Review Article

Investigating Cell Type Specific Mechanisms Contributing to Acute Oral Toxicity

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Abstract

The replacement of animals in acute systemic toxicity testing remains a considerable challenge. Only animal data are currently accepted by regulators, including data generated by reduction and refinement methods. The development of integrated approaches to testing and assessment (IATA) is hampered by an insufficient understanding of the numerous toxicity pathways that lead to acute systemic toxicity. Therefore, central to our work has been the collection and evaluation of the mechanistic information on eight organs identified as relevant for acute systemic toxicity (nervous system, cardiovascular system, liver, kidney, lung, blood, gastrointestinal system, and immune system). While the nervous and cardiovascular systems are the most frequent targets, no clear relationship emerged between specific mechanisms of target organ toxicity and the level (category) of toxicity. From a list of 114 chemicals with acute oral *in vivo* and *in vitro* data, 97 were identified with target organ specific effects, of which 94% (91/97) were predicted as acutely toxic by the 3T3 neutral red uptake cytotoxicity assay and 6% (6/97) as non-toxic. Although specific target organ mechanisms of toxicity could in some cases explain the false negative prediction obtained with the cytotoxicity assay, in general it is difficult to explain *in vitro* misclassifications only on the basis of mechanistic information. This analysis will help to prioritize the development of adverse outcome pathways for acute oral toxicity, which will support the assessment of chemicals using mechanistically informed IATA.

1 Introduction

Acute systemic toxicity after oral, dermal, or inhalation exposure requires that the substance becomes bioavailable at the target site and induces lethality through general toxicity or a specific mechanism. This means that kinetic factors, and mainly absorption, are important determinants of toxicity (EURL ECVAM, 2015). In addition, if the damage involves interference with homeostatic mechanisms at the organ system level, non-exposed tissues and vital organs can also be affected (Gennari et al., 2004; Andrew, 2013).

The assessment of acute systemic toxicity is a core component of the safety assessment of substances in the context of EU and international legislations (Hamm et al., 2017). Information requirements vary depending on the type of substance subject to regulation and the region (EU, 2006, 2008, 2009a,b, 2012). The regulatory landscape in the USA was reviewed during two workshops cosponsored by the NTP Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM), the People for the Ethical Treatment of Animals (PETA) International Science Consortium Ltd, and the Physicians Committee for Responsible Medicine (PCRM) (Hamm et al., 2017; Clippinger et al., 2018a; Strickland et al., 2018). The relevant information is available at the PETA website[1]. In preclinical drug development, however, these studies are no longer required by default to support first clinical trials in humans (Robinson et al., 2008; ICH, 2009; Chapman et al., 2010).

One of the main uses of acute systemic toxicity data is classification and labelling (Seidle et al., 2010; Graepel et al., 2016; Buesen et al., 2016; Strickland et al., 2018). Within the EU, the CLP (Classification, Labelling and Packaging) Regulation (EU, 2008) is used to classify chemicals on the basis of acute oral toxicity into four toxicity categories (categories 1 to 4 of the United Nations Globally Harmonised System of Classification and La-

[1] https://www.piscltd.org.uk/acute-systemic-toxicity/ (accessed 28.06.2018).

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belling (UN GHS)). While CLP does not require animal testing, the classification criteria are based on data derived from animal tests (conducted, for example, under other pieces of legislation), including reduction and refinement methods for the oral, dermal, and inhalation routes (OECD TGs 402, 403, 420, 423, 425, 433, 436). Most of the standard *in vivo* tests use lethality as the endpoint, even though this has been widely criticized both on animal welfare and scientific grounds (Zbinden and Flury-Roversi, 1981; Hoffmann et al., 2010; Prieto et al., 2013a).

Basal cytotoxicity is certainly a key factor in many prevalent toxicological modes-of-action associated with acute health effects. It covers many general mechanisms of toxicity common to most cell types that can lead to organ failure including, for example, disruption of cell membrane structure or function, inhibition of mitochondrial function, disturbance of protein turnover, and disruption of metabolism and energy production (Gennari et al., 2004; NIH, 2009; Andrew, 2013). This is the reason why the utility of *in vitro* cytotoxicity assays to predict acute oral toxicity has been extensively investigated (Ekwall, 1999; Halle, 2003; NIH, 2006; Prieto et al., 2013a,b). Recently, Vinken and Blaauboer (2017) proposed the application of an adverse outcome pathway (AOP) framework for basal cytotoxicity consisting of three consecutive steps, i.e., initial cell injury, mitochondrial dysfunction, and cell death. The outcome of the basal cytotoxicity was then suggested by the authors as the first step of a tiered strategy aimed to evaluate the toxicity of new chemical entities. Further, in a second step, more specific types of toxicity could be evaluated.

One of the better known and standardized *in vitro* methods for basal cytotoxicity is the 3T3 Neutral Red Uptake (NRU) assay (DB-ALM protocol 139[2]; Stokes et al., 2008). The use of data from the NRU cytotoxicity assay within a Weight-of-Evidence (WoE) assessment is one of the choices for adapting the standard information requirements for acute oral toxicity, as described in the last update of the ECHA's guidance on Information Requirements and Chemical Safety Assessment. This WoE adaptation proposed by ECHA applies primarily to low toxicity substances (i.e., those that are not to be classified for acute toxicity) and it is based on an in-depth analysis of the REACH database (Gissi et al., 2017; ECHA, 2017). Nevertheless, the limitations of the *in vitro* cytotoxicity assay, such as the lack of metabolic competence of 3T3 cells and difficulty to capture specific mechanisms of action relating to interaction with specific molecular targets in certain tissues, need to be considered when building a WoE case for the purposes of REACH (Buesen et al., 2018; Gissi et al., 2018).

In addition to the assessment of basal cytotoxicity, it is also important to identify cell types and *in vitro* endpoints that are indicative of cell-type specific toxicities, with a view to incorporating such endpoints into integrated approaches to testing and assessment (IATA), as proposed in the EURL ECVAM strategy to replace, reduce, and refine the use of animals in the assessment of acute mammalian systemic toxicity (EURL ECVAM, 2014). As defined by the OECD, IATA are pragmatic, science-based approaches for chemical hazard or risk characterization that rely on an integrated analysis of existing information in a WoE assessment coupled with the generation of new information, if required (OECD, 2016a). An iterative approach that preferably relies on mechanistic information or available AOPs is followed to answer a defined question in a specific regulatory context, taking into account the acceptable level of uncertainty associated with the decision making (OECD, 2016a; Sachana and Leinala, 2017). The importance of understanding the mechanisms of acute toxicity was further recognized during an international workshop in which a group of experts discussed alternative approaches for identifying acute systemic toxicity (Hamm et al., 2017; Clippinger et al., 2018a). A better theoretical and mechanistic understanding of acute systemic toxicity would be useful to developers of test methods and other predictive tools as well as to validation and regulatory bodies.

Mechanisms involved in cellular failure and susceptible functions compromised in organ failure were discussed at an ECVAM workshop on strategies to replace *in vivo* acute systemic toxicity testing (Gennari et al., 2004). Several fundamental cellular processes common to many organ systems were identified, including energy production and metabolism (mitochondrial function and glycolysis), transportation of molecules, membrane integrity and secretion of molecules (enzymes, proteins, hormones, neurotransmitters). A number of key events associated with acute human poisoning were further identified in an ICCVAM/ECVAM/JaCVAM workshop on acute chemical safety testing (NIH, 2009) and it was agreed that mechanistic information could be used to develop more predictive *in vitro* test methods. A report, commissioned by the US Department of Defense, lists several of the cellular targets or molecular targets that are often associated with the acute lethal or debilitating effects of chemicals. This includes changes in neurotransmission function, altered ion flow, increased permeability of cellular membranes, altered bioenergetics, altered oxygen transport, oxidative stress and reactive oxygen species (ROS) formation, damage to DNA and subcellular systems, and immune-mediated effects (NRC, 2015). Hamm et al. (2017) and Clippinger et al. (2018b) have also reported some of the known mechanisms involved in acute systemic toxicity as part of ongoing activities in the US.

However, despite all the efforts made over the past 20 years in the area of acute systemic toxicity, relevant AOPs, mechanistically informed alternative methods, and IATA for acute systemic toxicity have not been adequately developed. This is partially due to the lack of a complete mechanistic understanding of the key acute toxicity pathways in humans specific for different cell types (e.g., neuronal, cardiac, liver, or kidney).

This study describes the analysis of mechanistic information collected on eight potential organs (i.e., nervous system, cardiovascular system, liver, kidney, lung, blood, gastrointestinal system (GI), and immune system) identified as relevant for acute systemic toxicity and using a set of chemicals inducing acute toxicity after oral exposure. This work will support the development of AOPs and IATA in the area of acute systemic toxicity,

[2] https://ecvam-dbalm.jrc.ec.europa.eu/ (accessed 28.06.2018).

and will inform the development and application of mechanistically relevant new approach methodologies.

2 Materials and methods

Collection of mechanistic information

Information was collected on the eight potential target organs identified as relevant for acute systemic toxicity during the ECVAM workshop on acute systemic toxicity (Gennari et al., 2004): liver, blood, kidney, cardiovascular system, central and peripheral nervous system (CNS/PNS), lung, immune system, and GI. In safety pharmacology studies, the cardiovascular, respiratory, and central nervous systems are assessed in a core battery since they are considered vital organs or systems, the functions of which are acutely critical for life (ICH, 2000).

In order to approach the ambitious task of mapping mechanisms specific for these potential target organs, a three-step approach was taken to identify the potential pathways of target organ toxicity.

1. Based on a literature review using *target organ* and *acute toxicity* and *mechanism* as key words, commonly recognized pathways of toxicity were identified for each target organ/system. Information was derived from published literature, toxicology handbooks, short descriptions of reference compounds used in the EU FP6 project ACuteTox[3], and internet databases (HSDB[4], INCHEM[5], PubChem[6], PubMed[7], Scopus[8], Google Scholar[9]). The pathways were then organized and visualized according to the target organ/system, the cell type, the effect, and the mechanism. In this context, the effect refers to any adverse reaction that could be observed or measured (*in vivo*) and the mechanism refers to the molecular or cellular process that is interrupted by chemical stressors and leads to the observed adverse effect.
2. In the second phase, the "completeness" of the theoretical pathways of toxicity that were developed in phase 1 was probed. In order to do so, we consulted the in-house database and selected chemicals that were shown to be acutely toxic. For these chemicals, a thorough literature search was conducted to identify the target organ and mechanism of toxicity, searching first for *chemical* AND *acute toxicity* and *mechanism*, followed by *chemical* AND *target organ*. These mechanisms were then added into the generated "maps" if they were not already present. Chemicals for which both *in vivo* acute oral toxicity data and *in vitro* cytotoxicity data were available were selected. On the basis of reference *in vivo* oral LD_{50} data and of the 2000 mg/kg body weight threshold introduced by the CLP Regulation, all compounds with an acute LD_{50} mean value below or equal to 2000 mg/kg were identified as acutely toxic, whereas those with an acute oral LD_{50} mean value above 2000 mg/kg were identified as non-acutely toxic. Only the chemicals that fell into our group of toxic chemicals were considered in this second phase.
3. In a third phase, chemicals from the group of non-toxic chemicals that, nevertheless, had been assigned a harmonized classification (Annex VI of EU CLP Regulation) were identified and selected.

The mechanisms collected and shown in this report are not intended to be exhaustive.

Selection of chemicals with in vivo *LD_{50} values and* in vitro *cytotoxicity data*

In the in-house database 178 test chemicals had oral LD_{50} values[10] that were collected from publicly available databases (e.g., ChemIDplus, IUCLID, RTECS, and HSDB), Merck index, EU Risk Assessment Reports, Sax's Dangerous Properties of Industrial Materials, and the published literature. According to the calculated mean LD_{50} values, 112 test chemicals were assigned to an EU CLP acute oral toxicity category and 66 remain as non-toxic (i.e., no category assigned because the LD_{50} was higher than 2000 mg/kg). Eleven out of the 66 non-classified chemicals had an official acute oral classification.

In vitro cytotoxicity data were available for 177 test chemicals that had been screened in the following international projects: NICEATM/ECVAM validation study (NIH, 2006), the EU FP6 project ACuteTox (Prieto et al., 2013a), and the ECVAM validation study (Prieto et al., 2013b). The list of chemicals used in each study is available through the JRC Chemical Lists of Information System (CheList[11]).

When the two sets were compared, *in vitro* cytotoxicity data were not available for two compounds, formaldehyde and carbon tetrachloride, and *in vivo* oral LD_{50} data were not found for benz(a)anthracene. Therefore, the final common set contained 176 chemicals.

