Comment

Human-based Systems in Drug and Chemical Safety Testing — Toward *Replacement*, the 'Single R'

Robert A. Coleman

Independent Consultant, Falmouth, UK

Summary — The Three Rs was a concept originally conceived as a means of reducing the suffering of laboratory animals that are used largely in identifying any potential safety issues with chemicals to which humans may be exposed. However, with growing evidence of the shortcomings of laboratory animal testing to reliably predict human responsiveness to such chemicals, questions are now being asked as to whether it is appropriate to use animals as human surrogates at all. This raises the question of whether, of the original Three Rs, two — *Reduction* and *Refinement* — are potentially redundant, and whether, instead, we should concentrate on the third R: *Replacement*. And if this is the best way forward, it is inevitable that this R should be based firmly on human biology. The present review outlines the current state-of-the-art regarding our access to human biology through *in vitro*, *in silico* and *in vivo* technologies, identifying strengths, weaknesses and opportunities, and goes on to address the prospect of achieving a single R, with some suggestions as to how to progress toward this goal.

Key words: *human-based testing,* in vitro, in vivo, in silico.

Address for correspondence: *robt.coleman@btinternet.com*

Introduction

Over 50 years ago, Russell and Burch (1), concerned about the suffering caused to laboratory animals in medically-related research, proposed the Three Rs. From an ethical standpoint, this was a hugely laudable proposal. However, since that time, it has become clear that there is another dimension to the issue. It is now widely appreciated that the use of non-human species as surrogates for humans in the exploration of disease aetiology and the potential safety and efficacy of chemicals, such as medicines, cosmetics and industrial pollutants, is critically flawed as a result of important inter-species differences. This reliance on non-human biology to predict human responsiveness is not only causing hugely wasted expenditure, but is also endangering human lives and well-being. Hence the urgent need to find realistic alternatives, putting the spotlight firmly on *replacement*. While *reduction* and *refinement* might address the ethical issue, they ignore the fundamental weakness of the relevance argument, and a focus on them may even serve to propagate the *status quo*.

So, if we are to replace the current paradigm, there seems to be no logical alternative to a focus on human biology, and *in vitro*, *in silico* and *in vivo* approaches should all be considered. There has long been scepticism in many quarters with regard to the ability of any such human-based approaches to substitute for whole animal testing, and this has been an argument for the perpetuation of the widespread use of laboratory animals. However, non-human species, even if closely related to humans, can never be wholly satisfactorily substitutes. With an ever-expanding range of human-based technologies at our disposal, it is surely only a matter of time before the hitherto seemingly impossible becomes commonplace. Although there is a need for more human-based approaches to establish both efficacy and safety, it may be argued that the most pressing need is in the better detection of potential toxicity. The following overview is particularly focused on this aspect of their role.

Availability of Human Biomaterials

If we are to expand and extend our use of human-based approaches, of fundamental importance is the availability of human cells and tissues suitable for experimental purposes. While this has always been a difficult area, both on the grounds of ethics and logistics, these issues are increasingly being overcome (2). A certain amount of experimental work has long been conducted on cells and tissue samples obtained from human subjects, be these

healthy, sick or deceased, but the regulations governing such work were ill-defined or absent. This resulted, at one extreme, in difficulty in obtaining the necessary materials, and at the other, frankly illegal activities, ending in high profile public scandals (3). Such problems led to the publication, in 2004, of the *Human Tissue Act* (4). While this Act is not without its critics and calls for changes, it has provided a framework within which human tissue work can be undertaken; issues that hitherto were in murky legal and/or ethical waters, are at least now resolved in terms of what is legally and ethically acceptable and what is not. The fact that regulations are now in place, does not necessarily mean that obtaining the necessary specimens is a straightforward process — indeed, it is not. It still depends absolutely on the presence of champions at every stage of the process, and many of these champions are within the healthcare system and already have extraordinarily demanding roles, without the extra burden of facilitating sample access for would-be human tissue researchers. However, none of the bottlenecks that remain are insuperable, if there is sufficient commitment.

