ReproTracker: an animal-free platform for developmental toxicity testing

The realization that chemicals can cross the placenta and inflict irreversible damage to the foetus, triggered concern for human health and made scientists and regulators strive for greater understanding of developmental toxicity to protect future parents and children. A very careful analysis of reproductive and developmental safety of a new drug is an important part of safety assessment during drug development. For pharmaceuticals and chemicals, the ICH S5 and the OECD 414, 421/422 and 443 guidelines, outline dedicated batteries of non-clinical developmental and reproductive toxicological (DART) studies to further assess developmental toxicity before allowing these compounds on the market.

In the DART testing of chemicals and pharmaceuticals, per type of compound, up to 4000-4500 animals on average are used. There is an urgent call from society for animal-free testing while ensuring safety for mother and child at the same time. Standing guidelines allow animal-free alternatives. To date, however, the use of animal-free techniques in registration dossiers is extremely limited.

Over the past decades, several alternative *in vitro* assays have been developed, but these often suffered from low predictability and the inability to provide a mechanistic understanding of developmental toxicity. We developed ReproTracker (Jamalpoor et al., 2022), a human induced pluripotent stem cells (hiPSCs)-based biomarker assay that can identify the teratogenicity potential of new pharmaceuticals and chemicals and signify the outcome of *in vivo* test systems. The assay is based on the differentiation of hiPSCs into functional cardiomyocytes, hepatocytes, and neural rosettes. Proper stem cell differentiation is investigated by morphological profiling and assessment of time-dependent expression patterns of cell-specific biomarkers. In this system, a decrease in the expression of the biomarker genes and morphology disruption of the differentiated cells following compound treatment indicates *in vivo* teratogenicity (Figure 1).

The assay has a number of key advantages compared to other current *in vitro* methods as it combines the functional/morphological evaluation with the power of gene expression analysis to assess the developmental toxicity of chemicals. Moreover, it incorporates multilineage differentiations of hiPSCs into cardiomyocytes, hepatocytes and neural rosettes to ensure the detection of a wide range of chemicals inducing specific teratogenic effects. We are also validating a protocol to differentiate the hiPSCs into osteoblasts (bone cells) as a fourth lineage to ReproTracker, to investigate the induction of skeletal malformations in humans.

