GSH content
Classified as hepatotoxic if	SVM predicted probability of toxicity > 0.5	MOS = IC_{50}/Cmax < 50	One readout positive <100 x Cmax	TC50 of one readout <100 x Cmax

Table 3.25: Summary of the classification of mutual compounds for different *in vitro* test systems

PHH: primary human hepatotoxicity. Ambiguous: call differed for the two tested replicates. Compounds with divergently assumed true human hepatotoxicity status are written in italics. HepG2: Hepg2 cell line LOEC: lowest observed effective concentration for PHH or HepG2, hLiMT: human liver microtissues; FN: false negative, FP, false positive, TN: true negative, TP: true positive, N/A compound not included in the data set.

Compound	Abbreviation	PHH	HepG2	LOEC	Proctor PHH	Proctor hLiMT	Xu	Khetani
Acetaminophen (therapeutic)	*APAP*	FP	FP	FP	TP	TP	TP	N/A
Allopurinol	Allo	TP	TP	TP	N/A	N/A	TP	N/A
Amiodarone	AMIO	FN	TP	TP	TP	TP	TP	TP
Aspirin	*ASP*	TP	TP	TP	FN	FN	TN	TN
Atorvastatin	AVS	FN	FN	FN	FN	FN	N/A	N/A
Benzbromarone	BZB	TP	TP	TP	TP	TP	N/A	TP
Benztropine	BZT	TN	TN	TN	FP	FP	N/A	N/A
Bosentan	BOS	TP	TP	TP	FN	TP	N/A	TP
Buspirone	BPR	TN	TN	TN	TN	TN	TN	TN
Busulfan	BUSF	FN	TP	FN	N/A	N/A	TP	N/A
Carbamazepine	CBZ	TP	TP	TP	FN	TP	N/A	TP
Chlorpheniramine	*CHL*	FP	TN	TN	FN	FN	TP	N/A
Chlorpromazine	CMZ	FN	TP	TP	TP	TP	TP	N/A
Ciprofloxacin	CIPRO	TP	TP	TP	N/A	N/A	N/A	TP
Clonidine	*CLON*	TN	TN	TN	N/A	N/A	FN	N/A
Clozapine	CZP	TP	TP	TP	TP	TP	TP	TP
Cyclosporin A	CsA	TP	TP	TP	N/A	N/A	FN	N/A
Diclofenac	DFN	TP	TP	TP	FN	TP	TP	TP
Digoxin	Digi	FP	FP	FP	FP	TN	TN	N/A
Diphenhydramine	DPH	FP	FP	FP	N/A	N/A	TN	N/A
Erythromycin	*Ery*	TP	TP	TP	N/A	N/A	TN	N/A
Famotidine	FAM	TN	TN	TN	N/A	N/A	TN	N/A
Fluconazole	FCA	TP	TP	FN	N/A	N/A	FN	N/A
Fluoxetine	*FLT*	FN	TP	TP	FN	TP	TN	TN
Imipramine	IMP	TP	TP	TP	FN	FN	FN	TP
Indomethacine	Indo	TP	TP	TP	FN	FN	FN	N/A
Isoniazide	INAH	TP	TP	TP	N/A	N/A	FN	TP
Ketoconazole	KC	TP	TP	TP	TP	TP	N/A	TP
Labetalol	LAB	TP	TP	TP	N/A	N/A	TP	N/A
Leflunomide	LFM	TP	FN	TP	N/A	N/A	FN	TP
Levofloxacin	*LEV*	TP	TP	TP	FN	FN	TN	N/A
Melatonin	MEL	TN	TN	TN	N/A	N/A	TN	N/A
Methotrexate	MTX	TP	TP	TP	FN	FN	FN	N/A
Nevirapine	NVP	TP	TP	TP	N/A	N/A	N/A	TP
Nifedipine	NDP	FN	TP	TP	FN	FN	FN	FN
Nimesulide	NIM	TP	TP	TP	TP	TP	TP	N/A
Nitrofurantoin	NFT	TP	TP	TP	TP	TP	N/A	N/A
Paroxetine	*PXT*	TP	TP	TP	FN	FN	TN	N/A
Phenacetine	PHNA	FN	FN	FN	N/A	N/A	TP	TP