Cells and tissues can be retrieved from several sources, each of which presents its own opportunities and limitations (see Table 1).

Uses of Human Biomaterials

Human biology can be utilised in various ways, each of which has its own problems and possibilities. Although, historically, much work has been performed by using two-dimensional (2-D) cell culture in static dishes, it is a format that has limited resemblance to the physiological state. In recent years, considerable advances have been made in overcoming this limitation through various formats of three-dimensional (3-D) culture (6) and the heterologous co-culture of relevant cell types (7). While various co-culture approaches have been used in attempts to retain the relationships between different cell types that occur in native tissues, the simplest way of ensuring the presence of cells in their natural heterogeneous relationships is to use whole sections of tissues. These can be prepared as cubes, strips, rings or precision-cut slices, depending on the particular tissue and the intended assay format. The use of small strips (and rings for tubular structures, such as blood vessels and airways) maintained in warmed, oxygenated physiological fluid, has been commonplace for pharmacological studies for decades, and is particularly useful where changes in muscle length, vessel diameter or contractile force are the objective (5).

One drawback of using human tissue strips is anoxia within the centre of the tissue, which in effect means that much of the tissue is dead. This can be largely overcome by the use of precision-cut tissues slices (PCTS), which, due to their large surface area:volume ratio, suffer much reduced anoxia and therefore increased viability. Although other *in vitro* methods with human material are successfully employed to predict absorption–distribution–metabolism–excretion (ADME) and toxicity, the use of PCTS is the only one that fully represents the tissue architecture and multi-cellularity, combined with overall tissue sample viability. There is ample available literature showing the potential of liver, intestinal and lung slices to represent the *in vivo* metabolic functions of these organs, and several comprehensive reviews have been published recently, highlighting the value of human PCTS for the study of drug metabolism and toxicity (8, 9).

Where the objective of a study is to determine ion flux or drug transport across a cellular membrane, isolated mucosal layers or other such biological membranes can be mounted in Ussing chambers or similar formats, where the bathing solutions on each side of a membrane are separated by that membrane. Ion flux can then be measured as changes in current, and drug transport across the membrane can be assessed by the appearance of compound in the fluid on the other side of the membrane. This type of approach is particularly useful for membranes that have a natural barrier function, such as epithelia and endothelia (10, 11).

One challenge to studying isolated cells and tissues is the assessment of function. Many functions, such as smooth muscle contraction, cell movement and electrical activity, are simple to monitor; others are rather more complex, and require purpose-built devices, such as microphysiometry (measuring drug/chemical-induced changes in metabolic activity; 12, 13) and fluorometric imaging plate reader systems (FLIPR, e.g. for measuring calcium fluxes; 14), both of which can be used to explore both efficacy and toxicity of test compounds.

In addition, there is increasing interest in high-content assays involving human cells, and these promise great utility in the identification of therapeutic potential and side-effect liability. Such technologies may identify patterns of drug-induced injury, and the release of proteins and other mediators (15, 16), including transcription factors (17). The perceived value of such approaches has resulted in at least two high-content technologies — Attagene's Cis-Factorial™ multiplexed transcription reporter technology, and BioSeek's mechanism of action signature technology, BioMAP®, which is included in all three phases of the US EPA's ToxCast programme (18).