3 Results

3.1 Overall analysis of mechanistic maps

The mechanistic information collected following the three-step strategy was visualized in maps according to the eight organs/systems. The layout and structure across organs and systems

3 http://www.acutetox.eu/ (accessed 28.06.2018).

4 https://toxnet.nlm.nih.gov/newtoxnet/hsdb.htm (accessed 28.06.2018).

5 http://www.inchem.org/ (accessed 28.06.2018).

6 https://pubchem.ncbi.nlm.nih.gov/ (accessed 28.06.2018).

7 https://www.ncbi.nlm.nih.gov/pubmed (accessed 28.06.2018).

8 https://www.elsevier.com/solutions/scopus (accessed 28.06.2018).

9 https://scholar.google.it/ (accessed 28.06.2018).

10 Rat oral LD50 values (mg/kg body weight) collected for 178 chemicals in the context of the following international projects NICEATM/ECVAM validation study (NIH, 2006), the EU FP6 project ACuteTox (Hoffmann et al., 2010) and the ECVAM validation study (Prieto et al., 2013b): doi:10.14573/altex.1805181s1

11 http://chelist.jrc.ec.europa.eu/ (accessed 28.06.2018).

was harmonized and analyzed as shown below. Three maps were created per target organ/system. A first map illustrated the mechanisms found based on information collected from literature (step 1 under methods). The second map was an updated version based on the information collected from the in-house list of selected compounds (steps 2 and 3 under methods). The final harmonized version of each organ/system was shown by the third map (Fig. 1-8).

Information on mechanisms of toxicity was collected for 114 out of the 123 oral acutely toxic chemicals (see Methods). In terms of target organ/systems, the overall analysis summarized in Figure 9 shows that, according to the information found, the nervous and cardiovascular systems are the most frequent targets (67 and 39 chemicals, respectively) followed by liver, kidney, lung, gastrointestinal system, blood and immune system (31, 30, 24, 18, 11, and 3 chemicals, respectively). Twenty-six chemicals appear to target single organs, in particular the nervous systems (12 chemicals). Seventy-five chemicals affect more than one organ/system and thirteen chemicals affect all organs (non-specific target organ effects) (Fig. 10). Indirect effects were reported for 9 chemicals with multi-organ/system effects: 6 on the lung, 1 on the kidney, and 4 on the cardiovascular system (Fig. 9).

General cytotoxicity mechanisms were cited for 72 chemicals and target organ/system specific effects for 40 chemicals (11 chemicals acting on a single organ/system and 29 on multiple targets) (see Tab. 1). For pentachlorobenzene and tetramethylthiuram monosulfide, the specific mechanism of acute toxicity was not found.

Tables 2-9 provide an overview of the specific target organ/system mechanisms leading to acute toxicity according to the information collected from the literature. The list of mechanisms shown is neither exhaustive nor definitive.

Referring to organ specific mechanisms of toxicity, interference with neurotransmitters and/or neurotransmission and impairment of propagation of electrical activity are among the main reported mechanisms for chemicals that target the nervous system. In particular, many chemicals interfere at the level of receptors and ion channel function.

Chemicals that target the cardiovascular system often interfere with ion balance/signaling/membrane potential of the cell and with intracellular signaling mechanisms.

For many of the chemicals that damage the liver after an acute insult, mechanisms such as depletion of free radical scavengers, ROS production, lipid peroxidation (grouped under oxidative stress induced inflammation), and necrosis were reported.

Alterations in kidney tubule cell structure (accumulation in proximal tubular cells, loss of tubular epithelial barrier, and/or tight junctions), alterations in tubule cell metabolism (interference with ion balance), tubular obstruction (impaired Na^+ and water reabsorption, distal cast formation, crystal deposition), and alterations in cell viability (necrosis) are among the most reported mechanisms leading to acute renal failure.

The *in vivo* classification for acute oral toxicity (i.e., the assigned CLP acute oral toxicity categories based on the collected mean oral LD_{50} values) of the chemicals acting via specific mechanisms of toxicity at organ/system level was evaluated in view of the information found in the literature for each chemical. Table 10 summarizes the outcome of this analysis, confirming that the nervous and cardiovascular systems are the most frequent targets for chemicals inducing acute oral toxicity.

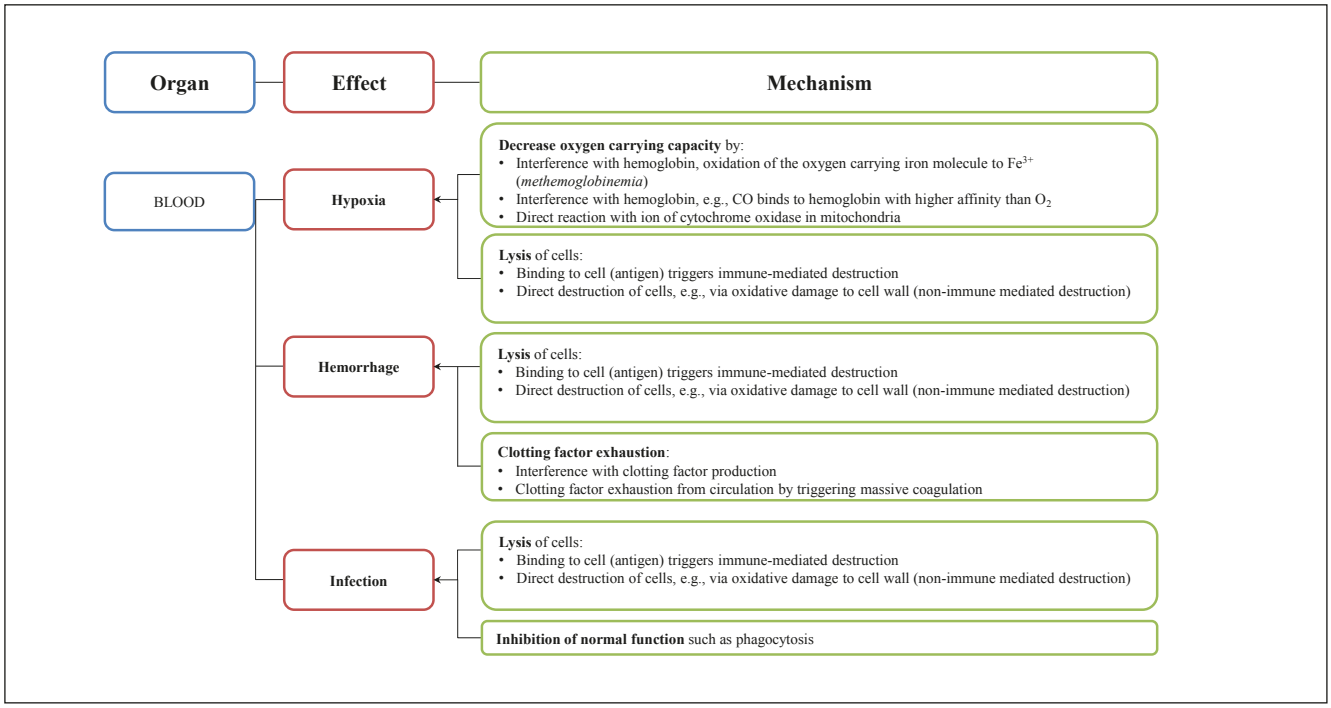


Fig. 1: Target organ blood – visualization of mechanisms leading to acute systemic toxicity
CO, carbon monoxide

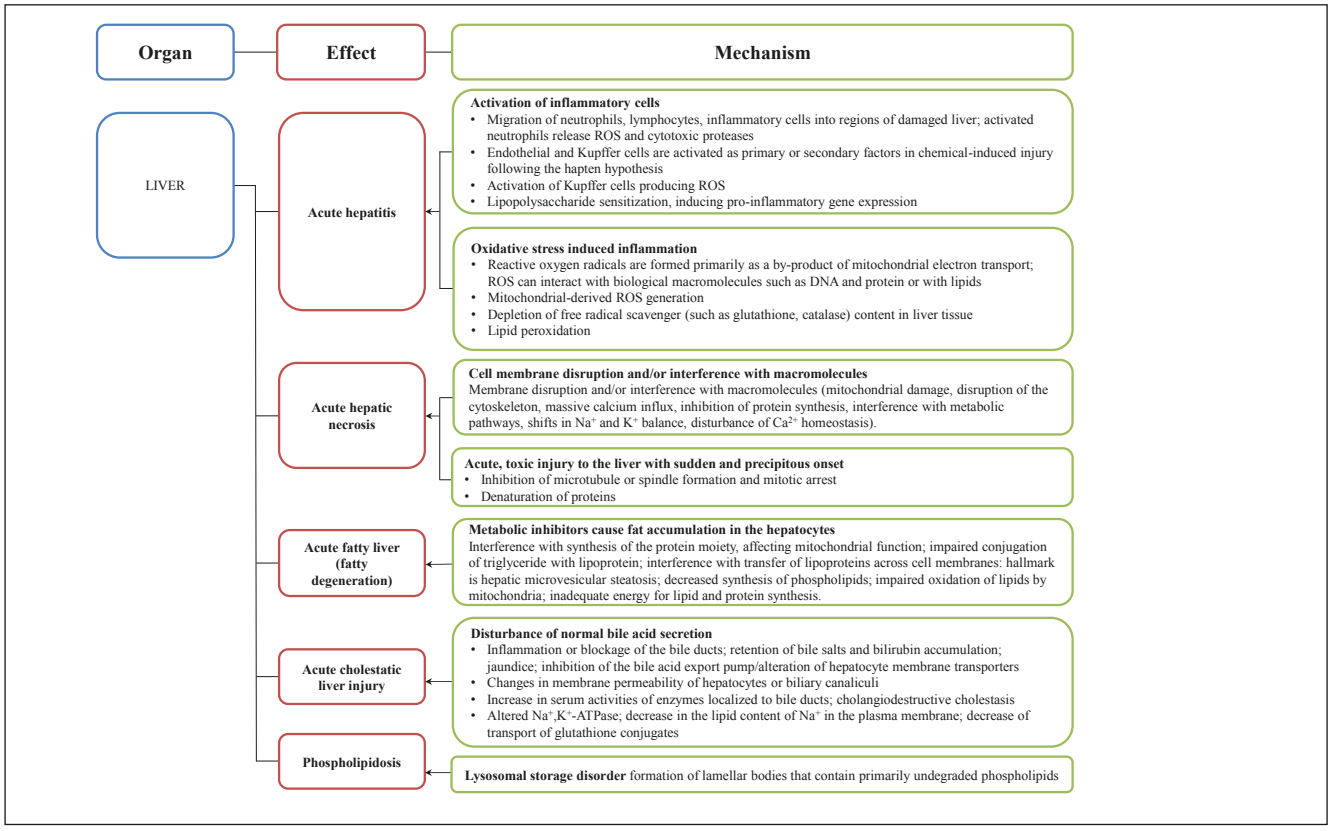


Fig. 2: Target organ liver – visualization of mechanisms leading to acute systemic toxicity
ROS, reactive oxygen species

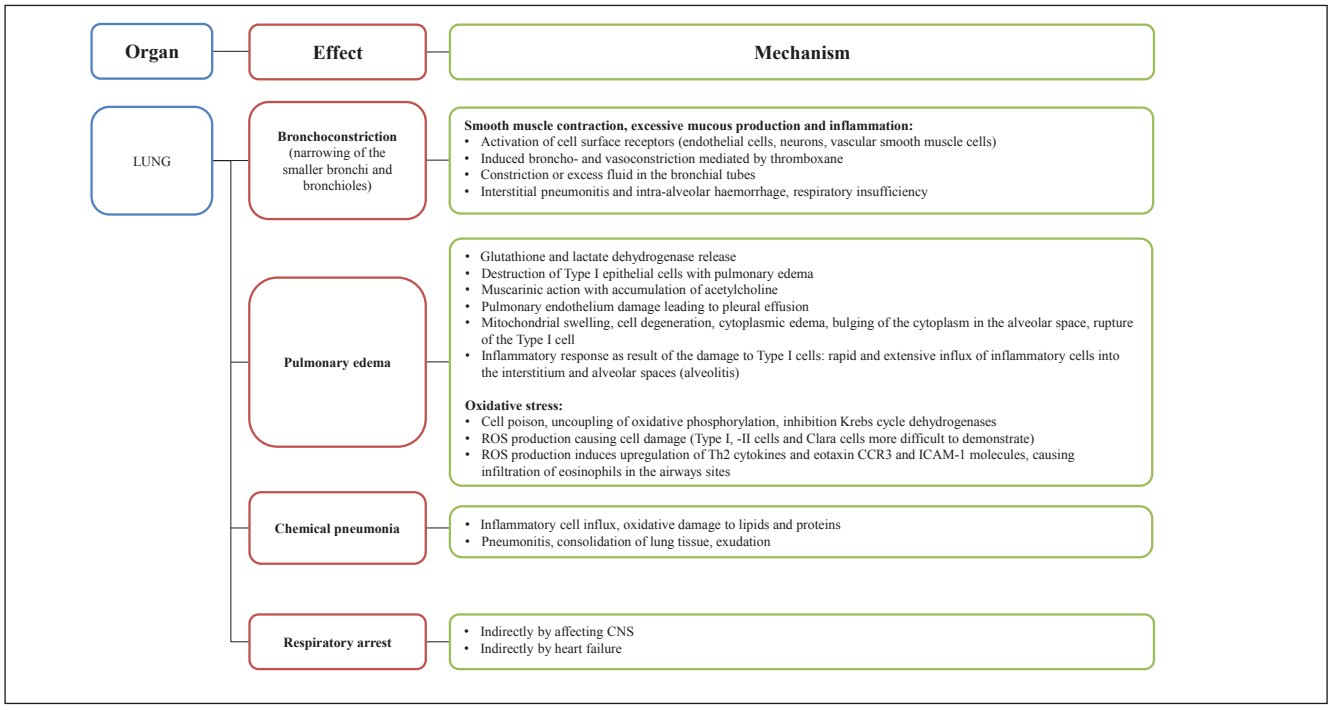


Fig. 3: Target organ lung – visualization of mechanisms leading to acute systemic toxicity
Th2, T helper type 2 cells; ROS, reactive oxygen species; CCR3, C-C motif chemokine receptor 3; ICAM-1, Intercellular Adhesion Molecule 1; CNS, central nervous system