Compound	Abbreviation	PHH	HepG2	LOEC	Proctor PHH	Proctor hLiMT	Xu	Khetani
Phenylbutazone	PhB	TP	TP	TP	N/A	N/A	TP	TP
Pindolol	PIN	TN	FP	TN	N/A	N/A	TN	N/A
Pioglitazone	*PIO*	TP	TP	TP	FN	FN	TN	N/A
Primaquine	Prima	FP	FP	FP	N/A	N/A	TN	N/A
Primidone	PRI	FP	FP	FP	N/A	N/A	TN	N/A
Promethazine	PMZ	TN	TN	TN	N/A	N/A	TN	N/A
Propranolol	PPL	FP	FP	FP	N/A	N/A	TN	TN
Pyridoxine	PDX	TN	TN	TN	N/A	N/A	TN	N/A
Rosiglitazone	*RGZ*	TP	TP	TP	FN	TP	TN	N/A
Rosuvastatin	ROS	FN	TP	TP	FN	FN	N/A	N/A
Simvastatin	SIM	FN	FN	FN	FN	FN	TN	N/A
Sitaxentan sodium	SXS	TP	TP	TP	FN	TP	N/A	N/A
Theophylline	THE	FP	FP	FP	TN	TN	N/A	N/A
Tolcapone	Tolc	TP	TP	TP	TP	TP	N/A	TP
Valproic acid	VPA	TP	TP	TP	FN	FN	N/A	Ambi-guous
Verapamil	VERA	TP	TP	TP	FN	FN	N/A	N/A

4 Discussion

4.1 An *in vitro/in silico* approach for the prediction of human hepatotoxicity in relation to oral doses and blood concentrations

In this thesis a combined *in vitro/in silico* approach for the prediction of human hepatotoxicity in relation to oral doses and blood concentrations is described. This approach combines an *in vitro* cytotoxicity assay, physiologically based pharmacokinetic modelling and support vector machine based classifiers. During the development of this approach two novel metrics for test system development were applied as described in Albrecht et al. 2019. These indices eliminate the need of an arbitrary selection of the relevant *in vitro* effective and *in vivo* concentrations, while allowing for continuous refinement of the approach and evaluation of further *in vitro* read outs.

The resulting *in vitro/in silico* test system should ultimately alert the user whether a certain compound poses an increased risk of causing hepatotoxicity at likely reached *in vivo* concentrations. A further intended application of the approach is the derivation of acceptable daily intakes for compounds with unknown exposure scenarios. These applications are of relevance for prioritizing potential drug candidates for further testing and development or in the regulatory context for chemicals.

4.2 Development of the in *vitro/in silico* test strategy

As starting point for the test battery development primary human hepatocytes were chosen as a gold standard for *in vitro* hepatotoxicity assessment and a commercial available well-established cytotoxicity assay was chosen as a readout. In a first step, the feasibility of the approach was tested by choosing a small subset of 28 compounds with mostly well-defined human hepatotoxicity status. For acetaminophen and ethanol information about an exposure scenario with and without an increased risk for hepatotoxicity were available, leading to a total of 30 *in vitro/in vivo concentration* pairs. The set of compounds also contained famotidine, for which the toxicity status is not as clear compared to the other compounds. Reports of clinically apparent liver injury are rare and the reported serum transaminase elevation rates in clinical studies were similar in the placebo group (LiverTox). In addition, famotidine is listed as lessDILI concern compound in DILIrank based on liver damage being listed in the adverse effects section of the drug label not necessitating proof of a causal relationship (DILIrank). Taking this into account, famotidine was considered as non-hepatotoxic in agreement with Proctor et al. 2017 and Albrecht et al. 2019.

After establishing the best combination of *in vitro* and *in vivo* concentrations as judged by the degree of separation between toxic and non-toxic compounds a SVM based leave-one-out classification was carried out. As Go/No-go criterion an accuracy of ≥ 70% was chosen. The accuracy was selected as performance metric since both specificity and sensitivity are important applying this approach for prioritization of the development of potential drug candidates. **Figure 4.1** illustrates the strategy for test system development.

Discussion

The obtained accuracy for the training set compounds was 93.3% with two false positives, showing that the approach is in general feasible. This led to two separate follow up research questions: 1) Can the *in vitro* test battery be improved by addition of a further readout? And 2) Is the cytotoxicity assay only approach still feasible when applied to a larger compound set?

