**ILAR J**

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***Technology, the Three Rs, and Pharmaceutical Development***

**Kinter and DeGeorge.** [**Scientific Knowledge and Technology, Animal Experimentation, and Pharmaceutical Development**](https://academic.oup.com/ilarjournal/article/57/2/101/2806918/Scientific-Knowledge-and-Technology-Animal)**, pp. 101-108.**

Domain 3: Research

SUMMARY

Introduction: Pharmaceutical development is arguable the oldest of the biomedical sciences with origins >3500 years ago. In the 20thcentury CE 4 major transformations have dramatically impacted this process: (1) anesthesia, analgesia, and antisepsis, (2) medicinal chemistry, (3) regulatory toxicology, and (4) targeted drug discovery. This article serves to review pharmaceutical development since ancient times, describe its coevolution with animal experimentation, and attempts to predict future transformations.

Ancient Pharmaceutical Development and Animal Experimentation: The Ebers Papyrus (1536 BCE), one of the earliest pharmaceutical records, describes over 800 treatments for ailments. Ancient pharmacists also implemented antagonism and tolerance using chronic low doses of venom and poisons. Mithridates (120-63 BCE), the most influential ancient pharmacist, developed a “universal antidote” containing 60 or more ingredients, which he used to protect himself from poisoning by rivals. After his death, this “Antidotum Mithridaticum” was sought after through the Middle Ages. Renaissance pharmacists were often required to formulate their concoctions (mithridates) in public, a harbinger of Good Manufacturing Practice regulations.

The use of animals to advance medical knowledge also dates from antiquity. Aristotle (4th century BCE) was the first to perform live animal experiments, while Galen (2nd century CE) became known as the “Father of Vivisection” due to his extensive animal experiments. Many other scientists conducted animal dissections and experiments as academic and public demonstrations through the 19th century. Human experimentation (self-experimentation and testing in convicted criminals) was used through the mid-20th century, which avoided algometric scaling, dose extrapolation, and interspecies response differences. These practices led to the development of inoculation of small pox-contaminated sera to confer life-long protection against the disease. Animal experimentation was focused on expanding scientific knowledge; concepts of animal welfare and the 3Rs were nonexistent before the 20th century.

20th Century Transformations in Pharmaceutical Development

*Anesthesia, Analgesia, and Antisepsis:* During the 19th century, three discoveries combined to spare humans and animals pain and distress associated with dissection, surgery, inflammation, and infection: (1) The use of ether, chloroform, and IV barbiturates and analgesics to produce surgical anesthesia, analgesia, and amnesia; (2) establishment of causal relationships between pathogenic organisms and diseases; (3) advancement of antiseptic technique and the development of antibiotics. These technologies transformed surgery and other invasive procedures from being last resort to mainstays of medical practice. Beginning in the 1950s, knowledge of pathogenic organisms and disease led to the development of “pathogen-free” rodents, health surveillance programs, and refined facility design and husbandry procedures. Anesthesia, analgesia, antisepsis, and antibiotics were initially associated with increases in animal use, but they provided the basis for refinement and reduction in animal experimentation.

*Medicinal Chemistry and Recombinant DNA Technologies:* A second transformation in 20th-century pharmaceutical development occurred with the knowledge and technology for isolation of individual chemicals from mixtures, identification of chemical structures, organic chemical synthesis, and deciphering the genetic code and recombinant DNA technologies. The rise of medicinal chemistry in the first half of the 20th century produced an unprecedented number of new chemicals that needed screened for therapeutic utility, which involved useful animal bioassays. Also during this period, the dedicated breeding and supplying of animals for laboratory research began.

*Regulatory Toxicology*: Government regulation leading to prospective safety evaluation of new drugs marked a third pharmaceutical transformation in the 20th century. Regulation dates back to Renaissance pharmacists being required to prepare mithridates in public and the earliest recent attempt came after the American-Mexican War (1846-1848), where deaths were blamed on tainted medications. Neither the 1848 US Drug Importation Act nor the 1902 US Biologics Control Act had significant impacts on drug development, but reflected the expectation of the American public that their medications be effective and safe. In response to the 1937 Elixir of Sulfanilamide and 1950s thalidomide debacles, Congress passed the 1938 US Food, Drug, and Cosmetics Act and the 1962 Kefauver Amendment, requiring sponsors to demonstrate drug efficacy and safety prior to market. Subsequent regulatory guidance introduced new standard practices for animal bioassays including: (1) testing in multiple species and sexes, (2) testing for acute and chronic durations, (3) testing drug effects on specific functions, and (4) Good Laboratory Practice standards.

*Target-Directed Drug Discovery and Molecular Pharmacology*: Scientific advances in biochemistry, enzymology, receptor biology, and other disciplines in the early 20th century coalesced in the 1970s to introduce a fourth transformation of pharmaceutical development: target-directed drug discovery and molecular pharmacology. In target-directed drug discovery, medicinal chemistry and recombinant DNA technologies are directed against specific molecules. Animal use in drug discovery dropped drastically until by the 1990s some candidate drugs were entering regulatory toxicology testing without previously having been administered to an intact animal. Animal experimentation in discovery phase shifted from a primary tool to a secondary tool to 1) confirm that targeted mechanism and pathway modulation was associated with anticipated changes in tissue biology or disease pathophysiology and 2) identify potentially adverse effects on critical organ systems. In contrast, animal safety bioassays and phenomenological study endpoints have remained the mainstay of regulatory toxicology for transition to human clinical trials. Currently, there are complex in vitro systems being developed that have the potential to transform pharmaceutical development by replacing some animal bioassays. Additional knowledge and technology expanding molecular toxicology will enable avoidance of safety concerns in discovery, improve pharmaceutical development efficiency by reducing drug failures for safety concerns, and over time reduce or even eliminate animal safety bioassays.

Summary and Conclusions: Advances in pharmaceutical development have been intimately linked to advances in scientific knowledge and technology and to animal experimentation and regulation throughout history. The explosive pace of growth of this knowledge and technology in the 20th century supported four distinctive transformations: (1) anesthesia, analgesia, and antisepsis, (2) medicinal chemistry, (3) regulatory toxicology, and (4) targeted drug discovery. Animal bioassays identifying drug-induced life-threatening effects, birth defects, and cancer have largely eliminated those risks in modern pharmaceutical development. A transformation reducing or eliminating animal safety testing will encompass new knowledge and technology permitting a robust evaluation of regulatory toxicology in vitro, and sufficient time and experience to confirm that some or all animal bioassays no longer contribute to safety. This transfer, application, and replacement in regulatory toxicology will be a slow process due to public expectations that new drugs be ever-more effective and safe, and the conundrum for regulators who endure public castigation for either delaying patient access to new medications or too quickly approving medications that are subsequently shown not safe enough. We may be on the emergent edge of a new, fifth transformation in pharmaceutical development that will yield dramatic reductions in animal use while bringing forth safer and more effective therapeutics to treat diseases.

QUESTIONS

1. What are considered to be the 4 distinctive transformations of pharmaceutical development in the 20th century?

2. The “Father of Vivisection” is:

a. Galen

b. Mithridates

c. Aristotle

d. Bob Barker

3. The US Food, Drug, and Cosmetics Act gave authority to what government agency to oversee pharmaceutical development?

a. USDA

b. FWS

c. ATF

d. FDA

 4. True or False: Drug discovery phase relies heavily on animal bioassays.

ANSWERS

1. Anesthesia, analgesia, & antisepsis; medicinal chemistry; regulatory toxicology; targeted drug discovery

2. a

3. d

4. False

**Kaufman et al. Data Standardization, Pharmaceutical Drug Development, and the 3Rs, pp. 109-119**

Domain 3: Research, T3: Design and conduct research

Domain 5: Regulatory responsibilities, T2, Advocate for humane care and use of animals

SUMMARY: This article reviews the FDA’s requirement for standardized electronic subject-level data for nonclinical submissions which can expedite and improve drug development and contribute to the 3Rs.  Only a small number of studies have methodically compared findings from animal toxicology studies with those from human clinical trials. This is part because the lack of easy access to data and the need for extensive data curation.  Food and Drug Administration (FDA) has been created a comprehensive electronic regulatory environment designed to improve drug development, understand the translation of nonclinical studies, and facilitate submission review.  These efforts fall under the Prescription Drug User Fee Act (PDUFA). PDUFA, mandate electronic standards (FDA 2014b) for regulatory submissions.  Study Data Tabulation Model (SDTM) developed by the Submission Data Standard working group of the Clinical Data Interchange Standard Consortium (CDISC).   The study Data has two implementation guides: human clinical, (SDTM) and animal nonclinical studies, Standard for Exchange of Nonclinical Data (SEND).   Data tabulations are datasets in which each record is a single observation for a subject.  SDTM and SEND provide FDA reviewers with direct electronic access to subject / animal-level submission data for analyses.  It will be possible to query data across studies, species, compounds, and companies. Examples for types of submission that must submitted electronically are: Certain INDS (Investigational New Drug), NDAs (New Drug Application), and certain BLAs (Biological Licensing Application). Exemptions from the electronic submission requirements Noncommercial INDs. SEND will capture electronically all individual animal data associated with toxicology studies, from protocol (planned events) to actual findings, and is intended to replace individual animal data tables in final reports. Both SDTM and SEND are built on domains. Domains are grouped topically into General Observations and Special Purpose Datasets.

