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REVIEW ARTICLE

Investigative safety strategies to improve success in drug development

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Abstract: Understanding and reducing attrition rate remains a key challenge in drug development. Preclinical and clinical safety issues still represent about 40% of drug discontinuation, of which cardiac and liver toxicities are the leading reasons. Reducing attrition rate can be achieved by various means, starting with a comprehensive evaluation of the potential safety issues associated to the primary target followed by an evaluation of undesirable secondary targets. To address these risks, a risk mitigation plan should be built at very early development stages, using a panel of *in silico*, *in vitro*, and *in vivo* models. While most pharmaceutical companies have developed robust safety strategies to de-risk genotoxicity and cardiotoxicity issues, partly driven by regulatory requirements; safety issues affecting other organs or systems, such as the central nervous system, liver, kidney, or gastro-intestinal system are less commonly addressed during early drug development. This paper proposes some de-risking strategies that can be applied to these target organ systems, including the use of novel biomarkers that can be easily integrated in both preclinical and clinical studies. Experiments to understand the mechanisms' underlying toxicity are also important. Two examples are provided to demonstrate how such mechanistic studies can impact drug development. Novel trends in investigative safety are reviewed, such as computational modeling, mitochondrial toxicity assessment, and imaging technologies. Ultimately, understanding the predictive value of non-clinical safety testing and its translatability to humans will enable to optimize assays in order to address the key objectives of the drug discovery process, i.e., hazard identification, risk assessment, and mitigation.

Keywords: safety attrition; drug development=target organ strategies=on and off target effects=hazard identification=risk assessment=mitigation plans

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Received: June 1, 2016; **Accepted:** June 2, 2016; **Published Online:** July 29, 2016

Citation: Atienzar F, Delaunois A, Brouta F, *et al*. 2016, Investigative safety strategies to improve success in drug development. *Journal of Medicines Development Sciences*, vol.2(1): 4–4; . http://dx.doi.org/10.18063/JMDS.2016.01.001.

1. Introduction: Understanding the Attrition Challenge in Drug Development

Despite major breakthroughs for some diseases in recent decades, many of the most common human diseases are not effectively treated by existing therapies. Drug development is a science-driven, research-intensive, long lasting, and high-risk endeavor[1]. It is well-established that R&D productivity is a particularly difficult challenge to overcome in the pharmaceutical sector[2]. The cost of inventing new drugs has greatly increased since 1970, whereas the overall output of investigational new drug products has remained relatively constant whilst return on in-

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vestment is going down[1–3]. Attrition rates of clinical stages have risen sharply in the last decade, especially in late-phase clinical trials. Thus, understanding the root causes of why compounds undergo attrition, and reducing these rates are crucial to better understand pharmaceutical industry performance and improve drug development efficiency[4–6]. Only 15% of the molecules that enter clinical trials receive marketing approval, and drug success rates differ across different stages of development[7,8]. It has been reported that around 30%–50% of the projects that are in phase 3 clinical trials fail to be launched on the market[9]. According to recent reports, Phase II, with an attrition rate higher than 50%, has the highest attrition frequency in the drug development process[10,11]. Many factors contribute to attrition, and drug development success rates vary dramatically between different therapeutic areas[12]. A survey revealed that molecules undergoing attrition during the preclinical stage are withdrawn from further clinical development for various causes, such as lack of efficacy and safety issues, poor absorption, distribution, metabolism and elimination (ADME) properties, market competition, and commercial interests[9,12].

More specifically, over the last two decades, both preclinical and clinical safety have remained the major cause of drug attrition during clinical development and of drug withdrawal from the market, accounting for 35%–40% of all drug discontinuation[13,14]. Although the safety related reasons for attrition have evolved over the years, cardiac and hepatic toxicities have remained the leading reasons for attrition[15,16] followed by, to a much lower extent, neurological, renal, and gastro-intestinal related toxicities. It must be stressed that target organ toxicities can be functional and/or structural in nature. Furthermore, the relative contribution to adverse drug reactions, attrition, withdrawal, and labeling implications vary from one type of toxicity to another and from one organ system to the other[13].

Numerous solutions have been proposed, but the mindset of reducing attrition in development should be in place from the earliest stages of discovery. Some approaches include, but are not limited to, improving pre-clinical testing (efficacy, safety, ADME)[11], designing proof-of-concept clinical trials[12], formulation and drug delivery technologies, use of appropriate pharmacokinetic/pharmacodynamic (PK/PD) models[9], and identifying predictive biomarkers[17]. The current approaches to reducing safety related attrition in drug development are presented and discussed in this review article.

2. How to Reduce Attrition Rate

2.1 Target Related Safety Issues

Besides the well implemented *in vitro* and *in vivo* safety testing of promising compounds during the drug development process, an upfront *in cerebro* evaluation of the target can be beneficial to anticipate important potential safety risks at any stages of drug development. Indeed, for the last years, the proportion of target-related safety closures rose substantially in the clinical phases and this was responsible for almost half of the safety-related project closures[6]. Unintended adverse effects can arise as a consequence of the intended primary pharmacology in organs other than those related to the indication or can arise from exaggerated secondary pharmacology[18]. To anticipate potential safety liabilities directly or indirectly linked to the target, Target Safety Evaluation (TSE) is becoming a standard practice within pharmaceutical companies. This TSE provides an in-depth review of the target and may include different sections covering the biology (gene, protein, function, pathway, expression profile, tissue distribution, disease, etc), human genetic phenotype, transgenic animal genotype and phenotype (knock-out [KO], mutants), potential safety risks and its associated mitigation plans as well as competitive intelligence and differentiation criteria. It is a key step in trying to understand the safety risks associated with the target and helps to determine upfront which endpoints could be measured to mitigate the safety risks identified. In the case that too many safety risks are identified compared to the potential benefit, the decision can be taken not to pursue the target for a particular indication. In less severe cases, this TSE may guide in part the development of the compound based on the identified liabilities. Examples of key questions that need to be addressed within such an evaluation are: what is known about the target or a close structurally related target? Are there any known safety implications related to the targeted pathways? How is the target regulated with respect to agonists and/or antagonists? Are there any known on-target and/or off-target toxicities? What information can be extracted from existing KO animals or models overexpressing the target? There are however some challenges to take into account with KO data. Firstly, some gene deletions are embryo-lethal. Secondly, the

observed phenotypes may be due to developmental effects and not to the acute effects of the absence of the target[19]. Finally, adaptive changes during development can provide a “workaround” to circumvent the missing gene. One way round these problems is to use conditional KO. Of added value as well is a description of the information that is available regarding competitor activities in relation to the safety of the particular target or the therapeutic indication.

Potential safety biomarkers should also be identified and deployed for use in the preclinical studies (e.g., blood markers and/or tissue gene expression profiles) together with the identification of the most relevant preclinical species. Based on all the acquired safety information, if necessary; dedicated experiments or specific endpoints within the *in vivo* studies can be put in place in order to evaluate the identified target-related safety issues. If available, tool compounds are extremely useful in these experiments because they can help in the interpretation of severity of the safety risks identified. The TSE assessment is built through information mining and analysis of public and internal databases and through consultations with experts in the target specific field. During the lifespan of the project the TSE document needs to be updated regularly using the latest available literature information as well as in the meantime generated *in silico*, *in vitro*, and *in vivo* data. If necessary the project can be redirected based on this newly available safety information.

2.2 Off Target Related Safety Issues

Besides the (desired or unwanted) effects related to the action of a drug on the primary therapeutic target, interactions with targets other than the primary target can also lead to undesirable secondary effects, also named “off-target” effects[20]. It has been shown that compounds with a target hit rate (i.e., percentage of a panel of at least 50 targets for which more than 50% binding is noted at 10μM) of 20% or more have a higher attrition rate[21]. Hopefully, off-target effects can be easily predicted from the *in vitro* pharmacological profiling that is conducted at early stages of discovery projects. Such profiling includes screening of compounds against a variety of receptors, ion channels, enzymes, and transporters that are known to be associated with safety issues in humans.

Except for the human ether-a-go-go-related gene (hERG) channel associated with QT prolongation and delayed ventricular repolarization, which is covered by the International Conference on Harmonization (ICH) S7B guidance, there is no mandatory regulatory requirement on the list of targets that must be screened before moving a drug into the clinic[22]. Nevertheless, receptor binding profiling is mentioned, but not required, in the ICH S7A[23] and drug abuse guidance[24]. However, some off-target effects may lead to specific regulatory actions, based on their potential risk for human safety[25]. Bowes *et al*.[21] published a list of 44 recommended targets that provides an early assessment of the potential hazard of a compound or a chemical series. For secondary pharmacology profiling, some pharmaceutical companies apply a step-wise approach including at very early stage (e.g., lead optimization phase) a minimal panel of 10 to 20 targets followed by one or two successive panels with extended list of targets (50 to 150), used for deeper characterization of lead or candidate drugs. The early profiling will identify liabilities associated with a chemical series and improve compound design, while the expanded profiling will provide a comprehensive understanding of the potential side effects expected in preclinical studies. Measurement of direct binding affinity of the drug for these targets should be followed by the assessment of its functional response on the hit targets (agonism, antagonism, inhibition, etc.) in a cell or tissue assay. Table 1 provides a list of targets that are associated with toxicity issues (due to off-targets effects). For example, binding of a compound to 5-hydroxytryptamine receptor 2B ($5HT_{2B}$) is not an issue if it exhibits antagonistic activity, while it should be avoided in case of agonistic activity, due to the risk of cardiac valvulopathy[26]. Determination of the potency of the compound, expressed as EC_{50} or IC_{50} (half maximal efficacy and inhibitory concentrations, respectively), is also necessary to evaluate its selectivity (ratio between EC_{50} or IC_{50} values for primary and secondary targets). As a general rule, a 100-fold selectivity ratio is recommended to provide a sufficient safety margin[27] although lower safety margins can be considered for instance when targeting kinases[28].