Table 1: Sources of human fresh isolated tissue for research

Sources of human fresh isolated tissue	Organisation/ provider	Typical applications	Advantages	Disadvantages
Post mortem	Pathology department or biobank.	Target identification and validation, rather than functional studies.	A wide range of tissues are available that are difficult or impossible to obtain from surgery.	*Post mortem* interval (PMI) for collection of tissue is typically hours rather than minutes; tissue quality might be compromised. Not suitable for most functional assessments, but might be the only available access route.
Residual surgical	Surgical or pathology departments, or biobank.	Target identification and validation; studies in target disease tissue; functional studies of safety and efficacy.	Rapid collection of tissues; wide range of diseased tissues available which are a valuable model for drug.	Main purpose of collection is diagnosis, so aligning collection procedure with requirements for research can be difficult; often the leftover tissue sample following pathologist's analysis.
Non-transplantable organs	Organ procurement organisation (OPO) or biobank that liases with OPO.	Functional studies of safety and efficacy; may also be used for development of surgical implants or medical devices.	Freshness and volume of tissue; ability to receive healthy control tissues.	Frequency of tissue is much less than for residual material and costs are much greater.
Clinical biopsies	Clinical research organisation; clinical research department of some hospitals.	Biomarker studies as part of a clinical trial; collection of diseased tissues from specific patient groups, e.g. psoriasis or atopic dermatitis.	Collection conditions can be specified exactly. Allows more extensive investigations of safety and efficacy in patients during a clinical trial, e.g. measurement of surrogate markers of clinical effects.	Time and costs involved in organising and planning the study, very small amounts of tissue may be available and may not be practical to retrieve the tissue of interest.

Modified from Reference 5.

Use of Stem Cell Technologies

Stem cells have huge potential in permitting human-based *in vitro* toxicity testing, with significant advances already made in the areas of cardiotoxicity, hepatotoxicity and neurotoxicity (19). The protocols for deriving human induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs) are now well established, and result in cells exhibiting spontaneous rhythmic beating, which can be maintained in culture for long periods. Such cells are amenable to electrophysiological measurements through patch clamp and multi-electrode array (MEA) technologies, and also to fast kinetic fluorescence imaging with calcium-sensitive dyes and assays for mechanical contraction. The ability of such cells to detect clinical cardiotoxicity has been demonstrated through a study of a range of reference drugs with known cardiotoxic liability (20). It has also been shown that hiPSC-CMs have utility in demonstrating patient-specific sensitivity to cardiotoxicity (21), and in identifying risk of drug-induced arrhythmias (22, 23).

While stem cell-based assays to test for potential hepatotoxicity are at an earlier stage of development, proof-of-concept studies are encouraging. By using a high-throughput approach, Sirenko *et al.* (24) examined a number of assays and phenotypic markers, and developed automated screening methods to evaluate the effects of a diverse library of 240 known hepatotoxicants on hiPSCs. The establishment of robust protocols to derive metabolically active hepatocytes, and the co-culturing of these hepatocytes with other non-parenchymal cells, such as endothelial cells, stellate cells and Kupffer cells in 3-D microfluidic liver platforms, are foreseen to better-model liver physiology for effective toxicity assessment (25).

Although, for obvious reasons, assays for CNS toxicity have been more challenging to establish than those for toxicity of the heart or liver, significant progress has been made. Human iPSCs can provide subtype-specific neurons and glial cells, and their vulnerability to drug-induced toxicity can be tested *in vitro* by means of assays such as neurite outgrowth and synapse activity. By using a standard patch clamp or MEA platform, electrical excitability in established neuronal networks can be used to assess neurotoxicant activity that is not detected by more conventional methods (26). Further examples of mitochondrial-dependent neurotoxicity and developmental neurotoxicity have been reported (20, 27).

ADME

Although ADME problems *per se* are no longer a major cause of drug failure in clinical testing, metabolism through enzyme families such as cytochrome P450s (CYPs) makes a major contribution to drug side-effects and toxicity. CYPs are the major family of drug metabolising enzymes, and their distribution and function are not only highly species-specific, but also show considerable inter-individual differences (28). It has been suggested that up to 62% of drugs withdrawn from the market, or given black box warnings on the basis of unexpected toxicity, were associated with metabolism-derived reactive products (29). The impact of drug metabolism on drug safety, the lack of predictive power of animal models, and the marked inter-individual distribution of drug metabolising enzymes in humans means that not only do we need more human-based models, but we also need these models to be capable of taking this inter-individual variation into account. Promising technologies such as high-content screening are expected to play an increasing role, and some highly encouraging results have been reported (30, 31). In fact, the approach is now widely used in industry (32). The application of stem cell-based models using patients' own cells opens up the possibility of determining patient-specific problems (33).

