Testing Times in Toxicology – *In Vitro* vs *In Vivo* Testing

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Summary

Initiatives such as the "Human Toxome Project," which aims to map the "Pathways of Toxicity" (PoT) in man illustrate a trend that moves away from our current reliance on high-dose animal toxicity studies to a wide range of new tools such as functional genomics, proteomics, metabolomics, high data content screening, pharmacokinetic modeling, and systems biology to study the effects of chemicals on cells, tissues, and organisms in a rapid and cost-efficient manner. These technologies are also paving the way to improve the evaluation of health risks posed by chemicals found at low levels in the environment. This convergence of factors, coupled with the need to evaluate the safety of an increasingly large number of chemicals and their mixtures, has prompted a call for a fundamental paradigm shift in toxicology testing.

In this scenario, we are seeing a sure and fast transition in toxicology testing from animal tests to in vitro methods, virtual tissues, robotic automation, and beyond, where mathematical modeling, structure activity relationships of chemical molßecules, and computational toxicology are being used to predict toxicity with precision and high throughput.

This brings us back to the eternal philosophy that says the ultimate search for truth culminates in nonviolence. As Indians we cannot but honor the Great Mahatma, who said, "I abhor vivisection with my whole soul. All the scientific discoveries stained with innocent blood I count as of no consequence." The science of alternatives is to know that humane science is better science, being precise, predictive, and pain-free.

Keywords: toxicity testing, in vitro, in vivo, animal alternatives

1 Introduction

"*Absence of evidence is no evidence of absence*" – Carl Sagan, US astronomer (1934-1996)

In the context of animal toxicity testing Hartung and McBride (2011) evoked the above quote to explain the fallacies we see in live animal testing. However, for reasons unknown or maybe due to the lack of better and more humane testing strategies, animal testing was in the past and, unfortunately, continues to be the gold-standard in the toxicity evaluation of pharmaceuticals, cosmetics, agrochemicals, and industrial chemicals. Even with the evolution of science to more robust, precise alternatives, both "humane and human," the shift has been slow despite the fact that regulatory authorities, both in the USA and the EU, have withdrawn close to 50 important drugs from the market in the last 50 years, even after they had been proved safe/efficacious, having been tested with the whole gamut of animal studies and clinical trials as prescribed by regulatory agencies. The worst and biggest tragedies resulted from the use of thalidomide and sulfanilamide. A more recent drug (drotrecogin alfa), once believed to be useful in treating patients with severe sepsis, has been withdrawn from the market because new evidence revealed – 10 years after the drug was approved by the US Food and Drug Administration (FDA) – that it is not effective for severe sepsis after all. This drug was withdrawn by Eli Lilly and Company on October 25, 2011.

False negatives have been one of the biggest and seemingly insurmountable problems in animal testing. A negative animal test could mean nothing or so many things. A different species or simply a different experimental variation may yield positive results. Animals have different defense mechanisms that may lead to false or skewed results, and the stress of being caged, being held in unnatural conditions, and being handled, in itself, could be responsible for result vagaries. Furthermore, the rodent to human extrapolation factor, which is intrinsic and inevitable to animal toxicity data, must be considered. Physiological responses to stress may be an add-on to a positive result, camouflage a toxicity indicator, pose variations to a toxicity pathway, exaggerate, or maybe just trigger a toxicity pathway that may be absent in the absence of stress or another variable.

The scientific understanding of how genes, proteins, and small molecules interact to form molecular pathways that maintain cell function has evolved rapidly, thanks to advances in molecular and computational tools. This knowledge gives non-animals methods the edge over *in vivo* testing, taking away that adjunct of variations that come in animal testing due to the pain, stress, and distress that laboratory animals suffer during

an experiment. This unassessed and un-quantified variable of stress interferes with physiological processes and disrupts critical pathways, interfering with the experimental design, results, and observations, thereby underplaying or exaggerating the ultimate inference of the level of toxicity. The absence of this variable in non-animal testing makes the tests more robust, adding high throughput and high precision, rendering a better prediction potential. Reconstructed human tissues, "neurons on microchips," "organs-on-a-chip," and "brains-in-a-test tube," computational toxicology, and "-omics" have taken toxicology testing to greater heights in human risk assessment, allowing scientists to work with real-world human exposure levels and real-life predictable, human results.