To address the first question, the glutathione depletion assay was chosen. Hypothesizing that glutathione depletion may provide a more sensitive readout than cytotoxicity alone, it was assumed that the separation of toxic and non-toxic compounds measured by the Toxic Separation Index (TSI) or the estimation of the toxic concentration for the toxic compound indicated by the Toxic Estimation Index (TEI) might be improved. The glutathione depletion assay was performed for the training set compounds, but neither the GSH depletion alone nor the combination of this assay with the cytotoxicity assay improved the metrics. Therefore, it was concluded that the GSH assay does not improve the *in vitro* readout for PHH.

For the future applicability of the *in vitro/in silico* approach it is important that the approach is feasible for are large variety of chemical compounds. As a first step in scaling up the assay 52 further compounds were chosen for the cytotoxicity assay in PHH. The extended compound set included in total 80 different compounds with 82 different *in vitro/in vivo* concentrations pairs. The selection of new compounds included misclassified compounds from three previously published *in vitro* test systems for human hepatotoxicity utilizing PHH. Examples for these compounds are atorvastatin, benztropine, cyclosporine A, indomethacine, methotrexate and nifedipine (Xu et al. 2008; Khetani et al. 2013; Proctor et al. 2017). Furthermore, compounds with diverse proposed toxicity mechanisms e.g. busulfan, cyclosporin A, fluconazole and methotrexate were selected. The selected compounds cover a diverse range of clinical presentations, indications and clarity of hepatotoxicity status. For the five compounds clofibrate, dipyridamole, sodium phenylbutyrate, tolbutamide and vancomycin the decision was made to classify them as having an unclear hepatotoxicity status. This meant they were not included in our calculation of performance metrics, but served as a case study for classification, with the classifier built for the 77 concentration vectors of compounds with defined hepatotoxicity status (**Supplement 1, Supplement 3**).

Prior to the SVM based classification, the selection step for the *in vitro* and *in vivo* parameters based on the TSI and TEI was repeated for the extended compound set. During this analysis it became evident that the maximal EC_{20} led to a better TSI compared to the median EC_{10} leading to the best TSI for the 28 compound set. This might be due to a better robustness against experimental variance. Interestingly, out of all 25 *in vivo* parameters investigated the 95% confidence interval of the total, systemic peak blood concentration following a single dose still led to the best TSI. Again, a cut-off of an accuracy of ≥ 70% for the SVM based leave-one-out classification was set (**Figure 4.1**). The observed accuracy was 70.1% fulfilling this criterion, but clearly lower than the accuracy obtained for the initially tested set. Taking into account the previously published misclassification of some of the compounds and the diversity of toxicity mechanisms, the drop in performance was to be expected.