General Observation Has Three Subclasses

I.  Interventions:  Exposure

II. Findings: BG: Body Weight Gain; BW: Body Weight; CL: Clinical Observations; DD: Death Diagnosis; EG: ECG Test Results; FW: Food and Water Consumption; LB: Laboratory Test Results; MA: Macroscopic Findings; MI: Microscopic Findings; OM: Organ Measurements; PM: Palpable Masses; PC: Pharmacokinetics Concentrations; PP: Pharmacokinetics Parameters; SC: Subject Characteristics; TF: Tumor Findings; VS: Vital Signs

III.  Events: DS: Disposition.

Special Purpose Datasets Has Three Subclasses

1. Doman datasets: CO: Comments; DM: Demographics; SE: Subject Elements;

II.   Trail Domains: TA: Trial Arms; TE: Trial Elements; TX: Trial Sets; TS: Trial Summary;

III.  Relationships: RELREC: Related Records; SUPP–: Supplemental Qualifiers; POOLID:

Pooled Definition: RELREC domain can be used to show macroscopic and microscopic correlation.  Supplemental Qualifier domains can also be used to identify biologically significant results in the Findings. POOLDEF domain is used for pooled animal observation data or for group-housed animals or for laboratory test or pharmacokinetics results from pooled samples.

FDA solicited stakeholder input develop standardized clinical and nonclinical data terminology and implementation guides. Because SEND and SDTM are based on the same model, it will be possible to analyze and compare data across clinical and nonclinical disciplines routinely to evaluate translation. Easy access to in vivo toxicology data can play a critical role validating in vitro models. Access to historical control data makes it possible to explore the use of Bayesian methods and associated reductions in the numbers of animals needed for control groups. The imposed framework of SEND, SDTM, and associated standards creates opportunities to improve science, accelerate drug development, and promote the 3Rs for all stakeholders.

QUESTIONS

1.  Which of the following is UNTRUE about “SEND”?

a.  SEND and SDTM are based on the same model

b.  SEND will capture electronically all individual animal data

c.  SEND format submission is voluntary

d.  SEND is intended to replace individual animal data tables in final reports

2.  Which of the following IS NOT part of the General Observation?

a.  Demographics

b.   Animal body weight

c. Pharmacokinetics Concentrations

d.  Disposition

3.  SDTM and SEND provide FDA reviewers with which of the following information

a.  Query data across studies

b.  Query data across species

c.  Query data across compounds

d.   Query data across companies

e.  All of the above

4.  Which of the following is exempted from electronic submission?

a.   IND (Investigational New Drug)

b.  NDA (New Drug Application)

c.  Certain BLAs (Biological Licensing Application)

d.  All Noncommercial INDs

5.  T/F. Data tabulations are datasets in which each record is a single observation for a subject.

ANSWERS

1.  c. Is mandated by the prescription drug user fee act (pdufa).

2.  a. Is under special purpose datasets

3.  e

4.  d

5.  T

Domain: 3 Research

SUMMARY: This article discusses the need for consistent data standard across clinical and nonclinical research to reduce the development of data silos, which become obstacles to data sharing and maximizing the value of animal and human data leading to a better methodically compared findings from animal toxically studies to those from human trials. Furthermore it explores how the FDA’s requirement for standardized electronic subject-level data for nonclinical submission can expedite and improve drug development, contribute to the 3Rs in pharmaceutical research, and be used by multiple stakeholders in addition to the FDA to address fundamental and unresolved assumptions and questions within nonclinical safety.

The discipline of nonclinical toxicology studies is predicated on the assumption that the results of in vivo studies can translate to the clinic and predict human adverse reactions and safety in clinical drug trials. Despite efforts of cost saving and extensive use of animals, only a small number of studies have methodically compared findings from animal toxicology studies with those from human clinical trials. In the past the Food and Drug Administration (FDA) has been working with stakeholders to create a comprehensive electronic regulatory environment, designed to improve drug development, understand the translation of nonclinical studies, and facilitate submission review. To accomplish these goals, electronic standards like Standard for Exchange of Nonclinical Date (SEND) have been required. SEND is a nonclinical implementation of the Study Data Tabulation Model (SDTM) used for clinical FDA submission and developed by the Clinical Data Interchange Standard Consortium. SDTM and SEND provide FDA reviewers with direct electronic access to subject-/animal-level submission data for analyses.

The foundation for the regulatory background was laid in 2012, when FDA’s authority was expanded and the agency was allowed to specify and require electronic formats for certain submissions to the FDA’s Center for Drug Evaluation and Research leading up to the FDA’s requirements for SEND which covers single-dose and repeat-dose toxicology studies and carcinogenicity studies. SEND is a data transport format and requires additional electronic tools for visualization and analysis. It strives to capture electronically all individual animal data associated with toxicology studies, from planned protocols to actual findings, and is intended to replace individual animal data tables in finale reports. Thus it doesn’t include interpretative narratives or statistical analysis, accordingly, toxicology final reports remain critical for submissions. SEND does not only include individual animal data tables, but it includes metadata not usually found in reports, such as the date and time for each individual observation. For that reason, SEND submissions are typically created from electronic raw data and not final toxicology reports. SEND is built on domains which are usually grouped into general observations and special purpose datasets which subcategories in interventions, findings, evens, domain datasets, trial domains, and relationships. Controlled terminology is an important part of SEND, because it allows findings to be compared across studies, species and submissions. It undergoes extensive public review and is updated four times a year. FDA directly partners with stakeholders through the Pharmaceutical Users Software Consortium to develop standardized clinical and nonclinical data terminology and implementation guides. When a new standard or version is released, it must go through a multi-stage process before the FDA will require its submissions.

While data warehouses, data repositories, and tools for advanced mining and visualization are not new, SEND can expand the growth and use to aggregate study data across studies, data collections systems, and nonclinical and clinical disciplines into a central repository, regardless of whether studies were conducted in-house or at a contract research organization. A warehouse solution can be used to augment static SEND study data with scheduled feeds from internal and external transactional systems containing information that is periodically refreshed, such as clinical and nonclinical study results. Data visualization is the representation of numeric or nonnumeric data in a visual context, such as a graph or chart. Data represented in visual format allows scientists to readily extract patterns and relationships from the data. SEND has promoted the development of effective ways for scientists to visualize and communicate study data, both within individual studies and across multiple studies.

Bayesian statistics currently accepted for clinical trials are based on probabilities established on actual data, such as historical control data generally using the same species, gender, age, and supplier. The ability to make accurate predictions based on robust control data, together with Bayesian statistical approaches, can result in the reduction of animals in concurrent control groups. Until more statisticians are involved in the implementation of such methods, and working examples are widely shared, the uptake of Bayesian approaches for nonclinical trials may lag behind the clinical.

Faster review of FDA submissions can be expected relatively quickly once SEND becomes required, since the FDA is mandated to publish performance metrics to satisfy requirements for transparency and accountability, which will make comparisons of pre- and post-standards review timelines straightforward.  Using actual data nonclinical safety has not been proven to translate to the clinic. Because SEND and SDTM are based on the same model, it will be possible to analyze and compare data across clinical and nonclinical disciplines routinely to evaluate translation. Leveraging historical control data can also be critical for employing statistical procedures, such as the Bayesian methods, that can result in reduction of animals needed for control groups. The ability, to easily share data, has the potential to conserve animals, and standardized electronic study data are already fostering collaborations across organizations.

Having a consistent data standard across clinical and nonclinical, will discourage the development of data silos, which easily become obstacles to data sharing and maximizing the value of animal and human data.  The documentation and presentation of data will no longer be at the discretion of the individual scientist and contributing to the three Rs in pharmaceutical research.