In silico tools have been developed over the last decade to facilitate the interpretation and maximize the impact of pharmacological profiling data. Proprietary databases such as Bioprint™ or DrugMatrix™ provide access to large datasets which can include chemical structures of reference and marketed drugs, *in vitro* pharmacological profiling data (binding and/or functional) and ADRs observed in clinical trials. These tools can be used to compare a new drug structure to

Table 1. Examples of off-target related toxicities. Adapted from Bowes *et al.*[20,21] and Valentin *et al.*[13]. 5-HT: 5-hydroxytryptamine, CNS: central nervous system, hERG : human ether-a-go-go related gene, TdP: Torsades de Pointes.

Target involved	Organ system affected	Mechanism of action	Known functional effects	Known structural effects
hERG channel	Cardiovascular	Ion channel inhibition	Prolonged QT, TdP	Embryonic malformations and death due to reduced cardiac output and hypoxia
5-HT2B receptor	Cardiovascular	Agonism	Pulmonary hypertension	Cardiac valvulopathy
Adrenergic'beta'2 receptor	Cardiovascular	Agonism Antagonism	Tachycardia, hypotension Bronchospasm	Vascular smooth muscle necrosis, cardiac lesions (dog)
Dopamine transporter	CNS, reproductive system, skin	Inhibition	Effects on cognition and locomotor activity, drug abuse potential, depression	Rat specific uterine tumors, Acne
Tyrosine kinases	Various (cardiovascular, thyroid, blood,…)	Inhibition	Anemia, thrombopenia, hypothyroidism, hypertension,…	Congestive heart failure
Cyclooxygenase 1	Upper gastrointestinal tract	Inhibition	Increased acid secretion, decreased mucus production	Gastric bleeding, ulcers

structures of already profiled drugs, predict pharmacological activity on targets associated with safety issues, or help in the interpretation and understanding of side effects observed in *in vivo* studies[20].

2.3 Addressing Chemistry Related Safety Issues

2.3.1 Genotoxicity Safety Strategy

Genotoxicity remains an important reason for drug attrition during pre-clinical testing and represents about 10% of the safety-related failures[6]. Over recent years, companies have therefore focused on developing testing strategies to maximize genotoxic hazard identification at early stages of drug development (e.g., lead optimization). This approach will guide the selection and ranking of pharmaceutical development candidates and is crucial to improve the success rate of newly developed drugs. In this perspective, the regulatory test battery, aiming at detecting DNA damage, can be complemented with a significant number of *in vitro* screening tools of higher throughput and higher speed, low compound requirement and lower cost, making them better adapted for incorporation in early phases of drug development. Since these newly available assays, developed as alternative to established regulatory assays, generally show a good sensitivity and a high specificity, they provide a comprehensive initial assessment of the genotoxic potential of a molecule. As no single test is capable of detecting all genotoxic mechanisms which could ultimately lead to cancer, a battery of *in vitro* genotoxicity screening assays with different endpoints is considered to be the most reasonable approach to identify genotoxic hazard early on. However, today, no consensus on the best combination of genotoxicity screening assays to use has been reached. Nevertheless, most companies initially screen for bacterial mutagenicity using modifications of the regulatory Ames assay. Nowadays, a variety of assays is available each with their strengths and weaknesses[29]. Figure 1 gives an example of the strategies deployed by Lundbeck (Figure 1A) and UCB (Figure 1B) to triage compounds in different fields including genotoxicity. Some use the traditional bacterial strains in miniaturized agar or liquid format (e.g., mini-Ames, micro-Ames, Ames Multi Plate Format [MPF]); others use modified strains (e.g., Ames II) or non-traditional approaches (e.g., fluctuation assays, bioluminescence assays, SOS-response assays, or reporter gene assays). In addition, a number of bacterial mutagenicity assays based on DNA repair such as the SOS chromotest and the Salmonella SOS/umu assays have been developed. The initial bacterial assays are generally followed up by a mammalian cell assay to detect DNA damage. Several reporter gene assays using human-derived cell lines exist. They are based on the activation of DNA repair genes with luminescence (e.g., Bluescreen) or fluorescence (Greenscreen) detection[30,31]. Furthermore, also chromosomal damage can be evaluated in a higher throughput screening setting in mammalian cells by analyzing micronucleus formation using flow cytometry[32] or high content screening methods[33]. The performance of some models is presented in Table 2.

Over recent years, computational toxicology has gained a lot of importance in drug discovery and resulted in the development of several *in silico* structure-activity relationship (SAR) tools for the prediction of toxicity[34,35]. The use of computational tools to identify potential genotoxicity based on the chemical structures is part of most mutagenicity screening

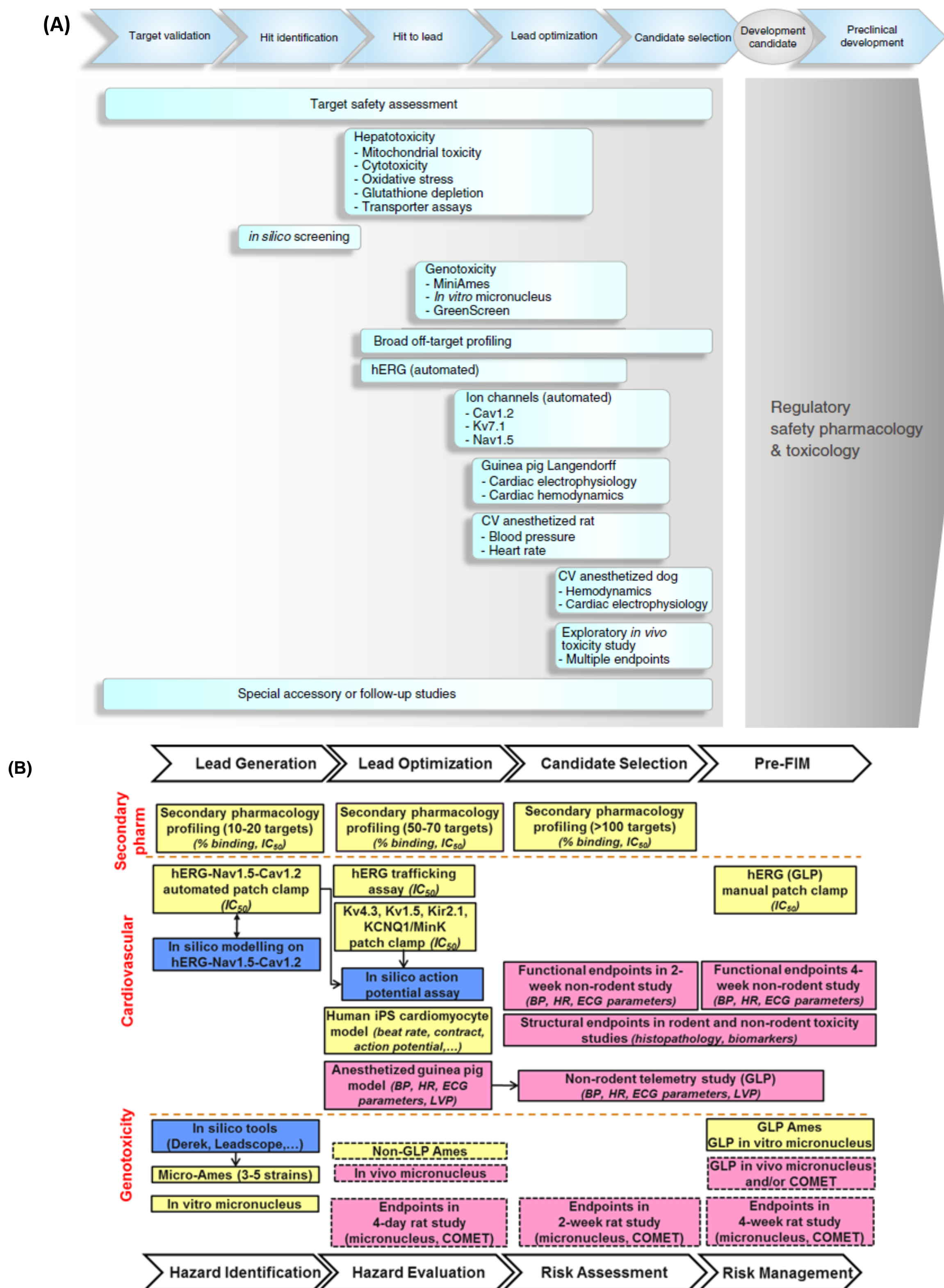

Figure 1. Examples of strategies used by pharmaceutical companies to select the most promising compounds. (**C**) Lundbeck[184],with permission from Elsevier. (**D**) UCB BioPharma SPRL. Blue, yellow and pink boxes refer to *in silico*, *in vitro* and *in vivo* approches, respectively. Abbreviations: BP: blood pressure; ECG: electrocardiogram; FIM: first-in-man; GLP: good laboratory practices; hERG: human ether-a-go-go related gene; HR: heart rate; IC_{50}: half maximal inhibitory concentration; iPS: induced pluripotent stem cells; LVP: left ventricular pressure.

Table 2. Performance of *in silico*, *in vitro* and *in vivo* approaches to predict different toxicity endpoints. Sensitivity and specificity data are reported. Values <50% are highlighted in red, 50% < values < 75 % are highlighted in yellow and those >75% appear in green. For more details, please refer to the original sources. NA: not applicable.

