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The new paradigm in animal testing – "3Rs alternatives"

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A R T I C L E I N F O

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A B S T R A C T

Regulatory studies have revolutionised over time. Today, the focus has shifted from animal toxicity testing to non-animal for regulatory safety testing. This move is in line with the international 3Rs (Replacement, Reduction, and Refinement) principle and has also changed the regulator's perspective. The 3R principle has stimulated changes in policy, regulations, and new approaches to safety assessment in drug development in many countries. The 3Rs approach has led to the discovery and application of new technologies and more human-relevant *in vitro* approaches that minimise the use of animals including non-human primates, in research and improve animal welfare. In 2016, the European Medicines Agency published the Guidelines on the principles of regulatory acceptance of 3Rs testing approaches, followed by a conceptual paper in 2023 to align with current 3R standards. Additionally, the United States Food and Drug Administration passed new legislation in 2023 that no longer requires all new human drugs to be tested on animals, which will change the current testing paradigm. This review paper provides the adoption of the 3Rs and the current regulatory perspective regarding their implementation.

Abbreviations

3D	three-dimension	IATA	Integrated Approaches to Testing and Assessment
3Rs	Replacement, Reduction, and Refinement	ICH	International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use
AI	artificial intelligence	MAD	Mutual Acceptance of Data
ALURES	Animal Use Reporting - EU System	MHRA	Medicines and Healthcare Products Regulatory Agency
AOPs	Adverse Outcome Pathways	MOST	Ministry of Science and Technology
ARRIVE	Animal Research: Reporting of In Vivo Experiments	MyCode	Malaysian Code of Practice
AVS	Animal & Veterinary Services	NACLAR	National Advisory Committee for Laboratory Animal Research
CAAT	Center for Alternatives to Animal Testing	NAM	New Approach Methodologies
CALAS	Chinese Association for Laboratory Animal Science	NC3Rs	National Centre for Replacement and Reduction of Animals in Research
CAM	chick embryo chorioallantoic membrane	NIH	National Institute of Health
C. elegans	Caenorhabditis elegans	OC	organ-on-chip
EC	European Commission	OECD	Organisation for Economic Co-operation and Development
EDA	Experimental Design Assistant	PETA	People for the Ethical Treatment of Animals
EDD	Embryo Development Days	QSAR	Structure-Activity Relationship
EMA	European Medicines Agency	SIT	Skin Irritation Test
FICAM	Finnish Centre for Alternative Methods	U.S. FDA	U.S. Food and Drug Administration
HI	Human Intelligence	WHO	World Health Organisation
IACUC	Institutional Animal Care and Use Committee		

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1. Introduction

Regulatory studies for drug marketing authorisation have evolved significantly over the past decade. Today, the focus has shifted from animal toxicity to non-animal testing for regulatory safety purposes. This move is in line with the international 3Rs (Replacement, Refinement, and Reduction) principle and this has also changed the regulator's perspective. The 3Rs principle has stimulated changes in policy, regulations, and new approaches to safety assessment in drug development in many countries.

For the last decade, pharmaceutical companies have been actively investing and developing more human-relevant *in vitro* models based on current needs and the advancement of technologies. *In silico* modelling is also one of the approaches that characterise toxicity profiles of new medicines in drug discovery, development, and approval. Acceptance of animal toxicology packages with more human-relevant *in vitro* data, artificial intelligence (AI), and *in silico* simulations by the regulatory authorities does promote the principle of 3Rs and has the potential to replace, refine, or reduce animal use in the future if not entirely (Schmeisser et al., 2023). The use of more predictive non-animal approaches (alternative methods) either *in vitro* and/or *in silico* human-relevant may serve as a future replacement for human safety (toxicology) testing.

New Approach Methodologies (NAM) is a widely used term that defines non-animal methods or novel approaches aimed at replacing the use of animals in assessing drug toxicity, thereby providing more accurate and relevant data on drug safety (Ram et al., 2022; Sengupta et al., 2022; Stucki et al., 2022). These methodologies also aim to enhance the speed and efficiency of toxicity testing compared to traditional animal studies. This encompasses various techniques, including the use of body-on-chip devices for testing drugs, which employ 3-dimension (3D) printing to create compartments replicating human organs such as the heart, lungs, kidneys, liver, intestine, and brain (NC3RS - www.nc3rs.org.uk).

Organisation for Economic Co-operation and Development (OECD) is committed to implementing the 3Rs principles by developing guidance documents and tools to apply these principles. The tools include **Quantitative** Structure-Activity Relationship QSAR **models** and Adverse Outcome Pathways (AOPs) as part of Integrated Approaches to Testing and Assessment (IATA). **IATA is an approach that involves multiple information sources, whether from *in vitro*, *in vivo*, *in silico*, or even omics technologies; to assess the safety of pharmaceuticals (Knudsen et al., 2019).**

Global harmonisation of animal toxicity testing within regulatory requirements is critical to avoid any barrier to the application of the 3Rs. The International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH), also played an indispensable role in enhancing 3Rs principles and reducing the use of other drug development resources. **If these methods are validated and accepted by all ICH regulatory authorities, they can replace the current standard methods, which mainly involve animal testing.** Regulatory bodies such as European Medicines Agency (EMA) and **United States (U.S.)** Food and Drug Administration (FDA) has developed guidance documents to cater to non-animal approaches concerning the 3Rs.

Animal testing is widely used to predict the toxicities and safety of substances in humans, and it is also often required as part of regulatory requirements for product licensing. Animal studies were commonly performed by the pharmaceutical industry as part of their research and development to identify potential safety issues with new drugs, **compounds**, and other substances before they are marketed to protect mankind. Although the chemical industry also uses animal studies to evaluate the safety of new chemicals, pesticides, and other products, this review only focuses on the pharmaceutical industry.

This review paper provides the adoption of the 3Rs and the current regulatory perspective regarding their implementation. It also examines the extent to which the 3Rs are applied in Asia and Western countries, **including European Union countries**. More consistent, efficient, rapid, reliable, and translatable non-animal models are required to fulfil the regulatory needs knowing that this will be the way forward in the next phase of pharmaceutical regulation.

Furthermore, the U.S. is the first-ever country that recently passed a bill for removing the animal testing requirement for new drugs, this has given the green light to the receiving authority to consider an alternative testing method that is more predictive of human responses and scientifically valid than using whole animals (Wadman, 2023). As a leading authority in drug regulation, the U.S. has set a new standard that has, precipitated a paradigm shift (Cosmetics Testing on Animals: EU Ban, 2011) in global drug development.

It is noteworthy that animal testing for cosmetics and cosmetics ingredients is banned in several regions around the world. Animal testing for the consumer safety of cosmetics and their ingredients was banned in the United Kingdom (U.K.) in 1998 (Cosmetics Testing on Animals: EU Ban, 2011). Several other countries, including Canada, India, Israel, New Zealand, Norway, and Switzerland have also implemented bans or restrictions on animal testing for cosmetics (Cosmetics animal testing FAQ, 2024). These bans vary in scope but generally aim to promote the use of alternative testing methods. Nonetheless, the U.S. has no federal ban on animal testing for cosmetics (Cruelty Free International, 2019). However, many cosmetics companies have voluntarily phased out animal testing in response to consumer demand for cruelty-free products. There are also states such as California and nine other states that have implemented bans on the sale of cosmetics tested on animals (CA 2023 Luxury Law Update: First State to Ban Animal Testing and Furs but Loses Pre-emption battle On Alligator/Crocodile Ban, 2023).

Animal testing has been there for over a century, with some of the earliest animal experiments documented dating back to ancient Greece and Rome. However, the modern use of animal testing in scientific research only began around the 19th century during the development of vaccines for rabies and anthrax by the famous French chemist Louis Pasteur (Cavaillon and Legout, 2022). Since then, numerous animal testing have been conducted and become a routine part of scientific research and drug development although they are not mandatory.

Animal toxicity testing became mandatory in the U.S. because of the 1938 U.S. Federal Food, Drug, and Cosmetic Act (FDA, 1938). This law was enacted in response to a public health crisis caused by contaminated medicines, which led to widespread illness and death. In 1937, a mass poisoning incident occurred in the United States that became known as the 'Elixir Sulfanilamide disaster' (Wax, 1995). The newly developed elixir Sulfanilamide was an antibiotic indicated for streptococcal infections. The sulfa antibiotic was not properly tested for safety and hence resulted in one hundred and seven deaths both in adults and children (Greek et al., 2012). The drug was later found to contain diethylene glycol, a toxic solvent shown to be toxic to humans and can cause serious fatal complications (Schep et al., 2009).

The Nuremberg Code was established in 1947, after World War II. It comprised ten principles for ethical research involving human subjects. Later in 1964, the Helsinki Declaration was developed by the World Medical Association, and it includes similar principles to the Nuremberg Code to safeguard human and ethical principles (Ethical Principles and Guidelines for the Protection of Human Subjects of Research, 1979). Nuremberg Code and the Declaration of Helsinki both stipulate that animal-based research should precede human experimentation (Greek et al., 2012).

Regulatory agencies in most countries require animal toxicity testing, also known as preclinical testing, as part of the drug registration process. The preclinical testing data is required for the agencies to evaluate the safety of the products and to protect public health. However, there has been growing interest in developing and implementing alternative testing methods that are more predictive of human responses and do not involve the use of animals. In recent years, regulatory

agencies have been working to reduce and replace animal testing with alternative methods wherever possible, following the principles of the 3Rs of animal testing.

In the United States, following the passing of the FDA Modernization Act 2.0 in January 2023, the FDA abolished animal testing before advancing to human clinical trials in the U.S. (Han, 2023; S.5002 - FDA Modernization Act 2.0 S-5002 117th Cong. Rec, 2022). FDA can now approve drugs that have not been tested in animals. Instead of animal studies, pharmaceutical companies are encouraged to use more relevant and predictive alternative testing methods such as *in vitro* testing and *in silico*, where appropriate to demonstrate the safety of the product before proceeding to clinical trials. The alternative testing method for any regulatory submission shall be scientifically validated and shown to be reliable in predicting safety and efficacy in humans.