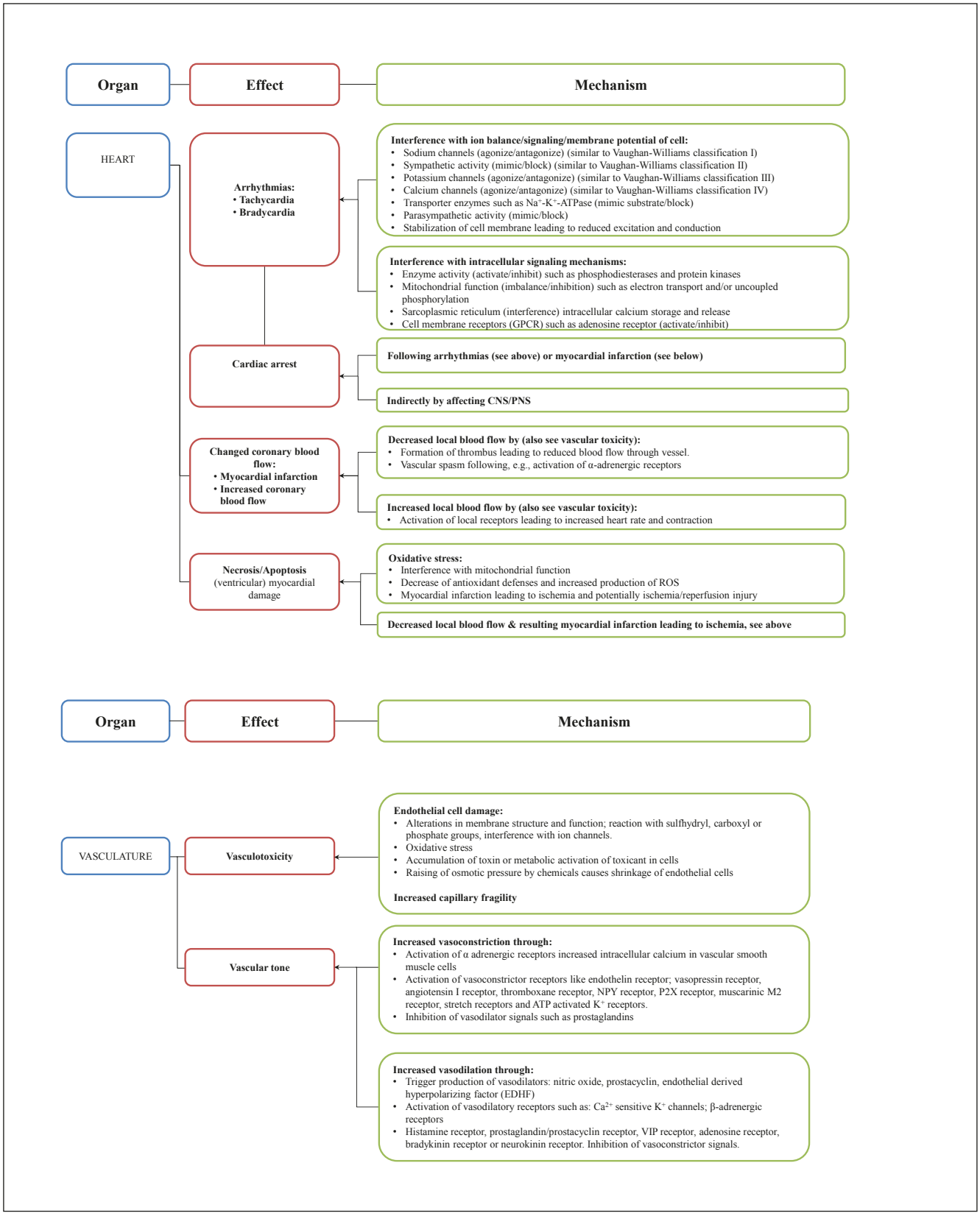
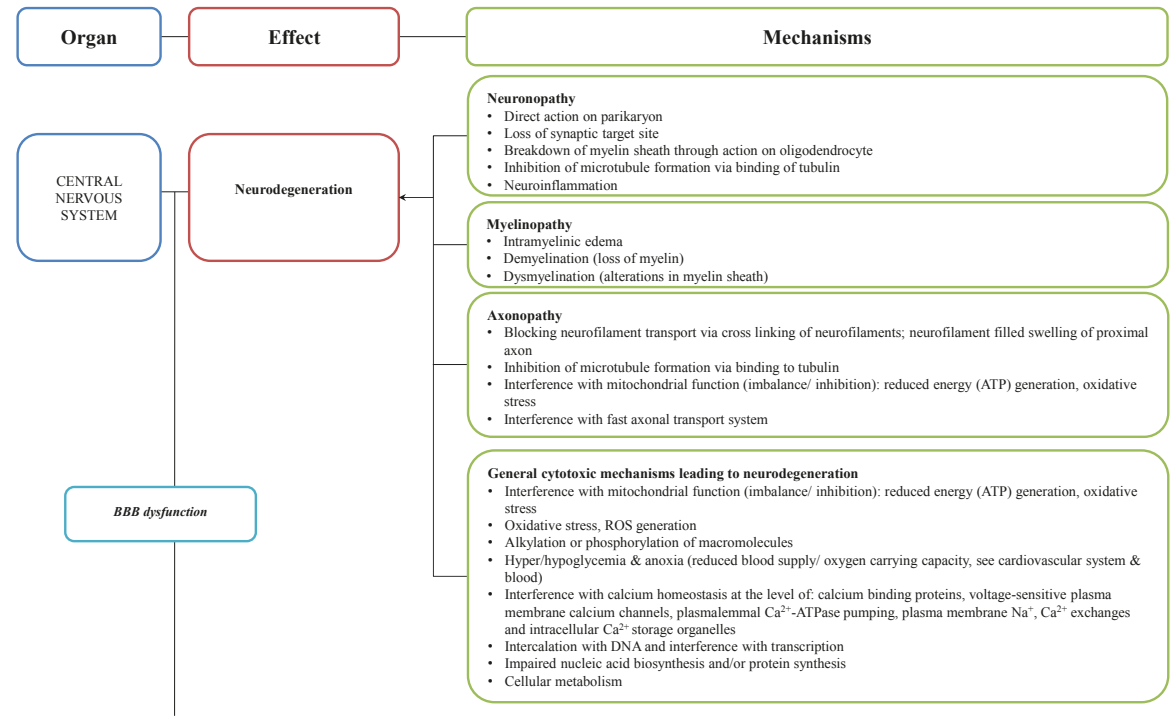
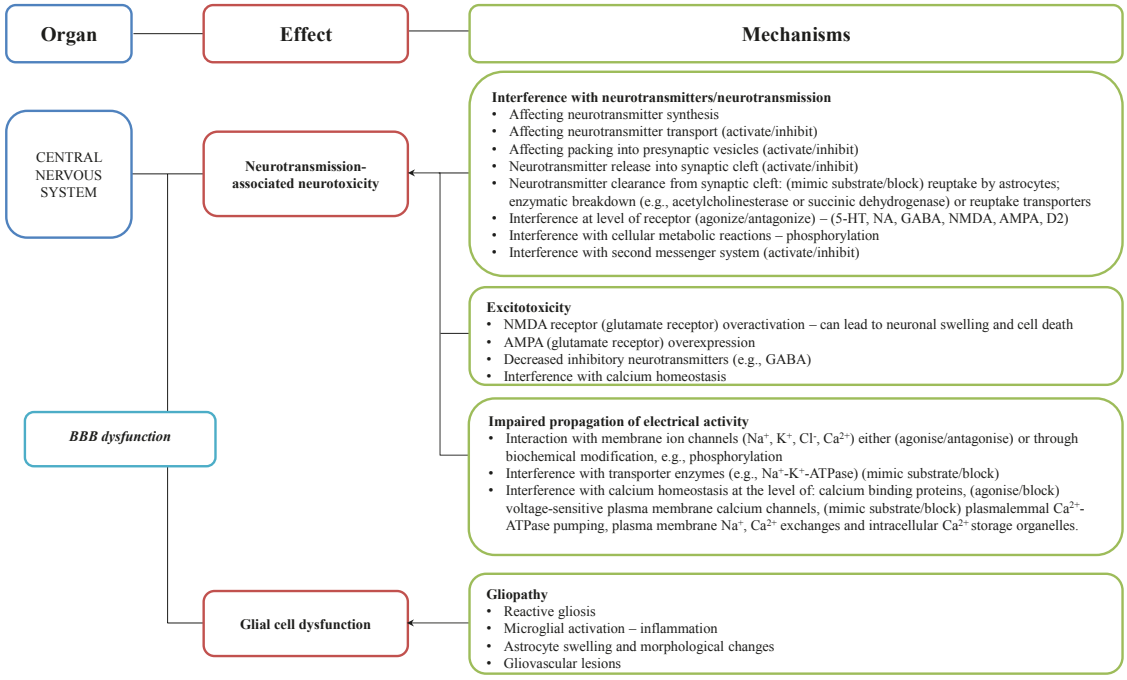


Fig. 4: Cardiovascular system – visualization of mechanisms leading to acute systemic toxicity

GPCRs, G protein-coupled receptors; CNS, central nervous system; PNS, peripheral nervous system; ROS, reactive oxygen species; NPY, neuropeptide Y; P2X, purinergic receptors; M2, muscarinic acetylcholine receptor; VIP, vasoactive intestinal peptide


Organ
Effect
Mechanisms
CENTRAL NERVOUS SYSTEM
Neurodegeneration
BBB dysfunction
Neuronopathy
• Direct action on parikaryon
• Loss of synaptic target site
• Breakdown of myelin sheath through action on oligodendrocyte
• Inhibition of microtubule formation via binding of tubulin
• Neuroinflammation
Myelinopathy
• Intramyelinic edema
• Demyelination (loss of myelin)
• Dysmyelination (alterations in myelin sheath)
Axonopathy
• Blocking neurofilament transport via cross linking of neurofilaments; neurofilament filled swelling of proximal axon
• Inhibition of microtubule formation via binding to tubulin
• Interference with mitochondrial function (imbalance/ inhibition): reduced energy (ATP) generation, oxidative stress
• Interference with fast axonal transport system
General cytotoxic mechanisms leading to neurodegeneration
• Interference with mitochondrial function (imbalance/ inhibition): reduced energy (ATP) generation, oxidative stress
• Oxidative stress, ROS generation
• Alkylation or phosphorylation of macromolecules
• Hyper/hypoglycemia & anoxia (reduced blood supply/ oxygen carrying capacity, see cardiovascular system & blood)
• Interference with calcium homeostasis at the level of: calcium binding proteins, voltage-sensitive plasma membrane calcium channels, plasmalemmal Ca2+-ATPase pumping, plasma membrane Na+, Ca2+ exchanges and intracellular Ca2+ storage organelles
• Intercalation with DNA and interference with transcription
• Impaired nucleic acid biosynthesis and/or protein synthesis
• Cellular metabolism