Approaches	Models/Assays (Species)	Safety endpoint to predict in animal or human	Sensitivity (%)	Specificity (%)	References
In silico	NA	Hepatotoxicity	68	95	[179]
	Commercial softwares (DEREK, Toxtree, MC4PC, Leadscope MA)	Ames test: public data	65.2–85.2	53.1–82.9	[175]
		Ames test: Roche data	17.4–43.4	77.5–93.9	
	NA	QT in rabbits (diverse ion channel databases evaluated)	60.0–91.4	27.6–80.8	[180]
In vitro	iPSCs (human)	Arrhythmia	81	84	[176]
	hERG patch clamp (human)	QT prolongation	82	75	[181]
	Ames (bacteria)	Carcinogenicity in rodent	60	74	[177]
	Micronucleus (hamster)	Chromosomal damage, carcinogenicity	94	85	[33]
	HepG2 (human)	Hepatotoxicity	82	36	[96]
	Primary hepatocytes (human)		83	46	
	Co-culture (dog)		78	73	
	Co-culture (human)		76	82	Unpublished data (manuscript in prep.)
In vivo	Rodent	General toxicity	43	NA	[51]
	Non-rodent		63	NA	
	QT interval (dog)	QT prolongation	83	86	[181]
	Cardiovascular telemetry (dog)	Blood pressure	36	93	[13]
		Heart rate	50	93	
		Contractility	92	67	[182]
	Motor activity (Zebrafish)	Seizure/convulsion	76	63	[178]
	Colonic motor activity (mouse)	Diarrhea/constipation	90	75	[183]

strategies before moving to testing, as it allows the quick evaluation of large numbers of compounds[36]. Commercial systems from various vendors (e.g., Lhasa Ltd., Leadscope Inc., MultiCASE Inc.) provide genotoxicity databases for structural lookup and predictive SAR systems. Often more than one method is available from each vendor, as the recent implementation of the ICH M7 (ICH M7, 2015) requires the use of two complementary methods, one rule-based and one statistics-based-method, for the assessment of potential genotoxic impurities (PGIs). The ICH M7 guideline states that in the absence of an experimental result, two negative predictions from complementary computational methods together with an expert opinion, is sufficient evidence for lack of mutagenic potential of a PGI. As such, the use of *in silico* tools in the risk assessment of PGIs marks a milestone for the use of computational methods as it is the first example of such analyses being both acceptable and actually required for Regulatory submissions.

2.3.2 Cardiotoxicity Safety Strategy

Cardiotoxicity is one of the three major safety reasons for project failure or drug attrition in preclinical or clinical phases[6]. Between 1975 and 2007, 21% of drug withdrawals were due to cardiovascular safety issues[37]. During the last 10–15 years, a lot of emphasis has been put on QT prolongation and Torsades de Pointes (TdP) risk, in accordance with the ICH S7B and E14 guidelines. This regulatory strategy is being revisited through the novel paradigm of CiPA (Comprehensive *in vitro* Proarrhythmia Assay) focusing on *in silico* and *in vitro* approaches to screen new compounds and better predict cardiac proarrhythmia risk in man[38–40]. However, QT prolongation and other arrhythmias are only one part of the iceberg, as they account for 23% and 4% of the cardiovascular issues, respectively[15,16]. Therefore, to increase the likelihood of success, an effective de-risking strategy should not solely cover proarrhythmia liability, but also integrate

hemodynamic and cardiac contractility assessment, and address both functional and structural aspects of cardiotoxicity. As exemplified by Figure 1, such strategy usually consists of a combination of *in silico*, *in vitro* and *in vivo* models. *In vitro* assays aim at selecting compounds devoid of major liability on c ardiac ion channels involved in arrhythmia. Datasets generated in these ion channel assays can serve to build *in silico* models that will predict liability of new chemically-related molecules and help refine the SAR in a chemical series. *In silico* models simulating action potential effects of new drugs in human or animal cardiac cells are also a growing field of development, as it is part of the CiPA paradigm[41]. *In vivo* cardiovascular safety studies are mostly stand-alone single-dose studies conducted in rodents and non-rodents. The anesthetized guinea pig has emerged as an interesting model to screen compounds for both proarrhythmia risk and hemodynamic or contractility effects[42]. The conscious telemetered dog or non-human primate is used at later stages to confirm the absence of cardiovascular risk of the candidate drug. Furthermore, cardiovascular functional endpoints can be incorporated in repeat-dose toxicology studies in order to assess time course, duration, and reversibility of sub-chronic to chronic effects[43]. For this purpose, non-invasive (jacketed), ambulatory telemetry systems have been developed for use in dogs and primates.

While pathophysiological and pharmacological mechanisms underlying functional cardiovascular effects such as QT prolongation, blood pressure changes, or inotropy are quite well understood, mechanisms explaining morphological damage to cardiomyocytes that could lead to cardiomyopathy or heart failure are less clear. Significant efforts are currently deployed to improve this understanding through the development of predictive cellular models combining various technologies. The use of stem cell-derived cardiomyocytes of human origin allows assessing, in a single assay, structural endpoints such as ATP depletion, calcium mobilization, mitochondrial membrane potential, and endoplasmic reticulum integrity[44], and functional endpoints like action or field potential, contractility, or beating rate.

The development, validation, and implementation of translational biomarkers providing further understanding of morphological and/or functional effects of cardiotoxicity have seen a sustained growth. One example of more established molecular biomarkers are the cardiac troponins[45]. These have seen their application gradually increasing in both preclinical and clinical settings[46]. Another example is the current investigations into the use of microRNA (miRNA) biomarkers (miR-208 and miR-1) as endpoints that further support the interpretation of cardiotoxicity[47,48]. Due to their novelty, further studies addresses whether these can be considered as biomarkers of cardiac morphological and/or functional changes. The use of biomarkers is also moving towards an application in *in vitro* models (such as stem-cell derived cardiomyocytes) to improve their predictive value when translating findings into the clinical setting[49,50].

In a study comparing the adverse effects of a series of new drugs in humans with those in animals, all electrocardiographic changes in humans were also detected in animals, and the Beagle dog appeared to be the best model[51]. Although the comparison of adverse cardiovascular effects of drugs between humans and laboratory animals needs further investigations, there is a reasonable overall correlation[52]. However, some exceptions have emerged recently. As an example, a number of anticancer drugs that target tyrosine kinases (e.g., trastuzumab and imatinib) have been associated with cardiac dysfunction in a small percentage of patients but were not predicted in laboratory animals[53,54].

Cardiovascular responses to injuries can be functional, structural, or both. Changes in cardiac work usually lead to structural morphological changes, which may ultimately lead to heart dysfunction. Morphological evaluation includes the four chambers (ventricles and atria) of the heart and its different structural (valves, large vessels) and histological components (myocardium, endocardium, epicardium and pericardium). Blood vessels inside and outside the heart should be also considered. The conduction system is rarely well represented in routine histopathological sections and to allow a m ore specific evaluation, a collection of additional materials that include the sinoatrial node, the atrioventricular node, the bundle of His and specialized conduction fibers are needed. Samples are routinely stained using hematoxylin and eosin (H&E), but special stains may be of interest to characterize microscopic alterations. Immunohistochemistry, *in situ* hybridization and ultramicroscopy can also be useful to better identify morphological changes. Finally, genetically modified animal models could be valuable tools for providing information on the pathogenesis of cardiovascular changes and the mechanism of action of various drugs[55].

2.3.3 Central Nervous System (CNS) Safety Strategy

Twenty five percent of the CNS projects are closed due to CNS liabilities[6]. According to a recent survey[56], the most frequent CNS issues encountered preclinically are seizures, gait abnormalities, tremors, emesis/salivation and sedation while in phase I clinical trials, emesis/nausea, fatigue, headache and dizziness are the most frequent ones. It is noteworthy that the last three effects are difficult to address preclinically. In addition to those CNS issues, liability abuses (already widely regulated) as well as suicidal ideation often represent CNS risks appearing at later stages of the development or post marketing. From a regulatory point of view, CNS safety investigations are driven by regional (FDA: Guidance for Industry: Assessment of Abuse Potential of Drugs; EMA: Guideline for the Non-Clinical Investigation of the Dependence Potential of Medicinal Products) or global guidance (ICH S7A: Safety pharmacology studies for human pharmaceuticals; ICH M3(R2): Guidance on non-clinical safety studies for the conduct of clinical trials and marketing authorization for pharmaceuticals) which mostly only address the *in vivo* safety and, this, at a quite late development stage (post candidate selection). These include standalone behavioral safety pharmacology studies and observation of clinical signs and brain histopathology in toxicity studies. Some sponsors opted for the inclusion of functional endpoints in repeat-toxicity studies, for scientific [New Biological Entities (NBEs)], long lasting effects, integration with toxicity and TK data…), ethical (National Center for Replacement, Refinement and Reduction of Animals in Research) and cost reasons[43]. However, this practice has shown some limitations (different laboratory conditions, dose levels, interference with the primary endpoints, gve0) that might reduce the quality of the signal[43].

At early stage, CNS toxicity assessment should focus on the physicochemical properties of the compound (e.g., lipophilicity, protein binding) which will drive the propensity of the compound to cross the blood-brain barrier (BBB)[57]. Importance should also be given to the targeted disease as some pathophysiological conditions are known to affect its permeability[58,59]. With 75% of preclinical safety closures being off-target, *in vitro* pharmacological profiling takes on great importance in the reduction of the attrition rate by identifying undesired off-target activities at the early development stage[21].

Amongst the CNS' adverse effects listed above, the industry mainly focuses on the investigation of seizures which represent a high risk of drug failure at the late stage of development or withdrawal from the market. Screening cascades were developed including early *in silico* investigation, pharmacological profiling and high or medium throughput studies such as brain slice preparations or zebrafish models preliminary to the *in vivo* investigation in rodents[60]. Electroencephalograms along with video recording allow a further characterization of the seizure risk in rodents or larger species; the monkey being the species of choice with a high translatability potential to human[61]. The tremendous progress made in this field over the last decade has led to better sensitivity, interpretation and reduction of cost and burden of the surgical implantation[62]. Nevertheless, the data should be interpreted with caution to ensure that the filter applied by all these tests is not too stringent and would potentially prevent some good compounds to reach patients[63]. Despite the paucity of the data comparing drugs-related adverse effects between animals and humans, animals are considered rather poor predictors of subjective neurological effects. Laboratory animals are much better predictors of structural effects[52]. In a review on the concordance of the toxicities in humans and animals for 150 compounds, 49 out of 221 human toxicity observations were neurological in nature. Eighteen of these compounds belonged to the neurologic therapeutic class and a significant percentage of these compounds did not progress to the market[51]. Over half of these adverse nervous system effects were judged to be related to the primary pharmacology.