2. 3Rs principles

The 3Rs principles are no longer something new. They were first proposed in 1959 by British scientists William Russell and Rex Burch in their book (Russell and Burch, 1959).

After it was first introduced, it has become quite popular and widely accepted in the scientific community but at a slower pace as the extent to which they are implemented can vary in regulatory settings between countries, institutions, and individual researchers (Scholz et al., 2013).

While the 3Rs Principles are not necessarily mandatory in all countries, many countries and organisations have incorporated them into their laws, regulations, and guidelines for animal research. The U.K. was one of the first to pass legislation to regulate the use of animals in scientific research, the Cruelty to Animals Act 1876 ("Cruelty to Animals Act," 1876).

On the other hand, the European Union (EU) has established the "Three Rs Centre" and has implemented regulations requiring the use of alternative methods to animal testing where possible, along with requirements for the ethical use and care of animals in research (Knudsen et al., 2019). There are 3Rs Centres in Denmark and Sweden. Norway has NORECOPA, Norwegian Consensus-Platform for Alternatives, while Finland has the Finnish Centre for Alternative Methods (FICAM). Additionally, the U.K. has National Centre for Replacement and Reduction of Animals in Research (NC3Rs), among many others. These centres are established to promote best practices in the use of experimental animals (Knudsen et al., 2019).

There has also been a growing movement to promote the use of the 3Rs Principles in animal research. Organisations such as the NC3Rs (www.nc3rs.org.uk) in the U.K., the John Hopkins Center for Alternatives to Animal Testing (CAAT) in the U.S., OECD are dedicated to advancing the use of alternative methods to animal testing and promoting the implementation of the 3Rs Principles in scientific research (Taylor and Alvarez, 2019).

In Europe, the European Parliament passed Directive 2010/63 on the protection of animals used for scientific purposes (Directive 2010/63/EU of the European Parliament and of the council of 22 September 2010 on the protection of animals used for scientific purposes, 2010). This Directive, adopted in 2010, replaced a previous Directive from 1986 and introduced new requirements for animal use in research, including a focus on the principles of the 3Rs (Replacement, Refinement, and Reduction). The passing of the Directive has had a significant impact on scientific research, particularly in studies involving animals. This has increased and harmonised animal welfare standards throughout Europe, ultimately leading to high-quality science.

It is noteworthy how the concept of the 3Rs has expanded, particularly in Europe. European Union Reference Laboratory for alternatives to animal testing (EURL ECVAM) was incorporated into EU legislation on the protection of animals used for scientific purposes to enhance collaboration and promote innovative non-animal approaches, thereby facilitating the use of non-animal methods in testing and research (European Commission, 2024). EURL ECVAM facilitates the validation of test methods by applying good practices and developing new methods through several validation projects, including EU-funded, international, and Joint Research Centre (JRC) projects. To promote the use of organ-on-chip devices for regulatory applications, EURL ECVAM created a catalogue of resources for developers and end-users to support the validation and qualification of these new technologies (European Union, 2024a). Similarly, the European Union Network of Laboratories for the Validation of Alternative Methods (EU-NETVAL) supports validation studies to assess the reliability and relevance of alternative methods that could replace, reduce, and refine the use of animals in scientific research, following Directive 2010/63/EU (Directive 2010/63/EU of the European Parliament and of the council of 22 September 2010 on the protection of animals used for scientific purposes, 2010). Given that most companies operate globally, achieving worldwide regulatory acceptance is crucial for the implementation of new methods. Thus, it is an international effort to expedite and standardise these validations globally. Notable work under this network includes the validation of methods for assessing thyroid hormone disruption and the Validation of a Transactivation assay for detecting (anti) androgenic activity of compounds ("European Union Network of Laboratories for the Validation of Alternative Methods (EU-NETVAL)," 2023). This joint effort by various countries aims to develop safer practices to improve public health.

Conversely, the International Cooperation on Alternative Test Methods (ICATM) is a collaborative effort involving countries such as Japan, Canada, South Korea, Brazil, China, and the U.K., along with governmental organisations like EURL ECVAM and Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM). ICATM plays a crucial role in fostering international cooperation in validation studies and facilitating independent peer review of test method validations. Through this collaboration, ICATM ensures the optimal design and conduct of validation studies, supporting national and international regulatory decisions regarding the utility and limitations of alternative methods proposed for regulatory testing. Harmonising alternative test methods for regulatory purposes is essential for drug companies to get their regulatory submissions internationally accepted. ("International Cooperation on Alternative Test Methods (ICATM)," 2024). These organisations are essential in validating, promoting, and achieving regulatory acceptance of alternative methods, making the 3Rs an internationally recognised and widely accepted approach ("European Union Network of Laboratories for the Validation of Alternative Methods (EU-NETVAL)," 2023; (European Union, 2024b).

ICCVAM operates within the framework of the National Toxicology Program's Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM), which has a strategic roadmap aimed at encouraging U.S. federal agencies and stakeholders to adopt new approaches to safety and risk assessment of chemicals and medical products, to enhance public health and safety ("A Strategic Roadmap for Establishing New Approaches to Evaluate the Safety of Chemicals and Medical Products in the United States," 2018). ICCVAM's mission is to develop and validate New Approach Methodologies (NAMs). The implementation plan includes four key elements: (1) defining testing needs, (2) identifying available alternative tests and computational models, (3) developing integrated approaches to testing and assessment, and (4) addressing both scientific and regulatory challenges, including international harmonisation. The roadmap specifically targets acute systemic toxicity, eye and skin irritation, and skin sensitisation ("A Strategic Roadmap for Establishing New Approaches to Evaluate the Safety of Chemicals and Medical Products in the United States," 2018). ICCVAM ensures that new and revised test methods are validated to meet the needs of U.S. federal agencies, while also promoting 3R wherever feasible (National Toxicology Program U.S. Department of Health and Human Services, 2024).

Countries like the U.S. have made the move by implementing new laws that allow alternative methods (non-animal tests) for

drug submission while not completely prohibiting animal testing. This certainly can reduce the use of animals for preclinical studies (animal studies) significantly (Han, 2023).

Passing the bill could signify better animal welfare, a more ethical way of handling animals, and reduce the number of animals used in animal experimentation, **especially in non-human primates which frequently relate to animal cruelty (Padrell et al., 2021).** The bill is also supported by the aspiration to develop more accurate and relevant *in vitro* models based on human biology, which can provide improved safety and toxicity data for human use, rather than relying on animal testing (Han, 2023). Animal testing for regulatory purposes can cost millions of dollars, and yet the results are difficult to extrapolate to humans as animals may react differently to drugs or other interventions due to physiological differences in men (Van Norman, 2019). Due to pressing reasons, the bill was passed in December 2022.

The 3Rs Principles have undoubtedly made significant contributions to the field of animal testing and research for the past decades. Without these guiding principles, the industry would not have progressed to develop the array of sophisticated and human-relevant tools, such as tissue engineering, *in silico,* and *in vitro* techniques. These alternatives offer greater relevance to human biology, yielding data with higher precision, as animal data can sometimes be incongruent with human response (**Ram, 2019**). By adhering to the 3Rs, researchers can obtain quality data while safeguarding ethical standards. Moreover, the adoption of the 3Rs helps mitigate the substantial cost, time, and labour associated with traditional animal testing methods (Hubrecht and Carter, 2019)

The 3Rs must adapt and enhance to meet the needs of the contemporary world, utilising remarkable advancements. The goal is for the 3Rs approach to not only keep pace with the modern world but also to transcend it, rendering it more pertinent and irresistible for scientists to adopt. Table 1 presents a modern strategy that has been formulated by the NC3Rs to implement all three components of the 3Rs. These principles not only have led to improvement in animal welfare but also enhanced the quality of the preclinical data to be submitted for regulatory submission.

2.1. Replacement

Replacement in the 3Rs approach aims to find alternative methods to animal testing. This can be achieved by using *in vitro* or *in silico* that simulate human physiology, using human tissues or cells in experiments, or using non-mammal (i.e. *Drosophila melanogaster*, *Caenorhabditis elegans (C. elegan*) species that can provide relevant data.

To achieve effective progress in replacement, it is necessary for scientists who possess a deep understanding of their field to take the lead and evaluate the appropriateness of replacement alternatives. To date, cell-based assays such as complex cell cultures, including stem cell technologies, 3D tissue engineering (e.g. micro physiological systems), organ-on-chip, and computational methods are some of the promising alternative methods to predict toxicities in conjunction with conventional ways.

The use of animals as nonclinical safety study models to support regulatory requirements raises concerns regarding their specificity and validity. The lack of specificity can result in unnecessary testing during drug development. On the other hand, false positive findings from less specific animal models could prevent a safe and effective compound from progressing to the next stage of drug development (Moreno et al., 2019).

Some important replacement strategies that need to be considered are the standardisation of testing requirements, promoting the sharing of data, and ensuring that new regulations do not mandate animal-based testing when *in vitro* methods are adequate (Richmond, 2002).

Table 1
Comparison between Russell & Burch's conventional 3Rs definition with contemporary approaches to promote scientific advancement.

	Conventional definitions	Contemporary approaches
Replacement	Substitution for conscious living animals of insentient material	Accelerating the development and use of tools relevant to the target species (usually humans) based on the latest technologies
Reduction	Reduction in the number of animals used to obtain information of a given amount and precision	Using appropriately designed and considered animal experiments that are robust and reproducible
Refinement	Decrease in the incidence or severity of inhumane procedures applied to those animals which still have to be used	Using new *in vivo* technologies that can have positive impacts on animal well-being and scientific research, which include techniques to reduce pain and suffering, and also to improve animal care, housing, handling, training, and utilisation

Adopted from National Centre for the 3Rs ("3R," 2023)

2.2. Refinement

This principle involves improving the welfare of animals used in experiments. This can be achieved by using less invasive procedures, minimising pain and distress during experiments, and providing appropriate housing and care for the animals, for example, environmental enrichment. Utilisation of suitable anaesthetics and analgesics is an example of refinement (EMA, 2016).