Organ
Effect
Mechanisms
CENTRAL NERVOUS SYSTEM
Neurotransmission-associated neurotoxicity
BBB dysfunction
Glial cell dysfunction
Interference with neurotransmitters/neurotransmission
• Affecting neurotransmitter synthesis
• Affecting neurotransmitter transport (activate/inhibit)
• Affecting packing into presynaptic vesicles (activate/inhibit)
• Neurotransmitter release into synaptic cleft (activate/inhibit)
• Neurotransmitter clearance from synaptic cleft: (mimic substrate/block) reuptake by astrocytes; enzymatic breakdown (e.g., acetylcholinesterase or succinic dehydrogenase) or reuptake transporters
• Interference at level of receptor (agonize/antagonize) – (5-HT, NA, GABA, NMDA, AMPA, D2)
• Interference with cellular metabolic reactions – phosphorylation
• Interference with second messenger system (activate/inhibit)
Excitotoxicity
• NMDA receptor (glutamate receptor) overactivation – can lead to neuronal swelling and cell death
• AMPA (glutamate receptor) overexpression
• Decreased inhibitory neurotransmitters (e.g., GABA)
• Interference with calcium homeostasis
Impaired propagation of electrical activity
• Interaction with membrane ion channels (Na+, K+, Cl-, Ca2+) either (agonise/antagonise) or through biochemical modification, e.g., phosphorylation
• Interference with transporter enzymes (e.g., Na+-K+-ATPase) (mimic substrate/block)
• Interference with calcium homeostasis at the level of: calcium binding proteins, (agonise/block) voltage-sensitive plasma membrane calcium channels, (mimic substrate/block) plasmalemmal Ca2+-ATPase pumping, plasma membrane Na+, Ca2+ exchanges and intracellular Ca2+ storage organelles.
Gliopathy
• Reactive gliosis
• Microglial activation – inflammation
• Astrocyte swelling and morphological changes
• Gliovascular lesions

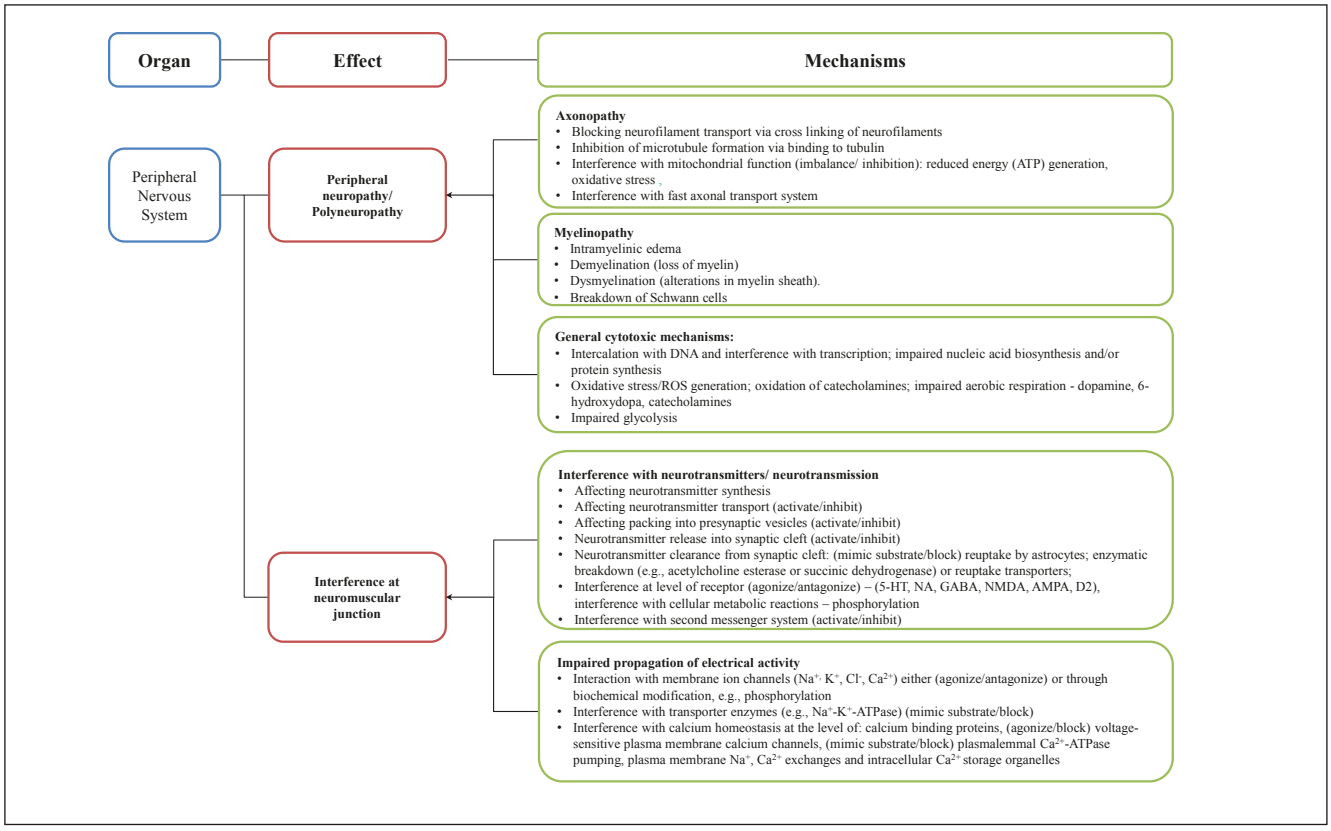


Fig. 5: Nervous system – visualization of mechanisms leading to acute systemic toxicity
ROS, reactive oxygen species; BBB, blood brain barrier; 5-HT, 5-hydroxytryptamine; NA, noradrenaline; GABA, gamma-aminobutyric acid; NMDA, N-methyl-D-aspartate; AMPA, α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid; D2, dopamine

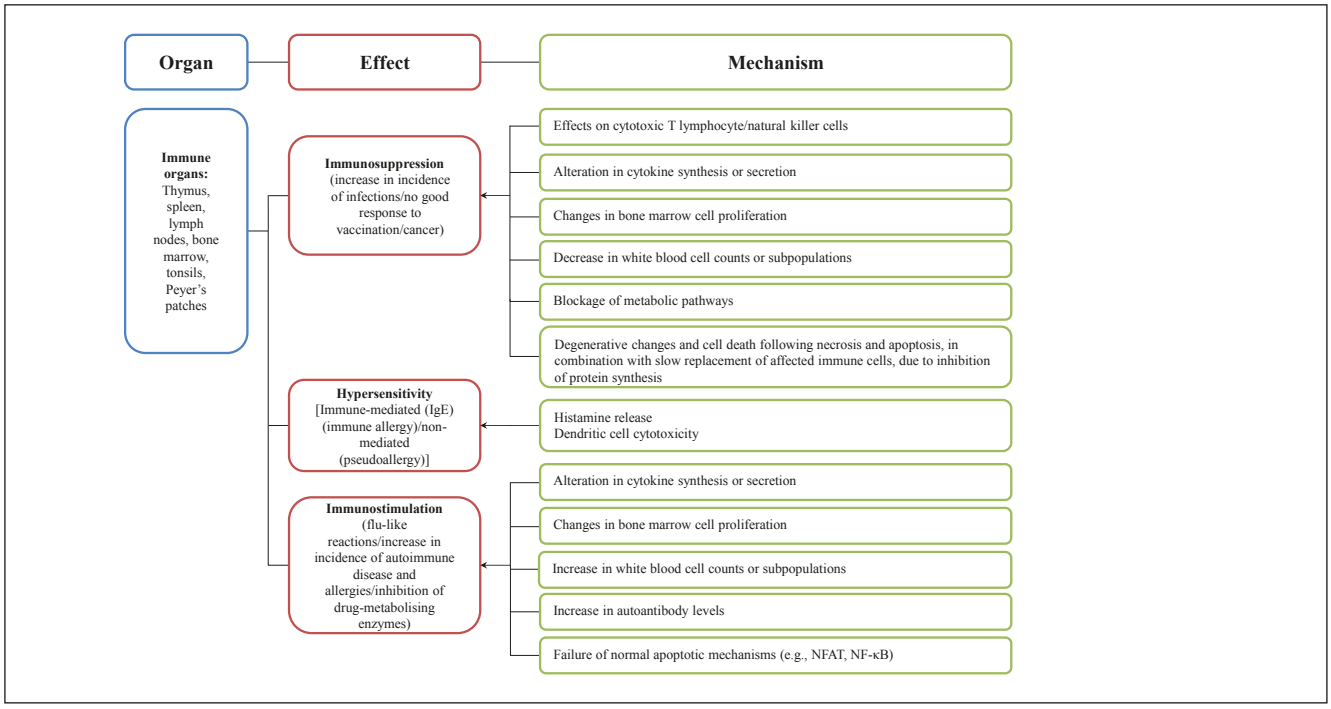


Fig. 6: Immune system – visualization of mechanisms leading to acute systemic toxicity
NFAT, nuclear factor of activated T-cells; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells

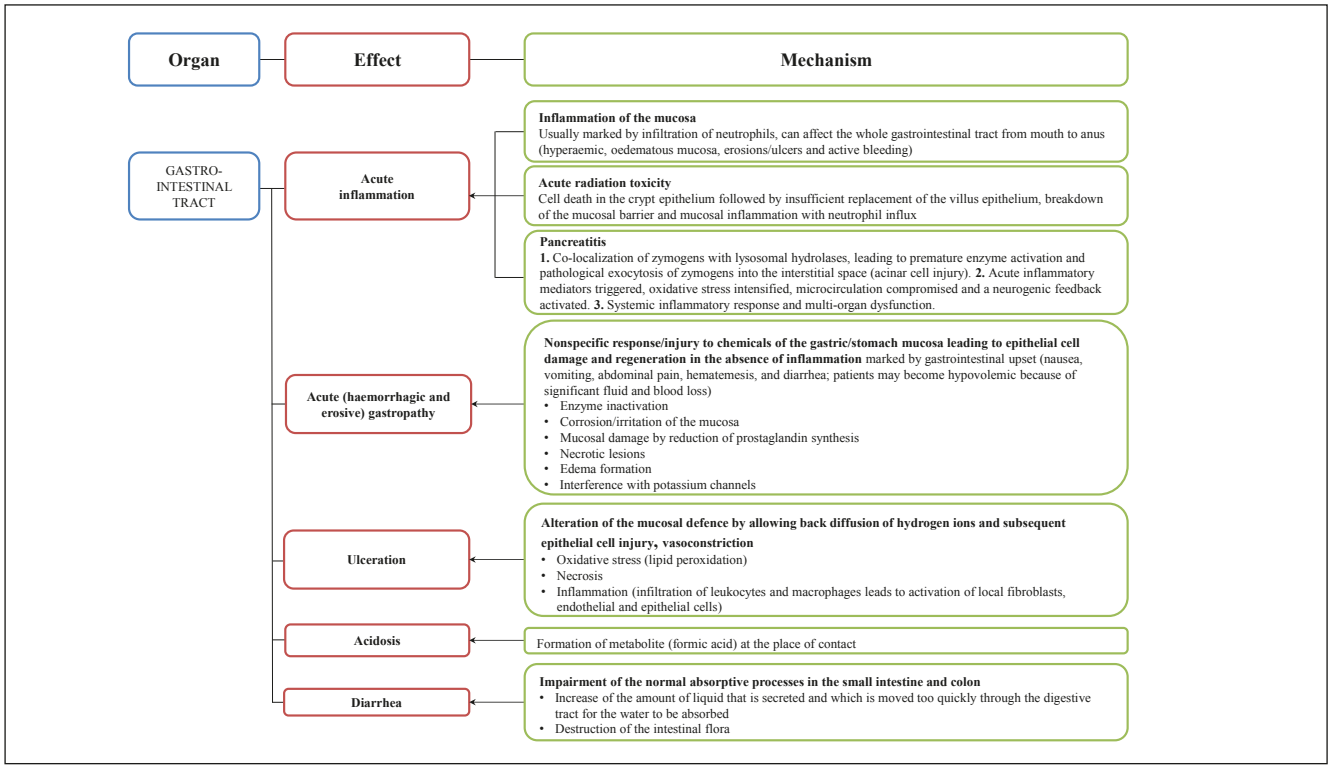


Fig. 7: Gastrointestinal system – visualization of mechanisms leading to acute systemic toxicity