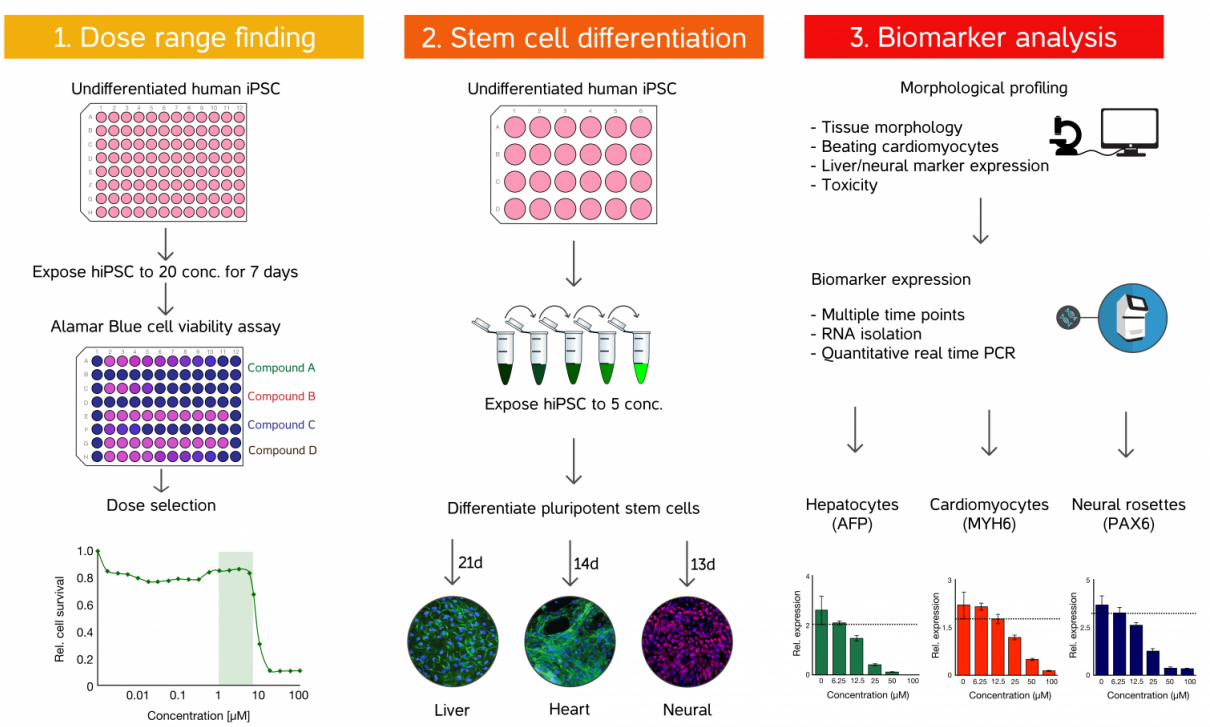

Figure 1. The ReproTracker assay overview. Step 1: *A dose range-finding experiment is performed using undifferentiated hiPSCs to identify optimal concentrations for test article evaluation.* ***Step 2:*** *HiPSCs are differentiated towards cardiomyocytes, hepatocytes, and neural cells in the presence of test article compounds at 5 concentrations, as well as positive and negative control compounds. The cell differentiation process is evaluated by quantifying mRNA expression of selected genes and observing cellular phenotypes. Correctly differentiated cardiomyocytes exhibit beating, hepatocytes show a columnar morphology and neural cells exhibit neural rosette-like structures.* ***Step 3:*** *The teratogenic properties of test articles are evaluated based on biomarker expression patterns and cellular phenotype observations.*

Contribution to the unmet need of human-relevant developmental toxicity testing

It is well known that animals cannot fully reproduce human responses as the effects of chemical exposure may differ between mammalian species, resulting in false predictions and social tragedies such as in the case of thalidomide. Thalidomide is a strong human teratogen where its developmental toxicity cannot be detected in murine-based *in vitro* assays or in mouse models. ReproTracker, however, showed to be very sensitive to thalidomide exposure and correctly captured the teratogenicity nature of thalidomide. Exposure of hiPSCs in ReproTracker to thalidomide led to a significant decrease in expression of the cardiomyocyte-specific marker *MYH6* and liver-specific markers *FOXA2* and *AFP* at clinically relevant plasma concentration ranges (1-6 µM). In a similar fashion, thalidomide markedly declined cardiac contractions and disrupted hepatocytes morphology. Thalidomide had had no effect on morphology nor on expression pattern of the neural rosette-specific biomarker genes (Figure 2).