There is a growing recognition that abnormally stressed animals make a poor experimental model, especially when the animals are not in their homoeostatic condition (e.g. temperature, humidity, lighting, ventilation, and noise) (MacArthur Clark, 2018). When an animal is stressed, it may exhibit abnormal repetitive behaviours (stereotypical, compulsive behaviour) and result in a variety of behavioural changes that affect the experimental outcome (Morris et al., 2011). As a result, it can affect the validity and reliability of the data as well as reproducibility between different laboratories (Lewis, 2019; MacArthur Clark, 2018).

In 1959, Russell and Burch in their book mentioned that the "best animal welfare" results in the "best science" (Russell and Burch, 1959). Therefore, besides ethical obligation being crucial in enhancing animal welfare, the scientific reasoning behind refinement is compelling. It is also important to keep in mind that animal welfare is compromised not just by causing unpleasant experiences but also by depriving them of pleasure experience. There is a growing consideration of increasing the animal quality of life through environmental enrichment. Several studies have demonstrated that incorporating enrichment minimises stress and abnormal behaviour in laboratory animals such as rats, guinea pigs, rabbits, and dogs. A study published by Loisy et al. conducted a 3-week mice study in an enriched environment with a running wheel, toys, and tunnels showed that a study using an enriched environment is important to provide valid and replicable data (Loisy et al., 2023). Similarly, another study by Slater et al. also used a toy, a running wheel as their enrichment tool. Thus, before initiating the study, a scientist may design and consider environmental enrichment that best suits their study for reliable data and quality of research (Slater and Cao, 2015).

In the past, it was believed that studies should be conducted in a standard or uniform manner to reduce variability. However, it is now increasingly evident that this is not the case as every animal may be different and may require different needs. Animals are a heterogeneous population just like humans and not merely a research tool. Various factors such as housing, environment, handling, diet, and gut microbiome can significantly affect the obtained data and its reproducibility (Lewis, 2019). The validity of animal studies can be increased by adopting many practices in clinical research namely randomisation and blinding studies to make the experimental design robust and minimise bias (Scott et al., 2008).

2.3. Reduction

The reduction in 3Rs involves minimising the number of animals used in experiments. This can be achieved by using statistical methods to design studies that require fewer animals (combined studies), harmonisation of testing requirements, sharing data (OCED MAD) (Mutual Acceptance of Data) and resources among researchers to avoid any unnecessary duplication of experiments, and employing more efficient experiment techniques that can produce reliable results with fewer animals (EMA, 2018).

It is almost impossible to get the number of animals used for scientific and medical research worldwide as many countries do not provide detailed statistics. Therefore, the figure here is just an estimation. Taylor et al. in their research article state that nearly 200 million animals were used in medical research worldwide with China being the highest user of experimental animals (20.5 million), followed by Japan and the U.S., 15 million and 14.6 million respectively. In European countries, for example, the U.K., Germany, and France, the use of animals was estimated at around 2 million each (Taylor and Alvarez, 2019).

According to another source by Animal Use Reporting – EU System EU Statistics Database On The Use of Animals for Scientific Purposes Under Directive 2010/63/EU (ALURES), in 2020, 7,938,064 animals were used for research purposes in the EU (including Norway), while in the U.S, it was estimated that 12–24 million animals were used for research purposes (European Union, 2020). In Israel, 402,412 animals were used for research purposes. In Canada, 5,067,778 animals were used, and in Switzerland, 556,107 animals were used in 2020. In South Korea, 4,141,433 animals were used for research purposes, and in New Zealand, 245,522 animals were manipulated. Approximately 10.7 million animals were used for research purposes in Australia in 2018 (Understanding Animal Research, 2023)("Number of animal used," 2023). However, there were no statistics on the number of animals used for research from Asian countries.

People for the Ethical Treatment of Animals (PETA) of the U.S., one of the largest animal rights organisations globally, reported that more than 110 million animals are used each year in the U.S. alone. The figures imply that animals are used not only for medical research but also for other purposes such as learning purposes, testing, experimentation, and training not solely for medical research purposes ("Facts and Statistics About Animal Testing," 2023).

It is interesting to note that according to the Annual Statistics of Scientific Procedures on Living Animals in Great Britain for 2021, 3.06 million scientific procedures involving living animals were carried out in Great Britain in 2021 with an increase of 6% on last year. There was an 8% decrease in procedures for creation and breeding and a 20% increase in experimental procedures. Mice, fish, birds, and rats, the most used species for more than a decade accounting for 96% of all procedures including both experimental and breeding purposes (GOV.UK, 2022).

While the number of scientific procedures on living animals in Great Britain increased by 6% to 3.06 million in 2021 compared to 2.88 million in 2020, it is also worth noting that there was a 15% reduction from the previous year, the lowest number of scientific procedures since 2004.

The results of most preclinical studies conducted before clinical trials are not translatable into human trials (Butler, 2008; Hackam and Redelmeier, 2006). A review conducted by Baker has raised significant concerns regarding the reproducibility issue in recent years. According to the survey, the majority of the over 1500 scientists who participated (90%), believe that the issue of reproducibility was a significant or slight crisis (Baker, 2016). Hunter claimed in an article that a reproducibility crisis occurs when the level of reproducibility falls below 50% (Hunter, 2017). Several reviews mentioned that the lack of reproducibility was due to a lack of quality raw data (Drucker, 2016; Miyakawa, 2020; Peng, 2015). In short, poor data is a contributing factor to the reproducibility crisis. This poses an ethical dilemma for the scientific community worldwide, particularly in cases where animals are involved in research.

Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines were developed to enhance the validity, quality, transparency, and reproducibility of animal experimental studies (Bayne and Turner, 2019; Kilkenny et al., 2010). The NC3Rs has created an online tool, known as the Experimental Design Assistant (EDA – https://eda.nc3rs.org.uk/), to assist scientists in designing and reporting their experiments. EDA is a tool provided by NC3Rs that includes a web application that assists in creating a robust study design that results in reliable and reproducible research outcomes (MacArthur Clark, 2018).

3. Alternatives to animal testing

The debate over toxicology testing in animals and preclinical animal studies in drug development has been ongoing for years and has intensified due to growing concerns over the poor prediction of in-human results. The 3Rs approach aims to minimise harm to individual animals and reduce the total numbers while obtaining reliable results. Ideally, the use of animals should be replaced altogether and that is where ‘Alternatives’ comes into the picture. ‘Alternatives’ can be divided into key categories, either completely replace animals (full replacement) or utilise animal tissues only (partial replacement).

It is important to remember to achieve a balance in utilising the 3Rs approach, without compromising the production of reliable and reproducible results. Some alternatives to animal testing, including human-relevant *in vitro* models including micro physiological systems, chick embryos (*in ovo*), *in silico*, and organ-on-chips are discussed in this review as in Fig. 1. These methods were chosen based on their prominence in contemporary research, their potential to replace or reduce animal usage, and their applicability across various scientific disciplines.

3.1. In vitro cell lines

The utilisation of models on human cells, tissues, or organs *in vitro* testing has the potential to enhance precision in identifying drugs that can cause adverse effects in humans. *In vitro* models primarily rely on two types of cell cultures: adherent monolayers and suspension cells. 3D organoid and micro physiological systems are two emerging technologies and improved cell culture models that serve as a cell culture platform to model the human-specific physiology of tissues or organs to improve the efficiency of drug development (Bai and Wang, 2020).

More precise tumour environment representation can be achieved by using advanced models such as 3D culture systems (Fontana et al., 2021a). **These 3D culture models, utilised in cancer studies to stimulate the growth and invasion of tumours *in vitro*, can be created by culturing cells in 3D spheroids.** In cancer research, cell-creating cell spheroids using cell culture techniques to simulate *in vitro* tumour growth and invasion is a trend, as it allows researchers to study the behaviour of cancer cells in a more controlled and reproducible environment. Cell spheroids are three-dimensional (3D) structures that mimic the complexity of tumours *in vivo* and they can be used to test new drugs or study the mechanisms of cancer cell invasion and metastasis (Katt et al., 2016). Solid tumours are heterogeneous, containing mutated cells as well as other different cell types. While 3D models using only cancer cells cannot fully replicate the genetic heterogeneity in the tumours, heterotypic multi-cellular models can partially replicate the cellular diversity observed in tumours (Proietto et al., 2023; Rodrigues et al., 2018; Thoma et al., 2014). The 3Rs facilitate the process of drug development and help realise the promise of advancing precision cancer medicines (Jean-Quartier et al., 2018; Katt et al., 2016; Thoma et al., 2014; Wang et al., 2023). Although 3D organoids can recapitulate key aspects of organ structure and function, they are valuable tools for investigating disease mechanisms and testing potential therapeutics. However, their limitations may include heterogeneity in organoid cultures and variability in differentiation efficiency. Additionally, the relevance of organoid results to human physiology may be influenced by factors such as tissue complexity and microenvironmental.