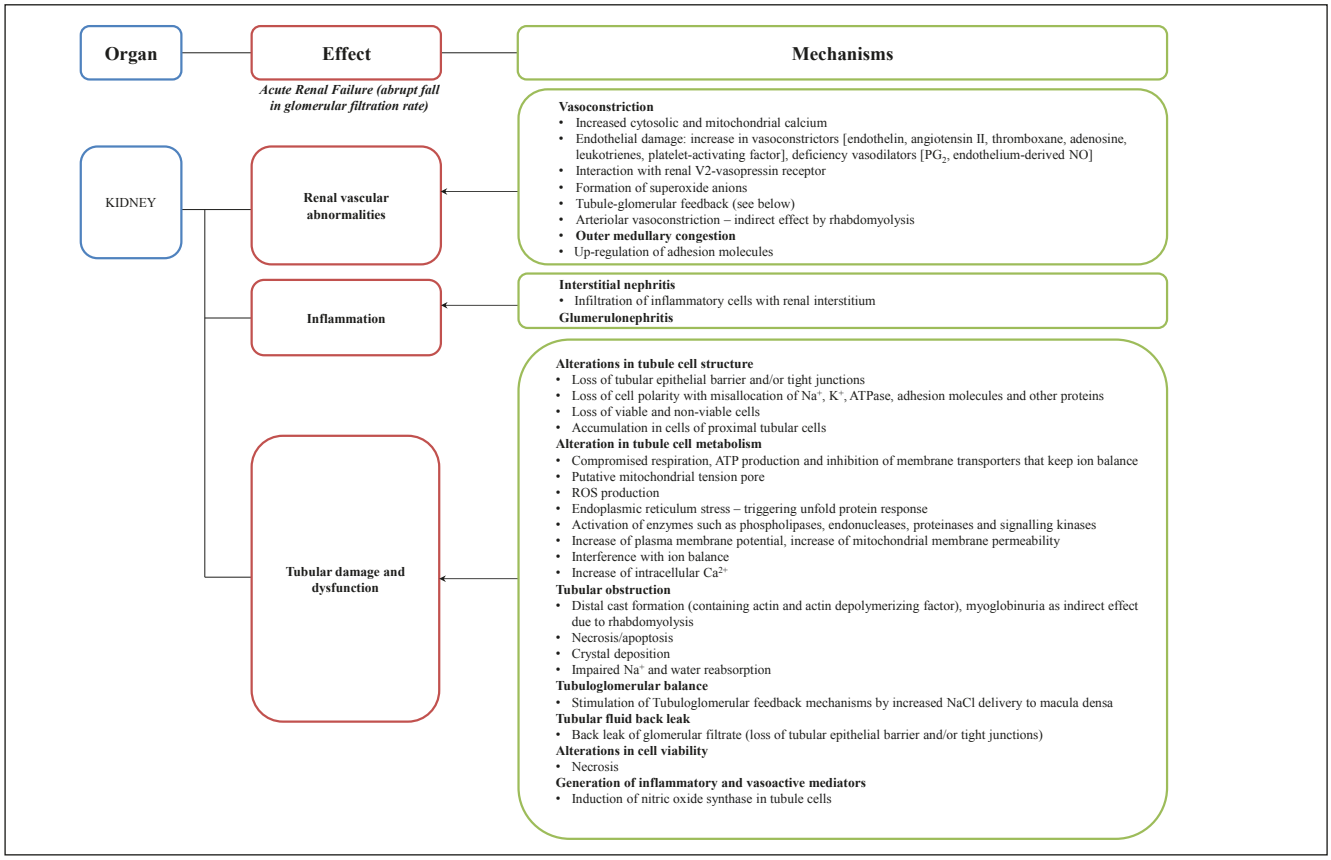


Fig. 8: Target organ kidney – visualization of mechanisms leading to acute systemic toxicity
ROS, reactive oxygen species

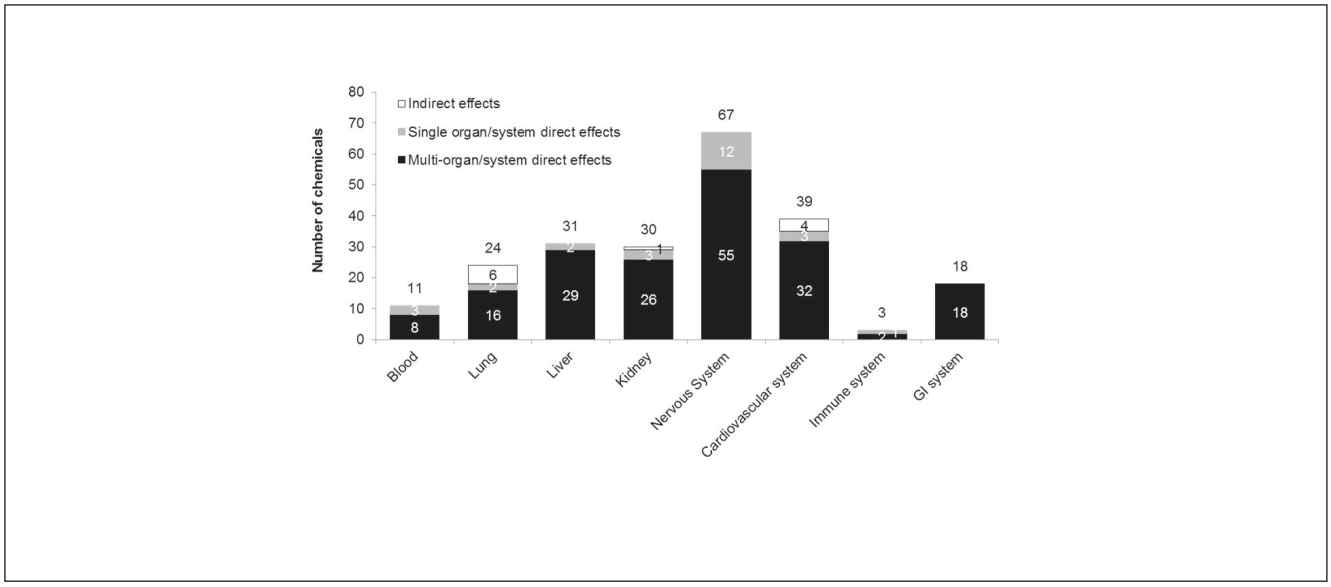


Fig. 9: Frequency of target organs/systems effects after acute oral insult
The number on top of each bar represents the number of chemicals affecting a particular organ/system. GI, gastrointestinal system

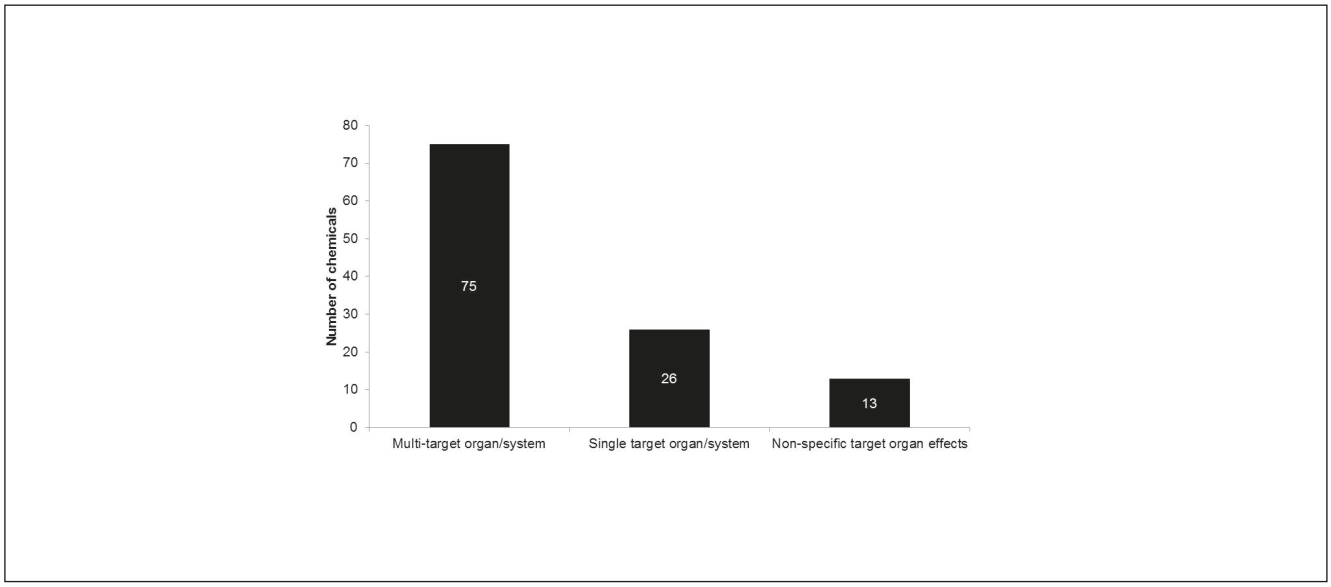


Fig. 10: Chemicals with specific target organs/systems effects (single and multiple) and non-specific effects
Indirectly acting chemicals are included under multi-target organ/system.

Tab. 1: Chemicals acting through general cytotoxic mechanisms and/or specific mechanisms of toxicity

General cytotoxic mechanisms		Specific mechanism of toxicity
(4-Ammonio-m-tolyl)ethyl (2-hydroxyethyl) ammonium sulfate	Endosulfan	(±)-Propranolol hydrochloride
1,2,3,4-Tetrachlorobenzene	Ethoxyquin	(±)-Verapamil hydrochloride
1,2,4-Trichlorobenzene	Ferrous sulfate	1-Naphthylamine
1,2-Dichlorobenzene	Formaldehyde	1-Phenyl-3-pyrazolidone
17α-Ethynyloestradiol	Glutethimide	2,4,6-Tris(dimethylaminomethyl)phenol
1-Phenyl-2-thiourea	Haloperidol	5,5-Diphenylhydantoin
2,4-Dichlorophenoxyacetic acid	Hexachlorophene	Acetophenone
4-Aminofolic acid	Isoniazid	Ammonium chloride
5-Fluorouracil	Lindane	Atropine sulfate monohydrate
Acetaldehyde	Maleic acid	Codeine
Acetylsalicylic acid	Malononitrile	D-Amphetamine
Aconitine	Maprotiline	Diazepam
Acrolein	Mercury II chloride	Diethylene glycol
Acrylamide	Nicotine	Digoxin
Amitriptilyne hydrochloride	Ochratoxin A	Diphenhydramine hydrochloride
Arsenic trioxide	Octyl 3,4,5-trihydroxybenzoate	Disopyramide
Barium chloride	Orphenadrine hydrochloride	Disulfoton
Benzaldehyde	Paraquat dichloride	Epinephrine hydrogen tartrate
Brucine	p-Benzoquinone	Ethyl chloroacetate
Busulfan	Pentachlorophenol	Ethylene glycol
Cadmium (III) chloride	Phenanthrene	Fenpropathrin
Caffeine	Phenol	Glufosinate-ammonium
Carbon tetrachloride	Potassium cyanide	Lithium carbonate
Chloral hydrate	Sodium arsenite	Lithium sulfate
Chloroform	Sodium cyanate	Malathion
Chloroquine bis(phosphate)	Sodium lauryl sulfate	Meprobamate
Chlorpromazine	Sodium oxalate	Methadone hydrochloride
cis-Diammineplatinum (II) dichloride	Sodium salt of chloroacetic acid	N-isopropyl-N'-phenyl-p-phenylenediamine
Colchicine	Sodium selenate	Paraldehyde
Copper sulfate	Sodium valproate	Parathion
Cupric sulfate pentahydrate	Strychnine	Phenobarbital
Cyclohexamide	Tert-butyl hydroperoxide	Physostigmine
Cyclosporin A	Thallium sulfate	Procainamide hydrochloride
Diallyl phthalate	Theophylline	Quinidine sulfate dehydrate
Dichlorvos	Triethylenemelamine	Resorcinol
Diquat dibromide	Valproic acid	Rifampicin
		Sodium pentobarbital
		Thioridazine hydrochloride
		Triphenyltin hydroxide
		Warfarin

Tab. 2: Specific mechanisms of acute blood toxicity

Mechanisms	Example of chemicals	References
Decrease oxygen carrying capacity		
• Interference with hemoglobin	N-isopropyl-N'-phenyl-p-phenylenediamine	Williamson et al., 1981
• Oxidation of the oxygen carrying iron molecule to Fe^{3+}	Resorcinol 1-Naphthylamine	NJ RTK, 2010 NJ RTK, 2004
Clotting factor exhaustion		
• Interference with clotting factor production	Warfarin	Hanley, 2004
Lysis of cells		
• Binding to cell (antigen) triggers immune-mediated destruction	Rifampicin Quinidine sulfate	POISINDEX® System[a]; Manika et al., 2013 Freedman et al., 1956
• Direct destruction of cells, e.g., via oxidative damage to cell wall	Copper sulfate	Franchitto et al., 2008

[a] https://www.micromedexsolutions.com/home/dispatch (accessed 09.07.2018). Login required.