Structural neuropathology remains the ''gold standard'' for the assessment of experimental toxic neuropathy even if special neurobehavioral assessment and electrophysiological tests help in the correlation of functional and morphological effects[64]. In conventional toxicity studies, immersion fixation of the brain, spinal cord and nerves in formalin-based fixatives followed by paraffin wax embedding and H&E staining is the best approach as a routine screening method. Nevertheless, perfusion fixation should be preferred when special neuropathological assessment aims to characterize drug-induced changes found in the brain[65]. Additional special histochemical staining is helpful to better detect, characterize or quantify pathological changes: cresyl violet (for neuronal features), Bielchowsky's of Bodian's stains (for axonal integrity), Luxol fast blue (for myelin integrity), amino cupric

silver (frozen sections required) or fluoro-Jade stains (for neuronal degeneration). Immunologic stains can be used to identify different cell populations. Glial fibrillary acidic protein is now classically used for the detection of glial cells in laboratory animals while markers for neurons include synaptophysin, NeuN, neurofilament protein, neuron-specific enolase and microtubule associated protein 2. Myelin associated glycoprotein and myelin basic protein can be used to stain oligodendroglia[66]. For microglial cells, Iba1 and CD68 are among the most widely used microglia and macrophage markers[67]. On certain occasions, ultrastructural assessment is needed to fully characterize pathological changes and typically requires dedicated investigations.

A number of international regulatory guidance documents for neuropathology assessment in preclinical toxicity studies are available[68–70] and describe the zones to be included because of their specificities (e.g., high glucose consumption, blood vessels with fenestrated endothelium). Finally, the development and implementation of CNS biomarkers is still in its initial stage. Several efforts have been made to develop fluid-based biomarkers and neuroimaging methodologies[71,72]. To this end, working groups such as those led by the Health and Environmental Sciences Institute are instrumental in assessing the utility of selected biomarkers in a translational manner[73].

2.3.4 Hepatotoxicity Derisking Strategy

Drug-Induced Liver Injury (DILI) is a major concern for the pharmaceutical industry as being one of the leading causes of drug withdrawals, non-approval, and regulatory actions. DILI is also a problem for care providers and patients because of its severity and sometimes fatal consequences. In addition, DILI represents a substantial part of ADRs, for which the total annual financial burden has been estimated to be as high as $177 billion in treatment costs in the United States[74]. DILI is generally divided into intrinsic (predictable) or idiosyncratic (unpredictable) categories. While intrinsic DILI results from drug-induced direct hepatotoxicity over the course of a few days, idiosyncratic DILI occurs in a minority of susceptible individuals with a prolonged latency. The most common example of a drug causing predictable DILI is acetaminophen while examples of idiosyncratic DILI include those related to amoxicillin/clavulanate, nonsteroidal anti-inflammatory drugs, and isoniazid[75]. With a low occurrence estimated to be within 1 in 10,000 to 1 in 100,000[76], idiosyncratic DILI remains a major challenge with regard to its prediction and prevention. This is due to multiple factors including the nature of its reactions which lack a definitive correlation to the drug's known pharmacological effects, the variation in temporal patterns with regard to drug exposure[77,78], and large dependence on individual susceptibility of affected patients.

Pharmaceutical companies have taken various measures during preclinical phases of drug development that were intended to decrease the risk of DILI, by steering away from drug candidates that are perceived to be at "high risk". Figure 1A illustrates the strategy set up by Lundbeck to discard hepatotoxic compounds. When clearly hepatotoxic agents are discovered from animal testing, they are usually rejected and not allowed to enter into clinical phase[79]. As a consequence, there are limited data available for a systematic assessment of the predictive value of animal findings for DILI in humans. Most of the drugs that were found to cause severe DILI in humans did not cause significant hepatotoxic effects in animals[79]. In contrast, there are examples of drugs that caused significant liver injury in animals[80] but are still considered very safe to the human liver. In a study by Olson *et al.*[51], 55% of drugs known to be hepatotoxic to humans were correctly classified using standard animal models. However, many pharmaceutical companies still continue to rely on animal studies to predict DILI, which bears the risk of unnecessary termination of potentially safe and effective medications due to adverse animal findings.

DILI is challenging to predict in humans because many mechanisms may lead to hepatotoxicity as illustrated in Figure 2. For more details on the different mechanisms please refer to the publications by Lee[81] and Godoy *et al.*[82]. Among them, chemically reactive metabolites have been claimed to be often associated with hepatotoxicity because of their potential to irreversibly bind to and modify cellular macromolecules[83]. However, studies seeking to determine the predictive value of reactive metabolites and covalent binding have shown conflicting results[84–86]. The direct link between the production of reactive metabolites and occurrence of DILI is quite complex as it is the balance among bioactivation, detoxification, and defense mechanisms that determine whether a reactive metabolite elicits a toxic effect[87]. A recent study found that while *in vitro* covalent binding levels in human hepatocytes failed to predict DILI, multiplying

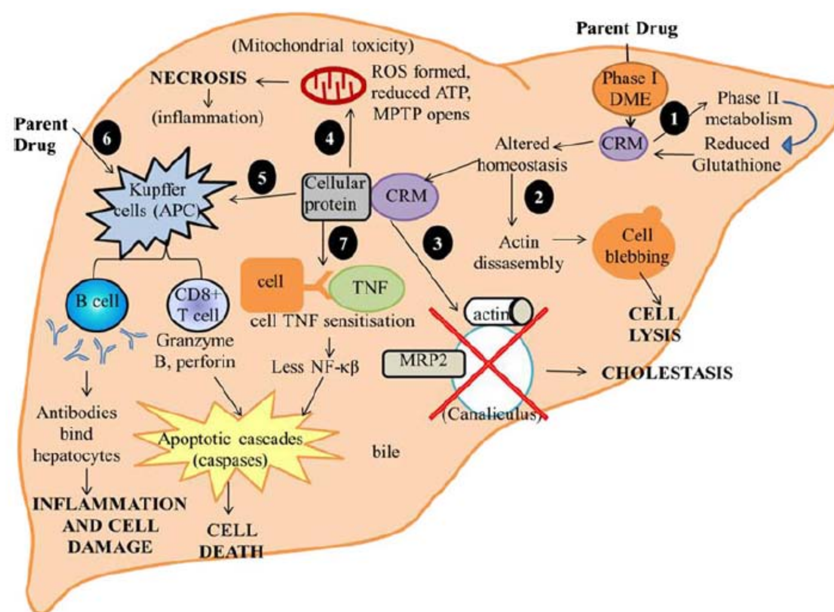

Figure 2. Overview of mechanisms of DILI. Figure extracted from Godoy *et al*.[82], with permission from Springer. (1) Detoxification: conjugation with glutathione. (2) Altered calcium homeostasis. (3) Reactive metabolites may bind to transport pumps or actin around the bile canaliculi preventing bile export. (4) Reactive metabolites binding to mitochondrial proteins may reduce ATP formation, produce ROS, and open the MPTP causing apoptosis. (5) Immune stimulation via the hapten or prohapten mechanisms leading to either humoral (B cell) or cell-mediated (Tcell) reactions. (6) Immune activation (PI mechanism with parent drug). (7) TNF receptor sensitivity may be heightened increasing responsiveness to TNF, leading to apoptosis. For more details, please refer to Godoy *et al*.[82].

the covalent binding amount by the maximum daily dose was able to discriminate between drugs that were considered hepatotoxic and those that were not[84]. Although additional studies are required to confirm these initial observations, this apparent relationship between the doses of medications and DILI may assist in our understanding of and potential escape from DILI during drug development and patient usage.

In vitro assays using cell cultures have been tested for prediction of DILI but their predictivity is variable[88–90]. A very serious hurdle is the lack of standardization of these *in vitro* models, which certainly limits our understanding of how to best use them and the need for validation for potential use in regulatory submissions[91]. In addition, scientists may also question the relevance of measuring general cytotoxicity markers in comparison to mechanistic endpoints. For instance, different studies investigated whether improved sensitivity of DILI prediction can be obtained by combining data provided by diverse assays assessing differing mechanisms. This approach has yielded very encouraging results[92], as have approaches that combine *in vitro* assay data with physicochemical properties of drugs, and/or *in vivo* plasma exposure data[93,94]. If *in vitro* data are used for decision making, such models need to be validated very carefully because there is always the risk of terminating safe drugs for no valid reasons. Nevertheless, the scientific community is developing more sophisticated human models including organ on a chip[95] and co-culture[96] models that have the potential to better detect hepatotoxicity in humans at the early stages of drug development. Moreover, the value of approaches such as *in silico* models[97], structural alert systems, and toxicogenomics[98,99] for the prediction of DILI risk during drug development is still highly controversial. These approaches have indeed suffered from a lack of specific and sensitive biomarkers, scarcity of toxicological data, and lack of concrete unequivocal understanding of underlying mechanisms of most known hepatotoxic drugs[97].

In parallel to the ongoing request for new biomarkers and a better understanding of genetic and immunologic factors accounting for predisposition to DILI, some drug makers have also implemented strategies in clinical development to use the information from early clinical trials to better predict potential risk of drugs for DILI. Indeed, it has become increasingly evident that milder forms of liver injury occurring in clinical trials, when evaluated properly, may significantly en-

hance the ability to predict the drug's potential to cause more severe liver injury in further development. Many drug makers have adopted strategies using Hy's law [based on the use of well-established biomarkers such as alanine aminotransferase (ALT), aspartate aminotransferase (AST), bilirubin, and alkaline phosphatase], causality assessment of individual hepatic cases and adherence to strict hepatic discontinuation rules to prevent unnecessary early discontinuation of the study drug[100].

The use of translational molecular biomarkers in the context of hepatotoxicity strategies is characterized by the wide application of well-established markers such as ALT, AST, bilirubin and the deployment of novel biomarkers ranging from proteins, such as high-mobility group Box 1 (HMGB1) and colony stimulating factor 1 receptor (CSF1R), to miRNAs such as miR122[101,102]. The former are widely used in both preclinical and clinical settings for risk assessment and management[103]. The latter, although showing the potential to be more precise and robust, still require further validation in the clinical setting before full implementation and investigations into their back-translational potential[103,104].