2 The evolution of testing strategies in pyrogen testing – An example of the development of a "humane and human" alternative

Twenty years ago, if one spoke of "fever in a test tube," it would have been seen as the proverbial rabbit-from-the-black-top-hat kind of magic, rather than a scientific discovery. In the late 1990s – somewhere between the rabbit and the test tube in the history of pyrogen testing – came the Horseshoe Crab Assay (Limulus Amoebocyte Lysate Assay or LAL), which was an effective replacement for the rabbit pyrogen test. It was based, however, on the hemolymph of the horseshoe crab. In 2009, the evolution of a non-animal alternative in pyrogen testing took another step forward in replacing the ubiquitous rabbit pyrogen test. This time, the world saw a ground-breaking discovery with the use of cryopreserved human whole blood as an effective and efficient tool for detecting fever-causing bacteria. No rabbits – with or without a magic hat – are used in this test.

In this *in vitro* pyrogen test, where one tests fever in a test tube, the sample is incubated with fresh or cryopreserved human whole blood, and the proinflammatory cytokine interleukin-1β (IL-1β) is detected by an enzyme-linked immunosorbent assay (ELISA). In addition to detecting pyrogenic contaminations in aqueous samples, e.g., parenteral drugs, adaptations allow the assessment of lipidic, toxic, or immuno-modulatory substances; detection of low-grade contaminations in large-volume parenterals, e.g., dialysis, water, and fluids; pyrogenicity assessment of solid materials, e.g., medical devices; and evaluation of airborne pyrogenic burden. It is superior to both the rabbit pyrogen test and the Limulus Amoebocyte Lysate (LAL) test in that it also detects nonlipopolysaccharide pyrogens, and the procedure takes 21-35 h to complete (Daneshian et al., 2009).

3 Toxicity testing and the use of animal alternatives – The 21st century scenario

3.1 The ToxCast program

The United States Environmental Protection Agency (EPA) launched ToxCast[1] in 2007 to develop ways to predict potential toxicity and to develop a cost-effective approach for prioritizing the thousands of chemicals that need toxicity testing. ToxCast uses advanced science tools to help understand how human body processes are affected by exposure to chemicals, and it helps determine which exposures are most likely to lead to adverse health effects. ToxCast testing methods include more than 650 state-of-the-art rapid tests (called high-throughput assays) that can screen more than 2,000 environmental chemicals for potential toxicity. Phase I, "Proof of Concept," was completed in 2009, and it profiled more than 300 well studied chemicals (primarily pesticides). Phase I chemicals have more than 30 years' worth of existing toxicity data, since they have been tested already using traditional toxicology methods (primarily animal studies). Data from animal studies can be searched and queried using EPA's Toxicity Reference Database (ToxRefDB) that stores nearly $ 2 billion worth of studies. Phase II currently is screening 1,000 chemicals from a broad range of sources, including industrial and consumer products, food additives, and drugs that never made it to the market, to evaluate the predictive toxicity signatures developed in Phase I. Data from the high-throughput assays is available via the ToxCast Database. Toxicity signatures from ToxCast are defined and evaluated by how well they predict outcomes from mammalian toxicity tests and identify toxicity pathways relevant to human health effects.