QUESTIONS

1.  True or False: Findings from all animal toxicology studies compare closely to those from human trials.

2.  What does SEND stands for?

a.  Standard Exchange of Non-human Data

b.  Standard Exchange of Non-clinical Data

c.  Standard Exchange of Negative-clinical Data

ANSWERS

1.  False

2.  b

**Berridge et al. Technological Advances in Cardiovascular Safety Assessment Decrease Preclinical Animal Use and Improve Clinical Relevance, pp. 120-132**

Domain 1: Management of Spontaneous and Experimentally Induced Diseases and Conditions;

T3. Diagnose disease or condition as appropriate; TT1.12. Diagnostic procedures

SUMMARY: Animal studies are an important part of assessing pre-clinical drugs, especially cardiovascular drugs. This article reviewed advancements of four technologies that are improving the assessment of cardiovascular drugs in preclinical studies:

1.  Implantable Instrumentation:Advancements in implantable instruments (i.e. telemetry) have provided very sensitive measurements of important functional parameters in unrestrained animals. These measurements include blood pressure, heart rate, ECG, and contractility (ionotropy) following a single administration of a novel compound at varying doses. Study animals with implantable instruments are now routinely reused in Latin square study design, thus reducing the number of animals needed for research. Also, there was a study that demonstrated that results from chronically instrumented dogs accurately and consistently detected inotropic effects at plasma concentration that are known to cause similar effects of these same drugs in human patients.

2.  Imaging: Improvements in imaging modalities have allowed a more integrated in vivo assessment of CV structure and functions that aligns to clinical norms. MRI and echocardiography have been the most useful imaging modalities for assessment of cardiovascular drugs in preclinical studies. The stress test and strain analysis have enhanced the sensitivity of imaging modalities. The stress test involves administering a drug with known inotropic and chronotropic effects via intravenous infusion to slowly increase contractility and/or heart rate in the animal. Echocardiography is used to examine the heart across a range of functionality. Strain imaging uses conventional images to track myocardial wall motion throughout systole and diastole.

3.  Biomarkers: The detection of novel circulating biomarkers has offered sensitive insights into health and disease, enabling minimally invasive ways to follow progression or regression of changes in structure or function. This articles discusses the following 3 cardiac biomarkers:

a.   Cardiac troponins I and T (cTnI &cTnT) are proteins within cardiac myocytes that are released in the circulating blood after an injury. Elevated cardiac troponins in the blood is recognized as the hallmark for diagnosis of acute coronary syndrome. There are numerous commercially available immunoassays for measurement of cTnI and a few for measurement of cTnT. Most assays for measurements of cardiac troponins require a sample volume that is too large for rodents (ng/mL quantities). However, there have been recent advances in cardiac troponins assays that can quantify smaller samples (pg/ul quantities).

b.  MicroRNAs (miRNAs) are short single stranded noncoding RNAs that regulate gene expression at the post-transcriptional level.  An atlas of miRNAs has been established for the heart of rats, dogs and monkeys.

c.   Nanoparticle/Microparticles are extracellular membrane vesicles that are biomarkers for organ injury and are measured via flow cytometry. Microparticles are membrane vesicles (100-1000 nm) that are released into the blood following cellular processes, like activation and apoptosis. Exosomes (nanoparticles) are small particles (30-100 nm) that are released from the cells by reverse budding of multivesicular bodies.  Although cardiac myocytes are not traditionally described as secretory cells, stimuli can these cells to release microvesicles and exosomes.

4.   In Vitro Technologies: New in vitro technologies have allowed the use of more human relevant samples in more biologically relevant context for more effective pre-animal screening. The most promising in vitro technology are human iPSC (induced pluripotent stem cells). Human iPSC are derived from skin or blood cells that have been reprogrammed back into an embryonic-like pluripotent state that enables the development of an unlimited source of any type of human cell needed for therapeutic purposes.  Human iPSC can be prodded into becoming cardiac myocytes to evaluate structural cardiotoxcity, cardiac arrhythmia and cardiac myocyte contractility.  Even with significant improvements in *in vitro* technologies brought on by the development of human iPSC derived cardiac myocytes, there are still limitations with current 2D monoculture. Future studies hope to use human iPSC as a starting point to develop a multi-cell type heart model with 3D architecture or in other words a ‘heart in a dish.’

QUESTIONS

1. What experimental design has significantly reduced the number of animal used by reusing chronically instrumented animals?

2.  What imaging modalities are the most useful for assessment of cardiovascular drugs in preclinical studies?

a. Radiography

b.  Echocardiography

c. Magnetic Resonance imaging (MRI)

d. Positron emission tomography (PET)

e.   b and c

f.   All the above

3.  An elevation in which biomarker is considered the hallmark for diagnosis of acute coronary syndrome?

a.  MicroRNAs

b.  Nanoparticle

c. Microparticles

d. Cardiac Troponins

4.  Which assays measure microRNAs?

a.  qRT-PCR

b.  Southern Blots

c.  In situ hybridization

d.  Microarrays

e. High throughput sequencing

f.  Deep sequencing

5.  How can micropeptides and exsomes be measured?

ANSWERS

1.  Latin square study design.

2.  e. Echocardiography and MRI

3. d. Cardiac troponins

4.  b. Northern blots are used for the detection of RNA in a sample.

5.  Flow cytometry

**Strange. Drug Discovery in Fish, Flies, and Worms, pp. 133-143**

Secondary Species: Zebrafish (Danio rerio)

Tertiary Species: Invertebrates

Domain 3: Research. TT3.3

SUMMARY: Non-mammalian species have been used for drug discovery process. These models have advantages to other alternative methods as providing a whole animal context and allowing modelling human disease and toxicity assessment. In addition, they allow a high throughput screening. *Caenorhabiditis elegans*, Zebra fish (Danio rerio) and the fruit fly (*Drosophila melanogaster*) are by far the most representative examples used in research. *C. elegans*is a nematode (worm) that has a fully sequenced genome and a vast number of molecular/genetic tools that makes it a valuable. *C. elegans* is hermaphrodite and has a very-short life-span of 3 weeks. Petri dishes and agar media can be used for housing and E.coli as a source of food. C. elegans can be stocked in liquid nitrogen. Overall it is easy and cheap to maitaing. One of the main disadvantages is the presence of the intestinal cuticula that impedes drug absorption and the fact that only shares 40% of genes with humans. One of the main applications of C. elegans is the discovery of new anti-infective drugs. The main approach is to expose C. elegans to human pathogens and drug compounds and assess worm survival. Genetic manipulation allows for mechanism of action discovery by using wild-type and mutant worms (i.e. protection against bacterial pathogens). Genetic manipulation in C. elegans also allowed for discovery that anti-aging drugs exert their effects through the alteration of neurotransmission related to caloric intake. Neurodegeneration has been also studied in C. elegans models created by genetic manipulation (Parkinson and Alzheimer disease). In these models the outcome evaluated was swimming behavior (motility). D. melanogaster has 75% of orthologs genes with human and have a complex anatomy that makes it suitable for the study of many human disease. Ease of genetic manipulation has enabled the discovery of many human genes by creating knock-out models. Housing is simple and cheap by means of plastic or glass vials. Life cycles are 40-50 days and nowadays there are no methods for preservation of stocks (unlike C. elegans). D. melanogaster had a pivot role in cancer research by identifying genes (Ras1, Raf, Ret) involved in cancer development. Cancer recurrence with cancer stem cells, thyroid cancer (rough eye phenotype) and the creation of cancer-avatar using the analysis of patient genome are example of the use of this model in cancer research. Zebrafish have 70-85% of gene orthology, transparent embryo and similar organs and physiology to humans. Heart disease and regeneration is an example of zebrafish application in research. In contrast to worms and flies, housing and maintenance is more complex and its experimental use is subjected to ethics committee approval. Life-span is 2 years in the laboratory set up and genetic modification is easy to perform. Stocks can be cryopreserved. Zebrafish has been used for discovering new drugs to for cancer (genetically modified models) and for prevention of inner ear cells death induced by antibiotics using somatosensory cell of zebrafish lateral line. Non-mammalian models also have an application in the drug discovery in regenerative medicine. Drosophila, salamander (hind limb) and Zebrafish (caudal fin, lateral line, heart) have cells and tissues with a great capacity of regeneration.

QUESTIONS

1.  Which is false with respect to non-mammalian species?

a. Non-mammalian species are generally easy to maintain

b. Genetically modified models are mainly used for drug discovery

c. Only a limited number of small molecules can be assessed

d. Reduction of time and research-related costs compared to mice

2.  Which animal species are not subjected to IACUC policies?

a. C. elegans and Danio rerio

b. C. elegans, D. melanogaster

c. C. elegans, salamanders

d. Drosophila melanogaster, Danio rerio

3.  Which is true?

a. C. elegans are fed on powdered diets

b. Stocks can be cryopreseved for C. elegans, D. melanogaster and Danio rerio

c. C. elegans has 80% of human ortholog genes

d. C. elegans can be infected by human pathogens

4.  Which is true?

a. Behavior cannot be assessed in C. elegans

b. D. melanogaster and C. elegans have simple anatomy that limits drug discovery

c. D. melanogaster is hermaphrodite

d. D. melanogaster can express cancer-patient genes profile

5.  Which is false regarding use of non-mammalian species in research:

a. BRAF gene is expressed in zebrafish for the study of heart regeneration

b. Zebrafish have been used for the discovery of drugs with Doxorubicin cardioprotection effects

c. Acid retinoic have been identified as a molecule that regenerates prancreatic β-cells in zebrafish.

d. C. elegans expressing mutant form of human TAU is used for Alzheimer’s disease.