While microscopic evaluation on H&E stained formalin fixed paraffin embedded tissues can demonstrate most of the hepatocellular and biliary alterations, additional special histochemical staining are usually required to better detect, characterize or quantify pathological changes. Vacuoles and pigments are typically one of these examples and a set of staining can be applied to determine the nature of the content (e.g., periodic acid-Schiff, Sudan's dyes, or Oil-red-O). More elaborate techniques are sometimes required, such as immunohistochemistry (IHC) or electron microscopy (EM) to definitively demonstrate the nature of the material. For example, an immunohistochemical approach using lysosomal-associated membrane protein-2 or the demonstration of the characteristic ultrastructural multilaminated whorl of material in lysosomes by EM can aid for the differentiation of phospholipidosis from lipidosis. Pigments are a second example, as most pigments present a brownish-greenish yellow appearance when stained with H&E and it is sometimes difficult to determine their nature (see Section 3, second case study).

2.3.5 Other Target Organ Safety Strategies

Respiratory, renal and gastrointestinal systems are involved in only 3% to 9% of safety failures in preclinical or clinical phases[6]. Probably due to this low contribution in drug attrition rate, pharmaceutical companies rarely establish routine de-risking safety strategies for these target organ systems. Such strategies are applied on a case-by-case basis, when the primary target or the secondary pharmacology profile is associated with potential liabilities for these systems.

From a regulatory perspective, the respiratory system is part of the safety pharmacology core battery that investigates the effects of the test substance on vital functions (ICH S7A guideline). As a minimum, respiratory rate and tidal volume should be measured, as clinical observations are not adequate to properly assess respiratory function. Stand-alone respiratory studies can be performed using techniques such as the whole-body or head-out plethysmography. Respiratory functional endpoints can also be integrated in general toxicology studies, in particular in non-rodents. The respiratory inductive plethysmography using jacketed external telemetry (chest bands) allows an accurate and non-invasive measurement of respiratory function. Additional parameters such as hemoglobin oxygen saturation or blood gases might also provide useful information.

In contrast with respiratory function, renal and gastrointestinal (GI) functions are not considered as 'vital' and therefore, are not part of the safety pharmacology core battery. However, supplemental studies can be conducted to evaluate suspected adverse effects on these systems. GI side effects observed in the clinic are mainly nausea/vomiting and intestinal transit disturbances (diarrhea or constipation). Although not life-threatening, such side effects can greatly impact the quality of life. GI function assessment should cover GI motility, nausea and emesis liability, secretory function and absorption[105]. A variety of *in vitro* models allows studying the effects of substances on smooth muscle and enteric nervous system without the influence of external factors[106]. *In vivo* gastric emptying and intestinal motility can be investigated in simple models such as the charcoal meal transit test in rodents. New promising technologies like wireless devices (SmartPill™, Bravo™ capsule) provide non-invasive GI endpoints such as changes in temperature, pH or pressure, but to this point can only be used in large animals[107,108]. Assessment of nausea and emesis liabilities requires *in vivo* models, as no *in vitro* tool is capable of reproducing such complex physiological events. Emesis evaluation can be conducted in dogs, ferrets, primates or shrews. It usually consists of sim-

ple visual recordings of retching and vomiting episodes. More sophisticated techniques combining video-monitoring and telemetric measurement of abdominal pressure and electromyography can also be used[109,110].

Acute or chronic drug-induced kidney injury (DIKI) is mainly associated with small molecules and can consist of diverse pathological manifestations (interstitial nephritis, tubular cell toxicity, glomerulonephritis, rhabdomyolysis,"gve0)[111]. The mechanisms involved are poorly understood and histopathology remains the gold standard to detect DIKI. However, recent developments in the biomarkers field have emerged. Seven urinary biomarkers (kidney injury molecule-1, clusterin, albumin, total protein, β2-microglobulin, cystatin C and trefoil factor 3) have been qualified by the FDA for use in GLP rat studies[112,113]. Their appearance or excretion in urine offers the promise of greater sensitivity and utility to detect early stages of DIKI, before histopathology changes occur. In addition to these biomarkers, renal function is addressed through various *in vitro* and *in vivo* models. *In vitro* experiments can be conducted on isolated perfused kidney preparations, renal slices or different kidney cell lines[114]. *In vivo* assessment of renal function should cover glomerular function (measurement of glomerular filtration rate), tubular function (through plasma or urine markers such as creatinine, electrolytes), and hemodynamic function (renal blood flow, renal vascular resistance).

3. Mechanistic Investigations: Case Studies

Understanding the mechanisms of toxicity and assessing the associated risks can represent a key challenge when it comes to provide timely go/no go decisions that may result in the removal of a compound. Here, two examples of the successful integration of investigative toxicology experiments to support drug discovery process are provided. The first case study involves ticagrelor, an inhibitor of platelet aggregation, which is used together with aspirin to prevent atherothrombotic events as treatment of patients with acute coronary syndromes. Its antiplatelet and clinical activity is mainly mediated through its potent and reversible inhibition of the platelet $P2Y_{12}$ receptor[115,116]. Compared to clopidogrel (another contemporary antiplatelet agent), ticagrelor significantly reduces the risk of heart attacks. Moreover, data collected during ticagrelor clinical development showed that treatment with ticagrelor as compared with clopidogrel also significantly reduced the rate of all-cause mortality[117]. Dyspnea and ventricular pauses were observed in some patients treated with ticagrelor[117–119]. These observations suggested a likely additional mechanism of action for ticagrelor, i.e., adenosine mediation[120–122]. In the *in vitro* and *ex vivo* studies performed by Armstrong *et al*.[123], the authors further characterized ticagrelor pharmacology with respect to adenosine mediation. They examined ticagrelor effect on specific adenosine transporters using recombinant cells, performed receptor ligand binding and functional assays and assessed adenosine and ticagrelor effects in *ex vivo* guinea pig and rat C fiber preparations[123]. Thanks to these investigations, the authors showed that ticagrelor inhibited cellular adenosine uptake selectively via equilibrative nucleoside transporter (ENT) 1 inhibition at concentrations of clinical relevance. In addition, ticagrelor displayed low binding affinity and functional inhibition of adenosine receptors suggesting that a direct effect of ticagrelor on adenosine receptors was unlikely to be of clinical relevance. Hence, ENT1 inhibition by ticagrelor can potentially contribute to the overall clinical benefit of ticagrelor compared to clopidogrel (that had no effects on ENT1 transfected cells). Overall, this provides a comprehensive set of *in vitro/ex vivo* data that contributes to understand ticagrelor adenosine mediated mechanism of action, which help to explain the clinical picture of ticagrelor treated ACS patients.

The second case study describes an antiepileptic synaptic vesicle 2a ligand drug candidate that, when tested in 4-week rat and dog oral toxicity studies, elicited different liver findings depending on the species[124]. Indeed, as illustrated in Figure 3, dark pigment deposits were detected in the liver of high dosed (200 mg/kg/day) dogs while no adverse effects were observed in the rats at any dose tested (up to 1000 mg/kg/day). Of note, rats were exposed to higher parent drug levels compared to dogs [toxicokinetic (TK) data]. The morphology of the liver deposits, accompanied by increases in the plasma liver enzymes and a slight elevation in bilirubin, were suggestive of hepatic porphyria with accumulation of porphyrin and potential neurovisceral complications[125]. By conducting a thorough TK analysis and performing *in vitro* metabolism assays in primary hepatocytes from different species, Nicolas *et al*.[124] showed that the dog was more prone than the rat to oxidize the drug candidate into a porphyrogenic metabolite. *Ex vivo* tissue measurements did help elucidate the cascade of events

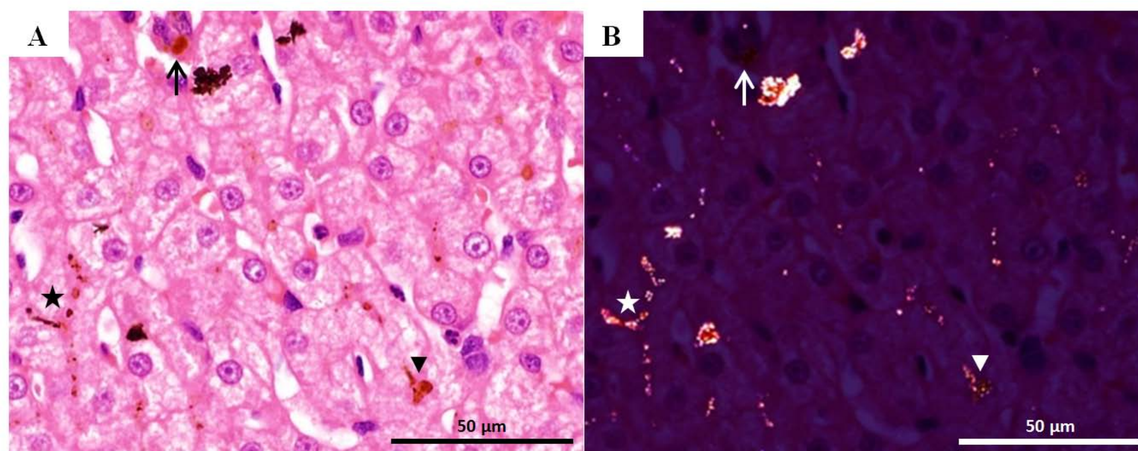

Figure 3. Liver section from a 200 mg/kg/day treated dog stained with hematoxylin and eosin (**C**) and under polarized light (**D**). Brown pigments were observed in bile canaliculi (star), and in the cytoplasm of Kupffer cells (arrow) and hepatocytes (arrowhead) (**C**). Porphyrin pigments are characterized by red birefringent properties (white star and arrowhead) under polarized light and can be differentiated from lipofuscin deposits (white arrow) (**D**). Original magnification: ×200.

underlying the porphyria observed in dog. Hence, the pigment in the liver of treated dogs was the result of protoporphyrin IX accumulation. The latter was linked to a decrease of hepatic ferrochelatase activity, as well as combined induction, and inactivation of cytochrome P450 CYP2B11. The causative agent for the disrupted heme biosynthetic cascade in treated dogs was identified by mass spectrometry as an N alkyl-protoporphyrin adduct formed by a reactive metabolite. Decisively, the authors assessed the translatability of these findings in humans and demonstrated that this particular metabolite was not produced in rats or in humans. Altogether, these findings enabled Nicolas *et al*.[124] to conclude that the protoporphyria elicited by the drug candidate in dog livers was not of clinical relevance. Therefore, the drug candidate should neither induce protoporphyria nor activate quiescent inherent porphyria in humans. This work also highlights the species variability that can be observed with drug induced porphyria and the consecutive challenge of extrapolating animal toxicity data to human. For this reason, *in vitro* approaches have also been described to elucidate porphyria species variability[126,127].