3.2 The Tox21 program

The robotic testing of a 10,000-compound library marks the beginning of a new phase of toxicity testing. An ongoing venture of the National Institutes of Health, the U.S. Environmental Protection Agency, and the U.S. Food and Drug Administration, referred to as Tox21[2], was launched on December 15, 2011. It signifies a collaboration that will move science forward. In this program 10,000 chemicals will be screened for potential toxicity by a high-speed robotic screening system. The project aims at protecting human health by improving the way chemicals are tested in the United States. The compounds cover a wide variety of classifications and include consumer products, food additives, chemicals found in industrial processes, and human and veterinary drugs. Each test compound will undergo a thorough chemical analysis to verify its identity and determine its purity, concentration, and stability. The goal of the testing is to provide results that will be useful for evaluating whether these chemicals have the potential to disrupt processes in the human body to an extent that leads to adverse health effects. The Tox21 partnership integrates advances in molecular biology, chemistry, and computer science, enabling fast and low-cost

[1] http://www.epa.gov/ncct/toxcast/
[2] http://www.epa.gov/ncct/Tox21/

screening of the thousands of chemicals in use, and it will help generate the toxicity data of these chemicals, which in turn will enable the production of safer drugs and also help identify unsafe drugs.

3.3 The Human Toxome project

Taking toxicity testing to a still higher platform are scientists who have embarked on a pioneering and novel project along the lines of the "Human Genome Project," in this case called the "Human Toxome" project, led by Dr Thomas Hartung, Director, CAAT, Johns Hopkins University. The project envisages the mapping of the "Pathways of Toxicity" (PoT) in man. PoT include pathways of endocrine disruption, perturbations of the hormonal system or other physiological disruptions that manifest themselves as adverse health effects, tumors, birth defects, developmental disorders, etc. The vision is to produce a suite of *in vitro*, sub-cellular, and *in silico* tools that comprehensively represent the human toxome. Once a PoT is identified, the construction of the test system will not be difficult (Hartung and McBride, 2011). Currently, toxicity testing typically involves studying adverse health outcomes in animals subjected to high doses of toxicants, with subsequent extrapolation to expected human responses at lower doses. At present, humans are potentially exposed to more than 80,000 chemicals for which no toxicity data exists.

According to the same authors, the challenge we face as scientists is to turn around the testing paradigm of regulatory safety assessments from phenotypical tests to tests based on a mechanistic understanding identified on the basis of known human toxicants. According to them, our current understanding of systems biology and a host of molecular, informational, and computational tools provide the potential to identify PoT to evaluate the effects of tens of thousands of chemicals at concentrations relevant to human exposure levels. "*In contrast to the currently used phenomenological "black box" animal testing, pathways of toxicity (PoT) will be identified in human in vitro systems to provide more relevant, accurate, and mechanistic information for the assessment of human toxicological risk*," they opine. The goal is to map the entirety of the human toxome. The concentration at which a substance triggers a PoT then can be extrapolated to a relevant human blood or tissue concentration and, finally, a corresponding dose by (retro-) PBPK (physiology-based pharmacokinetic) modeling, thereby informing human risk assessment. Perhaps more importantly, if a substance does not trigger any PoT, it may be possible, for the first time, to establish the lack of toxicity, i.e., safety of a substance at a given concentration. A comprehensive list of PoT, the mapped human toxome, can become a cornerstone of this new regulatory toxicology. Project "Human Toxome" will use integrated testing strategies that combine transcriptomics and metabolomics data with computational models with a view to creating a public database of PoT, enabling full access to researchers around the world. This understanding of toxicity pathways could serve as the background, rendering the basic cues for a humane or, more importantly, a "human" alternative in toxicity testing.

3.4 AXLR8

Advances made in molecular biology, biotechnology, and other fields are paving the way for major improvements in how scientists evaluate the health risks posed by potentially toxic chemicals found at low levels in the environment. These advances would make toxicity testing quicker, less expensive, and more directly relevant to human exposures. They also could reduce the need for animal testing by substituting more laboratory tests based on human cells.