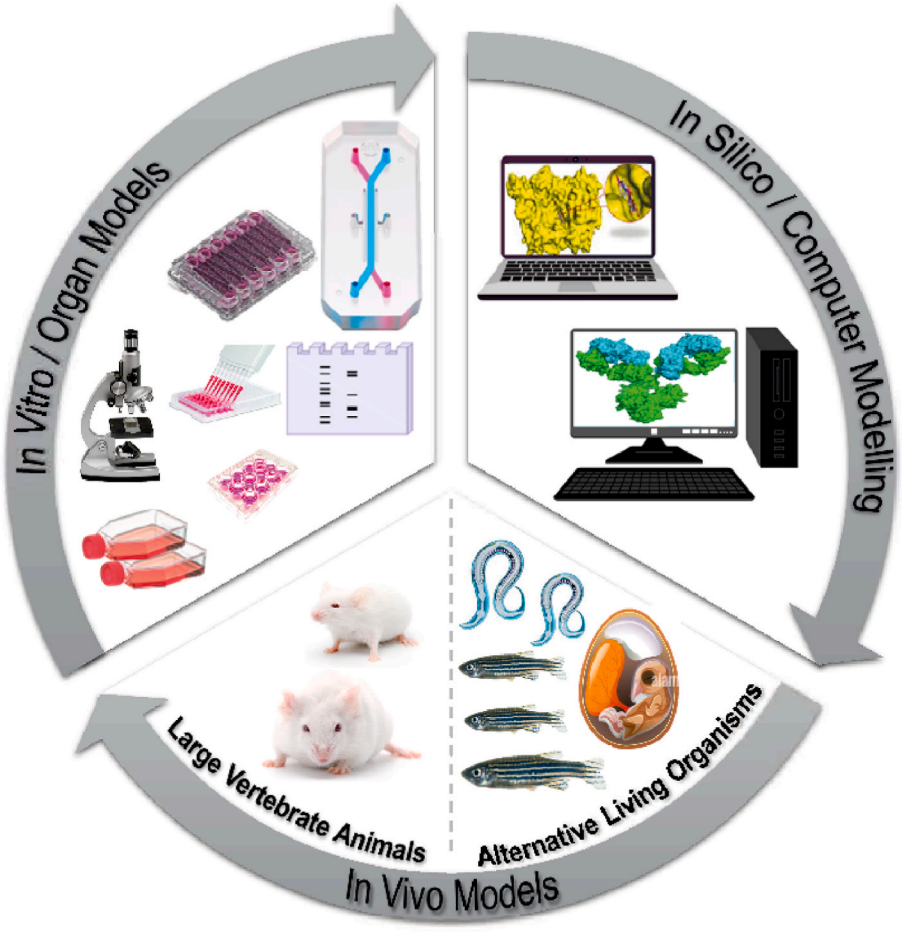

Fig. 1. Schematic illustration of drug development involving various models and techniques. Cells, organ models, alternative living organisms instead of larger vertebrate animals together with *in silico* and other *in silico* should be emphasised in 3Rs.

3.2. Invertebrates

Current scientific understanding suggests that certain invertebrates may lack the ability to experience pain, thus making them a potential partial replacement in the 3Rs framework. *Drosophila*, nematode worms, and social amoebae are invertebrates that are beginning to receive attention for their potential use in the laboratory for research purposes (NC3Rs – www.nc3rs.org.uk).

Caenorhabditis elegans a microscopic, non-pathogenic roundworm are slowly being recognised for their usefulness for regulatory toxicity tests. *C. elegans* possess specialised cells and tissues that function in ways that are similar to those of vertebrates, which means that they can help predict toxicity. Recently, the U.S. FDA has been evaluating previously developed *C. elegans* toxicity assays to assess their ability to produce correlative responses to developmental and reproductive toxins. **Hunt *et al.*, in their research paper, introduced a novel worm Development and Activity Test (wDAT) using *C. elegans*, which showed promise in predicting the developmental neurotoxicity (DNT) caused by heavy metals known to be neurotoxic to human development and associated with hyperactivity in children (Hunt et al., 2018). Some aspects of reproduction in *C. elegans* are conserved with humans, and these findings are consistent with several existing mammalian studies (Hunt et al., 2018), suggesting the potential to complement current *in vivo* research.**

The FDA continues to enhance their skills and experience in developing and implementing accurate and effective new technologies into the regulatory review process, helping to prioritise useful compounds while simultaneously reducing the need for extensive animal testing, thereby saving time and money ("The C. elegans Model in Toxicity Testing," 2022). Invertebrate models are known for their short generation time, and ease of genetic manipulation, while conserving the biological pathways. Despite its advantage, researchers may have to consider the variation in experiment conditions, environmental factors may affect reproducibility and interpretation of results across studies.

3.3. In silico

Drug discovery involves multidisciplinary stages, such as identifying and validating targets, discovering and optimising lead compounds, and conducting preclinical and clinical trials before the drug reaches the market (Shaker et al., 2021). This can be a costly and time-consuming endeavour. The process from promising drug candidates to submission to the FDA takes more than 9.5 years (114 months) on average and costs approximately 2.8 billion (Chang et al., 2022). However, computer-aided drug discovery (CADD) approaches, using *in silico* methods, can eliminate inefficient and toxic chemical compounds at an early stage, thereby saving significant amounts of money on less favourable drugs (Chang et al., 2022; Rognan, 2017; Shaker et al., 2021). Pharmaceutical companies favour of 'Failing early, Failing cheap' concept. Early predictions can significantly reduce costs by identifying potential issues before advancing to clinical trials (Loiodice et al., 2019; Van Norman, 2020). The term *in silico* or computational modelling pertains to conducting experiments or parts of experiments using *in silico* or simulations involving complex data sets (MacArthur Clark, 2018). Fig. 2 shows a

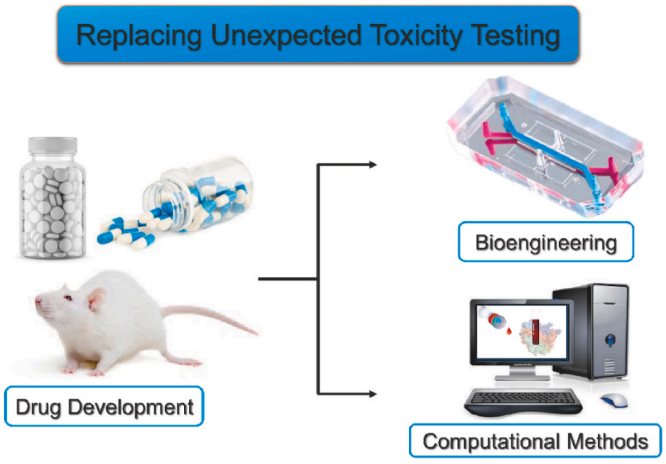

Fig. 2. Unexpected toxicity testing. Various technological advances allow the replacement of animal tests but are not limited to bioengineered organs and computational methods.

simple illustration of the combination of bioengineered organs and computational methods in 3Rs drug development for testing unexpected toxicity. The use of computer systems to support "Go" or "No-Go" drug development decisions has become increasingly popular. These systems are being used for predictive analysis, mechanism analysis of toxicities, and risk assessment with the help of comprehensive databases. The concept of Integrated Approaches to Testing and Assessment (IATA), developed by OECD member countries, employs high-content screening and high-throughput screening methods to generate and interpret data through the use of Adverse Outcome Pathways (Fontana et al., 2021b). Additionally, the OECD has issued Guidance on Reporting Defined Approaches Within IATA. This document aims to harmonise the reporting of various IATA components, such as Quantitative Structure-Activity Relationship (QSARs), chemical grouping, read-across strategies, and non-guideline *in vitro* methods ("Integrated Approaches to Testing and Assessment (IATA),").

QSAR, a computational methodology serving as a 3Rs alternative to animal testing, was pioneered by Corwin Hansch and his group as early as 1960. **Initially conceived as 2D QSAR, this method used graph theory derived relationship, with the affinity correlated to 2D pharmacophore of molecules. The concept then evolved into 3D QSAR, which incorporates spatial geometry to correlate affinity with the 3D structure of ligands. Recently, the methodology has been further advanced to include 4D QSAR, which represents ligands as an ensemble of configurations, taking into account conformation, orientation, and protonation state (Debnath, 2001; Lill, 2007; Roy et al., 2005).** QSAR serves dual purposes, initially devised for early toxicity profiling, and also recognised for its efficacy in virtual compound screening. Notably, QSAR models have gained acknowledgement from regulatory agencies as an alternative to animal testing. The QSAR Assessment Framework is outlined in the Series on Testing and Assessment No. 386 of the OECD (2013). While QSAR toolbox is a software tool developed by the OECD to facilitate the use of QSAR models in chemical hazard assessment and regulatory decision-making ("Integrated Approaches to Testing and Assessment (IATA),"; Mansouri et al., 2024). An example of QSAR utilised in regulatory decision-making is the case of nintedanib, a kinase inhibitor. QSAR analysis was employed to evaluate the impurities of nintedanib, assisting reviewers in assessing the primary pharmacology and toxicology data to support its drug approval (U.S. Food Drug and Administration, 2014)("Application Number: 205832Orig1s000 Summary Review (nintedanib)," 2014). Another example is tofacitinib citrate, a selective inhibitor of Janus kinases used in combination with methotrexate to treat active rheumatoid arthritis. QSAR predictions for the potential genotoxicity of impurities were considered reliable, as they were found to be consistent with results from two complementary *in silico* (DEREK and SARAH) systems (European Medicines Agency, 2017). Furthermore, the integration of clustering approaches, QSAR models, and *in vitro* constitute powerful New Approach Methodologies (Sewell et al., 2024). This integrated approach significantly reduces reliance on animal testing by directing focus toward the most promising molecules.

Molecular docking plays a crucial role in both academic and industrial drug screening and drug discovery processes, serving as an indispensable tool for high-throughput virtual drug screening, thereby contributing significantly to the advancement of pharmaceutical research (Zhang et al., 2022). While there are numerous docking software available, ranging from freely accessible to subscription-based platforms. Auto Dock, MEGADOCK 4.0, LeDock, and rDOCK are among the free software that are available online (Kamal and Chakrabarti, 2023). Moreover, the accessibility of enriched compound libraries has supported computational screening methods and fostered increased accuracy and effectiveness while facilitating data sharing among researchers. In essence, docking studies are computational simulations that forecast the interactions between small molecules (e.g. drugs) and larger biomolecules such as proteins or enzymes, enabling the prediction of molecular interactions within protein-ligand complexes based on their 3D structures (Huang et al., 2017, 2021).

Significant concerns have been raised about the poor specificity of *in silico* models, primarily because they lack the complexity of physiological systems or, are not closely related to human biology. Baheti et al., noted that while computational tools can predict the interaction between ligands and targets, their lack of specificity results in a low hit rate for high-throughput screening (HTS) (Baheti et al., 2021). According to Lloyd, the typical hit rate for an HTS assay ranges between 0.5 and 1 percent, and for more challenging targets, this rate can drop to below 0.1 percent (Lloyd, 2020).