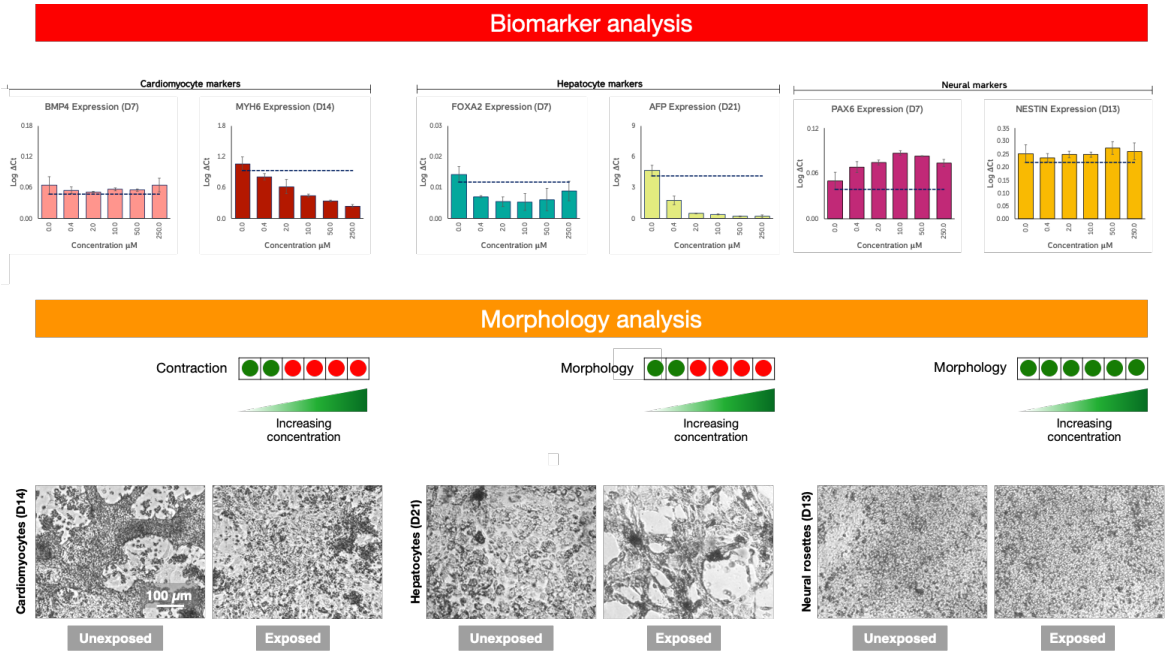

Figure 2. Testing thalidomide, an in vivo teratogen, in ReproTracker. *Gene expression patterns of BMP4 (mesodermal marker), MYH6 (cardiomyocyte-specific marker), FOXA2 (endodermal marker), AFP (hepatocyte-specific marker), PAX6 (neuroectoderm marker), and Nestin (neural rosette-specific marker) upon exposure to thalidomide. Representative bright-field microscopy images of contracting cardiomyocytes and morphology of hepatocytes and neural rosette following thalidomide treatment. Scale bar is 100 µm. The color red indicates the test compound stopped cardiomyocyte beating or disrupted hepatocyte and neural rosette morphology at the end of the differentiation protocol. The color green indicates the test compound had no effect on the cardiomyocyte beating or morphology of hepatocytes and neural rosette at the end of differentiation protocol.*

Proper validation is an essential step for every assay to be qualified in the regulatory spaces. In fact, both the OECD and ICH guidelines provide the possibility of using well validated alternative systems to minimise *in vivo* animal studies. ReproTracker has been extensively validated with various compound libraries (ICH, EURL ECVAM) of well-established teratogenic and non-teratogenic compounds (more than 150 compounds) with different mechanisms of action (Figure 3). ReproTracker identifies the teratogenicity potential of new pharmaceuticals and chemicals with an accuracy of 85% (sensitivity 85%, specificity 84%).

Chemical group	Compound name	CAS number	Therapeutic Cmax (µM)	FDA label	*In vivo* DART	ReproTracker classification	ReproTracker *in vitro* responses		
							Liver	Heart	Neural
Channel modulator	Carbamazepine	298-46-4	50	D			X	X	✓
	Topiramate	97240-79-4	40	C/D			X	✓	✓
	Trimethadione	127-48-0	300	n.d.			✓	✓	✓
Enzyme modulator	Aspirin	50-78-2	40	C/D			✓	X	✓
	Vildagliptin	274901-16-5	4.5	n.d.			✓	✓	✓
	Saxagliptin	361442-04-8	0.08	A			✓	✓	✓
DNA modifier	Cisplatin	15663-27-1	1	D			✓	✓	X
	Busulfan	55-98-1	0.5	D			✓	✓	X
Kinase modulator	Imatinib	152459-95-5	4	D			X	✓	✓
	Pazopanib	444731-52-6	40-110	D			X	X	✓
	Tacrolimus	104987-11-3	6.2-25	C			X	X	X
	Dasatinib	302962-49-8	0.1	D			✓	X	✓
Nucleoside modulator	Methotrexate	0059-5-2	1-5	X			X	✓	X
	Hydroxyurea	0127-7-1	130-680	D			X	✓	✓
Receptor modulator	Bosentan	147536-97-8	2	X			✓	X	✓
	Citrizine	83881-52-1	0.7	B			✓	✓	✓
Anticonvulsant	Valproic acid	99-66-1	400-1400	D			X	X	✓
	Phenytoin (Diphenylhydantoin)	57-41-0	50-80	D			X	✓	X
Transcription modulator	Acitretin	55079-83-9	1.3-2.4	X			X	X	✓
	Vismodegib	879085-55-9	31	D			X	X	X
Others	Thalidomide	50-35-1	2.5	X			X	X	✓
	Tretinoin (all-trans-retinoic acid)	302-79-4	1	D			X	✓	X
	Ribavirin	36791-4-5	3	X			✓	✓	✓

Legend
Teratogen
Non-teratogen

Figure 3. ICH(S5) compound screening results in ReproTracker. *Enough precision to take decision?*

The identifcation of human developmental toxicants is also correlated with their therapeutic plasma concentrations. Hence, for screening teratogens *in vitro*, it is of importance to compare the *in vitro* teratogenic concentration range with their maximum *in vivo* concentration (C_{max}) in human plasma following therapeutic dosing. In figure 4, we have demonstrated that ReproTracker can determine the developmental toxicity of test agents at doses that are relevant for human exposure. This is beneficial and critically important for *in vitro* to *in vivo* extrapolation (IVIVE) and predicting equivalent human administration dose or chemical safety margins.