Based on this premise, the AXLR8[3] program has been launched by the European Union. The European Commission currently is funding a number of research consortia to develop new 3Rs (replacement, reduction, and refinement) test methods and strategies as potential alternatives to the use of animals in safety testing. AXLR8 aims to fulfill this growing need by providing a focal point for dialogue and collaboration. Specifically, AXLR8 aims to:

- Organize a series of annual workshops to monitor research progress on alternative testing strategies.
- Provide a range of tools and opportunities for enhanced interdisciplinary and international communication, coordination, and collaboration in order to maximize the impact of available resources.
- Work to streamline regulatory acceptance procedures to provide for the uptake of validated 3Rs methods, including a smooth transition to 21st century systems as they become available.

The ultimate goals are to assess safety of a much larger number of substances and mixtures than is currently possible, more rapidly, efficiently, and cost-effectively than at present, and in systems that may be more relevant to toxicity in humans and that will be capable of identifying the cellular mechanisms at the root of toxicity and disease, using fewer animals and with the final aim of complete replacement of animals. Instead of focusing on signs of gross toxicity at high doses in living animals, AXLR8 will focus on the "21st century" approach advocated by leading scientific and regulatory authorities, working towards a mechanistic understanding of how chemicals interact with cellular response pathways in the human body at environmentally relevant exposure levels. As critical pathways are identified, human cell-based tests will be developed to study chemical interactions at key cellular and molecular targets within a pathway. Through robotic automation, cell-based *in vitro* methods will enable the high throughput testing of thousands of substances in a single day. Data from toxicity pathway assays then could be integrated and interpreted with the aid of systems biology tools controlling pathway function and be combined with pharmacokinetic modeling to relate *in vitro* conditions to real-world exposure levels.

[3] http://axlr8.eu/overview/

3.5 Sens-it-iv Project

Twenty-eight groups, of which nine represent industry, fifteen represent universities or research institutes, and four represent organizations in the EU, have joined hands to initiate the program Sens-it-iv[4]. The ultimate goal of this project is to develop an *in vitro* test strategy to predict the sensitizing potency of compounds, enabling the full replacement of animals in safety testing.

To date, there are no validated *in vitro* models for skin and respiratory sensitization. In addition, there is no widely accepted *in vitro* screening approach for early identification of potential sensitizers. Given the complex mechanisms involved in sensitization, it is unlikely that a single *in vitro* test will be able to substitute for the existing animal tests. Sens-it-iv attempts to overcome these limitations by exploring innovative approaches and integrating existing knowledge on the cellular and molecular mechanisms involved in sensitization based on assay systems that model sensitization, rather than irritation and toxicity of chemicals and proteins.

Moreover, since the cell culture models that will be used in Sens-it-iv are of human origin, they might prove to be more predictive than the current animal models of toxicity in man. This new knowledge also will help in designing safer drugs, vaccines, and many other materials of value to the EU.

Deliverables from Sens-it-iv will be *in vitro* tests that will be ready for formal validation according to international standards for subsequent international regulatory acceptance and, finally, for world-wide application in industry, regulatory establishments, and elsewhere.

3.6 Non-animal toxicity tests scientifically validated as alternative testing strategies for regulatory testing and biomedical research

Validation, in the context of non-animal toxicity testing, is the process by which the scientific prowess of a non-animal testing strategy is evaluated for the purpose of regulatory testing of agrochemicals, industrial chemicals, cosmetics, and pharmaceuticals. Validation criteria for new toxicological test methods in use today were developed by three organizations: the Organization for Economic Cooperation and Development (OECD), the European Centre for the Validation of Alternative Methods (ECVAM), and the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM). These organizations are sometimes called validation authorities.

More than 25 years ago, the OECD recognized the need to protect animals in general and, in particular, those used in experimental work. The progress in OECD on the harmonization of chemicals control, especially the agreement on Mutual Acceptance of Data (MAD), has helped greatly to reduce the number of animals used in testing by reducing duplicative testing. MAD states that data generated in the testing of chemicals in an OECD member country in accordance with OECD Test Guidelines and OECD Principles of Good Laboratory Practice (GLP) shall be accepted in other member and adhering non-member countries. These proactive Council Decisions still save thousands of animals every year, and an increasing number of non-OECD economies adhere to MAD.