In 2012, University of California, San Francisco researchers created an *in silico* that successfully predicted the side effects of 656 marketed drugs by comparing the chemical structures of these drugs to other molecules with known side effects, demonstrating its promise for early drug development (Lounkine et al., 2012; Van Norman, 2020). Initially, 151 drugs were linked to specific side effects. However, using in silico screening, researchers found that 26% (39 out of 151) of these drugs were not linked to the previously known side effects. This suggests that these 39 drugs might have therapeutic effects that were previously unrecognised, potentially allowing them to be repurposed for treating other diseases or conditions (Lounkine et al., 2012; Van Norman, 2020). While the findings from the *in silico* model were significant, it also exhibited poor specificity, similar to that seen in the animal studies (Van Norman, 2020). This is evident in that 46% of its predictions of adverse drug activity were found to be false positives (Van Norman, 2020). However, according to Luechtefeld et al. (2018), a computer comparison algorithm was able to accurately predict the toxicity of thousands of chemicals across nine different types of tests, including those related to inhalation injury and hazards to aquatic environments. The accuracy of the *in silico* was found to be comparable to that of animal models, and it was able to produce more consistent and reproducible results (Luechtefeld et al., 2018).

While computational methods are valuable, it is improbable that *in silico* can accurately predict the behaviour of drugs in complex physiological systems or human subjects. However, when computational simulations were combined with *in vitro* assay, it is possible to generate accurate and reliable outcomes (Alves et al., 2016; Lancaster and Sobie, 2016). Lancaster and Sobie achieved an accurate classification of 86 drugs as either torsadogenic or non-torsadogenic based on *in vitro* preclinical data combined with computational simulation, with a success rate of approximately 90% (Lancaster and Sobie, 2016) also concurred that by combining computational methods with *in vitro* and *in vivo* experimental approaches, more precise data can be obtained in

identifying combinations of targets that work together synergistically and in confirming polypharmacology (Kabir and Muth, 2022).

No doubt, *in silico* modelling, provides a cost-effective and efficient approach for predicting drug-target interactions, assessing toxicity, and optimising drug candidates. Nevertheless, they have limitations in data quality, model accuracy, and validation procedures that may impact the reliability and applicability of predictions. Expert review of computational toxicology assessments can enhance the reliability of *in silico* predictions.

There are two main types of QSAR models for genotoxicity namely, rule-based and statistics-based methodologies from Leadscope Inc. and Lhasa Limited (Tcheremenskaia and Benigni, 2021). Amberg et al. reported that 8.1% of non-mutagenic predictions for bacterial mutagenicity were false negatives using these QSAR methodologies. When both Leadscope Inc. (2017) and Lhasa Limited (2017) (Lhasa, 2017)predicted an impurity to be mutagenic, 60% were confirmed as true positives by the Ames test. Conversely, when the two systems disagree, the actual percentage of mutagens is 25% and 37% (Amberg et al., 2019). In such cases, an expert review is advisable to gather robust information and resolve inconclusive outcomes (Amberg et al., 2019; Benigni et al., 2020).

As the field is progressing towards advancement where the integration of *in silico* methodologies in research and development is inevitable. The benefits of *in silico* models outweigh their limitations, leading to continuous refinement and development of more human-relevant *in silico* models. Numerous guidance documents, such as those provided by the U.S. FDA, exist to aid the development and application of *in silico* models including modelling and simulation (M&S) during development, helping to minimise the risk of erroneous decisions based on computational predictions (Ahmed et al., 2023; "Promoting Innovation in Medical Product Assessment: A Risk-based Framework for Evaluating Computational Models for Regulatory Decision-Making," 2020). To facilitate this transition, the US FDA has provided substantial support by promoting advanced alternative policies and guidelines to enhance the effective use of *in silico* models ("Promoting Innovation in Medical Product Assessment: A Risk-based Framework for Evaluating Computational Models for Regulatory Decision-Making," 2020; "Success and Opportunities in Modeling & Simulation for FDA," 2022; U.S. Food & Drug Administration, 2020).

3.4. Chick chorioallantoic membrane assay (CAM)

The use of the CAM assay as a cost-effective, less sentient *in ovo* experimental model is growing international attention that can serve as an alternative to rodent experimentations. The CAM is anticipated to exhibit closer resemblances to rodents than invertebrates or fish due to the physiological similarities shared between chicks and rodents. Fig. 3 demonstrates the chorioallantoic membrane (CAM) in a fertilised egg. This includes similarities in the vasculature system and organogenesis (Siang Kue et al., 2015). Compared to animal models CAM assay is viewed as not innervated, thus experiments conducted on it do not cause pain perception in the embryo. In other words, the CAM assay is considered to have higher ethical standards and typically does not require ethical approval (Kunz et al., 2019). Additionally, in most developed countries, the CAM assay is not regarded as an animal model until the chick has reached Embryo Development Days (EDD) 17 or later (Dupertuis et al., 2015; Ribatti, 2016). The CAM assay is a useful cancer research model because it lacks mature immunity, including both B and T cell-mediated immune functions, until EDD 18 when the chicken embryo becomes immunocompetent (Dupertuis et al., 2015). A 3D image of the tumour that was grafted onto the CAM by using positron emission and computed tomography (microPET/CT) imaging which improved the reproducibility of the tumour grafting process by implanting tumour spheroid in the CAM (Dupertuis et al., 2015; Sokolenko et al., 2021).

A study by Sing Kue et al., indicated that there is a noteworthy correlation between the ideal LD50 (Lethal Dose) values produced using the CAM and those produced using mice. This suggests that conducting preliminary studies using the CAM as a means of saving time, reducing the amount of drug used, and minimising the number of animals used could be beneficial for acute toxicity studies in rodents (Siang Kue et al., 2015).

It is worth noting that CAM assay is also a useful model for investigating short-term skin grafts on the chorioallantoic membrane, which could have potential applications in the field of dermatology (Kundeková et al., 2021). Overall, CAM fulfils the 3Rs principles and it seems to be a practical model for numerous research fields (Kundeková et al., 2021). Some researchers have utilised the CAM assay as a promising tool for human bone regeneration research, serving as a valuable *in vivo* model for human tissue engineering (Moreno-Jiménez et al., 2016).

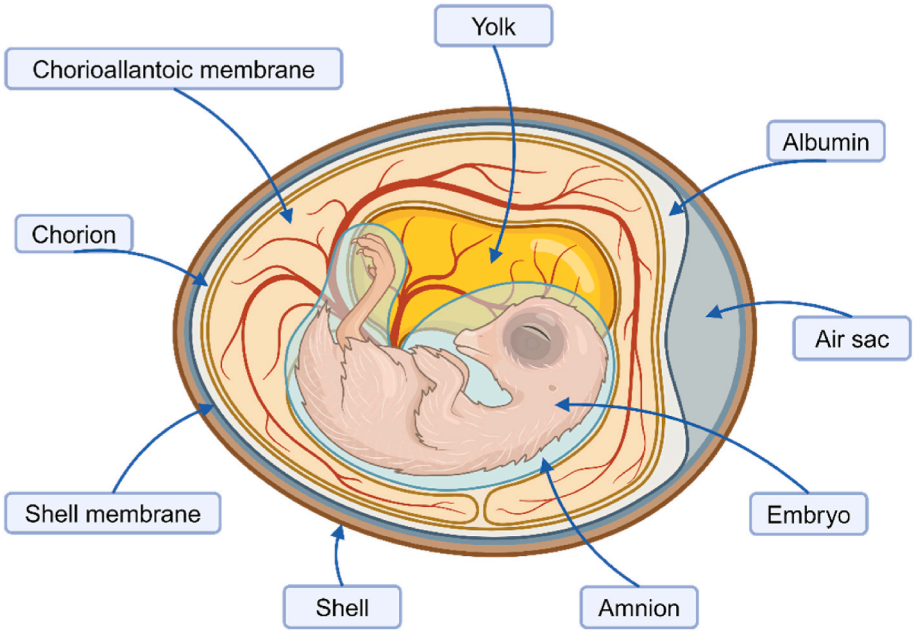

Fig. 3. Illustration of membranes and blood circulation system of an embryonic chick egg on Embryonic Development Day 14. Created with Biorender.com.

The use of chick embryos has gradually gained importance in regulatory contexts. The U.S FDA utilises the CAM assay for preclinical evaluation of drugs intended for approval in treating burn wounds and chronic cutaneous ulcers, following the FDA's guidance for industry on the development of products for the treatment of chronic cutaneous ulcers and burn wounds, published in June 2006 (Chen et al., 2021; Center for Drug Evaluation and Research, U.S. FDA, 2006). The CAM could be utilised to establish all stages of human wound healing, including inflammation, re-epithelialisation, angiogenesis, fibronectin deposition, and scar formation to study wound healing.

CAM is getting more attention due to its cost-effective and ethical alternative to traditional animal models for studying angiogenesis and tumour growth, however, the relevance of CAM results to human biology may be limited by species differences in terms of angiogenesis regulation.

3.5. Organ-on-chip

Tissue culture cannot demonstrate interactions between organs. However, the development of microfluidic technology combined with computer technology has led to the creation of organ-on-chip (OC) systems that can replicate human organ-level pathophysiology. Over time, OC has advanced to combine multiple organ chips to mimic whole-body responses, creating a "body-on-chip" (BC) system (Van Norman, 2020). Over the past five years, there has been notable advancement in OC and BC technologies. This progress has been attributed to the introduction of new technologies, including the "breathing" lung-on-chip developed at Harvard University (Huh et al., 2010). Over time, researchers such as Sengupta *et el.* (2022) have developed increasingly advanced lung alveolar organ-on-chip models, incorporating the specific phenotypes of alveolar type 1 (AT1) and 2 (AT2) cells, which believed to be more accurately represent the *in vivo*-like alveolar microenvironment (Sengupta et al., 2022). Additionally, other organ systems such as the kidney, liver, and heart have also been successfully developed (Clippinger et al., 2018; Leung et al., 2022; Van Norman, 2020).

Another recent study, conducted by Olson et al., found that regulatory testing using rodents and dogs correctly predicted only 71% of toxicities in humans by performing an analysis of 150 drugs (Olson et al., 2000). In addition, although there was relatively high consistency in predicting gastrointestinal, haematological, and cardiovascular toxicities, the ability to accurately predict drug-induced liver injury (DILI) was considerably lower (Jang et al., 2019). Thus, there is a pressing need to develop alternatives such as liver-on-chip technology to better predict drug effects in the liver.