In conclusion, these two case studies[123,124] perfectly illustrate how investigating the mechanisms of action can help to progress or stop a compound, even at a late stage of drug development. Moreover, as exemplified in both reports, successfully addressing key safety issues requires most often a combination of *in vitro*, *ex vivo* and *in vivo* approaches, at the time of an increasing debate regarding the clinical outcome prediction by animal toxicology testing[128–130].

4. New Trends in Investigative Safety

4.1 Computational Models Impacting Investigative Safety

The high attrition rates due to safety issues have been a strong driver during the past decade in developing and applying computational models for toxicological endpoints. Models, or other computational tools, can be applied in most stages during drug discovery and development, and should ideally be aligned to the experimental target organ strategies to support and complement these in an integrated fashion. Very early on, during the safety evaluation of the target, bioinformatic methods such as pathway analysis[131] are largely employed with chemo-informatic methods, becoming more applicable in the lead identification stage and onwards. The methods used in various stages of drug discovery depend on factors such as the abundance of available data, quality of those data, and the intended usage of the output. To a high degree, this correlates with the complexity and accuracy of the data generated in projects at various stages. High or medium throughput *in vitro* screening data are often used for the generation of machine learning models, such as quantitative structure-activity relationship (QSAR) models. The datasets are often rather large (thousands to tens of thousands of compounds) and can vary in origin from proprietary, public or a mixture of both. These models are usually comprised of diverse chemical compounds and are commonly referred to as “global” models. Ion

channels, such as hERG and Nav1.5 or some of the targets mentioned by Bowes *et al.*[21], are common to model using such methods, with the aim of selecting compounds for experimental screening or to prioritize which chemical series to pursue. Predicting potential off-target effects via the compounds' interaction(s) with other proteins can be done either on an *ad hoc* problem solving basis or as part of profiling of compounds/series. This is frequently done by looking at chemical similarity and drawing conclusions based on the targets that similar compounds interact with[132–136]. It is desirable in this context to also relate *in vitro* to *in vivo* data. Some work has been published relating the experimental interaction patterns of *in vitro* results to *in vivo* toxicology studies by looking at biological similarities[63]. "Structural alerts", chemical substructures that have been shown to be mechanistically relevant to a specific toxicology endpoint, are often used for endpoints of chemical toxicity. These include mutagenicity and skin sensitization but they may also be used in situations where the data sets are not of high enough quality or large enough to support quantitative modeling. Garcia-Serna *et al.*[137] recently published a methodology to link structural alerts and secondary target effects to human side effects. The comparison of chemical analogs, or "read across", is also a common practice for risk assessment of compounds for chemical toxicity endpoints. This entails drawing conclusions for untested compounds based on the structural comparison to similar compounds with associated experimental data. As mentioned previously, the use of computational tools is required in the context of PGIs and regulatory submissions through the application of the ICH M7 guidelines. Many of the methods mentioned above have been around for a number of years and are often well embedded into the preclinical discovery processes. The future of computational work in this field lies in the more complex relationships relating high content data (such as phenotypic and genotypic information) to toxicological outcomes. This will infer high demands on knowledge management and thorough understanding of the underlying mechanisms at hand, as it is far from trivial to connect molecular events to clinical events. It is however clear that there is room for improvement in this field and that computational tools are maturing and becoming embedded and aligned with the experimental target organ strategies.

4.2 Mitochondrial Toxicity Assessment

Organ toxicity strategies can be complemented by investigating a common underlying mechanism of those toxicities. For instance, mitochondrial toxicity has been reported to be one of the main primary causes of various organ toxicities induced by xenobiotics[138–140]. These include drugs that induce hepatotoxicity[141,142], cardiotoxicity[143–144], nephrotoxicity[145] and neurotoxicity[146]. Drugs were also reported to cause multiple organ toxicities due to mitochondrial dysfunction. For instance, the adverse effects associated with fialuridine, i.e., nausea, vomiting and painful paraesthesia with subsequent hepatic failure; pancreatitis, neuropathy, myopathy and lactic acidosis, are probably due to multisystem mitochondrial toxicity[147]. In addition, co-administration of drugs can increase the risk of drug-induced mitochondrial toxicity and the latter can also be associated with genetic predispositions[148]. Drugs can act in many distinct ways on the mitochondria. Some mitochondrial toxic compounds, such as rotenone (pesticide inhibiting complex I), act directly on the electron transport chain (ETC) and oxidative phosphorylation (OXPHOS). Other compounds may interact in an indirect way, for instance by reducing substrates needed for the ETC or damaging mitochondrial DNA (mtDNA)[139]. As an example, sodium valproate, an antiepileptic drug, indirectly affects mitochondrial function by inducing carnitine deficiency, leading to a depression of intramitochondrial fatty acid oxidation, and consequently the inhibition of OXPHOS[149]. Some physicochemical properties have also been linked to mitochondrial toxicity. For instance, uncouplers of OXPHOS are characterized as being hydrophobic weak acids (i.e., phenols or amides) with a pKa of 5–7[139]. Recently, Nelms *et al.*[150], developed an *in silico* profiler for mitochondrial toxicity based on structural similarity and molecular mechanism of mitochondrial toxicity. A high-throughput screening platform was also developed using isolated mouse liver mitochondria in order to detect global mitochondrial membrane permeabilization (swelling), inner membrane permeabilization (transmembrane potential), outer membrane permeabilization (cytochrome c release), and alteration of mitochondrial respiration driven by succinate or malate/glutamate[142]. Around 90% of oxygen consumption by mammals occurs in the mitochondria with the ultimate objective of synthesizing ATP, oxygen consumption can be used as an indirect readout of mitochondrial function[140,151]. A kinetic readout for cell energy metabolism applicable for screening purposes can be assessed with the extracellular flux (XF) ana-

lyzers (Seahorse Inc.). XF analyzers concurrently measure oxygen consumption rate (OCR) and extracellular acidification rate (ECAR), indirect measures of OXPHOS and glycolysis, respectively[152,153]. In addition, specific parameters of mitochondrial function can be deduced through the addition of known mitochondrial stressors[149]. For example, the consecutive addition of oligomycin, FCCP, and a mixture of antimycin and rotenone after exposure to the compound will provide the effect of the compound on the OCR associated with ATP production, maximal respiratory capacity, proton leak and spare respiratory capacity[154]. Finally, mitochondrial toxicity should be assessed throughout the development of new chemical entities and the implementation of biomarkers in pre-clinical and clinical studies is important. Reported side effects of drug induced mitochondrial toxicities can help us to define biomarkers. For instance, hyperlactic acidemia, lipid accumulation (microsteatosis), hypoglycemia, hypoxemia and heat production are observed with ETC and substrate inhibitors[139].

4.3 Impact of Technologies

The pharmaceutical industry is under massive pressure from economical, regulatory, public and R&D point of views. There is a crucial need to implement innovative approaches and technologies as failure to innovate in drug development will render the "big pharma" model unsustainable[155]. The use of groundbreaking technologies should not only reduce the attrition rate due to efficacy and safety reasons, but also, avoid the huge cost associated to the development of well advanced drug candidates[155]. Different examples are provided in the following paragraphs to illustrate how the use of innovative technologies may greatly impact drug development. Although the technologies/approaches described in the next sections carry many advantages, there are also some limitations associated as with any technology (not described in this manuscript). Finally, to implement such technologies in the pharmaceutical industry, it is important to demonstrate that they have some added value compared to the traditional methods currently used in the drug development paradigm.

In the field of Alzheimer's disease, the use of positron emission tomography ligands, volumetric magnetic resonance imaging and fluid biomarkers has allowed to better characterize the different steps of disease progression at molecular, functional, and structural levels. Nevertheless, the development of reliable markers to quantitatively assess cognitive domains that are subtly changed before memory is an urgent need since it is important to detect subjects at risk as early as possible. Leurent and Ehlers[156] provided a new concept on how emerging mobile, computer, and device-based cognitive tools are converting classically noisy, non-objective, data-poor clinical endpoints associated with CNS disease assessment into a richer, scalable, and objective set of measurements. In summary, the prospect for transformative efficiency and accuracy in testing novel therapeutics in neurodegenerative disease may rest in the devices we each day carry in our pockets[156].

Compound PK, PD and transport data in the different tissues collected from animal studies are crucial during the early stages of drug development. Traditionally, the abundance and distribution of drugs have been assessed by well-validated methodologies including, for instance tissue homogenization with liquid chromatography/mass spectrometry (LC/MS) analysis and/or whole-body autoradiography (WBA). Unfortunately, LC/MS, despite its remarkable sensitivity, does not provide information on spatial distribution and WBA does not make any distinction between parent compound and metabolites. In contrast, mass spectrometry imaging (MSI) can discriminate drug and its metabolites and endogenous compounds, while simultaneously reporting their distribution[157]. MSI data are influencing drug development and are currently used in investigational studies in areas such as compound toxicity[157]. Hence, MSI data generated from animal studies results may soon be used to support new drug regulatory applications, although clinical trial MSI data will need more time for the validation and incorporation into submissions.