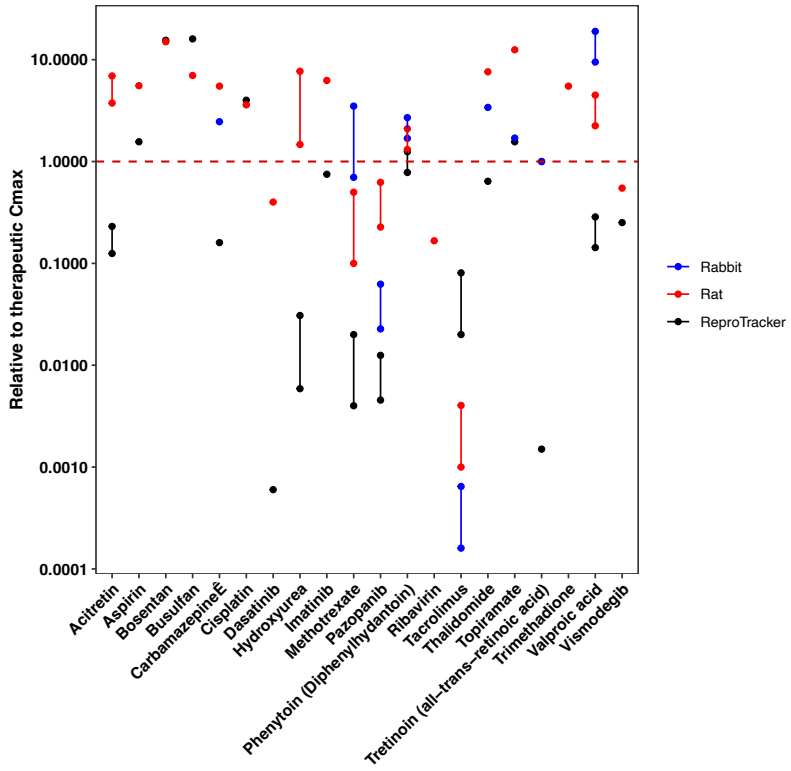

Figure 4. *Correlation between lowest observed adverse effect level (LOAEL) of teratogens in ReproTracker, rat, and rabbit with their therapeutic plasma concentrations (C_{max}). Red horizontal-dotted line represents compound therapeutic C_{max}.* ReproTracker *is more sensitive than rat and rabbit models and determines the developmental toxicity of test agents at doses that are relevant for human exposure.*

Although guidelines and legislations provide the possibility of using alternative systems, there is a lack of clear implementation approach in the guidelines (Fentem et al., 2021). Consequently, in submitted registration dossiers for chemicals and pharmaceuticals the alternative approaches are currently less used. This could be due to 1) lack of experience with *in vitro* safety data among industry and regulator, when applied for regulatory purposes, and/or lack of confidence exists in how well a small set of *in vitro* assays can predict the complexities of the broad spectrum of developmental toxicities that can occur.

Our team at Toxys is committed to providing human health-relevant information while replacing animal testing by demonstrating the usability and applicability of the animal-free approaches and developing a publicly supported values framework and facilitating (regulatory) acceptance of the animal-free approaches. Over the past two years,

ReproTracker has been applied as an early drug safety testing platform by the pharmaceutical, chemical and cosmetics industries. Furthermore, data generated from ReproTracker are utilized by these industries as a weight-of-evidence for registration dossiers required by health authorities.

Moreover, Toxys and the Safety and Environmental Assurance Centre (SEAC) at Unilever started a collaboration to further validate and expand ReproTracker for animal-free developmental toxicity assessment of cosmetic reagents. The intent of the collaboration is to design a teratogenicity strategy in line with the next generation risk assessment (NGRA) framework that integrates new approach methodologies (NAMs) to ensure product safety without generating any animal data (Figure 5) (Rajagopal et al., 2022). Through this collaboration, we aim to further position ReproTracker as a method to improve developmental toxicity testing without the use of animals. All data generated in this collaboration will be soon published in peer-reviewed journals and be available in the public domain. This will result in developing a publicly supported value framework in which the degree of uncertainty is weighed up against the acceptance of the animal-free approaches. Moreover, this will allow to develop a broader normative framework for the responsible implementation of preclinical models in DART and make explicit possible normative presuppositions behind the current practice of (the lack of) using animal-free models in DART studies.