ANSWERS

1. c
2. a
3. d
4. d
5. a

**Brannen et al. Alternative Models of Developmental and Reproductive Toxicity in Pharmaceutical Risk Assessment and the 3Rs, pp. 144-156**

Domain 3

SUMMARY: Use of alternative toxicology models can be used for safety testing with early, simple, and inexpensive methods.  The 3Rs, first described in The Principles of Humane Experimental Technique, considers the use of alternative approaches and replacing animals with nonanimals systems.  One system, embryo-fetal developmental (EFD) toxicity studies are required to evaluate pharmaceuticals in 2 preclinical species.  Alternative assays could help reduce the number of animals needed for these studies.  Some alternative that have been developed include rodent cell, tissue, and whole embryo culture, as well as hydra, frog embryo teratogenesis assay, and zebrafish.  Ideally, these processes should be translatable with clear results, high predictivity, high throughput, low cost, minimal compound requirement and short turnaround time.  Rodent whole embryo culture (rWEC) has been used to assess teratogenic potential using postimplantation conceptuses in appropriate conditions.  A limitation is that teratogens affecting only late stage gestation may not be observed.  Mouse embryonic stem cell (ESC) assay has been used for testing potential toxicity by culturing day 3 cell lines for 10 days.  Limitation include no maternal component and low biological complexity, but after initial isolation of the cells there is no animal usage and the pluripotent cells can be maintained in vitro for extended periods of time.  Zebrafish are another developmental toxicity model.  Their development is very fast and they are transparent.  Potential disadvantages include that they are nonmammalian and live in water which can make it difficult to use low aqueous solutions.  The eggs are also enveloped and some researchers will remove the chorion prior to use, while some don’t.  This introduces an extra variable.

Another set of in vitro testing models include reproductive toxicity testing.  Spermatogenesis models using dispersed tubular cells in Matrigel matrix has shown logical relationship in vitro as in vivo.  Seminiferous tubule cultures have been used as well as post-meiotic spermatids in explanted pieces of testis.  These assays are still in development.  Epididymis culture has also been tested in limited amounts.  On the female side, a model has emerged that recapitulates hormonal changes and tissue responses over 28 days using follicles, oviductal tissue, uterine, and vaginal explants.

Early testing of compounds can help identify toxicity hazards and help in selection or subsequent testing.  The compound may be “de-risked” prior to development.  WEC, ESC, and zebrafish assays have fairly robust predictivity and recently there has been interest in developing tiered or integrated strategies using these assays together.  Currently, there is no standardized alternative assay for assessment of embryo-fetal effects, but with increasing technology these assays may someday make up the bulk of safety assessment work.

QUESTIONS

1. Where were the 3Rs first described?
2. What are some in vivo assays currently is use for both embryo-fetal toxicology studies and reproductive toxicities?
3. What should one be looking for when deciding on using in vitro models in toxicology studies?

ANSWERS

1. The 3Rs, first described in The Principles of Humane Experimental Technique, considers the use of alternative approaches and replacing animals with nonanimals systems.
2. Embryo-fetal: embryo-fetal developmental (EFD) toxicity, rodent cell, tissue, and whole embryo culture, hydra, frog embryo teratogenesis assay, and zebrafish; Reproductive: spermatogenesis models using dispersed tubular cells in Matrigel matrix, Seminiferous tubule cultures, post-meiotic spermatids in explanted pieces of testis, Epididymis culture, and follicles, oviductal tissue, uterine, and vaginal explants
3. The processes used in vitro should be translatable with clear results, high predictivity, high throughput, low cost, minimal compound requirement and short turnaround time.

**Harstad et al. Balancing Blood Sample Volume with 3Rs: Implementation and Best Practices for Small Molecule Toxicokinetic Assessments in Rats Balancing Blood Sample Volume with 3Rs: Implementation and Best Practices for Small Molecule Toxicokinetic Assessments in Rats, pp. 157-165**

**Primary Species:** Rat (*Rattus norvegicus*)

SUMMARY: Improved small molecule bioanalytical sensitivity and concomitant decreased sample volume requirements provide an opportunity to reconsider how toxicokinetic\* (TK) data are collected in rat toxicity studies. Toxicokinetics may be performed either in all or a representative proportion of the animals used in the main study or in special satellite groups. Normally, samples for the generation of toxicokinetic data may be collected from main study animals, where large animals are involved, but satellite groups may be required for the smaller (rodent) species. Often, satellite groups\*\* of rats are designated to separate procedural effects of TK blood collection from the primary toxicity evaluation. Blood microsampling (i.e., ≤50 μL) decreases the blood volume collected such that TK samples can be collected from toxicity groups without impacting toxicity assessment. Small plasma sampling uses slightly higher blood volumes (i.e., 200 μL) with comparable technical feasibility and, importantly, allows multiple analyses with no negative impact on study interpretation. We review the state of knowledge in bioanalytical and blood sampling techniques and support the paradigm whereby TK sampling of main study animals significantly decreases the overall number of rats required for toxicity assessments and refines study interpretation with additional data options. These efforts maintain a commitment to the 3Rs (replacement, reduction, and refinement) while maintaining high-quality TK evaluations on toxicity studies.



CMS capillary microsampling, DBS dried blood spot, VAMS volumetric absorptive microsampling devices

Within the last several years, we have witnessed several methods striving to be the “preferred” bioanalytical technique for microsampling. As with most new technologies, nothing appears to be definitive and superior in all instances. There are times when each of the approaches excels over one another or even over the traditional liquid plasma sampling, which is by far still the most prevalent sample matrix for bioanalysts.

**DBS** has been utilized for detection of genetic diseases for over 50 years and has been considered in situations where logistic constraints conflict with traditional liquid sample handling. DBS outside of clinical applications became popular just before 2010 for the intention of wider uses, such as bioanalysis for TK assessments.

The next example of microsampling utilizes hematocrit tubes for blood collection and was termed “capillary microsampling.” **CMS** became popular shortly after DBS and focused on using liquid samples and staying with the more commonly used plasma matrix as opposed to blood. Using a single matrix throughout drug development negates the need to “bridge” data from one matrix to another to assess exposure across rodent or nonrodent preclinical species and humans. CMS comes in two slightly modified procedures, with one method cutting the hematocrit tube and transferring known amounts of plasma to collection tubes, while the second method uses a special plug to “push” the plasma out of the tube.

A third example of microsampling, **VAMS**, is actually a newer modified version of DBS that has reduced the larger cards and limited the absorptive tip size to collect only 10 μL by capillary action. The single-use “wicks” are more suited for automation, easier to collect compared to DBS, and are free of blood hematocrit effects on drug quantitation. Unfortunately, VAMS still does not address the need to bridge exposure data from blood samples to plasma samples, if they are used in other species during drug development. Although in vitro assessments such as blood to plasma ratios are determined during development, these ratios have not been widely accepted as appropriate to correct for exposure adjustments when different matrices across multiple species are utilized. This need to “bridge” data with validated methods for each matrix and species quickly becomes prohibitive based on costs, further study complexity, and animal numbers required. Similar to DBS, VAMS may also limit the sample volume too much, making LLOQs difficult to obtain. As with any dry sample extraction technique prior to analysis, DBS and VAMS may not produce uniform recovery of drug related material compared to liquid samples to allow quantitative metabolite identification from toxicology animals.

Current microsampling technologies enable innovative TK sampling strategies and illustrate future potential for additional reduction, refinement, and replacement of animals in toxicity studies. As an industry, we should capitalize on these advances and continue to strive for the most robust yet prudently scaled toxicity evaluation in animals to ensure we accurately characterize and investigate safety issues in a manner that ethically delivers life-changing therapies to patients. Utilizing advances in bioanalysis, blood sampling techniques, and strategic study design, it is possible to balance the deliverables of rat toxicity assessments with 3Rs principles by reducing overall animal use with small plasma samples from main study groups.