The OECD is committed to implementation of the 3Rs principles (Replacement, Reduction, and Refinement), as first laid down by Russell and Burch (1959). Probably the most noteworthy achievement is the deletion of the much criticized Test Guideline 401 on Acute Toxicity Testing and its replacement with Test Guidelines 420, 423, and 425, introducing reduction and refinement. Other examples include the Local Lymph Node Assay (Test Guideline 429) introducing refinement and reduction compared to Test Guideline 406, and the Test Guideline 428 on "*Skin Absorption: In Vitro Method*," offering an alternative method to Test Guideline 427, among others.

Another example of the commitment of the OECD to the implementation of the 3Rs principles into regulatory toxicity testing is the development of the Guidance Document No. 19 on "*the Recognition, Assessment and Use of Clinical Signs as Humane Endpoints for Experimental Animals Used in Safety Evaluations*" from 2000. Guidance Document No. 19 gives practical guidance on how to apply the 3Rs principles, with an emphasis on refinements when performing OECD Test Guidelines.

The validated alternatives that can be used in regulatory and biomedical research are elaborated in Table 1**.** The INVITTOX database provides tried and tested *in vitro* toxicity assays that can be used in biomedical research. The aim of INVITTOX protocols is to present precise and up-to-date technical information on the performance of the *in vitro* techniques currently in use and under development, their applications, advantages, and drawbacks. The information is obtained directly from those scientists already employing such methods. Each protocol includes a detailed methodology sufficient to enable another researcher to carry out a procedure; experimental data, where available; the rationale for choice of technique and endpoint; and critical assessment comments about the accuracy of the system, its sensitivity, ease of implementation, shortcomings, etc. It is envisaged that making all this information available in one document will help scientists to select the systems most appropriate to their needs.

4 Ban on animal use in toxicity testing for cosmetics in the EU

An amendment to the European Union's Cosmetics Directive (76/768/EEC) phases out the use of animals in testing for any acute toxic effects of beauty products and toiletries.

The Cosmetics Directive was introduced in 1976 to enforce high safety standards for cosmetics across the EU Member States. It was amended in 1993 to phase out the use of animals in testing, but the amendment was never implemented because

[4] http://www.sens-it-iv.eu/

Tab. 1: Non-animal toxicity tests validated as alternative testing strategies for regulatory testing and bio medical research

Test	Alternative (Replacement/Reduction/Refinement)
Eye corrosion	Bovine Corneal Opacity and Permeability (BCOP) assay and Isolated Chicken Eye (ICE) assay
Acute systemic toxicity	3T3 NRU assay / NHK NRU assay
Acute Oral Toxicity	Up-and-Down Procedure(UDP) / Fixed Dose Procedure (FDP) / Acute Toxic Class method (ATC)
Embryotoxicity/Teratogenicity	Embryonic stem cell test (EST) / Micromass test / Whole rat embryo culture
Phototoxicity	3T3 Neutral Red Uptake Phototoxicity Test
Skin corrosion	EpiSkin® – *in vitro* human skin model / Rat Skin Transcutaneous Electrical Resistance (TER / SkinEthic) – *in vitro* human skin model Corrositex® – noncellular membrane / Vitrolife-Skin RHE
Skin irritation	Reconstructed Human Epidermis Test – EpiSkin™ / EpiDerm™ SIT (EPI-200) / SkinEthic™ RHE
Pyrogenicity	Human cryopreserved whole blood IL-1 / Limulus Amebocyte Lysate (LAL) test
Skin sensitization	Local Lymph Node Assay: BrdU-ELISA
Reproductive toxicity	"Two-generation" animal test replaced by "extended one-generation" method
Endocrine disruptor screening assay	(ER)-alpha Transcriptional Activation Assay / H295R Steroidogenesis Assay/ Aromatase Inhibition Assay/ BG1Luc ER TA Test Method
Batch potency testing of vaccine	ELISA test for erysipelas vaccines / ELISA test for human tetanus vaccines/ Toxin binding inhibition (ToBI) test for human tetanus vaccines
Monoclonal antibody production	*In vitro* MAb production systems
Genotoxicity testing	Mammalian Cell Micronucleus Test / Bacterial Reverse Mutation Test (Ames Test)* / Genetic Toxicology: Saccharomyces cerevisiae Gene Mutation Assay* / *In vitro* Mammalian Chromosome Aberration Test* / *In vitro* Mammalian Cell Gene Mutation Test* / *In vitro* Sister Chromatid Exchange Assay in Mammalian Cells* / DNA Damage and Repair, Unscheduled DNA Synthesis in Mammalian Cells *In vitro**