Tab. 3: Specific mechanisms of acute liver toxicity

Mechanisms	Example of chemicals	References
Disturbance of normal bile acid secretion	17α-Ethynyl estradiol	Wan and O'Brien, 2014; Davis et al., 1978
• Changes in membrane permeability of hepatocytes or biliary canaliculi	Chlorpromazine	Jennings et al., 2014
Fat accumulation in hepatocytes	Barium chloride	Ananda et al., 2013
Membrane disruption and/or interference with macromolecules	Isoniazid	Saukkonen et al., 2006; Boelsterli and Lee, 2014
Inflammation induced by oxidative stress		
• Lipid peroxidation	Carbon tetrachloride	El-Hadary and Ramadan Hassanien, 2016

Tab. 4: Specific mechanisms of acute lung toxicity

Mechanisms	Example of chemicals	References
Pulmonary endothelium damage	1-Phenyl-2-thiourea	Scott et al., 1990; Henderson et al., 2004
Increase capillary permeability	Dichlorvos	Li et al., 1989
Muscarinic action	Dichlorvos	Li et al., 1989
Destruction of Type I epithelial cells	Cadmium chloride	INCHEM, 2017
Inflammation	Chloroform	de Oliveira et al., 2015
Induced broncho- and vasoconstriction mediated by thromboxane	Tert-butyl hydroperoxide	Olafsdòttir et al., 1991
Intra-alveolar hemorrhage	Paraquat	Dinis-Oliveira, 2008
Deposits of calcium oxalate crystals in lung parenchyma	Ethylene glycol	Pomara et al., 2008; Leth and Gregersen, 2005
Irritation to respiratory tract	Acrolein	Bein and Leikauf, 2011
Lung accumulation through the polyamine uptake system	Paraquat	Dinis-Oliveira, 2008

Tab. 5: Specific mechanisms of acute cardiovascular toxicity

Mechanisms	Example of chemicals	References
Interference with ion balance/signaling/membrane potential of cell		
• Mimic substrate/block transporter enzymes such as Na^+-K^+-ATPase	Digoxin	Nicolas et al., 2015; Prassas et al., 2011
	Thallium sulphate	Riyaz et al., 2013
• Interference with sodium and/or potassium channels	Thallium sulphate	Riyaz et al., 2013
	Barium chloride	Bhoelan et al., 2014
	5,5-Diphenylhydantoine	Ekwall et al., 1998
	Quinidine sulphate dehydrate	Kim and Benowitz, 1990
	Disopyramide	Kim and Benowitz, 1990
	Procainamide hydrochloride	Kim and Benowitz, 1990
	Amitriptyline hydrochloride	Woolf et al., 2007
• Interference with Ca^{2+} channels	Aconitine	Sun et al., 2014
• QT interval prolongation	Thioridazine hydrochloride	Beach et al., 2013
	Haloperidol	Raudenska et al., 2013; Henderson et al., 1991
• Calcium channel blocker and binding to the cytosolic surface of the channel	Verapamil	Nicolas et al., 2015; Meister et al., 2010
• Stabilization of cell membrane leading to reduced excitation and conduction	Chloroquine bis(phosphate)	Ekwall et al., 1998
• Mimic/block parasympathetic activity	Atropine sulphate	Ekwall et al., 1998
• Prevention of the reuptake of heart noradrenaline	Amitriptyline hydrochloride	Dollery, 1991; Ekwall et al., 1998
• Pronounced negative chronotropic and inotropic effect and a quinidine-like effect	Propranolol	Kerns et al., 1997
Interference with intracellular signaling mechanisms		
• Interference with adenosine receptors	Caffeine	Ekwall et al., 1998
Increased vasoconstriction		
• Activation of β1-adrenergic receptors, β2-adrenergic receptors in blood vessels	Epinephrine hydrogen tartrate	Zhang et al., 2011
Increased capillary fragility	Warfarin	Hanley, 2004

Tab. 6: Specific mechanisms of acute neurotoxicity

Mechanisms	Example of chemicals	References
Interference with neurotransmitters/ neurotransmission		
• Inhibition of glutamine synthetase and glutamate decarboxylase	Glufosinate ammonium	Lluís et al., 2008
• Inhibition of the dopamine transporter	Chloral hydrate	Kreuter et al., 2004; Sabeti et al., 2003
• Slowing down catecholamine metabolism by inhibiting monoamine oxidase	D-amphetamine	Fitzgerald and Bronstein, 2013
Neurotransmitter release into synaptic cleft		
• Stimulation of glutamate release which can activate glutamate receptors to initiate excitotoxic processes	Potassium cyanide	Patel et al., 1993
• Stimulation of the release of norepinephrine and dopamine from stores in adrenergic nerve terminals	D-amphetamine	Fitzgerald and Bronstein, 2013
• Attenuation of glutamate release and reduction of activation of glutamate receptors	Chloral hydrate	Kreuter et al., 2004

Mechanisms	Example of chemicals	References
Neurotransmitter clearance from synaptic cleft		
• Inhibition of acetylcholinesterase and accumulation of acetylcholine	Dichlorvos	Binukumar and Gill, 2010; EXETOXNET, 1999; Sachana et al., 2001
	Physostigmine	Gilman, 1985
	Disulfoton	ATSDR, 1995
	Parathion	Casarett and Doull, 2001
• Increased acetylcholine release at the neuromuscular junction	Phenol	Liao and Oehme, 1980
• Blockage of the neuronal reuptake of norepinephrine, serotonin, and dopamine	Amitriptilyne hydrochloride	Dollery, 1991; Ekwall et al., 1998
• Selective norepinephrine re-uptake blockade	Maprotiline	Jan et al., 2013; Baumann and Maître, 1979
• Depletion of gamma-aminobutyric acid (GABA)	Isoniazid	Casarett and Doull, 2001
• Increase of GABA by indirect mechanisms involving inhibition of the enzyme succinate semialdehyde dehydrogenase (SSA-DH) in the GABA shunt	Sodium valproate	Sztajnkrycer, 2002; Chateauvieux et al., 2010
Interference at level of receptor	Phenobarbital	Jana et al., 2014
	Theophylline	Nakada et al., 1983
• Blockage of the action of acetylcholine at muscarinic receptors	Atropine sulfate monohydrate	Ekwall et al., 1998
• Competitive antagonism of cellular adenosine receptors	Caffeine	Fredholm et al., 1999
• Antagonist at the glycine receptor	Brucine	Teske et al., 2011
	Strychnine	Teske et al., 2011
• Blocking the release of inhibitory neurotransmitters such as GABA and acetylcholine	Codeine	NCIt, 2018; Takahama and Shirasaki, 2007
• Down-regulation of GABA receptors	Diazepam	Casarett and Doull, 2001
• Antagonizing chloride ion transport in GABA receptors	Endosulfan	Jang et al., 2016
• Interaction with GABAA receptors in a barbiturate-like fashion	Meprobamate	Rho et al., 1997
• Inhibition of NMDA receptors	Meprobamate	Rho et al., 1997
• Blockade of the GABA-receptor coupled sodium channel	Lindane	POISINDEX® System[a]
• GABAA receptor agonist	Sodium pentobarbital	Dollery, 1991
• Inhibition of the reuptake of GABA into the glia and nerve endings	Valproic acid	TOXNET, 2015[b]; POISINDEX® System[a]
• Interference at the level of GABAA receptors	Chloroform	Dick, 2006; Greenblatt and Meng, 2001
	Phenobarbital	Jana et al., 2014
	Valproic acid	Sztajnkrycer, 2002; Chateauvieux et al., 2010
• Anticholinergic effects	Quinidine sulfate dehydrate Disopyramide	Kim and Benowitz, 1990
• Competitive antagonism of acetylcholine at the neuroreceptor sites	Orphenadrine hydrochloride	POISINDEX® System[a]; Rejdak et al., 2011
• Blockade of the H1-receptors	Diphenhydramine hydrochloride	Pragst et al., 2006
• Direct stimulation of α- and β-adrenergic receptors	D-amphetamine	Fitzgerald and Bronstein, 2013
• Glutamate receptor activation	Glufosinate ammonium	Matsumura et al., 2001
• Dopamine receptor antagonism	Chlorpromazine	Haddad and Winchester, 1990
• Blockage of dopamine D2 receptor	Thioridazine hydrochloride	POISINDEX® System[a]
• Competitive blockade of postsynaptic dopamine (D2) receptors	Haloperidol	Raudenska et al., 2013

Mechanisms	Example of chemicals	References
• NMDA antagonism and inhibition of serotonin/norepinephrine reuptake	Methadone	Zorn and Fudin, 2011; Jamero et al., 2011
• Agonist at nicotinic cholinergic receptors	Nicotine	Williams and Robinson, 1984
Impaired propagation of electrical activity		
• Interaction with membrane ion channels (Na^+, K^+, Cl^-, Ca^{2+})	5,5-Diphenylhydantoin	Ekwall et al., 1998
	Aconitine	Chan, 2009; Peng et al., 2009
	Fenpropathrin	Xiong et al., 2016; Spencer et al., 2001
• Interference with transporter enzymes (e.g. Na^+-K^+-ATPase) (mimic substrate/block)	Thallium sulfate	Osorio-Rico et al., 2017; Ekwall et al., 1998; Casarett and Doull, 2001
	Aconitine	Peng et al., 2009
• Interference with the normal flux of Na^+ and K^+ ions across the axon membrane as nerve impulses pass	Lindane	Vučević et al., 2009
Peripheral neuropathy/ Polyneuropathy		
Myelinopathy		
• Intramyelinic edema	Hexachlorophene	Persson et al., 1978; Casarett and Doull's, 2001
Axonopathy		
• Blocking neurofilament transport via cross linking of neurofilaments	Acrylamide	Le Quesne, 1985; LoPachin et al., 2003
• Neurofilament filled swelling of proximal axon	Acrylamide	Le Quesne, 1985; LoPachin et al., 2003
• Inhibition of microtubule formation via binding to tubulin	Colchicine	Gooneratne et al., 2014; Finkelstein et al., 2010

[a] http://www.thomsonhc.com
[b] https://toxnet.nlm.nih.gov/cgi-bin/sis/search/a?dbs+hsdb:@term+@DOCNO+3582

Tab. 7: Specific mechanisms of acute immune toxicity

Mechanisms	Example of chemicals	References
• Degenerative changes in combination with slow replacement of affected immune cells due to inhibition of protein synthesis	Ochratoxin A	Al-Anati and Petzinger, 2006
• Decrease in whole blood cell counts or subpopulations	Triethylene melamine Triphenyl tin hydroxide	Bickham et al., 1994 Vos et al., 1984
• Changes in bone marrow cell proliferation	Triethylene melamine	Bickham et al., 1994

Tab. 8: Specific mechanisms of acute gastrointestinal toxicity

Mechanisms	Example of chemicals	References
Epithelial cell damage	Colchicine	Iacobuzio-Donahue et al., 2001
• Corrosion/irritation of the mucosa	Ferrous sulfate	Yuen and Gossman, 2018
• Interference with potassium channels	Barium chloride	Bhoelan et al., 2014
• Enzyme activation	Theophylline	Barnes, 2013
Inflammation of the mucosa	5-Fluorouracil	Boussios et al., 2012
Formation of metabolite (formic acid) at the place of contact	Formaldehyde	Wood, 2014; Pandey et al., 2000; Eells et al., 1981

Tab. 9: Specific mechanisms of acute kidney toxicity

Mechanisms	Example of chemicals	References
Vasoconstriction		
• Arteriolar vasoconstriction indirect effect by rhabdomyolysis	Codeine Endosulfan Brucine Diphenhydramine	Pokorny and Saunders, 1994 Jang et al., 2016 Teske et al., 2011; Achappa et al., 2012 Pragst et al., 2006
• Deficiency vasodilators (PG2)	Acetylsalicylic acid	Ferenbach and Bonventre, 2016
• Endothelial damage with increase in vasoconstrictors	CsA	Bonventre, 2014
• Interaction with renal V2-vasopressin receptor	Lithium	Bonventre, 2003
Glumerulonephritis	Lithium	Naughton, 2008
Interstitial nephritis	Acetylsalicylic acid	Naughton, 2008
Alterations in tubule cell structure		
• Accumulation in cells of proximal tubular cells	Cisplatin Cadmium chloride Mercury chloride	Bulacio and Torres, 2013; Kuhlmann et al., 1997 Ozbek, 2012 Bonventre, 2003; Zhou et al., 2008
• Loss of tubular epithelial barrier and/or tight junctions	Ochratoxin A	Gennari et al., 2004
Alterations in tubule cell metabolism		
• Interference with ion balance	Ammonium chloride	McEvoy, 2006
Tubular obstruction		
• Impaired Na^+ and water reabsorption	Cisplatin	Safirstein, 2004
• Distal cast formation	Diethylene glycol Ethylene glycol	Fowles et al.,2017 Fowles et al., 2017; Hess et al., 2004; Huhn and Rosenberg, 1995; Pomara et al., 2008
• Crystal deposition and tubular obstruction	Sodium oxalate	Pawar and Vyawahare, 2017
Generation of inflammatory and vasoactive mediators	Cisplatin	Bonventre, 2003
Alterations in cell viability		
• Necrosis of tubular epithelium	Mercury chloride 4-ammonio-m-tolyl)ethyl (2-hydroxyethyl)ammonium sulfate Cadmium chloride Brucine	Zhou et al., 2008 EPA TSCATS[a] Bonventre, 2003 Liu et al., 2015

[a] https://bit.ly/2QkpbQ5

3.2 Analysis of mechanistic information and *in vitro* 3T3 NRU cytotoxicity results

A total of 97 chemicals, for which *in vivo* and *in vitro* cytotoxicity data were available, have been identified with target organ/system specific effects, of which 91 were predicted as acutely toxic by the 3T3 NRU cytotoxicity assay ($LD_{50} \leq 2000$ mg/kg). Table 11 summarizes the prediction of the acute oral toxicity (EU CLP toxicity categories) by the *in vitro* cytotoxicity assay for these chemicals.