\*Toxicokinetics (often abbreviated as 'TK') is the description of what rate a chemical will enter the body and what happens to it once it is in the body. It is an application of pharmacokinetics to determine the relationship between the systemic exposure of a compound in experimental animals and its toxicity. It is used primarily for establishing relationships between exposures in toxicology experiments in animals and the corresponding exposures in humans. Direct biological comparison of exposure and adverse events in the same animal is limited by the volume of blood required for analysis – typically around 200µl per time point. For small molecules, bioanalytical methods exist that allow drugs to be measured in blood samples of less than 50µl per time point. This provides the opportunity to take microsamples of blood from the main study group without the need for satellite animals, giving scientific as well as 3Rs benefits. Removing the need for specific groups of rodents for the sole purpose of toxicokinetics represents the single biggest opportunity to reduce the use of animals in regulatory toxicology studies – providing up to a 55% reduction for some studies.

\*\*Toxicokinetic analysis identifies the level of drug exposure which elicits an adverse event in animals. Most short and long-term toxicity studies include ‘main study animals’ which are used to determine potential adverse effects, plus ‘satellite animals’ for toxicokinetics. Satellite groups of animals included in the design and conduct of a toxicity study are treated and housed under conditions identical to those of the main study animals, but used primarily for toxicokinetics.

QUESTIONS

1.  When are satellite animals normally required in TK studies?

2.  Name the one liquid and one dry microsampling technique

ANSWERS

1.  Satellite groups have traditionally been required for the smaller (rodent) species given maximum blood volume limitations

2.  Liquid- CMS, hemolysed blood sample. Dry – dried blood spot, VAMS (Please refer to table 1)

**Hopper. Automated Microsampling Technologies and Enhancements in the 3Rs, pp. 166-177**

Domain 3: Research

SUMMARY: Data collected in vivo is essential for drug screening and development and basic research; animals are used extensively for acquiring experimental measurements.  Traditionally, collection has been invasive, stressful to animals, labor intensive, time-consuming, costly, and required many animals when using small models.  Automated microsampling (AMS) alone or in an integrative pharmacology approach to simultaneously evaluate multiple physiological, pharmacokinetic, and pharmacodynamic endpoints in the same animal accomplishes multiple experimental goals.  Use of AMS robotics can assist in achieving significant reduction and refinement of animal use.  Automatic robotic instrumentation can be used to provide better quality pharmacokinetic and pharmacodynamic data, reduce time, provide more data with less variability, reduce animal use, and refine animal models to reduce pain and stress.  Microsampling (small biological samples in volume of 5-100 µL) can facilitate reduction in animal numbers while minimizing stresses associated with excessive fluid removal.  Integrative pharmacology designs utilizing AMS result in many benefits including: decreased completion time for composite data collection, decreased personnel resources, lower costs, improved safety, higher quality and multiple data-sets, and improvements in the aspects of the 3Rs.

QUESTIONS

1. Which of the following is FALSE regarding capillary microsampling?

a. Automated method

b. Nonautomated method

c. Microsamples are collected in precise volume capillary tubes from freely flowing blood

d. Samples can be used for liquid chromatography/mass spectrometry or dried blood spot methods

2. Simultaneous collection of blood, urine, feces, and microdialysis perfusates when also collecting physiological parameters is an example of \_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_.

1. Integrative physiology
2. Integrative pharmacology
3. Integral physiology
4. Integral pharmacology

3. Which of the following is an example of a blocked study design wherein the same animals are used multiple times in rotating blocks?

* 1. Latin Circle design
	2. Latin Triangle design
	3. Latin Square design
	4. Latin Diamond design

4. The \_\_\_\_\_\_\_\_\_\_\_ has been the most widely utilized model for automated microsampling studies for many different purposes.

* 1. Pig
	2. Dog
	3. Monkey
	4. Rat

5. Which of the following instruments can automatically collect blood, bile, metabolites, and dialysates from awake and freely moving laboratory animals?

* 1. Culex NxT
	2. DiLab AccuSampler
	3. Instech ABS2
	4. All of the above

6. Which of the following is NOT one of Russell and Burch’s 3Rs?

* 1. Replacement
	2. Reduction
	3. Reuse
	4. Refinement

7. \_\_\_\_\_\_\_\_\_\_\_\_\_ refers to methods that minimize the number of animals used per study.

* 1. Replacement
	2. Reduction
	3. Reuse
	4. Refinement

8. \_\_\_\_\_\_\_\_\_\_\_\_\_ seeks “to reduce to an absolute minimum the amount of distress imposed on those animals that are still used.”

* 1. Replacement
	2. Reduction
	3. Reuse
	4. Refinement

9. Which of the following is an example of a refinement technique?

* 1. Use of anesthetic/analgesic treatments to prevent or alleviate pain
	2. Use of freely moving animal subjects in their home cage to decrease restraint and stress
	3. Replacing invasive with noninvasive procedures
	4. All of the above

10. Which of the following is NOT a benefit of using integrative PK/PD designs utilizing automated microsampling?

* 1. Increased time for study completion
	2. Decreased personnel resources
	3. Improvements in aspects of the 3Rs
	4. Higher quality and multiple data-sets

11. Which of the following is a limitation of utilizing automated microsampling and/or integrative measurements in animal subjects?

a. Require surgical procedures for catheters/data sensors

b. Technical issues with equipment (e.g. collection lines, computers, etc.)

c. Relatively high initial investment required

d. All of the above

12. Which of the following IS NOT a membrane-based microsampling techniques?

a. Microdialysis

b. Dried blood spot

c. Ultrafiltration

d. All of the above

ANSWERS

1. a

2. b

3. c

4. d

5. a. Other two instruments only collect blood

6. c

7. b

8. d

9. d

10. a. Should be decreased time for study completion

11. d

12. b. Deposited on specialty paper

**Zuberi and Lutz. Mouse Models for Drug Discovery. Can New Tools and Technology Improve Translational Power?, pp. 178-185**

Domain: 3 Research

Primary Species: Mouse (Mus musculus)

SUMMARY: Trends in utilization of genetic mouse models have shifted over time from spontaneous and chemically induced mutations to reverse genetics studying gene knockouts. 75% of drug development funds are spent on candidate drugs that are not approved. Two factors may also force withdrawal of approved drugs due to lack of sufficient efficacy or unanticipated toxicity associated with adverse drug reactions (ADR). After in-vitro development, drugs move into small scale animal efficacy studies concurrent with in-vivo pharmacokinetic and pharmacodynamics studies in which a drug’s ADME (gastrointestinal absorption, body distribution, drug metabolism, and drug excretion) is determined. Finally large scale efficacy studies are performed for therapeutic range.

Previous tools of genetic engineering to maximize the likelihood of successfully translation of pre-clinical data to clinical data have been the use of: inbred mouse strains, spontaneous mouse mutants, transgenic mouse models, ES cell models, Cre-LoxP and Flp-FRT. CRISPR-Cas9 appears to shift the paradigm of genetic engineering as a very simple, cheap, effective technique requiring minimal expertise and equipment compared to other genetic engineering tools.

To minimize the chance of ADR, new mouse models allow for adding a large amount of genetic diversity into the preclinical testing phase. Collaborative Cross (CC) mice are the result of an 8-way cross of inbred strains with 3 wild-type derived inbred lines. The strains represent the three major Mus musculus subspecies and together capture 90% of the genetic variation in the laboratory mouse. Diversity Outbred (DO) mice are generated using breeding strategies on CC mice to maintain balanced founder genomes, avoid allelic loss , as well as inbreeding. Each DO mouse is genetically unique, and therefore pre-clinical testing in these mice mimic human phase 1 trials. To humanize mice for patient derived xenografts and avatar studies, the authors highly rate the NSG mouse, as its mutations prevent B and T and NK cell development, block IL-2 signaling, eliminate hemolytic complement, and reducing macrophage/dendritic cell function. Finally by using mice which express human Neotatal Fc Receptor (FcRn), the ADME studies in mice have demonstrated increased predicative ability for clinical half-life of circulating antibodies in humans, which decreases the need for non-human primate studies for these purposes.

QUESTIONS

1.  What of the following choices represent the two ways to approach understanding the mechanisms contributing to Adverse Drug Reactions (ADR?)

a. Collect DNA from unrelated patients that experienced an ADR and scan the genome for common genetic mutations

b.  Use of monoclonal antibodies and Fc fusion proteins in humanized mice.

c.  Introduce greater genetic diversity into the pre-clinical testing phase.