The liver chips developed by Emulate Inc. successfully identified 87% of different drugs that had been cleared for human testing based on animal studies but later were withdrawn or recalled due to liver toxicity (Ewart et al., 2022; Van Norman, 2020). The chips did not produce an incorrect positive result for toxicity in drugs that were not toxic (Wadman, 2023). Norman et al., in their research evaluated the effectiveness of their chip using drugs that passed animal testing but were subsequently terminated during human trials due to toxicity. It was discovered that the chip discovered by them accurately predicted liver toxicity in human subjects (Van Norman, 2020). This discovery is particularly fascinating since drug-induced liver injury is the most common cause of the withdrawal of drugs from the market (Lasser et al., 2002).

Although many successful organ-on-chip discoveries offer advantages such as the ability to replicate the complex microenvironment of human organs, providing more physiologically relevant models for drug testing and disease modelling, but they do have challenges such as replicating full complex human organs, including the absence of certain cell types or biological functions.

3.6. Zebrafish

Zebrafish serve as a valuable vertebrate model that bridges the gap between *in vitro* assays and mammalian *in vivo* studies, offering an effective platform for investigating potent human diseases such as osteoporosis, osteoarthritis and scoliosis (Adhish and Manjubala, 2023). This efficacy stems from the substantial genetic homology of 70% between humans and zebrafish, coupled with the ease of gene manipulation in zebrafish (Adhish and Manjubala, 2023). It is noteworthy, according to the European Commission Directive from 2010, experiments involving the earliest life stages of certain animals, including zebrafish, are not regulated as animal studies (Commission Implementing Decision 2020/569 of 16 April 2020, 2020). For zebrafish, the stage of independent feeding, which begins around five days post-fertilisation, is considered the first stage subject to regulations for animal experimentation. Consequently, research involving zebrafish embryos or larvae under five days post-fertilisation can be considered as a viable alternative for animal experimentation.

Zebrafish embryos have been validated as an accepted alternative assay for assessing fish acute toxicity ("Test No. 236: Fish Embryo Acute Toxicity (FET) Test," 2013) and ongoing efforts explore their potential as a replacement for one of the regulatory *in vivo* mammalian embryofoetal development toxicity studies, particularly in light of the impending revision of the ICH S5 guideline on the detection of the toxicity to reproduction for human pharmaceuticals (ICH, 2020; Song et al., 2021). Notably, publications on zebrafish in toxicology have increased over 4-fold in the last decade (2007–2018) (Cassar et al., 2020). In recent years, research has primarily focused on interrogating the development, hepatic, and nervous systems of zebrafish for toxicity assessment (Cassar et al., 2020).

Furthermore, researchers at the University of Manchester, led by Addelman et al., have leveraged larval stage zebrafish (5 days post fertilisation) to induce gene mutation resulting in brain haemorrhage. These larvae exhibited spontaneous brain haemorrhage at two days old, presenting a promising model for drug screening in therapeutic applications for stroke (The University of Manchester, 2023). Zebrafish, with their genetic and physiological, resemble humans, making them valuable for studying development, genetics, and disease. However, it is noteworthy that while zebrafish offer substantial utility in biomedical research, they may have a shortfall in fully replicating the complexity of certain human diseases or physiological processes.

4. Regulatory requirements

The development of a new drug involves extensive research in drug discovery, preclinical, manufacturing processes, and clinical trials. Regulatory agencies worldwide have a significant responsibility to scrutinise the validity of the data that supports a new drug's safety, effectiveness, and quality, thereby ensuring public health. Regulatory requirements are developed to ensure products including

Table 2
The legal and institutional organizational environment regarding worldwide regulations for the impact on alternatives to animal testing.

Law, regulation, and programs	Organisations	Level
OECD guidelines	Organization for Economic Cooperation and Development	International consensus entity
ICH agreements	International Conference on Harmonisation	International consensus entity
Helsinki Declaration	Ethical Agreement in Clinical Testing	Physician associations
Animal welfare laws and acts	Nation, Country	States
Local or regional laws and jurisdiction	Cities to Countries, Courts	Public

Adapted from (Garthoff, 2005).

pharmaceutical and industrial sectors comply with established standards and regulations to protect public health and the environment. Several international documents and guidelines are internationally accepted and adopted. The rules and laws surrounding alternatives are controlled by international agreements, organisations that work towards an agreement, global institutions like the OECD, and national laws, and directives as shown in Table 2. OECD developed animal testing (preclinical) guidelines and methodology for government, industry, and non-clinical laboratories, Helsinki Declaration is for clinical study and ICH guidance covers both clinical and non-clinical practices. These international guidelines need to be adhered to fulfil specific regulatory requirements and to obtain regulatory clearance.

For several decades, it has been necessary to use both rodent and non-rodent animal models to evaluate the safety of new drug candidates (EMA, 2013; Jang et al., 2019). Key regulatory bodies like the U.S. FDA and EMA would heavily rely on animal studies (i.e. repeated dose toxicity studies, reproductive toxicity, carcinogenicity) to determine if a drug is safe before testing on humans. Undoubtedly, animal experiments have demonstrated remarkable precision in predicting safe dosage employed in clinical trials, detecting possible organs that may be affected by toxicity, and helping to identify appropriate monitoring for adverse effects. Without animal studies, the progress of safe and efficient medical products would not have been possible.

Nevertheless, animal testing is well known to be very costly and time-consuming (approximately 10 years to complete all required animal studies) (Van Norman, 2019). Take vaccines, for example, which have an average cost of €900 million to develop a new vaccine. A two-generation reproductive testing alone can be expensive, costing up to €285,842, worldwide and €318, 295 in Europe. To add to that, reproductive testing is not the only requirement. Drug evaluators also require pharmaceutical companies to provide preclinical studies, including but not limited to, repeated dose toxicity studies, skin/eye irritation, skin sensitisation, carcinogenicity, reproductive, and developmental and toxicokinetic. These studies can further increase the cost of animal studies (Barrow and Clemann, 2021; Meigs, 2018).

The data required and accepted for toxicity studies can differ significantly depending on the regulatory authorities in various countries. Countries like the U.S. and the U.K. have embedded the 3Rs principles into their regulatory framework to reduce animal usage for safety and toxicity by using 3Rs alternative predictive methods within the regulatory review process. Recently in December 2022, the U.S. made a 'big shift' by enacting new legislation that no longer mandates animal testing before human trials. This in turn will change the regulatory landscape globally.

4.1. Implementation of 3Rs in the conduct of scientific research

4.1.1. United States

In line with the 3Rs implementation, various agencies and institutes in the U.S. have developed and validated alternative methods that reduce or replace animal testing. For example, the National Institute of Health (NIH) has implemented a program to reduce the number of animals used in research. The FDA has developed guidelines to improve the welfare of laboratory animals, including standards for animal care and housing.

The FDA's New Alternative Methods Program, launched in June 2022 aims to align with the 3Rs principle by reducing, replacing, and refining the use of laboratory animals. This initiative is driven by ethical considerations, scientific advances, regulatory requirements, international collaboration, and expectantly to improve public health outcomes. By promoting alternative methods such as *in vitro* assays and computational modelling, the FDA seeks to enhance the efficiency, accuracy, and ethical integrity of regulatory toxicology while advancing drug development practices (Han, 2023; Endpoints News, 2023).

4.1.2. United Kingdom

In the U.K., the NC3Rs were established in 2004. It is a scientific organisation that leads the discovery and application of new technologies and approaches that minimise the use of animals in research and improve animal welfare. Furthermore, in the U.K. animal experiments are regulated by the Animal (Scientific Procedures) Act 1986 which requires researchers to reduce the number of animals used in scientific research (rspca.org.uk). Besides, the U.K. Home Office also developed a Code of Practice for the Housing and Care of Animals Used in Scientific Procedures, outlining standards to ensure the welfare of laboratory animals.

4.1.3. Sweden

Within the framework of the EU, the governance of laboratory animal welfare and utilisation for research purposes in Sweden adheres to a common legal framework established by Directive (2010)/63/EU (Directive 2010/63/EU of the European Parliament and of the council of 22 September 2010 on the protection of animals used for scientific purposes, 2010), harmonising regulations across EU member states. In alignment with this directive, Swedish legislation underwent adaptation in 2013 to ensure compliance with EU standards (Animal Welfare Ordinance. United Nations Environment Programme, 1988; Karolinska Institutet, 2024).

In Sweden, research involving animals is regulated by the Swedish Board of Agriculture (SBA). The Board of Agriculture is in charge of issuing licences for using and breeding laboratory animals and ethical approval is required before an animal experiment begins. Facilities conducting animal research must obtain permits from SBA, which assesses compliance with regulations and ethical standards (The Swedish Board of Agriculture, 2023). Under Swedish rules and regulations, research facilities are mandated to submit a comprehensive report annually ("Planning and reporting of animal studies," 2024). These reports include details such as the quantity and species of animals utilised, the objectives of their involvement, and the measures implemented to safeguard their welfare. Competent authorities in Sweden also inspect animal research facilities yearly to verify compliance with regulations and ethical standards. In Sweden, the 3Rs principles are implemented through legislation, guidelines, and regulatory requirements established by various government bodies including the SBA ("3R," 2024) and the Swedish Medical Products Agency (Swedish Medical Products Agency, 2024). Sweden has also established its Swedish 3R centre to promote best practices and advise on animal breeding, care, animal welfare, and use of research animals. It certainly encourages and facilitates the development of the 3Rs alternative methods (Knudsen et al., 2019)

4.1.4. Netherlands

As another member of the European Union, the Netherlands adheres to Directive 2010/63/EU, which plays an important role in promoting the 3Rs and the development of animal-free toxicology (Directive 2010/63/EU of the European Parliament and of the council of 22 September 2010 on the protection of animals used for scientific purposes, 2010). In the Netherlands, institutions seeking to conduct animal research must obtain permits following the Animal Testing Act from the Nederlandse Voedsel-en Warenautoriteit (Dutch Food and Consumer Product Safety Authority), with project licencing granted by the Central Committee on Animal Experiments (CCD) (Central Committee on Animal Experiments, 2024). Additionally, the ethics committee provide ethical approval for any research involving animals (Dutch Act on Animals University Utrecht, 1977; Langbein et al., 2022). It is also the investigator's responsibility to annually submit a report on the animal research conducted to the Netherlands Food and Consumer Product Safety Authority (NVWA) (Netherlands Food and Consumer Product Safety Authority, 2024). The NVWA monitors research facilities to ensure they meet legal requirements based on a risk-oriented approach. Consequently, inspections are conducted more frequently in facilities with a higher risk of violations. The principles of the 3Rs are firmly

incorporated within the laws and regulations governing animal research in the Netherlands, particularly under the Animal Experiments Act, the primary legislation in this domain. This act mandates that researchers consider and apply the principles of Replacement, Reduction, and Refinement in their research protocol. Oversight and promotion of compliance with the 3Rs are carried out by the CCD, which plays a vital role in ensuring ethical standards are upheld. Moreover, to actively promote the reduction of laboratory animal use in research and education, the 3Rs Centre Utrecht (3RCU) was established. Serving as a centralised hub for 3Rs initiatives and tools, the 3RCU fosters the development, implementation, and adoption of these principles. It collaborates closely with the Transition to Animal-free Innovations (TPI) Utrecht facilitating cutting-edge research and innovation in this area (University Utrecht, 2024).