*not validated but accepted by OECD/EPA

no alternative, animal-free tests had been approved. A stricter amendment developed in 2003 (Directive 2003/15/EC) forced a ban on the use of animals in the testing of finished cosmetics products within one year and imposed two further deadlines to phase out animal testing on any ingredient in a cosmetic product – regardless of whether alternative tests were available. As of March 11, 2009, the first of the two deadlines outlaws the use of animals in seven mandatory tests of toxicity following a single application. These are tests for skin irritancy, sensitivity to light, corrosivity, and absorption through the skin, genetic toxicity, eye irritancy, and acute toxicity. The amendment also bans the import of cosmetics containing ingredients that have been tested on animals in this way after the deadline.

The second deadline, March 11, 2013, would see a ban on eight tests designed to establish longer-term toxicity following multiple applications, for example their ability to cause cancer or birth defects. This deadline may be renegotiated, however.

Europe is a world leader in cosmetics, with global sales approaching € 80 billion (US $ 102 billion). That is nearly half of the world market. There are around 2,000 cosmetics manufacturers in the European Union, including some of the world's largest, such as L'Oréal and Estée Lauder, and they sell five billion items every year.

Alternatives to four of the seven tests banned today have been validated and approved by the European Union, and work on the other three has advanced. During this time, the cosmetics industry will not be able to introduce new products that include chemicals not tested before the cut-off date. The European Union has been pumping € 35 million into efforts to develop alternative methods every year since the Cosmetics Directive was amended. EU Member States are estimated to put in a combined total of a further € 25 million per year. The European Union has implemented a systematic pan-European research program to find alternatives, coordinated with the smaller efforts that are going on elsewhere in the world (Abbott, 2009).

The cosmetics industry itself has contributed € 25 million for developing alternatives, which was matched by a further € 25 million by the European Union.

5 The Socio-scientific perspective to adopt alternatives and the Indian Scenario

"The thinking person must oppose all cruel customs no matter how deeply rooted in tradition or surrounded by a halo…. We need a boundless ethic which includes the animals also." (Albert Schweitzer, Philosopher and Physician, Nobel Peace Prize Winner 1952)

Albert Einstein went on to say "*Our task must be to free ourselves – by widening our circle of compassion to embrace all living creatures and the whole of nature and its beauty.*"

Nonviolence has been an echoing and boundless truth, in all religions and in every philosophy, transcending every human culture, race, or profession.

The question posed here is: Today when we are in knowledge and convinced with evidence that alternatives to animal testing far excel in precision and prediction, does this not bring us back to history and the eternal philosophy that says the ultimate search for truth culminates in 'Non-violence'? At this juncture, as Indians we cannot but honor Mahatma Gandhi, the "Father of our Nation," who said more than half a century ago, "*I abhor vivisection with my whole soul. All the scientific discoveries stained with innocent blood I count as of no consequence*." Every sage, saint, and philosopher spoke about "ahimsa" as the ultimate truth that goes beyond just being a way of life but that which has a bearing on every aspect of our lives, be it societal, environmental, or educational. "Ahimsa" is a truth that seems to go beyond scientific reason and mathematical algorithms.