In Silico Approaches

While an ever-increasing array of human *in vitro* approaches to assess compound efficacy, toxicity and metabolism are available, there is a similar expansion in the field of *in silico* technologies, which are undoubtedly going to play a major role in the future for drug safety prediction. While *in silico* approaches are, by definition, virtual, they depend critically upon clinical and experimental data. Many of the currently available *in silico* safety prediction products are based extensively upon non-human data, and while some of these products are undoubtedly relevant, others are less so. Therefore, in the future, considerably more attention must be directed toward building human biology into the knowledge-base, for which the main limitation is the availability of data. Until industry understands and accepts the need for pre-competitive data sharing (34), no one will be able to access the breadth of information necessary to make this a possibility.

An additional and unrelated *in silico* approach that is fast gaining recognition is the construction of virtual organs. These incorporate a wide range of aspects of structure and function of the organ in question, and allow the modelling of responses to agents that interact with known processes within that organ. This approach has been particularly well developed in modelling the human heart — in terms of single cardiomyocytes and also the whole heart (35). This is a hugely complex exercise, which requires in-depth understanding of cardiac

architecture and of the elements contributing to the electrophysiological control of cardiac function. Despite this, considerable progress has been made, and models can now provide an impressively accurate reflection, not only of basic cardiac function, but also of the effects of known cardio-active compounds. As with *in silico* approaches to drug activity and metabolism, the current cardiac models are largely based on non-human data — however, the ultimate aim is to effectively model the complete human organ.

Special Cases

The lung

The respiratory system presents particular challenges, as it might be exposed both to drugs administered by the inhalation route and/or to potential toxicants present in the atmosphere. For this reason, it warrants separate consideration, and specific tests are required to explore potential adverse effects relevant to inhaled substances (36). Current animal-based models are inadequate, so there is undoubtedly a need for human-based *in vitro* models of the respiratory system. There are many relatively simple cell-based methods, involving primary cells, immortalised cell lines and stem cells, all of which have some value, but what are really needed are models that permit determination of the likely exposure of all of the various lung cell types under normal respiratory conditions. The use of precision-cut lung slices addresses some of the issues, but does not permit any investigation into the effects of respiration and associated bronchial air flow. Whole perfused, ventilated lungs are a possibility (37, 38), but while those from non-human species are readily accessible, and have been used for experimental purposes for decades, maintaining whole and viable lungs from humans represents a far greater problem. Some form of modelling is probably therefore required, through the co-culture of relevant cell types, along with constant superfusion. A particularly exciting approach is represented by the so-called organ(or body)-on-a-chip technology, which allows representative cultured cells from various parts of the body to be located (usually on a glass slide), in such a way that they can be serially perfused by means of etched channels (39). A particularly exciting development is the lung of a lung-on-a-chip developed at the Wyss Institute. This model not only permits the study of the relationship between bronchial epithelial cells and vascular endothelium, but also incorporates fluid and air flow, as well as simulated respiratory movements (40). How robust and generally practicable such models can be, remains to be determined, but they certainly represent a highly exciting approach.

The GI tract

The gut is another area of specific interest, as, like the lung, it is a primary site of exposure to xenobiotics. As with the lung, there are many models based on cultured cells, but unlike the lung, sections of viable human gut suitable for experimental work are far more accessible, whether as a by-product of surgery or from heart-beating organ donors. The gut has also been a focus of development by the Wyss Institute of organ-on-a-chip technology, incorporating gastrointestinal (GI) mucosal epithelial cells and vascular endothelial cells, and featuring simulated peristaltic movements. Furthermore, this model can also incorporate gut bacteria to simulate a GI microbiome, a development that apparently greatly enhanced physiological-like functionality (41), as gut bacteria themselves have a great propensity to produce toxic metabolites (42).