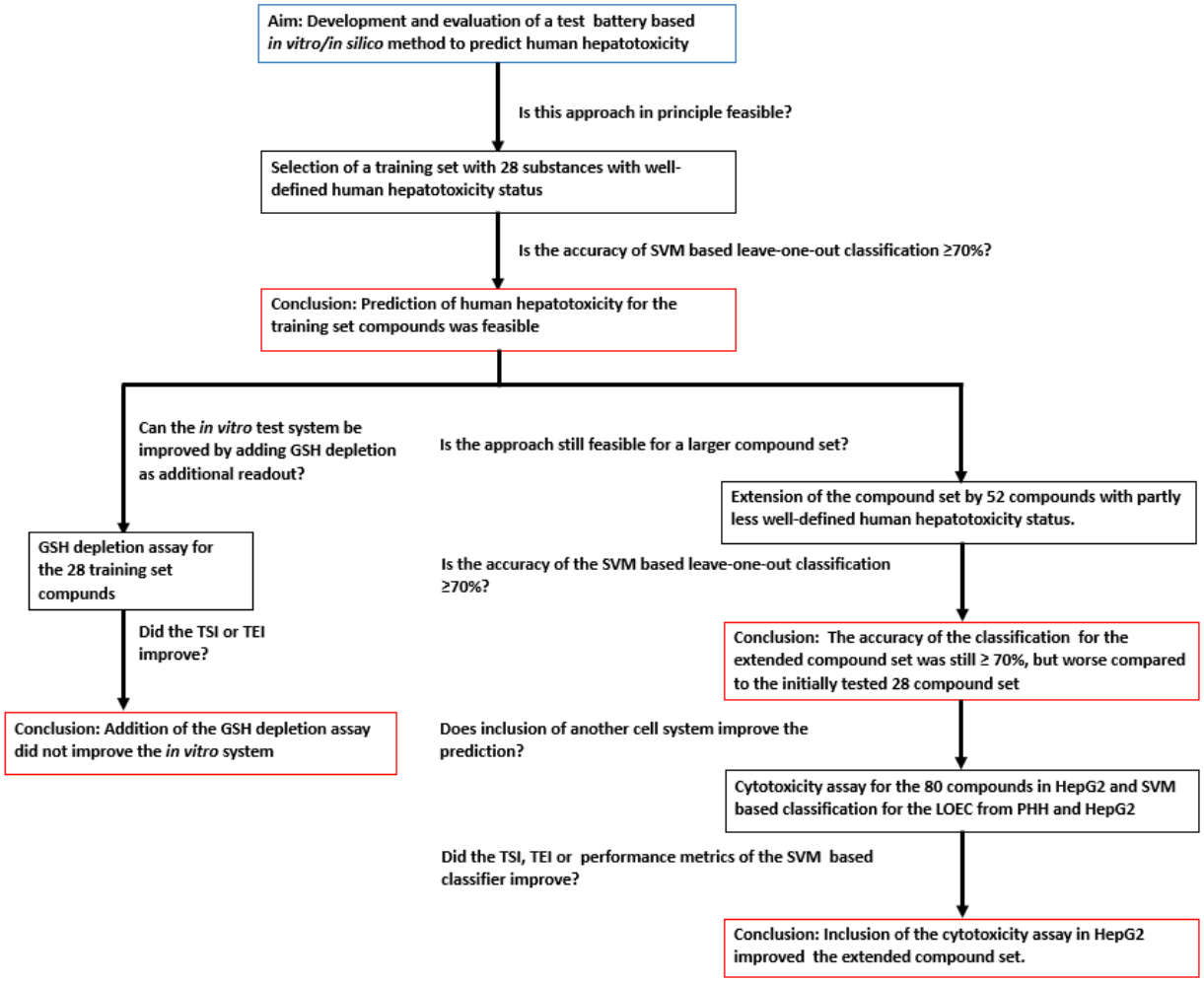

Figure 4.1: Overview of the test strategy

The initial assay was the cytotoxicity assay in PHH. HepG2: HepG2 cell line, LOEC: lowest observed effect concentration, PHH: primary human hepatocytes, SVM: support vector machine, TSI: toxicity separation index, TEI: toxicity estimation index.

In the next step, it was investigated whether the performance of the extended compound set could be improved by including the results of cytotoxicity assays in the HepG2 cell line. After an initial optimization step, it became evident that the best TSI and TEI were achieved by utilizing the minimal EC_{10}. The cytotoxicity assay in HepG2 cells outperformed the cytotoxicity assay in PHH for this compound set. This may be due to the mitotic activity of the HepG2 cells or due to possible differences metabolism, excretion or detoxification of and needs to be subject of further experimental validation with a more extensive compound set.

Since the combination of the cytotoxicity assay in PHH and HepG2 using the LOEC of these systems as *in vitro* parameter further improved the TSI and TEI, a SVM based leave-one-out classification was carried out. The obtained accuracy was 77.9% showing that the combination of the two cell systems did improve the prediction.

4.3 Comparison of the classification based on PHH and HepG2 data

The comparison of the cytotoxicity assay in PHH and HepG2 for the extended compound set revealed that the assay in HepG2 outperformed the assay in PHH in terms of TSI and TEI. Furthermore, the SVM based leave-one-out classification based on the cytotoxicity data of the extended compound set resulted in better performance metrics when using the data obtained in HepG2 cells compared to the PHH data. Possible reasons for this could be the sustained mitotic activity in culture of HepG2 and differences in expression or activity of transporters or key enzymes for the metabolism of xenobiotics. For the training set compounds the PHH cytotoxicity assay provided a better TSI and TEI compared to the HepG2 cell line results.