4.1.5. Japan

Japan has established a legal and regulatory framework for the ethical care and use of animals in scientific research, testing, and education, which includes a self-regulating system. The primary law governing animal welfare is the Act on Humane Treatment and Management of Animals 1973 (1973). The legislation also mandates that alternative approaches should be taken into account and every possible effort must be made to minimise the number of animals used. The law further requires the use of experimental and sacrifice methods that minimise the pain and distress inflicted on animals. In 2005, the law was amended to establish new fundamental principles for animal testing known as the 3Rs.

The Science Council of Japan has published the Guidelines for Proper Conduct of Animal Experiments which recommends that institutions appoint a director with overall responsibility for the proper conduct of animal experiments. The guidelines also recommend establishing in-house regulations for animal experimentation and define the role of the Institutional Animal Care and Use Committee (IACUC) as an advisory body to the director as well as reviewing protocol by IACUC (Kurosawa, 2008).

4.1.6. China (mainland)

In China, regulations, guidelines, laws, and standards at both national and provincial levels are used to oversee and manage animal research. China was first introduced to the principles of the 3Rs and the concept of animal welfare during the 1990s (Cheng et al., 2017). Chinese laws and regulations (MOST, 2006) mandate the consideration of the 3Rs when creating and evaluating animal study protocols (Bayne and Turner, 2019; Kong and Qin, 2010). The guidelines advocate the principles of the 3Rs and necessitate every institution to create an IACUC or ethics committee responsible for supervising their programme for animal care and use (Ogden et al., 2016). Given the need to inform the international community about the compliance requirements for animal studies in China, the Guidelines for the Ethical Review of Laboratory Animal Welfare were translated into English as of September 1, 2018 (MacArthur Clark and Sun, 2020). The principles of the 3Rs were mentioned in the guidelines.

The Ministry of Science and Technology (MOST) is the government agency in charge of developing regulations related to research conduct in China (Bayne and Turner, 2019; Bayne and Wang, 2014; Cheng et al., 2017; Ogden et al., 2016). To promote the sharing of research findings and minimise animal usage, the MOST at both the national and provincial levels in China provided funding in 1900 for the creation of animal testing information databases. For example, databases such as the Chinese Association for Laboratory Animal Science (CALAS), which is an information network (www.lascn.net), encourage data sharing (Bayne and Wang, 2014). By sharing data, duplicate studies can be avoided, and the number of animals needed can be reduced. The 3Rs principles were promoted in guidelines by MOST and each institution is required to form a local committee to oversee all aspects of the laboratory including animal husbandry and experimentation.

In less than 20 years, China has made significant progress in developing alternative methods and applications. China only became more aware of the 3Rs in early 2000, largely due to international collaborations that have required compliance with these principles. October 18, 2018, was a remarkable day as The NC3Rs of the UK and the China Rural Technology Development Centre of the China MOST signed an agreement to collaborate in promoting scientific and technological innovations aimed to replace, reduce, and refine the use of animals in research (NC3Rs – www.nc3rs.org.uk).

4.1.7. Malaysia

The care and use of animals in experimentation is regulated by the Animal Welfare Act 2015 in Malaysia (Welfare Act). This act, which has been fully enforced since July 18, 2017, provides guidelines for the ethical treatment of animals used in scientific research, testing, training, and teaching (www.dvs.gov.my). The Act requires that all animal experiments be approved by the IACUC, which is responsible for ensuring that animal welfare is taken into consideration in the design and conduct of experiments and that the 3Rs principles are incorporated whenever possible. This act also requires facilities to obtain a license from the Department of Veterinary Services before using animals for scientific purposes. The License will only be granted if the facility can demonstrate that it has implemented measures to ensure the welfare of the animals used, which are subjected to yearly inspection (Gettayacamin et al., 2014; Retnam et al., 2016).

The Malaysian Code of Practice (MyCode) for the Care and Use of Animals for Scientific Purposes is regulated under the Animals (Scientific Procedures) Act 2019, which sets out the legal framework for the use of animals in scientific research. MyCode in Malaysia follows the 3Rs principles, which require that all activities consider the replacement of animals with alternative methods, the reduction in the number of animals used, and the enhancement of techniques to minimise any harmful effects on animals and as well as animal welfare (www.dvs.gov.my). These regulations have been adopted into the policies of all research institutions in Malaysia (Mohammadi et al., 2020).

4.1.8. Singapore

In Singapore, animal research facilities are required to obtain a licence from Animal & Veterinary Services (AVS) (nparks.gov.sg). The Animals and Birds Rules regulate the use of animals for scientific purposes in Singapore (NACLAR, 2022). The Act requires all animal research facilities to be licensed and meet specific criteria for the care and use of research animals, in accordance with established international guidelines. The National Advisory Committee for Laboratory Animal Research (NACLAR) provides comprehensive guidelines delineating the responsibilities of all parties involved in scientific animal care, aligned with international scientific, ethical, and legal standards. The implementation of the 3Rs principles is incorporated into the NACLAR Guidelines. Approval from IACUC is required in Singapore before commencing any experiments involving animals. The IACUC is responsible for ensuring adherence to pertinent legislation and the NACLAR Guidelines through periodic internal audits and annual facility inspections (Retnam et al., 2016). Animal research facilities in Singapore are required to report their activities in the form of annual reports to AVS (Reporting of notifiable diseases,). AVS conducts inspections of these facilities to verify compliance with regulations, and guidelines governing animal research (Animals in scientific research,; Retnam et al., 2016). The comparison of laws, policies, and standards between, the U.S., U.K., Sweden, Netherlands, Japan, China, Mainland, and Singapore are summarised in Table 3.

5. Impact of non-animal approach

As the global trend shifts towards animal-free research, significant efforts have been made to champion non-animal methods and models in research and regulatory frameworks as the emerging standard,

Table 3
Comparison of laws, guidelines, and standards between countries.

	US	UK	Sweden	Netherland	Japan	China	Malaysia	Singapore
License/ registration for animal research facility	Required[a]	Required[b]	Required	Required	Not required	Required	Required	Required
IACUC	Required	Required	Required	Required	Recommended in guidelines, but not required by law	Required	Required	Required
Reporting to government	None	None. Self-reporting	Annual	Annual	None, self-regulation	Annual	None	Annual
Government inspections	An annual, unannounced inspection	Annual	Annual	Annual[c]	None, self-regulation	Annual	Annual	Annual
Alternatives/3Rs	Included	Included	Included	Included	Included	Not currently included in regulations but included in guidelines	Not currently included in regulations but included in guidelines	Not currently included in regulations but included in guidelines

Abbreviations: IACUC, Institutional Animal Care and Use Committee; 3Rs, Reduce, refine, replacement.
[a] personal licence for each person carrying out procedures on animals/project licence for the programme work.
[b] personal licence for each person carrying out procedures on animals/project licence for the programme work and establishment licence of that place which the work is carried out.
[c] Risk-oriented approach. Inspections are conducted more frequently in facilities with a higher risk of violations.

particularly at the legislative level. Notably, while passing legislation does not eliminate animal testing from the regulatory assessment of drugs, medical devices, and treatments before human clinical trials, it provides clarity on the acceptance of alternative methods. These include cell-based assays, bio-printed models, organs-on-a-chip, and *in silico*, which are now recognised as valid data sources for new drug applications to the U.S. FDA.

In general, shifts in legislation, advancements in alternative methodologies, and evolving societal perspectives on animal welfare may potentially contribute to a reduction in animal utilisation in research and regulatory sciences. The current number of animals used in testing is considerably lower than in previous years, owing to the adoption of novel techniques that optimise their utilisation. For instance, there was a notable 10% decrease in scientific procedures involving animals in Great Britain in 2022 (2.76 million) (Home office UK, 2023). However, there is a lack of recent statistics on animal use in the U.S. The U.S. federal agency only provides data up to 2021, which indicates a 6% increase ("Number of Animals Used in Experiments in U.S. In, 2021 Rises by 6%," 2023). This data gap hinders a comprehensive understanding of the current state of animal utilisation post the enactment of the law that ended the requirement for potential drugs to undergo animals before receiving approval from the U.S. FDA in 2023. The commitment of government and regulatory bodies to enact laws promoting alternatives to animal testing within the scientific sector such as universities and industry catalyses researchers and industry stakeholders to quickly explore novel methodologies for studying diseases and treatments without relying on animal models whenever scientifically feasible. This proactive approach fosters innovative research and encourages more humane refinement of human-relevant tools, thereby potentially enhancing their direct applicability to improving human health outcomes. The U.S. FDA states that numerous initiatives aimed at reducing animal testing are currently in progress across different stages of development. Additionally, the federal budget has allocated $5 million towards a new FDA program that supports the development of alternative product testing methods, further demonstrating the commitment to minimising reliance on animal experiments (Coco Lederhouse, 2023).