In Vivo Testing

In an ideal world, testing potential medicines for any given species would involve largely, if not exclusively, testing in that species. With veterinary medicine, this poses no legal or ethical problem; however, in the case of medicines for humans, this is not the case. Despite this, it is undeniable that clinical data actually do play a considerable role, and there is reason to believe that this role could be extended without exposing human subjects to any increase in potential harm. First we must accept that exposing an individual, whether volunteer or patient, to a new chemical entity is never going to be risk-free — the onus is on developers and regulators to ensure that that risk is minimised. The fact that humans are already put at risk is demonstrated by the fact that most drugs that are judged sufficiently safe and effective to be allowed to enter clinical testing, fail in early trials; further failures appear after marketing approval has been granted, and those drugs are used in sick people. The truth is that, in such a system, humans, whether healthy volunteers or sick patients, are effectively acting as test subjects. Whether this could or should be officially recognised is a highly sensitive ethical issue. One way of mitigating the ethical dimension might be to consider drug testing in the 'recently dead'. While a review of this possibility has been conducted (43), the ethical issues are not inconsiderable (44), and this is not an approach that has been adopted.

Despite the obvious limitations to the use of living human volunteers rather than animals in

early development studies, the microdosing approach (alternatively known as Phase 0 testing) has been gaining acceptance over the last decade or so. This involves administering a single microdose of the test substance, defined as 1/100th of the pharmacological dose based on primary pharmacodynamic data, or a maximum dose of 100μg, whichever is lower. This approach has been accepted by international regulatory authorities, and it forms the basis of ICH M3 (R2; 45). Microdosing is usually associated with accelerator mass spectrometry (AMS) bioanalysis, which is capable of detecting metabolites in attogram (10^{-18}g) down to zeptogram (10^{-21}g) amounts, and permits the determination of relative metabolite/parent concentrations. Despite early concerns that it was not necessarily possible to scale up from microdoses to therapeutic doses (46), a range of studies have confirmed that, when the IV route is used, linearity between microdose and therapeutic dose is excellent, and this is far superior to the extrapolation of data from animals (47). Such linearity is less impressive following oral administration, where it appears to be compromised by the influence of the gut wall (48).

Overcoming Criticisms of Human-based Testing

A number of objections against human-based testing have been put forward to defend the necessity of continuing animal-based testing and the impossibility of replacing such testing with an approach based directly on human biology, particularly *in vitro* and *in silico* approaches. For *in vitro* technologies, the objections focus on:

— poor modelling of idiosyncratic toxicities and chronic toxicities;

— weak modelling of fully-integrated human body responses through a study of its isolated parts;

— problems in achieving the timely acquisition of sufficient and adequately-viable human biomaterials to allow routine testing;

— a lack of experience of the use of human tissues; and

— the availability of only a small number of validated tests.

While each of these undoubtedly represents a challenge, there is no reason to believe that any of them is insuperable. As far as idiosyncratic toxicities are concerned, these result from discrete biological disturbances, to which a minority of individuals appear to be particularly sensitive. This is not to say that basic biological disturbance does not occur in the general population, it is simply that, for whatever reasons, it only manifests itself as overt toxicity in a minority of individuals. It is likely that many toxicities result, not from the candidate compound itself, but from a metabolite of it, so with our knowledge of drug metabolic enzymes expressed in man, such a possibility can be readily predicted. Alternatively, it may result from a discrete immune reaction, but there is no reason why such responses cannot be modelled in *in vitro* systems.

As for toxicities that only appear after chronic treatment, again, these will result from discrete disturbances in certain biological pathways, and there is no reason to suppose that high-content type assays conducted to mimic acute exposure would not uncover indications of such disturbances, particularly if they were directed toward Adverse Outcome Pathways (AOPs). It also ignores the significant developments being made in extending the viability of certain *in vitro* assays, which are making chronic treatment increasingly feasible (49).

The timely acquisition of appropriate human biomaterials is really, for the most part, only a matter of general acceptance of the need, and, critically, of logistics.

A lack of experience of working with human tissue cannot be accepted as a legitimate reason for not using it, as this could be applied to any novel or alternative approach.