Figure 11 complements Table 11 by adding the collected mechanistic information. It also shows the percentage of chemicals identified *in vitro* as acutely toxic acting through cell type specific mechanisms of toxicity and via general cytotoxic mechanisms when the acute oral toxicity category was correctly predicted (50 chemicals), under-predicted (32 chemicals), and over-predicted (9 chemicals) by the *in vitro* cytotoxicity assay.

An overview of the information collected with regard to specific target organ/system and general cytotoxicity for the chemicals

Tab. 10: Contribution of specific target organ/system mechanisms of toxicity to the *in vivo* acute oral toxic category of chemicals

Organ specific mechanisms of acute toxicity	[a]CLP Cat. 1 *(5 chemicals)*	[a]CLP Cat. 2 *(15 chemicals)*	[a]CLP Cat. 3 *(26 chemicals)*	[a]CLP Cat. 4 *(52 chemicals)*
Neurotoxicity	3 (60%)	8 (53%)	13 (50%)	29 (56%)
Cardiovascular toxicity	0	5 (33%)	5 (19%)	21 (40%)
Liver toxicity	0	1 (7%)	4 (15%)	11 (21%)
Kidney toxicity	1 (20%)	4 (27%)	4 (15%)	10 (19%)
Lung toxicity	1 (20%)	0	7 (27%)	5 (10%)
Gastrointestinal toxicity	0	2 (13%)	5 (19%)	4 (8%)
Blood toxicity	0	2 (13%)	0	4 (8%)
Immune toxicity	1 (20%)	1 (7%)	1 (4%)	0

[a] Cat. 1: ≤ 5 mg/kg; Cat. 2: > 5 mg/kg, ≤ 50 mg/kg; Cat. 3: > 50 mg/kg, ≤ 300 mg/kg; Cat. 4: > 300 mg/kg, ≤ 2000 mg/kg

Tab. 11: Summary of prediction of EU CLP toxicity categories *in vivo* and *in vitro* for the set of chemicals classified for acute oral toxicity

Shadow cells indicate concordant predictions. EU CLP: EU regulation on classification, labelling and packaging of substances and mixtures; Cat: acute oral toxicity category; Cat. 1: rat oral LD_{50} ≤ 5 mg/kg; Cat. 2: 5mg/kg < rat oral LD_{50} ≤ 50 mg/kg; Cat. 3: 50 mg/kg < rat oral LD_{50} ≤ 300 mg/kg; Cat. 4: 300 mg/kg < rat oral LD_{50} ≤ 2000 mg/kg

3T3 NRU predicted toxicity (mg/kg)	Reference *in vivo* oral LD_{50} (mg/kg)			
	Cat. 1	Cat. 2	Cat. 3	Cat. 4
Cat. 1	0	0	0	0
Cat. 2	1	1	1	0
Cat. 3	0	6	9	8
Cat. 4	4	6	15	40

that are correctly assigned to the CLP acute oral toxicity category, under-predicted and over-predicted by the *in vitro* cytotoxicity assay, respectively, is provided in the supplementary information (Tab. S1, S2, and S3[12]).

Among the 50 correctly predicted chemicals, 29 act through some general mechanisms of cytotoxicity (58%) and 21 only via cell type specific mechanisms of toxicity (42%).

Among the 32 under-predicted chemicals, 20 act through a general mechanism of cytotoxicity (63%) and only 12 via cell type specific mechanisms of toxicity (38%).

Among the 9 chemicals with toxicity category over-predicted by the cytotoxicity assay, 6 act through a general mechanism of cytotoxicity (67%) and 3 only via specific mechanisms of toxicity (33%).

Among the 6 chemicals falsely predicted as non-classified (Fig. 12), two act through some mechanism of general cytotoxicity (33%) and four act only via cell type specific mechanisms of toxicity (67%), as shown in the supplementary information (Tab. S4[12]).

3.2.1 Acute oral toxicity category 1

From the compounds assigned *in vivo* to the acute oral toxicity category 1 (fatal if swallowed), three (i.e., brucine, disulfoton, and physostigmine) target the nervous system and act via specific mechanisms (e.g., inhibition of cholinesterase, antagonism of glycine receptor). Among the remaining compounds, 1-phenyl-2-thiourea has the lung as target organ and needs bio-activation, and triethylenemelamine affects the immune system. General mechanisms of cytotoxicity have also been reported for these two compounds. For brucine, necrosis was found as the mechanism responsible for kidney tubular cell damage. When *in vivo* and *in vitro* mean values are compared, all compounds are misclassified by the *in vitro* cytotoxicity assay. Triethylene melamine is under-classified by one toxicity category and the other four compounds by 3 toxicity categories.

3.2.2 Acute oral toxicity category 2

Among the compounds assigned *in vivo* to the acute oral toxicity category 2 (fatal if swallowed), only colchicine is correctly predicted by the cytotoxicity assay. Colchicine inhibits microtubule formation and, thus, effectively inhibits mitosis, which is a general mechanism of toxicity. This mechanism of toxicity is also reported as the one responsible for the toxicity at the level of the nervous system and the liver. Digoxin and aconitine, which were predicted as false negatives by the cytotoxicity assay, act via specific mechanisms of toxicity such as interference with transporter enzymes (e.g., Na^+-K^+-ATPase) and calcium channels. Digoxin targets the cardiovascular system while aconitine

[12] doi:10.14573/altex.1805181s2

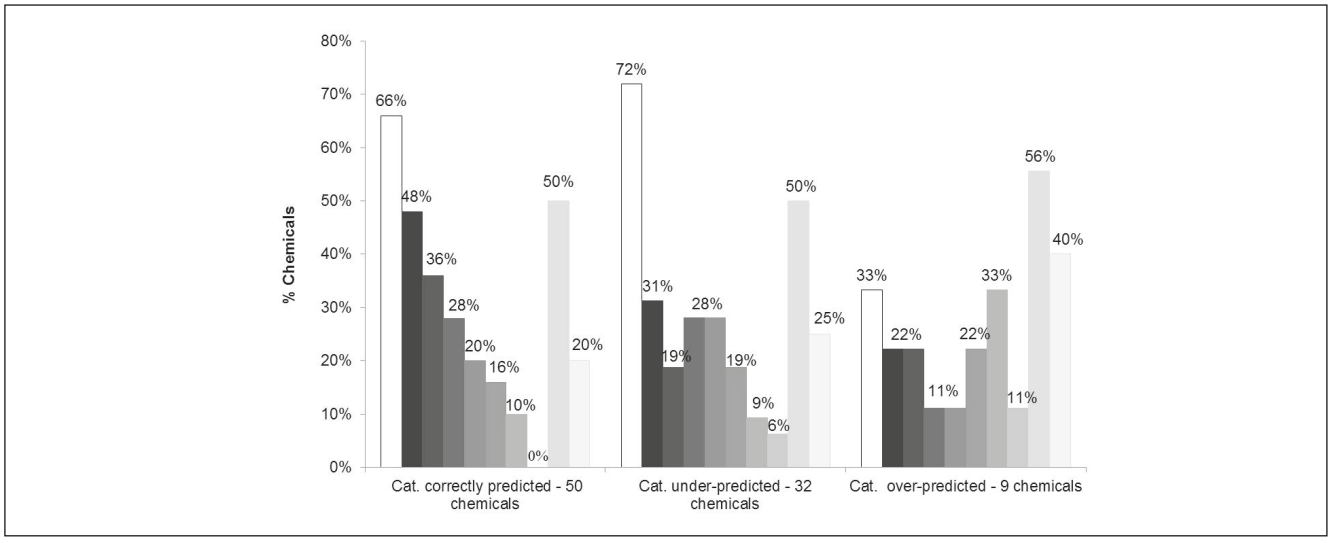


Fig. 11: Specific target organ/system toxicity and general cytotoxicity reported for the 91 chemicals predicted by the 3T3 NRU cytotoxicity assay as positive chemicals (i.e., $LD_{50} \leq 2000$ mg/kg)

Bars in each group represent from left to right: nervous system, cardiovascular system, liver, kidney, lung, gastrointestinal system, blood, immune system, general cytotoxicity, only specific target organ/systems. Cat., acute oral toxicity category

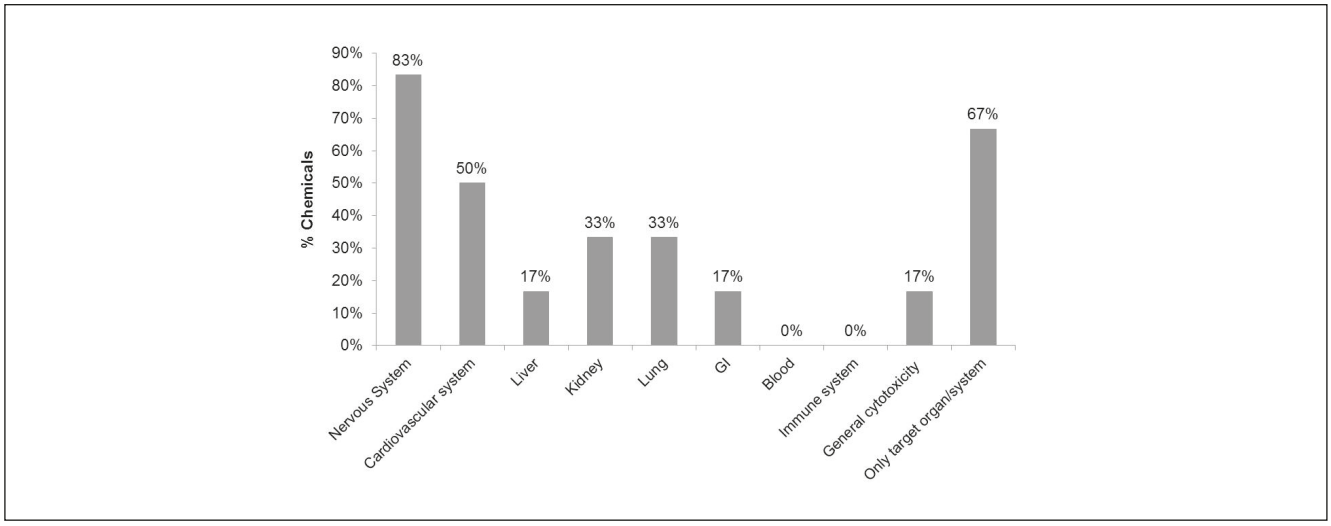


Fig. 12: Specific target organ/system toxicity and general cytotoxicity reported for the 6 chemicals falsely predicted as negatives by the 3T3 NRU cytotoxicity assay

GI, gastrointestinal system

acts on both the nervous and the cardiovascular system (Qiu et al., 2008; Chan, 2009; Prassas et al., 2011; Sun et al., 2014).

The compounds assigned to the toxicity category 2 *in vivo* but under-predicted *in vitro* act mainly via specific target organ toxicity mechanisms. For D-amphetamine, parathion, and strychnine, the central nervous system is the main target and specific mechanisms of toxicity were identified (i.e., inhibition of acetylcholinesterase, antagonism of glycine receptor, stimulation of the release of norepinephrine and dopamine). The kidney is the target for ochratoxin A, a well-known nephrotoxic agent acting on tubular cells (Ozbek, 2012). General cytotoxicity is also reported for ochratoxin A, and necrosis has been found as the mechanism underlying strychnine effects on kidney tubular cell damage. Epinephrine hydrogen tartrate targets the cardiovascular system, while warfarin targets both the cardiovascular system and blood cells (Hanley, 2004; Klaassen, 2001).