Non-animal research alternatives receive financial support. For instance, animal research in Maryland mandates payments to a fund supporting non-animal research methods, fostering their advancement. Facilities engaged in animal experimentations are required to contribute annually to the Maryland Department of Health, with payments ranging from $5000 to $75,000, depending on the scale of their operations, due by January 15th of each year. Failure to comply may result in penalties of up to $1000/day (National Geographic, 2024). Moreover, these funds can significantly support the development and implementation of non-animal approaches in scientific research, marking a proactive step towards reducing reliance on animal experimentation.

As the world increasingly embraces human-specific methodologies for expediting the development of safer and more ethically sound medicine, significant legislative efforts have been initiated. The European Parliament, in its 2021 action plan, outlined steps to phase out animal use (European Medicines Agency, 2021). Similarly, the U.S. FDA's commitment to exploring alternative methods to replace laboratory animals reflects the biomedical community's current thinking. Future drug development might be animal-free or involve fewer animals, with alternative methods fully validated and based on the best science (Nature Index, 2022).

6. Regulatory acceptance

Regulatory acceptance towards the 3Rs principles, which aim to minimise the use of experimental animals and promote the development of human cell and tissue-base method to enhance human safety in the presence of chemicals and to improve the safety and effectiveness of drugs has been increasing in recent years (Knudsen et al., 2019). Many countries have implemented laws and guidelines that require researchers to use alternative methods, reduce the number of animals used, and refine experimental techniques to minimise any harm to animals. It can be challenging to achieve regulatory acceptance and implementation of the 3Rs models, as it involves a multifaceted decision-making process in drug safety assessment.

Regulatory agencies such as the EMA and the U.S. Food and Drug Administration also encourage the use of alternative methods are require the consideration of the 3Rs principles in the design and conduct of animal experiments. The Directive 2010/63/EU requires the consideration of the 3Rs principles in the selection of testing approaches for regulatory testing (Directive 2010/63/EU of the European Parliament and of the council of 22 September 2010 on the protection of animals used for scientific purposes, 2010). To promote the development and use of 3R methodologies, the Guidelines on the Principles of Regulatory Acceptance (effective date: December 2016) and Reflection paper provide an overview of the current regulatory testing requirements for medicinal products for human use and opportunities for implementation of the 3Rs (effective date: October 18, 2018) (EMA, 2018) were

developed to encourage stakeholders and authorities to initiate and support their implementation.

Several countries, not limited to the UK, Norway, Netherlands, and the U.S. have already created strategic plans to eliminate animal experiments in regulatory contexts. To implement the 3Rs principle in basic science, the preclinical and regulatory settings can be assisted by using a combination of promising alternatives like organoid culture, organ-on-chip technology, and *in silico* approaches (van Berlo et al., 2021). While there has been a growing trend toward replacing animal experiments with alternative methods, such as *in vitro* cell-based models and computer simulations, it is unlikely that animal studies will be eliminated in the near future. MacArthur Clark et al., concur that animal testing will remain necessary if a compelling need exists (MacArthur Clark, 2018). Animals will continue to be an indispensable resource for conducting complex biomedical research, particularly in areas such as the study of complex diseases and the development of novel therapies.

Norman et al. stated that FDA adopts research using modelling and simulation approaches to predict clinical outcomes, determines clinical study design, provides efficacy data, and predicts the safety of the product (U.S.Food & Drug Aministration, 2022; Van Norman, 2020). Additionally, U.S. FDA has also approved the use of various alternative methods such as *in vitro* assays and computer simulations for the evaluation of drug safety and efficacy (Van Norman, 2020).

Generally regulatory testing is required in many countries. However, the requirements for testing may be different depending on the country. Some countries may have laws that mandate specific testing requirements (legislative), while other countries may have guidelines or recommendations for testing that are not legally binding (non-legislative). The 3Rs concept emphasises the ethical treatment of animals in scientific research by replacing animal use when possible, reducing the number of animals used, and refining procedures to minimise animal suffering. Although the concept of 3Rs originated in the Western region, because of the importance of harmonisation, the eastern part of the world such as Asia is also adopting the 3Rs concept. Through 3Rs harmonisation between Western and Asian countries, regulatory testing conducted in the West can be used for drug authorisation in Asia. This can eliminate the need to repeat animal regulatory testing.

Clearly, this change alters the licensure process for a biological product such as biosimilars, which typically requires assessment through non-clinical *in vivo* animal studies before initiating clinical studies. The signing of the FDA Modernization Act 2.0 has authorised the use of alternatives to animal testing, such as cell-based assays and *in silico*, streamlining the process of investing in the safety and effectiveness of drugs ("Information and Submission Requirements for Biosimilar Biologic Drugs," 2017). Similarly, regulatory bodies like the UK's Medicines and Healthcare Products Regulatory Agency (MHRA) and Health Canada have shifted their perspectives, recommending the removal of animal studies for biosimilar approval (GOV.UK; MOH-Canada, 2017). Furthermore, the latest (WHO) Guidelines issued in April 2022, clearly support the principle of 3Rs, which aims to minimise the use of animal testing. The guidelines state that animal studies are not considered necessary if the biosimilar product can prove high similarity to its reference product (WHO, 2022)("Guidelines on evaluation of biosimilars," 2022).

The other example is fixed-dose combination of drug, whereby the individual drugs have been authorised. When combined as a fixed-dose combination, they utilise *in vitro* studies, including but not limited to Pharmacokinetics drug interaction, primary pharmacology, and genotoxicity as part of the preclinical safety package provided to the regulatory authorities for market authorisation purposes (Therapeutic Goods Administration, 2016).

In addition to *in vitro* studies, for example, the EpiDerm Skin Irritation Test (SIT) method has been granted as a full replacement method for the *in vitro* Draize rabbit skin irritation test under the European Commission (EC) test method regulation (method B.46) and OECD test guidelines No. 439: In Vitro Skin Irritation: Reconstructed Human Epidermis Test Method (European Commission). Other alternative methods for toxicity testing (validated test methods on health effect) can be found at joint-research-centre.ec.europe.eu (European Commission).

Another noteworthy example involves the MHRA's acceptance of alternative methods to animal studies, underlining the importance of evaluating research relevance rather than defaulting to animal models. This principle was used in January 2019 when the regulatory body approved human trials for a cancer treatment developed by Achilles Therapeutics (Animal Free Research UK, 2023). Notably, the therapy, employing a patient's own immune cells to treat the cancer treatment, received approval despite lacking supporting animal data, thereby emphasising the limited relevance of such data in this particular context. The Coronavirus disease (COVID-19) vaccines serve as a notable example where global regulators departed from the standard requirement of completing all animal testing before initiating human clinical trials. Typically, full preclinical data must be available before proceeding to human trials. However, due to the urgent need for vaccines during the pandemic, regulators allowed preliminary animal study data to suffice for the commencement of human trials, with additional animal studies continuing in parallel (Animal Free Research UK, 2023).

7. Conclusions and recommendations

In the coming years, there will be increased efforts to develop new non-animal testing methods that are more cost-effective, robust, efficient, and provide consistent and translatable results to better predict human toxicity and accelerate innovation of new drugs supported by current science. To date, the acceptance rate of pharmaceutical dossiers utilising 3Rs methods, such as well-supported *in vitro* data, and validated *in vitro* models, has been positive, with many established regulatory bodies accepting these approaches with proper justifications and without rejections (Beken et al., 2016; Grimm et al., 2023).

The use of animals in medicine has certainly contributed to advances in human health, but it has limitations (i.e. high in cost, longer duration, interspecies translation issue) in predicting human safety. As a result, novel alternatives such as high-throughput and human-relevant *in vitro*, 3D cell culture, *in silico*, chick embryo, and organ-on-chip have emerged, providing better tools for decision-making in drug development.

Furthermore, recent legislative enactments require adjustments for researchers and industries. Many alternative methods remain underdeveloped and lack full validation. Moreover, the expense associated with validating these alternative methods poses a significant barrier. Lastly, the regulatory framework plays a pivotal role, drug manufacturers are required to comply with regulatory standards to obtain authorisation for their products. Flexibility within regulatory bodies regarding the acceptance of alternative methods could streamline this process for drug companies.

To accelerate the regulatory process, it is recommended that the industry takes a proactive role in identifying new methods and approaches that have the potential to be accepted by regulators. Big pharmaceutical companies can stay up-to-date with the latest scientific advances in safety pharmacology and agreed-upon best practices. This will also help to strengthen the connection between the industry and regulators, permitting greater communications and data sharing between the two parties. To meet current regulations and promote policy changes and practices, there should be ongoing discussions between the industry and regulators regarding the progress and challenges related to the development, validation, and implementation of alternative methods. Guidelines have to be regularly revised to stay in line with advancements in science and regulations of different countries.

For pharmaceutical companies to formulate safe and effective drugs more readily available to the public, they must incorporate a global harmonised development process and comply with global 3Rs regulatory standards. This will help minimise the need for conducting multiple or additional studies to meet country-specific requirements.

The 3Rs approach represents a promising direction for the scientific

community, leveraging innovative methodologies to develop predictive models aimed at reducing reliance on animal testing for safety and toxicity assessments. By incorporating alternative methods into the regulatory review process, we can advance our understanding of the potential risks associated with novel products, while safeguarding ethical standards in research.

This review reveals the obvious differences in the path toward 3Rs implementation concerning legal framework, regulations, policies, and guidelines. Despite these variations, the core principles of the 3Rs are preserved and the present review provides a concise summary of the key similarities and differences observed. In general, ensuring regulatory harmonisation across regions is paramount for the effective implementation of the 3Rs principles.

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Wen Tsin Poh: Writing – review & editing, Writing – original draft. **Johnson Stanslas:** Writing – review & editing, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Johnson Stanslas reports administrative support was provided by PutraMalaysia University. Johnson Stanslas reports a relationship with Putra Malaysia University that includes: employment. Johnson Stanslas has patent pending to Not applicable. The authors declare that there are no conflicts of interest. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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