2.  Diversity Outbred (DO) Mice are:

a.  Genetically unique

b.   Are good models for patient derived xenograft and avatar models

c.  Express human neonatal Fc receptor (FcRn)

d.   Were generated using CRISPR/Cas9 technology

ANSWERS

1.   a and c

2.  a

**Sistare et al. The Promise of New Technologies to Reduce, Refine, or Replace Animal Use while Reducing Risks of Drug Induced Liver Injury in Pharmaceutical Development, pp. 185-211**

Domain 3: Research (K12: Replacement, Reduction and Refinement techniques)

Domain 5: Regulatory Responsibilities

SUMMARY:In this extensive review, authors have discussed about the historical perspective of prognosis of drugs and novel toxicants causing liver injury, regulatory expectations for liver safety in drug development, current conventional and nonconventional approaches to predict liver injury and finally discussing novel tools/methods/approaches to predict liver injury and how scientific community, drug regulators (FDA) and pharma industry decision makers can utilize these tools in accomplishing rule of 3”R” for animal research. Drug-induced liver injury (DILI) appearing at all stages of drug discovery and development has historically been a major thorn in the side of pharmaceutical companies and drug regulatory agencies working to advance safe products to combat human disease. This liver safety liability is the number one cause among all toxicities, for significantly slowing the pace of successful drug development and leading to the waste of significant animal, human, and budgetary resources as promising drugs are discarded and safer alternatives are sought. New advances and novel approaches to better understand, earlier identify, and improve prediction of drug-induced liver toxicity are emerging at a rapid pace. The authors have attempted to review and summarize these exciting new approaches and model systems with the intent to spur readers to invest effort toward familiarization, understanding, and adoption. The insertion of such high performing, accurately predictive, well-qualified assays for DILI prediction at the right stage and in the appropriate context can favorably impact drug development to enhance success, shorten timelines, reduce the needless use of animals, provide more value from each animal study (and human trial) that is conducted, and help to reduce overall costs by spotting and weeding out compounds with liabilities earlier.

Novel In vitro Methods Described In Article Includes:

Hepatocyte co-culture systems:

* Primary hepatocyte and kuffer cell co-culture (HKCCS)
* Micropatterned co-culture (MPCC) model system
* hiPSC ( Human induced pluripotent stem cells) Hepatocytes

3D Liver Tissue Model Systems:

* 3D Spheroids
* 3D Bioprinted hepatic model systems

Microfluidic and dynamic Flow Culture Systems:

* 3-D Scaffolds with dynamic flow
* Cone-and-plate viscometer platform

In Vivo Models Described In Article Includes:

* + - * Humanized Liver Mouse models
			* Immune Checkpoint Modified Mice
			* Mouse Diversity Panel and Diversity Outbred Mice

QUESTIONS

1. Regarding drug induced liver injury (DILI) the following are TRUE EXCEPT:

a. Most common cause of drug induced acute liver failure in West (USA) is APAP (Acetaminophen).

b. Most common cause of drug induced liver injury in USA is antibiotics.

c. Degree of liver enzyme elevation correlates with severity of liver disease.

d. Clinical jaundice is a predictor of mortality.

e. Cholestatic pattern of DILI can be prolonged even on stopping the offending drug.

2. What is Hy’s law?

3. Conventional clinical chemistry gold standard for monitoring DILI in humans and animals:

a.  AST activity in serum

b. ALP activity in serum

c.  ALT activity in serum

d. Bilirubin levels in serum

e.  GGT levels in serum

4. ALT routinely used together with serum measurements of bilirubin, and alkaline phosphatase (or GGT) provides diagnosis/prognosis for

a. Damage to hepatocytes

b. Bile duct alterations

c. Cholestasis

d. Overall hepatobiliary function

e. All of the above

5. Mechanisms involved in DILI includes:

a.  Mitochondrial Injury

b.  BSEP (**Bile Salt Export Pump)** inhibition

c.  Innate Immune Activation

d.  Reactive Metabolite Formation

e.  All of the above

6. Emerging liver biomarkers for improving specificity (More specific than ALT) includes;

a.  GLDH (Glutamate dehydrogenase)

b.  HPD (4-hydroxyphenylpyruvate dioxygenase)

c.  Arginase

d.  Mir-122

e.  All of the above

ANSWERS

1. c

2. Hyman J. Zimmermann is widely credited for defining a prognostic rule of drug induced hepatotoxicity that predicted a case fatality rate of 10% or higher for drugs causing severe acute hepatocellular jaundice in the pre-transplant era. Hy’s law has been now been modified by the Food and Drug Administration, as bilirubin >2 mg/dL or and transaminases >3 upper limit of normal.

3. c

4. e

5. e

6. e

**Campbell et al. In Vivo Imaging in Pharmaceutical Development and Its Impact on the 3Rs, pp. 212-220**

Domain 3: Research

SUMMARY: Health costs have increased in the last decades. It is needed to increase the ability to identify no effective molecules or with safety issues prior to late-phase clinical development to reduce the costs and achieve more new therapeutics. In vivo imaging can provide biomarkers that supply information about biochemical, physiological, and anatomic processes. Repeated measurements can be made in the same animals, being the same group of animals used for all time points. Translation to humans also increases due to the fact that the methods used in preclinical studies are also available for clinical studies.

Ultrasonography (US), in which the echoes reflected from the tissues are detected with a transducer and converted into pixels is used for internal body structures and can also display the rate and direction of blood flow. Computed tomography (CT)is an X-ray based imaging modality which produces 3D images. It allows monitoring disease progression and evaluating therapeutic responses. Bone, lung and fatty tissues can be easily identified. Magnetic Resonance Imaging (MRI) is used taking profit of the spin properties of the nucleus of hydrogen atoms which are located in water and fat within tissues. When the magnetic field is applied the spins align and realign and send out radiofrequency signals providing information about the location and amount of the hydrogen spins in the body. A 3D image is reconstructed. This MRI signal can be made sensitive to vascular flow too. Positron emission tomography (PET) and single photon emission CT are nuclear imaging with provide information of tissue and organ function. It uses positron-emitting radioisotopes. After administration of a PET tracer a PET scanner measures its distribution by detecting the emitted photons in coincidence and a 3D image is constructed. PET is often used in combination with CT or MRI. Optical imaging includes bioluminescence imaging (BLI) fluorescence imaging and optical coherence tomography (OCT). BLI is based on detection of visible light produce by reaction of a substrate. It has higher throughput than other imaging modalities. Fluorescence imaging uses near-infrared fluorescence probes designed to reflect different biological processes. OCT captures micrometer-resolution 3D images just below the surface in biological tissues. It uses light which reflects and is detected.

Target engagement is important for increasing confidence, guiding dose selection and reducing risk. PET provides a noninvasive, quantitative measure of target distribution in tissues using a radioligand with a high affinity and selectivity for the specific target of interest. Combination of target occupancy curves with efficacy data ensure that target engagement is sufficient when the clinical proof of concept trial is conducted.

Pharmacodynamic (PD) effects can be objectively measured and lead to biomarker candidates with preclinical and clinical applicability. In vivo imaging allows for the direct measurement of a biomarker in tissue without the need of necropsy. The use of micro x-ray CT can measure the volume of tumors which are inaccessible without necropsy and monitor tumor progression and regression.

In vivo imaging is used also to assess potential safety-related treatment effects in drug development. Imaging is a powerful de-risking tool, where direct or indirect effects due to treatment are measured.

The mechanism of action of a drug can be measured also with the aid of in vivo imaging. Specific modes of action of a disorder can be visualized through the use of reporter genes, used to determine dose response, dose selection and needed drug levels to produce therapeutic response.

QUESTIONS

1. \_\_\_\_\_\_\_\_\_\_\_\_\_\_ are indicators of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention.

2. Regarding the 3Rs and in vivo imaging:

a. In vivo imaging allows for animal replacement

b. In vivo imaging allows for animal reduction

c. In vivo imaging allows for animal refinement

d. b and c

3. With regard to the advantages and disadvantages of the different in vivo imaging methods:

a. Ultrasonography allows for imaging tissues through bone or air.

b. Computed tomography uses magnetic waves to provide a detailed 3D image.

c. MRI an ultrasonography are sensitive to vascular flow.

d. PET yields 2D images

4. T/F: Auto fluorescence needs to be avoided in in vivo fluorescence imaging.

5. Ultrasonography is used normally to optimize the therapeutic window.

6. Tumor molecular cell surface targets can be imaged with PET.

7. The capacity of high penetration of optical imaging leads it suitable for safety assessment applications.

ANSWERS

* + - 1. Biomarkers
			2. D
			3. c

4. T

5. F

6. T

7. F

**Gribble Walker et al. Promoting Adoption of the 3Rs through Regulatory Qualification, pp. 221-225**

Domain: 3 Research

SUMMARY: This paper reviewed drug development tool (DDT) pathways for regulatory acceptance of novel DDTs and discuss examples of safety projects considered for regulatory qualification. Key concepts to be considered when defining the evidence required to formally adopt and potentially replace animal-intensive traditional safety assessment methods using qualified DDTs are proposed. Presently, the use of qualified translational kidney safety biomarkers can refine and reduce the total numbers of animals used in drug development. They propose that the same conceptual regulatory framework will be appropriate to assess readiness of new technologies that may eventually replace whole animal models.