India has surged ahead among nations in the last decade in promulgating laws favoring the welfare of animals and in promoting the science of alternatives (Shiranee, 2006). In the field of life science education, we have become a country to be emulated, with the University Grants Commission, Government of India, restructuring the Zoology curriculum with the ultimate aim of phasing out dissection (Akbarsha and Shiranee, 2010). Earlier, in 2004, the CPCSEA, the statutory body of the Government of India that controls and supervises the use of animals in experimentation, amended the "Breeding of and Experimentation of Animals Rules 1998," based on a sentience scale of animals (Anon., 2004; Shiranee et al., 2005). Five new principles included in elaboration of the 3Rs were formulated for the reduction of the use of animals: refinement by way of mandatory use of analgesics and anesthetics, guiding principles if animals have to be euthanized, and the mandatory rehabilitation of large laboratory animals after use was incorporated in the law governing laboratory animal use, and the concept of the 4th R *viz.*, "Rehabilitation" was made legally binding on scientists and researchers (Shiranee and Massimo, 2005).

6 Conclusions

The underlying and essential point here is to comprehend that science is evolving, and evolving to better models, precision, and higher throughput, simultaneously giving us the benefit of establishing humane standards in toxicity testing. A welcome and undeniable need is that these developments are in recognition of the desire for better medical care and safety standards for humanity. Higher understanding of toxicokinetic studies will help in making safer and better life-saving drugs and will help set robust standards for better health care. Such global initiatives to move from our current reliance on high-dose animal toxicity studies to a wide range of new tools such as functional genomics, proteomics, metabolomics, high data content screening, pharmacokinetic modeling, and systems biology, to study the effects of chemicals on cells, tissues, and organisms in a rapid and cost-efficient manner, is the sweeping change we observe in toxicology testing today.

High-dose animal toxicity studies and the application of extrapolation procedures focus on signs of gross toxicity, which results in uncertainty when used in human health risk assessment, as observed by tragedies associated with new drugs / therapeutics, and dozens of drugs that have been taken off the market in the last century due to their adverse side effects in humans but that were not manifested or predicted by animal studies. The sooner we realize this need to change the better. It may be simply to realize, as Dr Thomas Hartung succinctly observed, "*We are not 70 kg rats*."

The scientific understanding of how genes, proteins, and small molecules interact to form molecular pathways that maintain cell function has evolved rapidly, thanks to advances in molecular and computational tools. This knowledge gives non-animals methods the edge over *in vivo* testing, taking away that adjunct of variations that arises in animal testing due to the pain and distress that laboratory animals suffer during an experiment. This unassessed and unquantified variable of stress, which interferes with physiological processes and disrupts critical pathways, interferes with the experimental design, results, and observations and, thereby, undermines the ultimate inference of the level of toxicity, essentially underplaying or exaggerating the toxicity. The absence of this variable in non-animal testing makes the tests more robust, adding high throughput, high precision, and a better prediction potential. Reconstructed human tissues, "neurons on microchips," "organs-on-a-chip," and moving further ahead to computational toxicology and "-omics", have taken toxicology testing to greater heights in human risk assessment, giving scientists the possibility of working with real-world human exposure levels.

To promote this "humane science" of non-animal testing is to be able to understand that this is a science that needs to be promoted and practiced, not just for ethical reasons but in that it gives a three-fold advantage of being precise, predictive, and pain-free (the 3 Ps – Precision, Predictiveness, and Painlessness).

To quote William Russell and Rex Burch (1959): "*If we are to use a criterion for choosing experiments to perform, the criterion of humanity is the best we could possibly invent. The greatest scientific achievements have always been the most humane and the most aesthetically attractive, conveying that*

sense of beauty and elegance which is the essence of science at its most successful."

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