Finally, the use of label-free technologies applied to cell biology and drug discovery is receiving more and more attention[158]. Platforms based on acoustic resonance, electrical impedance, microcantilevers, nanowires, and differential calorimetry are beginning to appear, with commercially available products for post-high-throughput screening hit confirmation and mode-of-action studies[159]. The advantages of label-free detection include a simple homogeneous assay format, non-destructive methodology, reduced interference with normal cell function, kinetic measurement (Figure 4), and limited time for assay development. As an example, real-time cell analyzer (RTCA), a label-free technology based on impedance measurement, can be used to generate information on cell proliferation, migration, viability, and receptor-mediated signaling,

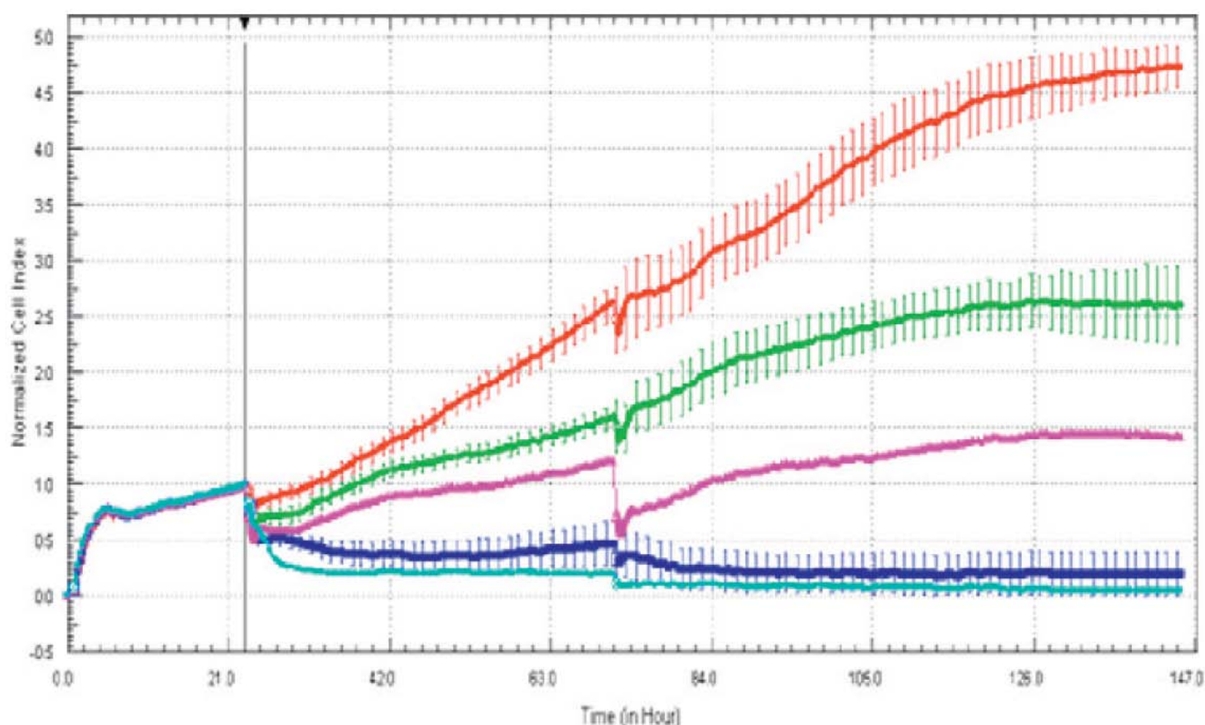

Figure 4. Example of label-free data (Real-Time Cell Analyzer). Figure extracted from Atienzar *et al*.[96], with permission from Elsevier. HepG2 cells were exposed to DMSO and/or troglitazone. Cells were exposed to 0 (0. 5% DMSO, red curve), 12.5 Cmax (green curve), 25 Cmax (purple curve), 50 Cmax (dark blue curve) and 100 Cmax (light blue curve) µM. Cmax refers to the maximal concentration of a given compound in human blood. Troglitazone is known to be hepatotoxic in human and the drug was withdrawn from the market. Cmax value (troglitazone) = 6.39 µM. Cell indexes were normalized with the last time point before compound addition. The normalized time point is indicated by the vertical line. For more details please refer to Atienzar *et al*.[96].

associated with very specific and well-defined changes in cell morphology and adhesion[158,160].

4.4 Impact of Cellular Models

For the development of new chemical entities, the current model in toxicology is mainly based on *in vivo* testing using rodent and non-rodent species to support studies in human. Unfortunately, animal studies are not optimal for predicting human toxicity[51], in part due to species-specific differences in ADME between human and animal models, as well as the use of healthy animals with limited genetic diversity for preclinical toxicology studies[96]. Consequently, it is of primary importance to have access to relevant *in vitro* models including but not limited to rat, dog and human cellular models. It has been estimated that up to 70% of assays developed for drug screening and discovery are cell-based assays[161]. However, it is important to show that the new cellular models are carefully validated with superior predictive values compared to traditional cellular models.

Organ-on-a-chip, which is becoming more and more popular, is a cell culture model with microfluidic channels[162] with the possibility to simulate activities and physiological responses of an entire organ. Different "organ-on-a-chip" devices have already been created. One of the first ones is the "lung-on-a-chip" model made by the Wyss Institute at Harvard (US)[163]. The following step for this approach is to make connections of many organ on-a-chip devices to create a "body-on-a-chip". This configuration could possibly allow researchers to investigate the effects of substances not only on the individual organs but to replicate the interactions between each component, providing a more comprehensive analysis which could ultimately revolutionize how drugs are developed[162].

To overcome the deficiencies of existing *in vitro* liver models (e.g., HepG2, primary hepatocytes), Khetani and Bhatia[164] developed a co-culture model constituted of hepatocytes and mouse fibroblasts. Primary human or rat hepatocytes in the co-culture models are viable, have functioning bile canaliculi network and sustained expression of metabolic enzymes, transporters and liver specific proteins for at

least four weeks[164]. Many investigations have already been performed to carefully study metabolic capacities as well as prediction to detect human hepatotoxic drugs[96,165,166]. In a recent study, a s et of 51 drugs including 40 hepatotoxic drugs and 11 non-hepatotoxic drugs was investigated in the in dog co-culture model[96]. Overall, the aforementioned studies indicate that the co-culture models may better mimic *in vivo* situations as they seem to represent relevant tools to perform chronic hepatotoxicity and metabolism studies. Sensitivity and specificity data with co-culture models are presented in Table 2.

Finally, a recent technological development allows obtaining human induced pluripotent stem cells (hiPSCs) from the skin, which can be used to generate patient-specific cardiomyocytes (CMs) under *in vitro* conditions. This means that each hiPSC produced from patient fibroblasts carries the relevant genetic information. hiPSCs have been used to recapitulate disease phenotypes of genetic cardiac diseases such as long QT (LQT), familial hypertrophic cardiomyopathy (HCM), and familial dilated cardiomyopathy (DCM)[167–169]. Patients suffering from LQT, HCM, and DCM syndromes are particularly sensitive to cardiotropic drugs and are vulnerable to fatal arrhythmias[170]. Recently, Liang *et al*.[171] characterized a library of hiPSC-CMs derived from patients with LQT, HCM, and DCM and screened them against a panel of drugs known to affect cardiac ion channels. Drug-induced cardiotoxicity profiles were recapitulated for healthy subjects, LQT, HCM, and DCM patients at the single cell level for the first time. The data clearly reveal that healthy and diseased individuals display different susceptibilities to cardiotoxic drugs[171]. hiPSC-CMs can detect drug-induced cardiac toxicity more accurately than the classical preclinical assays mandated by regulatory authorities. In summary, these investigations illustrate well the concept of personalized medicine through *in vitro* assays which allow assessing the genetic susceptibilities of distinct individuals to better predict clinical outcome[171]. This is certainly essential as the majority of cardiotoxic drugs have a low incidence of harmful effects for the general population but are toxic to specific patient populations with determined genetic traits. Finally, Figure 5 illustrates how iPSC could be used for drug and chemical safety assessment.

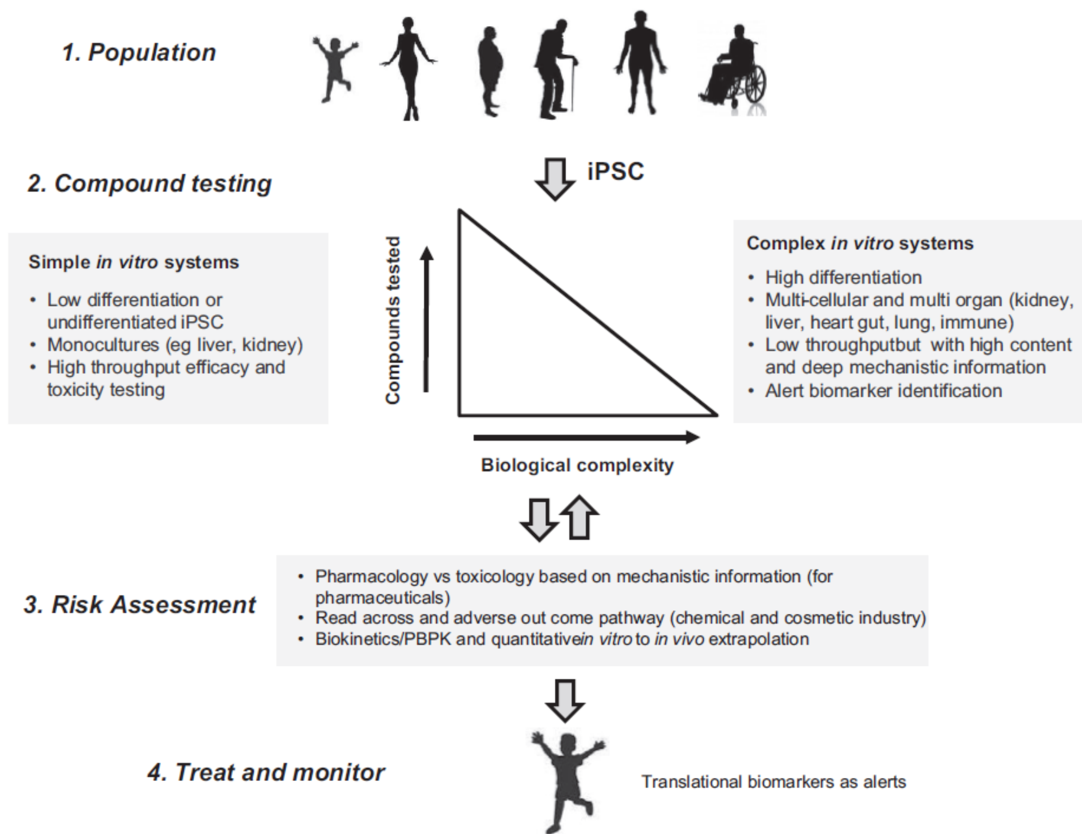

Figure 5. Potential use of iPSC for drug discovery and chemical safety assessment. Figure extracted from Jennings[185], with permission from Elsevier. Abbreviations: iPSC: induced pluripotent stem cell; PBPK: Physiologically Based Pharmacokinetic.