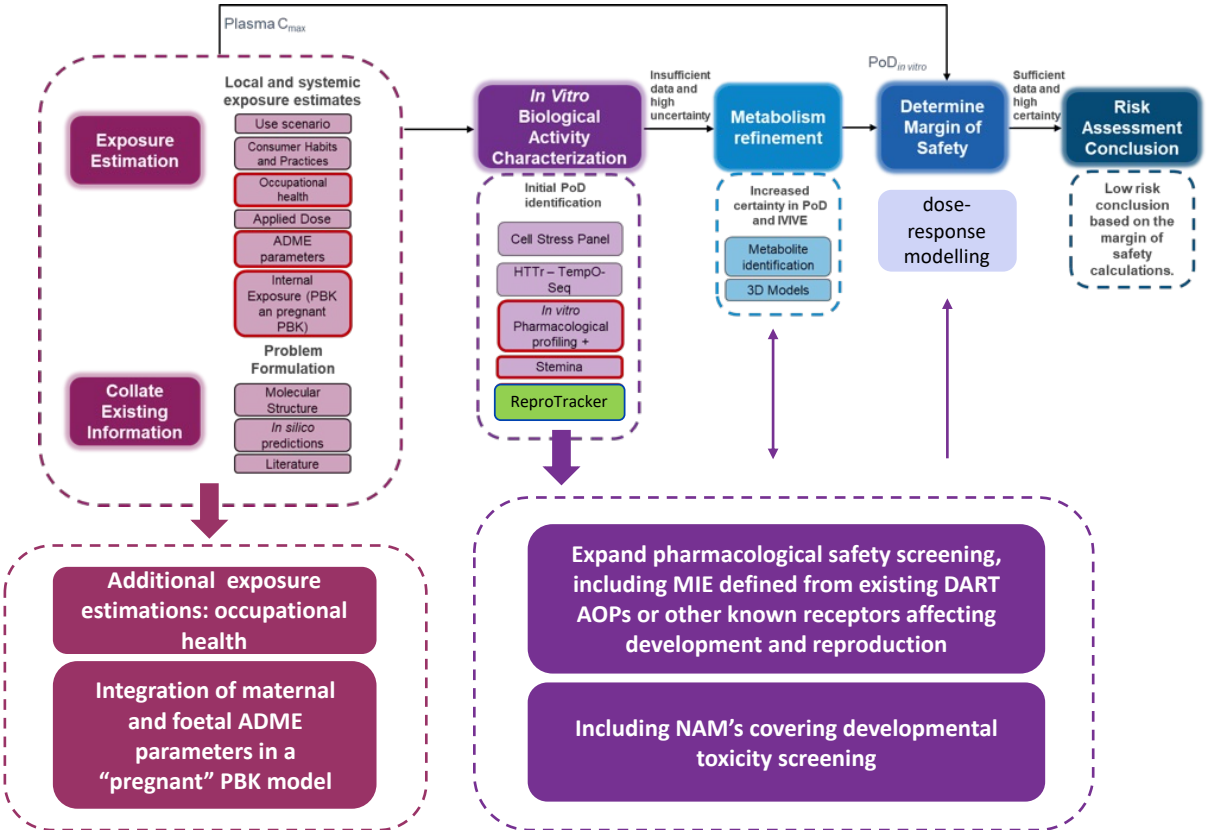

Figure 5. Integrating ReproTracker endpoints into Unilever's NGRA framework.

Yet another example that has greatly improved the implication as well as the application of the ReproTracker assay, is that ReproTracker can also be used as a late phase verification test platform for animal testing outcomes. This is particularly useful when the DART testing outcomes in the two most used preclinical animal species (Rat and Rabbit) are different. In figure 5, we have selected a few compounds where appropriate amount of clinical as well as non-clinical DART data were available. This practice allows us to analyse the outcome, retrospectively. For instance, warfarin is a strong human teratogen (FAD label-X) and its developmental toxicity cannot be detected in murine-based *in vitro* assays. Embryo-fetal developmental toxicity (EFD) testing of warfarin in both rat and rabbits showed a contradictory result, where warfarin was concluded as teratogenic in rats but not in rabbits. Exposure of hiPSCs in ReproTracker to warfarin affected the morphology and gene expression in all three lineage specific cell types at clinically relevant plasma concentration ranges. This demonstrates that ReproTracker utilizes human material (human iPSCs) to recapitulate human exposure, and therefore can be more predictive of chemical responses in humans and resolve the outcome differences in animal testing (Figure 6).