The individual compounds amiodarone, busulfan, chlorpheniramine, dimethyl sulfoxide, fexofenadine, nifedipine and rosuvastatin were misclassified when utilizing the PHH data, but for all of these compounds the prediction was correct using the classifier built with HepG2 data, or - with the exemption of busulfan - when using the classifiers built with the LOEC. In contrast, leflunomide, pindolol and triclosan were misclassified by the HepG2 data based classifiers, but correctly predicted by the PHH data based classifiers. In addition, leflunomide and pindolol were correctly classified when using the LOEC classifiers. Of note, fluconazole was wrongly classified when utilizing the LOEC, but correctly classified when using each cell culture system individually, while no compound was correctly classified solely by the LOEC classifiers. For the compounds with unclear human hepatotoxicity status only the prediction for vancomycin differed between the PHH classifier (toxic) and the HepG2 classifier (non-toxic). Contrary to the PHH the inclusion of the GSH assay in HepG2 improved both the TSI and TEI for the training set compounds. To further substantiate the present picture on performance for the assay, a follow-up study with a larger compound set is needed.

4.4 Limitations of the *in vitro/in silico* approach

For 14 compounds no correct classification was achieved regardless of the utilized classifier. 10 of these compounds (acetaminophen at therapeutic doses, digoxin, diphenhydramine, glucose, hydroxyzine, primaquine, primidone, propranolol, theophylline and vitamin C) were always wrongly classified as posing an increased risk of human hepatotoxicity. This is likely due to a suboptimal separation of toxic and non-toxic compounds utilizing the *in vitro* effective. In principle this should be remediable by the inclusion of further *in vitro* readouts covering a broader range of possible mechanisms of toxicity *e.g.* inhibition of bile salt transporters, hepatic steatosis, inflammation, damage to bile canaliculi or non-parenchymal cells. Of note, three of the four (atorvastatin, simvastatin and terbinafine) consistently misclassified toxic compounds have some immune system related component in the proposed mechanism of toxicity or clinical presentation. More detailed information for phenacetine was not available (**Supplement 2)**. Promising further *in vitro* readouts are expected to improve the TSI when combined with the cytotoxicity assay, since fewer hepatotoxic compounds would have non observed effective *in vitro* concentrations. This is important due to the necessity to apply a "penalty factor" for non-observed effective *in vitro* concentrations in the SVM based classification.

A general, limitation for an *in vitro* assessment of human toxicity are limits concerning the highest testable concentration. This is most often due to limited solubility in medium, DMSO or ethanol, but the pH value of the final treatment solutions as well as availability of testing compounds are also restricting factors.

The second general challenge lies in the definition of hepatotoxicity and the selection of compounds for training and validation of test systems. This is especially true for the selection of negative control compounds. The assessment of the potential to cause human hepatotoxicity may change over time due to increasing evidence for the possibility of hepatotoxicity, hence careful curation of the compound set and if necessary, refinement of the assumed true toxicity status during the entire project is required.

4.5 Comparison to published studies

The performance metrics for the SVM based leave-one-out classifications utilizing the cytotoxicity assay data for the extended compound set were compared to three previously published studies. The observed sensitivities ranging from 77.8% - 86.7% were higher than the sensitivities for the previously published test systems spanning from 40.6 - 65.7% (Xu et al. 2008; Khetani et al. 2013; Proctor et al. 2017). In addition, the obtained negative predictive values ranging from 65.6% - 80.0% were higher compared to the corresponding values from the literature in the range of 42.8 - 59.3 % (Xu et al. 2008; Khetani et al. 2013; Proctor et al. 2017). In contrast, both the specificity (59.4 - 65.6 %) and positive predictive values (72.9 - 78.0 %) for the *in vitro/in silico* approach were worse compared to the values for the published test systems ranging from 80.5 - 100 % for specificity and 79.3 - 100 % for the positive predictive value (Xu et al. 2008; Khetani et al. 2013; Proctor et al. 2017). For all studied compound sets and regardless of the *in vitro* readout for a number of toxic compounds no *in*

vitro effect was observed underscoring the limitations of *in vitro* assessment of toxicity discussed in **Section 4.4**.

The differences in sensitivity, specificity, PPV and NPV are likely due to the assignment of an effective *in vitro* concentration of 5 times the highest tested concentration for all compounds without an observed effect in this study. This leads to a toxic concentration for all tested compounds in the set and therefore likely increases both the number of true positives, which would otherwise be missed, as well as for false positives. In the previously published studies, compounds without an observed *in vitro* effect were classified as non-toxic increasing the likelihood of true negatives as well as false negatives. Across all approaches the accuracy was ranging from 57.3 - 77.9%. An overview of the performance metrics and data sets is provided in **Table 4.1**. Since the number of tested compounds (45 - 344) and the percentage of toxic compounds in these compound libraries (58.1 - 77.7%) varied between the studies (**Table 4.1)** a more detailed comparison was done considering only the mutual compounds.