Currently the conduct of studies in intact animal models to evaluate the safety of drug candidates prior to exposing humans is expected regardless of geographical region or regulatory agency. This expectation is based on the combined experience of both drug developers and regulators in the introduction of new chemical entities into first-in-human studies.

There are numerous examples in which animal models did not accurately predict toxicity in humans (false negatives).

Ultimately, the introduction of human biology-based in vitro assays, if successfully integrated into drug development, should reduce the reliance of safety assessment on intact animal studies. To develop this modernized approach to toxicology, we must first be able to accurately define toxicity in both humans and animals across a wide array of compounds.

However, the tools we use to define drug-induced target organ toxicity in humans are blunt compared with those we can use nonclinically. Thus, the first step in developing and applying a modernized toxicology approach must be the establishment of “novel” methodologies, such as translational biomarkers that are minimally invasive to better understand drug-induced toxicity in both animals and humans.

The first step in developing a modernized toxicology-based approach to drug safety assessment will require:

1. The introduction of safety biomarkers with greater predictive accuracy to identify organ toxicity in humans.

2. Second the use of regulators from the U. S. Food and Drug Administration (FDA), the European Medicines Agency (EMA), and the Japanese Pharmaceuticals and Medical Devices Agency (PMDA) have been engaged in the development of these novel biomarkers with the objective of ensuring they are appropriately characterized for use in clinical trials. A potential framework to understand when novel technologies are ready for use in drug development can be found in regulatory qualification.

3. “Critical Path Opportunity List”: Within this document, and building upon the experience of the Voluntary Exploratory Data Submission process at FDA, a framework was described whereby a submitter and the FDA could engage in a dialogue about a novel drug development tool (DDT), including biomarkers. Composed of initiation, consultation and advice, and review stages, the DDT qualification process results in formal regulatory guidance articulating specific conditions when and how a new biomarker can be used in a drug development program.

4. This pathway can be contrasted with the traditional mechanism to gain agreement with FDA regarding the appropriate use of a given biomarker.

5. The intended outcome of DDT regulatory qualification is to come to recommendations as to how any and all future sponsors may use a specific biomarker, rather than deliberations to identify a one-off use for a specific drug development program

* + - In contrast, once a biomarker is qualiﬁed by the FDA, it is considered appropriate for future use – by any sponsor – according to the guidance put forth that describes how it can be measured and interpreted.
		- A framework establishing the evidentiary considerations for assessing how and when novel technologies should be incorporated into drug development is being actively developed by many stakeholders.
		- Regulatory qualification hinges on the central concept of proposing novel tools for a specific “context of use” (COU) in drug development. At the beginning of a qualification research program, a COU can be an aspirational statement that identifies a need in drug development, for instance, in safety assessment. The use statement names the biomarker and its purpose for use in drug development; the conditions for qualified use then can provide conditions under which the biomarker is or is not qualified for use.

The qualification of safety biomarkers in the nonclinical space can directly reduce animal usage.

QUESTIONS

1. Several key considerations are under active debate toward matching the appropriate level of evidence to a desired “context of use” COU, including

a. Measurement methodology

b. A risk-benefit framework

c. The role of published literature

d. All the above

2. T or F. One mechanism to advance the application of novel safety assessment methodologies in drug development, including in silico or in vitro approaches that reduce the use of animals in toxicology studies, is regulatory qualification.

3. One mechanism to advance the application of novel safety assessment methodologies in drug development, including in silico or in vitro approaches that reduce the use of animals in toxicology studies, is regulatory qualification includes?

a. Standard Exchange of Non-human Data

b. Standard Exchange of Non-clinical Data

c. Standard Exchange of Negative-clinical Data

d. All the above

4. T or F. The qualification of safety biomarkers in the nonclinical space can directly reduce animal usage.

ANSWERS

1. d. First, the need for a well characterized and technically valid measurement methodology for a novel biomarker is uncontroversial. Another active area in the evidentiary considerations conversation is the utilization of a risk assessment framework to help determine the total data needed to support a COU. Third evidentiary consideration is how heavily to consider previously published data, whether it can be sufficient to qualify a biomarker, or whether prospective, original data will always be required of a submitter.

2. True

3. d

4. True

**Ford. Refinement, Reduction, and Replacement of Animal Toxicity Tests by Computational Methods, pp. 226-233**

Domain 3: Research

SUMMARY: Computational toxicology involves applying in silico approaches to predict, model, and explain toxicological mechanisms at the molecular level. As more computational models become available and validated, use of these programs promotes the application of the 3Rs through the reduction and replacement of animals used for toxicological research. Computational toxicology combines many scientific disciplines in order to apply computational and statistical methods to predict the toxicity of unknown compounds based on comparison with compounds for which toxicity data has been established. Currently, safety assessment of new drug candidates relies heavily on animal testing due to regulatory requirements, questions of the validity and relevance of in vitro/in silico models to actual in vivo behavior, and hesitation from pharmaceutical companies in investing in drug candidate development without a robust description of the in vivo safety profile. There are three main computational toxicology methods used to derive in silico data, including grouping approaches, structure-activity relationship (SAR) and quantitative SAR, and expert rules based systems. All of these methods are based on the hypothesis that compounds with similar structure have similar toxicological activity. There are also a number of freely available software tools to facilitate read-across of toxicological endpoints.

Increasing public demand for improved animal welfare has led regulatory agencies to encourage the use of computational toxicology models. Despite the rapid progress in the field, regulatory acceptance of these models has been slow, and in silico toxicology data are generally submitted on a voluntary basis. In silico data are not required for comprehensive toxicity evaluations and there aren’t many examples in the scientific literature of regulatory agencies granting approval of in silico methods as a means to replace animal toxicity tests. Regulatory groups such as the National Center for Computational Toxicology at the US Environmental Protection Agency and the Center for Drug Evaluation and Research at the US FDA actively construct and promote the use of in silico models for risk assessment. There are several regulatory programs in the US and Europe that serve to encourage the use of in silico data, including “Toxicity Testing in the 21st Century: A Vision and a Strategy” at the US National Research Council; EU Cosmetics Regulation Directive; REACH legislation passed by the EU (Registration, Evaluation, Authorization, and restriction of Chemicals); and US state legislation in states like California and New Jersey that have made it illegal to use animals for testing when alternative methods exist.

It is extremely challenging to state exactly how many animals have been reduced or replaced by the use of in silico data in drug development. For greatest impact, computational toxicology tools should be employed during the drug discovery phase, as this phase offers the greatest opportunity for reduction and refinement. Software programs currently exist to predict important toxicological endpoints such as mutagenicity, hepatotoxicity, eye irritation, skin sensitization, LD50, and carcinogenicity. These methods can help eliminate the need for animal-based testing such as the Draize test for eye irritation, the Buehler guinea pig test for skin sensitization, the rat oral acute toxicity test, and the rodent dominant lethal test for mutagenicity. In addition to replacing some animal testing, in silico methods can also help with in vivo study planning in accordance with the 3Rs:



Toxicity accounts for up to 50% of failures in preclinical drug development and the use of computational toxicology methods in this process presents the opportunity to reduce the numbers of animals used and refine procedures for those that are used in drug development.

QUESTIONS

1.   Which animal testing method is used to assess drug candidates for eye irritation?

a.  Rodent dominant lethal test

b.   The Buehler guinea pig test

c.   Guinea pig maximization test

d.   The Draize test

2.  Which of the following is NOT an advantage of in silico testing?

a.  A chemical toxicology expert is required to interpret and contextualize results

b.  Reduction of animals used and refinement of animal use procedures

c.   High throughput

d.  Less expensive, leading to overall cost savings

3.   True or False: Computational toxicology methods work on the assumption that compounds with similar structure have similar toxicological activity.

ANSWERS

1. d. The Draize test

2. a. A chemical toxicology expert is required to interpret and contextualize results

3.   True

**Sewell et al. Opportunities to Apply the 3Rs in Safety Assessment Programs, pp. 234-245**

Domain 3: Research, Tasks 1-3

SUMMARY: Before a potential new medicine can be administered to humans it is essential that its safety is adequately assessed, including live animal studies. This article outlines current and future opportunities to apply the 3Rs in safety assessment programs for pharmaceuticals, and the potential (scientific, financial, and ethical) benefits to the industry, across the drug discovery and development process.

Earlier and better screening have the potential to reduce late-stage attrition by improving compound selection that require testing in animals. It is important that approaches to safety assessment are continuously reviewed and challenged to ensure they are science-driven and predictive of relevant effects in humans.