Compound	Therapeutic Cmax (µM)	FDA label	Humans	Rodent	Rabbit	mEST	WEC	True classification	ReproTracker classification
Sitagliptin	1	B				n.d.	n.d.		
Thalidomide	1-6	X							
Warfarin	25	X					n.d.		
Imatinib	2-4	D	n.d.			n.d.	n.d.		
Bosentan	2	X	n.d.			n.d.	n.d.		

Figure 6. ReproTracker as a late phase verification test for animal testing outcomes.

A path towards regulatory awareness and acceptance

Currently, due to the lack of robust alternative methods, *in vivo* animal testing is considered the regulatory gold standard for predicting potential teratogens. ReproTracker (Toxys) addresses 3 key areas aiming to lift this deadlock and aims towards developing a broader normative framework for using animal-free models in the field of toxicology (Figure 7).

1) On a technical level; current *in vitro* developmental testing approaches do not consider exposure of the chemical or drug to the mother and foetus, which hampers adequate extrapolation of *in vitro* findings to relevant clinical dosing scenarios. We are integrating ReproTracker into physiology based kinetic modelling (PKPB) approaches to form a standardised exposure-informed approach to developmental toxicity testing.

2) Demonstrating the usability and applicability of the animal-free approach; despite a myriad of publications based on model compounds, companies still have limited confidence in the predictive value of *in vitro* data for their own specific compound portfolios. Also, regulators have limited experience in evaluating *in vitro* data. Therefore, utilising the developed exposure-informed approach, we are comparing ReproTracker data for “real-life compounds” with available *in vivo* testing data. The outcome of these studies will be discussed in safe-harbour sessions between regulator and industry, leading to a widely supported best practice and a whitepaper promoting the implementation and acceptation of animal-free DART testing.

3) Developing a publicly supported values framework and facilitating (regulatory) acceptance of the animal-free approach; different stakeholder values, including risk to mother and foetus, but also technical challenges, financial consequences, and legal liability, are impacting successful implementation of animal-free techniques. This can be seen from the detrimental experiences in the past (e.g., thalidomide). We are therefore, implementing a broader normative framework on preclinical safety studies in developmental toxicology and chart the stakeholder perceptions on uncertainty and acceptable risk for both pharmaceutical and chemical domains.

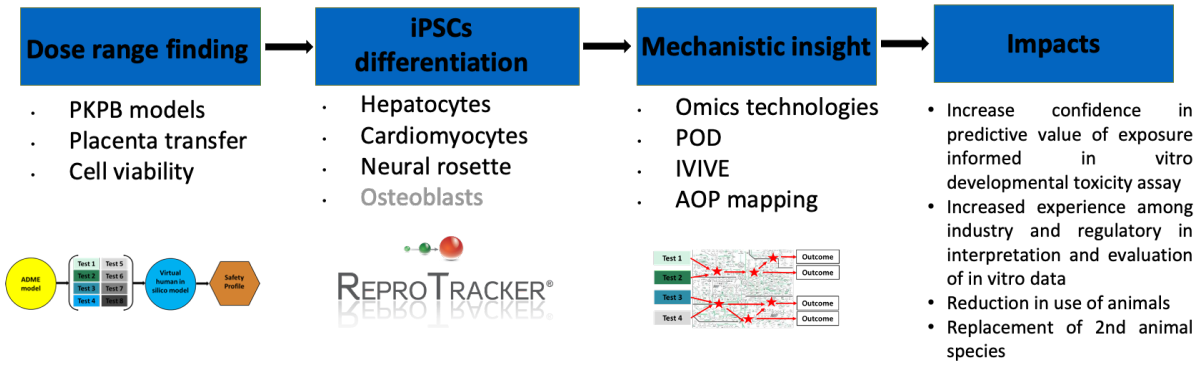

Figure 7. Representative scheme for paving the path towards 3Rs in DART field.

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The relevant ICH S5 (R3) guideline can be found at:

- https://www.ema.europa.eu/en/ich-s5-r3-guideline-reproductive-toxicology-detection-toxicity-reproduction-humanpharmaceuticals

The relevant OECD guidelines can be found at:

- https://www.oecd-ilibrary.org/environment/test-no-414-prenatal-development-toxicity-study_9789264070820-en
- https://www.oecd-ilibrary.org/environment/test-no-421-reproduction-developmental-toxicity-screeningtest_9789264070967-en
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