Table 4.1: Summary of compound sets and performance metrics across several in vitro test systems *HepG2: HepG2 cell line, hLiMT: human liver microtissues, LOEC: lowest observed effective concentration for PHH or HepG2, MOS: margin of safety, NPV: negative predictive value, PHH: primary human hepatocytes, PPV: positive predictive value. For Proctor et al. 2018 a MOS ≤100 was used as threshold for toxicity. In case of PHH, HepG2 and LOEC from this study acetaminophen and ethanol were considered both as toxic and non-toxic depending on the exposure scenario*.

	PHH	HepG2	LOEC	PHH Proctor et al. (2018)	hLiMT Proctor et al. (2018)	Khetani et al. (2013)	Xu et al. (2008)
Toxic compounds	45	45	45	69	69	35	200
Non-toxic compounds	32	32	32	41	41	10	144
Total compounds	77	77	77	110	110	45	344
Percentage toxic compounds	58.4%	58.4%	58.4%	62.7%	62.7%	77.7%	58.1%
Sensitivity	77.8%	88.9%	86.7%	40.6%	59.4%	65.7%	50.5%
Specificity	59.4%	62.5%	65.6%	85.4%	80.5%	90%	100%
Accuracy	70.1%	77.9%	77.9%	57.3%	67.2%	71.1%	71.2%
PPV	72.9%	76.9%	78.0%	79.3%	83.7%	95.8%	100%
NPV	65.6%	80.0%	77.8%	43.2%	54.1%	42.8%	59.3%

The study of Proctor and colleagues (Proctor et al. 2017) and this study shared 33 mutual compounds. Considering only the classification as toxic or non-toxic, between 11 and 17 (33.3 - 51.5%) of the compounds were classified divergently. The detailed comparison between all

permutations is given in **Table 4.2**. Of special interest are the compounds atorvastatin, aspirin, benztopine, indomethacin, levofloxacin, methotrexate, pioglitazone, rosuvastatin, simvastatin, theophylline and valproic acid. Atorvastatin and theophylline were misclassified by all three *in vitro/in silico* test systems in this work, while atorvastatin is correctly classified by the hLiMT system, and theophylline is correctly by both PHH and hLiMT from Proctor and colleagues. In contrast, aspirin, benztropine, indomethacine, levofloxacin, methotrexate, pioglitazone, and valproic acid were misclassified in both the PHH assay and the hLiMT assay by Proctor et al., but correctly classified by all test systems proposed in this thesis. Simvastatin is wrongly classified by all five approaches.

Table 4.2: Comparison with the data from Proctor et al. 2018 *HepG2: HepG2 cell line, hLiMT: human liver microtissues, LOEC: lowest observed effective concentration for PHH or HepG2, PHH: primary human hepatocytes. For Proctor et al. 2018 a MOS ≤100 was used as threshold for toxicity. For the classification it was differentiated only between positive and negative.*

Classifier/Test system	Number of divergently classified compounds	Percentage of divergently classified compounds
PHH versus PHH Proctor et al.	17	51.5
PHH versus hLiMT Proctor et al.	12	36.4
HepG2 versus PHH Proctor et al.	17	51.5
HepG2versus hLiMT Proctor et al.	11	33.3
LOEC versus PHH Proctor et al.	17	51.5
LOEC versus hLiMT Proctor et al.	11	33.4

There were 40 mutual compounds between the study of Xu and colleagues (Xu et al. 2008) and the extended compound set. Utilization of the cytotoxicity data in PHH or the LOEC of HepG2 and PHH led to 21 (52.5%) divergently classified compounds, while usage of the cytotoxicity data from HepG2 lead to 22 (55%) divergently classified compounds. The compounds cyclosporine A, digoxin, diphenhydramine, imipramine, isoniazid, methotrexate, phenacetine, primaquine, primidone and propranolol are especially interesting. While digoxin, diphenhydramine, phenacetine, primaquine, primidone and propranolol were always misclassified by the *in vitro/in silico* approach, they were correctly identified by Xu and colleagues. In contrast, the leave-one-out classifiers yielded correct classifications for cyclosporine A, imipramine, isoniazide and methotrexate, which were wrongly classified by Xu and colleagues.