Success rate for compounds tested in humans before animals (first in human or FIH) to registration or FDA approval shows success rate of only 10-11%. Biggest reasons for failure were failure of efficacy (30%) and clinical safety - (30%). The biggest causes of attrition from a safety perspective are cardiovascular and liver toxicities leading to the need for screening (animal and nonanimal) to predict these liabilities.

Even small improvements in the current failure rate would be helpful. Potential ex vivo, in vitro or in silico tools are described.

*In vitro*: 99% of in vitro tests are directed towards ADME (absorption, distribution, metabolism, excretion), safety pharmacology, and genotoxicity endpoints.  Ames, for DNA mutational risk assessment, and hERG (cell lines transfected with human-ether-a-go-go related gene), for cardiovascular arrhythmia risk assessment are commonly used. Pharmacological profiling (compound screening) is also widely used.  AOP concept: (Adverse Outcome Pathway) can improve safety assessment and reduce reliance on animal methods through a mechanistic approach. AOPs link a molecular initiating event (intended drug target, etc.) to an apical endpoint (e.g. unexpected side effect or anticipated treatment effect) through accepted data from multiple sources.

Predictive Computer Modeling: Development of computer modeling relies on mechanistic information but could integrate SAR (structural activity relationships) from databases to provide more predictive information earlier on in drug development, to avoid candidates with unacceptable safety profiles.

Human Tissue Models:Organ on chip, spheroid models, and human induced pluripotent stem cells (iPSC) are being more widely used for safety assessment. Human iPSCs can differentiate into a range of tissues and can be used as relevant early screens.  iPSC derived cardiomyocytes can be used in a variety of ways and may replace other assays based on primary myocytes from animals or cell lines overexpressing ion channels.

Invertebrate Species:Social amoeba (Dictyostelium), fruit flies (Drosophila), and nematodes (C. elegans) are increasingly used as early options to vertebrates.

Nonmammalian Vertebrates:*Danio rerio* has increased in capacity as small size and transparent body allows testing in a 96 well format, combining scale and throughput of in vitro systems with whole animal research.

GLP Standard Studies:Animal studies are used to determine a safe starting point for human clinical trials. The International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) has developed guidelines that have helped with harmonization across different countries for safety studies and toxicology studies. ICH guidelines are reviewed and revised, so a forum is available to question regulatory requirements that may be redundant or no longer have value.

Species Specificity: Reviews have shown that nonrodent data identifies additional toxicities to that detected in rodents, however, with appropriate justification, it can be relevant to provide rodent only data, or chronic-dosing studies in a single rodent species only, reducing the number of animals used.

Acute Toxicity Tests: ICH guidelines no longer require the determination of lethal dose and doses that cause major adverse effects, because the use of MTD (maximum tolerate dose) and other studies provided the dosing information necessary for clinical studies, as determined by an analysis by a working group in 2003.

Opportunities Within Current Framework: Study design that minimizes animal numbers exist, such as microdosing in exploratory clinical trials that require a reduced number of safety studies in animals. Microsampling, toxicokinetic satellites or off-treatment recovery animals can be used to vary and reduce total animal numbers needed.  The timing and design of developmental and reproductive toxicity studies are conducive to combination with or incorporation into other studies, which can reduce animal numbers. For example, combining the male and female fertility study and the embryo-fetal development (EFD) study can reduce rodent usage by 20% per compound. Recent ICH advocates propose the use of an enhanced pre/postnatal development study (eePPND) for NHPs which includes dosing from day 20 gestation to birth to combine EFD and pre/postnatal development PPND endpoints into one study.

Dose selection: there are five criteria for definition of the high dose in a toxicology study. These are MTD (max tolerated dose – based on clinical signs and body weight loss (BWL)), limit dose, top dose based on saturation of exposure, maximum practical dose, or dose providing a 50-fold margin of exposure. Usually a low, intermediate, and a high dose plus a control are all that are necessary. High dose should be selected to ID target organ toxicity or other nonspecific toxicity.  It is also important to establish the NOEL – no observed effect level.

LASA (Laboratory Animal Science Association) has worked with toxicologists in collaboration with NC3Rs (National Centre for the Replacement Refinement &Reduction of Animals in Research) to share advice for study directors and other toxicologists to maximize implementation of refinement in dose level selection for regulatory toxicology studies. There is opportunity in MTD especially to refine procedures and create endpoints that are valid yet consistent with better welfare (predictive weight loss percentages, etc.).

Microsampling to reduce animal numbers by potentially up to 42% depending on type of study, is a significant reduction, and used more often as clinical technology improves.

Social housing (during telemetry for instance) may be more appropriate to reduce variability caused by pathophysiology that isolation can cause. Technology is improving in this regard – automated cage systems for noninvasive behavioral assessments for instance, are being introduced.

QUESTIONS

1.  The International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) aims to:

a.  Implement the 3 R’s in safety studies in animals

b.   Override GLP requirements

c.  Inspect and accredit member countries on a voluntary basis

d.   Generate recommendations for punitive actions when approved drugs cause adverse effects in human populations.

2. Encouragement of social housing in animals used in toxicology research is an example of:

a.  Refinement

b.  Reduction

c.  Replacement

d.  Requirement

3.  *Dictyostelium*species can be used in assays that may replace some vertebrate-based assays. The common name for these are:

a. Fruit fly species

b.  Social amoeba species

c.  Nematode species

d.  Sea snail species

4.  *Danio rerio*have the following benefits for toxicology research:

a. Small size and opaque body allows testing in a 96 well format, combining scale and throughput of in vitro systems with whole animal research.

b.  Small size and livebearer status allows testing for enhanced pre/postnatal development study (eePPND), in a 96 well format.

c.  Small size and transparent body allows testing in a 96 well format, combining scale and throughput of in vitro systems with whole animal research.

d.  Small size and livebearer status allows testing for embryo/fetal development (EFD) in a 96 well format.

ANSWERS

1.  a

2.  a

3.  b

4. c

**Niemi and Davies. Animal Research, the 3Rs, and the “Internet of Things”: Opportunities and Oversight in International Pharmaceutical Development, pp. 246-253**

Domain 5: Regulatory Responsibilities

SUMMARY: Stages of drug (and vaccine) discovery and evaluation that involve laboratory animals increasingly occur via scientific collaborations across national borders and continents. Many of these research collaborations are between asset-rich institutions and others in less wealthy parts of the world. The care and use of laboratory animals in geographically disparate locations introduces new complexities, such as different oversight requirements and available resources, as well as diverse organizational and cultural milieus. These complexities can hamper the effectiveness of local animal welfare committees and regulatory compliance, as well as compromise good science and animal welfare. At the same time, new technologies are becoming available that offer greater transparency in how these collaborations and their animal subjects are faring in real time that, in turn, can enable progress towards the 3 Rs. The focus of this essay is to identify potential rewards and risks stemming from new techniques for producing and connecting data in preclinical pharmaceutical development and consider how further social scientific investigations have the potential to enhance the benefits of international research collaborations for both human health and animal welfare.

The “internet of things” (IOT) has transformative potential across the biological sciences: adding geolocation and micro-environmental information to data sets, connecting infrastructures across experimental sites, and redistributing roles and relations across international settings, giving researchers the capacity for technologically enhanced traceability, connectivity, and communicability. Given the opportunities and challenges that internet-connected devices offer to laboratory animal research, plans for their use should be deliberated in advance by IACUCs. Any potential benefits depend on the development of protocols for data collecting, sharing, accountability, and access, as well as adequate technology platforms to support data storage and exchange.

Potential negative consequences include adding regulatory burdens to cooperating institutions, overcoming resistance to the thought of providing “outsiders” with streaming access to one’s internal workings, the replacement of skilled workers with technology, and information safety, as transmitting digital information between parties renders that information more vulnerable to disruption or distortion. Pharmaceutical firms that oversee and sponsor large-scale clinical trials could serve as a helpful source of information on data collection and security, as they have experience with multi-site patient trials involving more than one nation, conducted under assorted institutional review boards and regulatory agencies.

QUESTIONS

1.  True/False. Greater transnational use of laboratory animals is being facilitated by the lower costs associated with animal research in low- and middle-income countries (LMICs), especially with nonhuman primates and especially in China.

2. True/False. The ARRIVE Guidelines are often over and above local institutional and national requirements for the care and use of laboratory animals.

3.  All of the following are given as examples of remote animal care and use monitoring applications, EXCEPT?

a. Ambient temperature

b.  Motion

c.  Welfare

d.  Colony health

ANSWERS

1.  True

2.  True

3.   c. “While some aspects of animal research can be automated for the benefit to animals and efficiencies, in other areas “a feel for the animal” remains important for both science and welfare.”