4.5 *In Vitro* Toxicology Assessment to Support Dkologics

For biologics, the main safety liabilities are related to the target itself and often reflect exaggerated pharmacological action[172]. The use of *in vitro* assays is mainly, but not exclusively, focused on the following areas at different stages of drug development. Before candidate selection, major biological target-related safety liabilities can be identified from known literature about the target, competitor drug liabilities and/or early experimental work. It may be of benefit to compare various candidates or even formats for the identified liability in an appropriate human *in vitro* system. As an example, FcγR-mediated cross-linking by an antibody targeting a cell-surface molecule may trigger an unwanted immunological effect such as antibody-mediated cellular cytotoxicity, unwanted cytokine release or platelet activation. In such cases, a head-to-head comparison of various formats (different Fc parts silenced for specific interactions or e.g., Fab fragments lacking the Fc parts) is useful to support candidate selection in a complex weighting with other parameters assessed to drive optimal selection.

During preclinical development *in vivo*, safety findings from preclinical *in vivo* assessments in relevant species often raise the question about translatability to man. In such cases, *in vitro* toxicological assessments in human in simple (e.g., cell line) to more complex settings (e.g., organoid culture) may help to understand this aspect. An in-depth understanding of the biology and function of the target across the species is an important prerequisite. An experimental approach often chosen is to try to recapitulate the *in vivo* findings in the toxicological species *in vitro* system to increase the confidence that potential effects in human can be predicted. Such assays may often have an impact on the minimum anticipated biological effect level approaches to determine the starting dose in man when the safety effect is directly related to the mode of action. Consequently, this may act both as sensitive pharmacodynamics and safety marker[173]. Another biologics-specific aspect is the generation of anti-drug antibodies that may cause through the formation of immune complexes various inflammatory responses including glomerulonephritis, vasculitis, thrombosis or even anaphylaxis, that are in rare cases found in man[174]. However, the frequency of anti-drug antibody responses observed preclinically is usually not translatable to man and pathogenic immune complexes may not form at the much lower human doses. The major goal of *in vitro* toxicology is in this case to exclude the direct functional target involvement in the preclinical findings. During clinical development, safety liabilities may become obvious which were not observed in the non-clinical species. This can have a variety of reasons: (i) the non-clinical toxicology species may not adequately predict all safety endpoints in man, due to limited similarity in expression patterns, functions, intercalating pathways, additional target functions in a species or sensitivity of the target action-related events, and (ii) the limited number of animals assessed *in vivo* does not allow to pick up rare safety events in man which are only discovered when large and more heterogeneous populations enter clinical trials as of Phase 3 and post-marketing. An *in vitro* toxicological species-comparative investment can highly increase the understanding of adverse events in the clinic and in the case of events in only a subpopulation of humans help to identify patients at risk (e.g., such bearing a specific allelic variant of the target).

5. Predictivity of the Models in the Different Target Organ Strategies

Over the last few years, data have been generated to assess the value of non-clinical tests to predict the potential drug effects in humans (but also in animals) by defining the parameters such as sensitivity and specificity of any given models (Table 2). The sensitivity of a model reflects the proportion of drugs whose effects in man are correctly identified by the model. A high sensitivity reflects a low rate of false negatives. The specificity of a model defines the proportion of drugs without an effect in humans that is correctly identified by the model. A high specificity reflects a low rate of false positives.

It is important to identify and eliminate, wherever possible, safety hazards during the early drug discovery phases, e.g., lead optimization[21,40]. Therefore, assays used during this phase should be highly sensitive, with great specificity. Table 2 gives examples of the performance of multiple approaches to predict toxicity effects in human (or in animals). Commercial *in silico* packages such as DEREK, Toxtree, MC4PC, and Leadscope MA performed relatively well to predict Ames data for compounds in the public domain and displayed reasonable specificity (77.5%–93.9%) but low sensitivity (17.4%–43.4%) with Roche chemical space[175]. This could be related to the fact that the

Roche chemical space is not well covered by the public chemical space which is not surprising. The hERG assay, which showed high sensitivity (82%) with 75% specificity is generally positioned during the early discovery phases despite the non-negligible rate (25%) of false positive (Table 2). Other in vitro assays (e.g., iPSCs) have better predictivity compared to the hERG assay but are not as thoroughly validated compared to the potassium channel assay[176]. The *in vivo* QTc assay in non-rodent species, which has overall good predictivity (ca 85%) is well positioned during the candidate drug selection phase assuming an adequate exposure range is tested (i.e., at least 30-fold)[129]. With regard to genotoxicity, the Ames assay which is part of the regulatory genotoxicity package to enter clinical testing displays sensitivity of 60% (i.e., 40% chance for false negative results) and specificity of 74% (i.e., 26% chance of generating false positive results) (Table 2). Based on the publication of Tilmant *et al*.[33], the Chinese hamster ovary micronucleus test performs better (Table 2) but it is worth mentioning that other publications have reported a much lower specificity in mammalian assays[177]. Finally, measuring motor activity in zebrafish allows to relatively well predict seizure and convulsions with sensitivity and specificity close to 70%[178]. Many other models and assays are presented in Table 2 but will not be discussed further in this section. To conclude, understanding the predictive value of nonclinical safety testing for humans enables to optimally align assays to address the key objectives of the drug discovery process, i.e., hazard identification and elimination, risk assessment, management and mitigation.

6. Conclusion and Final Remarks

Understanding and reducing attrition rate remains a key challenge in drug development. Preclinical and clinical safety issues still represent about 40% of drug discontinuation, of which cardiac and liver toxicities are the leading reasons. One would hope that increasing the extent of safety testing early during the drug discovery phases should enable to identify and eliminate safety hazards, therefore leading to the development of safer medicines with fewer and less severe ADRs and acceptable risk/benefit ratios in a given disease and patient population setting. Reducing attrition rate can be achieved by various means, starting by a comprehensive evaluation of the potential safety issues associated to the primary target as well as to undesirable secondary (off targets) pharmacological activities. To address these risks, a risk mitigation plan should be built at very early development stages, using a panel of *in silico*, *in vitro* and *in vivo* models. While most biopharmaceutical companies have developed robust safety strategies to de-risk genotoxicity and cardiotoxicity issues, partly driven by regulatory requirements, safety issues affecting other organs or systems, such as the liver, kidney, the central nervous, or gastro-intestinal systems, are less commonly addressed during early drug discovery. Some early discovery strategies can be applied to these target organ systems, including the use of novel biomarkers that can be easily integrated in both preclinical and clinical studies. The present manuscript highlights the importance and impact of investigative experiments to understand the mechanisms underlying toxicities and their human relevance that arise either in non-clinical chronic toxicology studies or clinical trials, thus enabling to form an integrated risk assessment, and to develop risk management and mitigation plans. Novel trends in investigative safety have been also reviewed, such as computational modeling, mitochondrial toxicity assessment, and imaging technologies that may become an integral part of drug safety testing. Assessing and integrating novel technologies and the latest scientific advancements as well as shaping and implementing emerging and future regulatory requirements may enable to further enhance our confidence in designing, and selecting drug candidates that have an increase likelihood of becoming successful medicines benefiting patients throughout the world.

Conflict of Interest and Funding

No conflict of interest was reported by the authors.

Acknowledgements

The authors want to thank Dr. J-M Nicolas for his support during the writing of this review.

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Appendix 1: List of Abbreviations

Abbreviation	What it stands for
ADME	absorption, distribution, metabolism and elimination
ADRs	adverse drug reactions
ALP	alkaline phosphatase
ALT	Alanine aminotransferase
AST	Aspartate aminotransferase
BBB	blood-brain-barrier
CiPA	Comprehensive *in vitro* Proarrhythmia Assay
CMs	cardiomyocytes
CNS	Central Nervous System
CSF1R	colony stimulating factor 1 receptor
DCM	dilated cardiomyopathy
DIKI	drug-induced kidney injury
DILI	drug-induced Liver injury
ECAR	extracellular acidification rate
EMA	European Medicines Agency
ETC	electron transport chain
EM	electron microscopy
ENT	equilibrative nucleoside transporter
FDI	Food and Drug Administration
GI	gastrointestinal
HCM	hypertrophic cardiomyopathy
H&E	hematoxylin and eosin
hERG	Human Ether-a-go-go-Related Gene
hiPSCs	human induced pluripotent stem cells
HMGB1	high-mobility group Box 1
ICH	International Conference on Harmonization
KO	knock-out
LC/MS	liquid chromatography/mass spectrometry
LQT	Long QT
miR	microRNA
mtDNA	mitochondrial DNA
MSI	mass spectrometry imaging
NBEs	New Biological Entities
OCR	oxygen consumption rate
OXPHOS	oxidative phosphorylation
PGIs	Potential Genotoxic Impurities
PD	Pharmacodynamics
PK	Pharmacokinetics
QT	Duration of the QT interval of the electrocardiogram
RTCA	Real Time Cell Analyser
SAR	Structure Activity Relationship
TdP	Torsades de Pointes
TSE	Target Safety Evaluation
TK	toxicokinetics
WBA	whole-body autoradiography
XF	extracellular flux
$5HT_{2B}$	5-Hydroxytryptamine receptor 2B