From 21 mutual compounds with the study of Khetani and colleagues (Khetani et al. 2013) 5 compounds (23.8%) were classified divergently when compared to the data from either PHH or HepG2, while 6 compounds (28.5%) were classified divergently when compared to the LOEC

data. Khetani and colleagues obtained correct classifications for phenacetine and propranolol, which were always misclassified in this work, while the obtained data for VPA was ambiguous.

4.6 Conclusions

The *in vitro/in silico* approach in general was feasible for classification of the 75 compounds with defined human hepatotoxicity status according to their potential to cause DILI at realistic exposure scenarios. The current cytotoxicity assay based test system necessitates only short term 2D monocultures of HepG2 and PHH in contrast to published test systems relying on long term cell cultures, 3D cell cultures and co-cultures. Despite this, the obtained accuracies were comparable between the different approaches (Xu et al. 2008; Khetani et al. 2013; Proctor et al. 2017). While the results are promising, it is clear that the inclusion of further *in vitro* readouts is necessary to cover a broader range of toxicities and pathomechanisms resulting in DILI. This is expected to improve separation of toxic and non-toxic compounds in relation to expected blood concentrations and, therefore, to enable a more accurate classification according to human hepatotoxicity status.

For the extended compound set presented in this study a combination of observed effective *in vitro* concentrations in PHH and HepG2 cells was providing the best separation of compounds according to their hepatotoxicity and the best estimation of toxic concentrations. This finding has to be validated and further investigated with an extended compound set.

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6 Appendix

The supplement is provided in digital format on DVD.

Supplement 1: Compound information

Supplement 2: Donor information

Supplement 3: Pharmacokinetics

Supplement 4: Raw data

Supplement 5: Concentration response curves

Supplement 6: Processed data

Supplement 7: Toxicity Separation Index and Toxicity Estimation Index

Supplement 8: SVM classifier

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9 Publications

9.1 Original articles

Comparing in vitro human liver models to in vivo human liver using RNA-Seq. Gupta R, Schrooders Y, Hauser D, van Herwijnen M, **Albrecht W,** ter Braak B, Brecklinghaus T, Castell JV, Elenschneider L, Escher S, Guye P, Hengstler JG, Ghallab A, Hansen T, Leist M, Maclenan R, Moritz W, Tolosa T, Tricot T, Verfaille C, Walker P, van de Water B, Kleinhans J, Caiment F. **Archives of Toxicology (95):573-589 (2021)**

Handling deviating control values in concentration-response curves. Kappenberg F, Brecklinghaus T, **Albrecht W,** Blum J, van der Wurp C, Leist M, Hengstler JG, Rahnenführer J. **Archives of Toxicology (94): 3787-3798 (2020)**

The EU-ToxRisk method documentation, data processing and chemical testing pipeline for the regulatory use of new approach methods. Krebs A, van Vugt-Lussenburg BMA, Waldmann T, **Albrecht W**, Boel J, ter Braak B, Brajnik M, Braunbeck T, Brecklinghaus T, Busquet F, Dinnyes A, Dokler J, Dolde X, ExnerTE, Fisher C, Fluri D, Forsby A, Hengstler JG, Holzer A-K, Janstova Z, Jennings P, KisituJ, Kobolak J, Kumar M, Limonciel A, Lundqvist J, Mihalik B, Moritz W, Pallocca G, Ullola APC, Pastor M, Rovida C, Sarkans U, Schimming JP, Schmidt BZ, Stöber R, Strassfeld T, van de Water B, Wilmes A, van der Burg B, Verfaille CM, von Hellfeld R, Vrieling H, Vrijenhoek NG, Leist M. **Archives of Toxicology (94): 2435-2461 (2020)**