3.2.3 Acute oral toxicity category 3

General mechanisms of toxicity were also described for the chemicals correctly assigned *in vitro* to the acute oral toxicity category 3 (toxic if swallowed). Only for ethyl chloroacetate no general mechanisms of toxicity were reported, although

it is a moderate irritant via the oral route. For the compounds under-predicted *in vitro* by only one toxicity category, mechanisms of general cytotoxicity were reported. For sodium salt of chloroacetic acid and pentachlorophenol also the mechanisms identified at target organ level were general mechanisms of toxicity such as interference with mitochondrial function (CNS), membrane disruption and/or interference with macromolecules, depletion of free radical scavenger (such as glutathione, catalase) content in liver tissue, and compromised mitochondrial respiration of tubular cells (kidney). Dichlorvos and theophylline act through general mechanisms of cytotoxicity at CNS level, and also via specific mechanisms of toxicity. Seventy-three per cent of these under-predicted compounds are linked to the CNS as the target system (i.e., GABAA receptor agonist, blockade of the GABAA-receptor coupled sodium channel, interference with the normal flux of Na^+ and K^+ ions across the axon membrane during neuronal signaling, antagonism of N-methyl-D-aspartate (NMDA) receptors and inhibition of serotonin/norepinephrine reuptake, agonist of nicotinic cholinergic receptors, neurotransmitter clearance from synaptic cleft). Verapamil, barium chloride, and theophylline act at the level of the heart by different mechanisms (blocking the calcium channel and binding to the cytosolic surface of the channel; interference with potassium channels, interference with intracellular signaling mechanisms, such as enzymatic activity, e.g., phosphodiesterases and protein kinases). Fat accumulation in hepatocytes was also described as the liver specific mechanism of toxicity of barium chloride (Ananda et al., 2013).

3.2.4 Acute oral toxicity category 4

General mechanisms of toxicity are reported for 49% of the compounds correctly assigned to the acute oral toxicity category 4 (harmful if swallowed). For several of these compounds, general cytotoxicity was identified at the target organ/level alone or in addition to other specific mechanisms of toxicity (e.g., acetylsalicylic acid uncouples mitochondrial oxidative phosphorylation and also inhibits Krebs cycle dehydrogenases at CNS level (Ekwall et al., 1998); valproic acid alters the activity of the GABA neurotransmitter by increasing the inhibitory activity of GABA through inhibition of GABA degradation, inhibition of GABA transaminobutyrate, increased GABA synthesis, decreased turnover and inhibition of the GABA reuptake by the glia and synaptic mechanisms. It also interferes with cellular metabolic processes, interacts with membrane ion channels (Sztajnkrycer, 2002; Chateauvieux et al., 2010), and induces oxidative stress by compromising the antioxidant status of the neuronal tissue (Chaudhary and Parvez, 2012); caffeine in the CNS competitively antagonizes adenosine receptors, inhibits phosphodiesterase, stimulates catecholamine release, and increases free calcium and intracellular cAMP (Fredholm et al., 1999); orphenadrine chloride competitively antagonizes acetylcholine binding at the neuroreceptor sites and induces necrosis in liver (Sangster et al., 1978; Ekwall et al., 1998)). The nervous and the cardiovascular system appeared as targets for 70% and 54% of the harmful compounds, respectively. Among the compounds acting via specific mechanisms of toxicity, 65% (13/20) targeted both the nervous and the cardiovascular system.

Four harmful compounds were falsely predicted *in vitro* as non-acutely toxic. Of those, isoniazid and paraldehyde act via specific mechanisms such as interference at the level of CNS receptors and ion channel function, depletion of GABA (isoniazid), impairment of the propagation of electrical activity in the CNS (paraldehyde), and inflammation of the GI mucosa (paraldehyde) (Gilman, 1985; Carpentier et al., 1992). The harmful effects of ethylene glycol mainly result from the accumulation of its more toxic metabolites (Hess et al., 2004). Diethylene glycol is metabolized to 2-hydroxyethoxyacetaldehyde by alcohol dehydrogenase oxidation, then to 2-hydroxyacetic acid (HEAA) by aldehyde dehydrogenase. HEAA causes acidosis, renal failure, and neurologic dysfunction. It is thought that the parent compound is toxic as well (Schep et al., 2009). The formation of toxic metabolites will be missed in the *in vitro* cell system due to the lack of metabolic competence of the 3T3 cells. This could explain, at least in part, the misclassification by the *in vitro* approach.

Many of the harmful compounds are extensively or rapidly metabolized in the liver and toxic metabolites were reported for five compounds (quinidine sulfate dehydrate (Kim and Benowitz, 1990), chloroform (HSDB, ACuteTox project; Hodgson, 2004), chloral hydrate (Beland, 1999; Pershad et al., 1999; Dogan-Duyar et al., 2010), sodium valproate (ACuteTox project; Sztajnkrycer, 2002), and malathion (ACuteTox project; Simoneschi et al., 2014)).

4 Discussion

The exercise reported here served several purposes. First of all, the mechanistic information collected was visually summarized, allowing direct comparison across target organs/systems. Secondly, by organizing acute toxic effects by their mechanism and cell type(s), we could start to associate known acutely toxic compounds with the different mechanisms. Finally, organizing information in this manner should facilitate the development of AOPs and IATA, for example by identifying properties that are requisites of *in vitro* testing systems for specific target organ toxicity testing.

This work also aimed to identify specific (complementary) mechanisms of acute toxicity that are perhaps not covered by the validated 3T3 NRU cytotoxicity assay. Therefore, in our analysis we tried to address: (i) whether chemicals can be identified and classified based on either positive or negative specific effects on target organ(s) (according to the 2000 mg/kg threshold) using the 3T3 NRU assay; (ii) which organs are the most frequent targets; and (iii) whether the triggered pathways of toxicity are conserved across organs.

In the overall analysis, of the 97 chemicals identified with target organ specific effects, 94% (91/97) were predicted as acutely toxic by the *in vitro* cytotoxicity assay and 6% (6/97) as non-toxic. When comparing the positive (i.e., acutely toxic) and negative (i.e., non-acutely toxic) *in vitro* predictions with those of the *in vivo* study, it turned out that all six negatives were false predictions (false negatives), while 55% of the positive predictions were correctly predicted, according to a CLP acute toxicity category,

35% under-predicted and 10% over-predicted. When evaluating the performance of any alternative approach for the purposes of regulatory classification, it is also necessary to consider the uncertainty associated with both the *in vivo* and the *in vitro* data. Actually, the analysis of consistency in classification published by Hoffmann et al. (2010) showed that conventional *in vivo* acute oral toxicity tests are intrinsically imprecise themselves and, about 44% of the substances would ambiguously occur within the limits of two adjacent classification categories (with at least 90% probability). A discussion of *in vivo* and *in vitro* data variability is outside the scope of this paper. Therefore, for the purpose of the mechanistic analysis shown and discussed here, the assignment of the compounds to the CLP acute oral toxicity categories was made based on the collected mean values (*in vivo* and *in vitro*). Another major source of uncertainty, not analyzed here due to lack of information, is the role of ADME (absorption, distribution, metabolism, and excretion) in determining the acute toxicity category. ADME has also been identified as a source of uncertainty in many OECD IATA case studies that are based on new approach methodologies. Several regulatory bodies have published guidance on the identification, characterization, and reporting of uncertainty (SCHEER, 2018).

A closer look at the chemicals acting through the specific target organs has not revealed a clear pattern with regard to which specific mechanisms of target organ toxicity are representative of compounds in the different CLP acute oral toxicity categories. For instance, approximately the same percentage of compounds acting through mechanisms of neurotoxicity was found in each acute oral toxicity category (i.e., 55% of the highly toxic chemicals allocated to toxicity categories 1 and 2, 50% of the toxic chemicals in category 3, and 56% of the harmful chemicals in category 4). A similar situation holds true for chemicals that act through mechanisms of cardiovascular toxicity, which were allocated to toxicity categories 2, 3, and 4 (33%, 19%, and 40%, respectively). Mechanisms of nephrotoxicity were also found for chemicals in all toxicity categories. Mechanisms of liver, lung, and blood toxicity were described for a small percentage of the highly toxic compounds (8%-21%). Based on these results, it can be concluded that mechanisms of toxicity specific for each organ can be triggered by compounds that belong to the different CLP acute oral toxicity categories. This is not surprising since the CLP categorization is based on potency, which can result from both toxicokinetic and toxicodynamic factors.

From the information collected and the analysis presented it can be concluded that general cytotoxicity is an important determinant of acute systemic toxicity. Overall, the majority of the analyzed chemicals (63%) causing acute lethal toxicity act via some general (rather than organ specific) mechanisms of toxicity. The nervous and the cardiovascular systems are the most frequent targets, with changes in neurotransmission and altered ion flow being important mechanisms often associated with acute neurotoxicity and cardiotoxicity, respectively.

It is well recognized that the use of basal cytotoxicity alone to determine the acute toxicity of a chemical may not always be enough and, furthermore, it depends on the chemical's kinetic behavior and/or its specific mechanisms of toxicity. These features may need to be considered in order to correctly estimate the *in vitro* concentration causing toxicity that could be compared with the concentration that the target cells *in vivo* would be exposed to (EU FP6 project ACuteTox). *In silico* tools such as the Virtual Cell Based Assay (VCBA) can be used to simulate the distribution of the chemicals in the *in vitro* system (Zaldivar Comenges et al., 2017). By comparing the simulated and the nominal IC_{50} concentrations of the dissolved chemical, the influence of the *in vitro* kinetics on the cytotoxicity result may be anticipated. In addition, *in vivo* kinetics is an important determinant of acute systemic toxicity that requires further investigation (Graepel et al., 2017; Duarte Lopes Mascarenhas Proença et al., 2017). *In silico* tools such as Physiologically Based Kinetic (PBK) models (Paini et al., 2017) can be used to simulate the kinetics and distribution of chemicals *in vivo*.

Although specific target organ mechanisms of toxicity could in some cases explain the false negative prediction obtained with the cytotoxicity assay, in general it is difficult to explain *in vitro* misclassifications only on the basis of mechanistic information. Therefore, in addition to kinetic considerations, *in vitro* misclassifications could be also linked to the number of acute oral toxicity categories under CLP and the associated LD_{50} ranges, which are not based on a particular mechanistic rationale. Indeed, the outcome of the classification analysis carried out in the context of the EU FP6 project ACuteTox indicated that it is challenging to make a clear distinction between acute oral toxicity categories 1, 2, and 3 based on *in vitro* concentration-response data (Kinsner-Ovaskainen et al., 2013) and, therefore, three levels of toxicity (i.e., level 1: combination of categories 1 to 3, level 2: category 4, and level 3: non-classified) were proposed (Prieto et al., 2013a). A similar grouping was also considered by Norlén et al. (2012) in an investigation of the predictive performances of five alternative approaches for the assessment of acute oral toxicity. Overall, the value of the CLP classification into four acute oral toxicity categories could be challenged.

Building on all the collected/generated information it would be worth trying to develop an alternative way of classifying chemicals for acute oral toxicity based mainly on cytotoxicity and kinetic information, and complemented, if needed, with relevant organ specific mechanisms of toxicity. Based on the mechanistic knowledge discussed in this paper, we propose to integrate *in vitro* assays anchored to the most frequent mechanisms of acute toxicity specific for each organ (CNS, heart, liver, and kidney) into an IATA. An IATA can include defined approaches, i.e., formalized decision-making approaches that apply fixed data interpretation procedures to data generated with a defined set of information sources (OECD, 2016b). In this regard, an *in vitro* cytotoxicity assay would be used together with specific target tissue toxicity mechanisms tested by assays permitting evaluation of neurotoxicity (as the most sensitive), followed by cardiotoxicity, hepatotoxicity, and kidney toxicity. Such a battery of tests should be designed to allow assessment of the compounds based on their cytotoxicity (e.g., based on the 3T3 NRU assay) and organ specific mechanisms. In the broader context of IATA, these *in vitro* mechanistic data should be integrated with additional sources of information (QSAR, read-across, *in chemico*, human data, *in vivo* data, etc.) including, where appropriate, exposure and ADME information.

The development of AOPs relevant to acute neurotoxicity, cardiotoxicity, hepatotoxicity, etc., is already ongoing. However, as indicated, they are at different stages of development (Tab. S5[12]). It is worth noting that some of the relevant AOPs are not specific to acute toxicity but nevertheless include key events that are relevant to acute exposure effects. Interestingly, some of the chemicals identified in these AOPs as triggers of molecular initiating events overlap with the chemicals reviewed in this report. The mechanistic information provided in this paper should inform the development of AOPs relevant to acute systemic toxicity, as well as AOP-informed IATA. The further development of AOPs and IATA should focus on the major target organs identified, i.e., the CNS, heart, liver, and kidney.

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Conflict of interest

The authors declare that they have no conflict of interest to disclose.