Prediction of human drug-induced liver injury (DILI) in relation to oral doses and blood concentrations. Albrecht W, Kappenberg F, Brecklinghaus T, Stoeber R, Marchan R, Zhang M, Ebbert K, Kirschner H, Grinberg M, Leist M, Moritz W, Cadenas C, Ghallab A, Reinders J, Vartak N, van Thriel C, Golka K, Tolosa L, Castell JV, Damm G, Seehofer D, Lampen A, Brauning A, Buhrke T, Behr A-C, Oberemm A, Gu X, Kittana N, van de Water B, Kreiling R, Fayyaz S, van Aerts L, Smedsrød B, Ellinger-Ziegelbazer E, Steger-Hartmann T, Gundert-Remy U, Zeigerer A, Ullrich A, Runge D, Lee SML, Schiergens TS, Kuepfer L, Aguayo-Orozco A, Sachinidis A, Edlund K, Gardner I, Rahnenführer J, Hengstler JG. **Archives of Toxicology (93): 1609-1637 (2019)**

Toxicogenomics directory of rat hepatotoxicants in vivo and in cultivated hepatocytes. Grinberg M, Stöber RM, **Albrecht W**, Edlund K, Schug M, Godoy P, Cadenas C, Marchan R, Lampen A, Brauning A, Buhrke T, Leist M, Oberemm A, Hellwig B, Kamp H, Gardner I, Escher S, Taboureau O, Aguayo-Orozco A, Sachninidis A, Elliginger-Ziegelbauer H, Rahnenführer J, Hengstler JG. **Archives of Toxicology (92): 3517-3533 (2018)**

Relevance of the incubation period in cytotoxicity testing with primary human hepatocytes. Gu X, **Albrecht W**, Edlund K, Kappenberg F, Rahnenführer J, Leist M, Moritz W, Godoy P, Cadenas C, Marchan R, Brecklinghaus T, Tolosa Pardo T, Castell JV, Gardner I, Han B, Hengstler JG, Stoeber R. **Archives of Toxicology (92): 3505-3515 (2018)**

9.2 Review

Road Map for Development of Stem Cell-Based Alternative Test Methods. Sachinidis A, **Albrecht W**, Nell P, Cherianidou A, Hewitt NJ, Edlund K, Hengstler JG. **Trends in Molecular Medicine. (25):470-481 (2019)**

9.3 Contributions on congresses

Talk: **Prediction of human drug-induced liver injury (DILI) in relation to oral doses and blood concentrations. Albrecht W,** Kappenberg F, Brecklinghaus T, Edlund K, Gardner I, Rahnenführer J, Hengstler JG. 5th German Pharm-Tox Summit, German Society for Experimental and Clinical Pharmacology and Toxicology (DGPT), Leipzig, Germany, 04.03.2020

Talk: **Prediction of human drug-induced liver injury (DILI) in relation to oral doses and blood concentrations. Albrecht W**, Kappenberg F, Brecklinghaus T, Edlund K, Gardner I, Rahnenführer J, Hengstler JG, Tag der Chemie, TU Dortmund University, Dortmund, Germany, 07.02.2020

Poster: **Case study 6: In vitro based prediction of human hepatotoxicity. Albrecht W, Brecklinghaus T,** Kappenberg F, Stöber R, Gu Xiaolong, Edlund K, Tolosa Pardo L, Castell JV, van Aerts L, Escher S, Leist M, van de Water B, Rahnenführer J, Hengstler JG. EU ToxRisk General Assembly, Egmond, The Netherlands, 12.-14. 02. 2019

Poster: **Case study 6: In vitro based prediction of human hepatotoxicity.** Stöber R, **Albrecht W**, Gu X, Kappenberg F, Escher S, Leist M, van de Water B, Gardner I, Rahnenführer J, Hengstler JG. EU ToxRisk General Assembly, Egmond, The Netherlands, 20.-23. 02. 2018

Poster: **Case studies 1 and 6: Relevance of repeated exposure and co-incubation with fatty acids on in vitro cytotoxicity in primary human hepatocytes.** Stöber R, **Albrecht W**, Gu X, Kappenberg F, Escher S, Leist M, van de Water B, Gardner I, Han B, Rahnenführer J, Hengstler JG. EU ToxRisk General Assembly, Egmond, The Netherlands, 20.-23. 02. 2018

Poster: **Acute and repeated dose toxicity testing of valproic acid and structure analogues in primary human hepatocytes.** Stöber R, Gu Xi, **Albrecht W**, Escher S, Kappenberg F, Rahnenführer J, Hengstler JG. EU ToxRisk General Assembly, Egmond, The Netherlands, 20.-23. 02. 2018

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