Finally, it is indeed true that there are few validated tests, but even where there are such tests, there is often a reluctance to utilise them. Alternatively, if validated tests are used, they are sometimes used in parallel with animal-based tests in a 'belt-and-braces' fashion. This, of course, is wholly against not only the spirit of regulatory instructions, but it is arguably illegal.

The particular concern relating to *in silico* approaches is that any such virtual system is built on what we know. It is, at best, of limited value in dealing with chemical structures, biological targets and pathways of which we are unaware or of which our understanding is limited. To a degree, this is a criticism that could be levelled at science generally, and, as in other fields, the more we discover, the better our virtual models become.

Determination of Fitness-for-purpose

It is now worth considering the issue of validation. Reluctance to move away from animal-based tests, stems, at least in part, from the widespread belief that there are no suitable validated tests. And while there is undoubtedly merit in this argument, all is not as it seems. There are systems in place, and bodies established, specifically for the validation of alternatives, e.g. EURL ECVAM in Europe and ICCVAM in the USA. However, despite the existence of such organisations, it is a fact that the

review of a proposed new method takes about five years, and few approvals are granted. Those that have been granted, concern a limited number of areas, largely relating to topical issues (50). This could be taken to reflect the unrealistic aspirations of those advocating non-animal approaches, until it is understood that none of the existing animal-based methods has itself ever been exposed to such scrutiny (i.e. has undergone 'validation'). If they had, few, if any, would be expected to receive such approval. I would suggest that any validation process that expects a new test to fulfil all the success criteria currently laid down is overly demanding and unrealistic. If we consider how advances are made in any other area of technology, we find that progress is almost invariably stepwise, with small increments being achieved, and these subsequently built upon to achieve the next small increment. Technological advances are seldom, if ever, achieved by identifying the desired end-product, and then rejecting all attempts, however positive, that fail to reach it. This is a recipe for standing still. The other general feature of development in key technological areas is that there is a positive drive, which might be due to competition (e.g. personal computers) or imposition by regulation (e.g. reduction of car exhaust emissions). In the area of drug safety testing, it appears that advances are viewed by the industry in the context of needing to defend the *status quo* (for example, in statements from *Understanding Animal Research*), and scepticism about proposed alternatives. Sadly, even where alternatives exist — and regulations actively require them to be used when available — they are either not used, or they are only used in addition to, rather than instead of, the animal-based methods that they have been developed to replace.

Fortunately, things are gradually changing. There is a groundswell of dissatisfaction with the animal-based *status quo*, and there are proposals of how to do things better (51–53). There are perhaps two principal ways of tackling the issue, one involving the identification of AOPs or Pathways of Toxicity (PoT), the identification of which will provide specific targets against which to evaluate new molecules (54). These proposals provide a practical roadmap with which to better establish whether a test compound is liable to represent a hazard to human health.

An alternative and complementary approach is to attempt direct comparisons of human-based methods with those animal-based methods on which the industry currently largely relies, to identify any evidence of superiority of the human-based methods that will allow us to advance in an albeit limited way toward an improved system. This approach makes considerable use of existing data, and may be termed 'pragmatic'. There are undoubtedly a number of ways in which such an approach may be taken, but two have so far been proposed. The first of these (55) involves the identification of drugs that have been marketed and therefore judged, on the basis of preclinical testing, to be without obvious toxicity, but have subsequently proved to cause serious adverse effects in patients and have been withdrawn. For each of these drugs, a partner is identified that is structurally and/or functionally similar, but does not cause the same toxicity. Such drug pairs are subsequently subjected to testing in a range of relevant human-based test methods to see whether any of these tests, on its own or in combination with others, could identify the known clinical toxicity. The inclusion of the non-toxic (or less-toxic) partner acts as a key negative control.

A second approach involves the determination of 'Likelihood Ratios' (LRs). LRs were originally introduced by Altman and Bland in 1994 (56) to establish the predictive value of clinical trials, but have subsequently been used to evaluate and question the value of animal models in predicting clinical toxicities (57, 58). While there have been expressions of concern about bias in the data that are the bases of the studies, the fact remains that the LRs generated apply to a set selection of compounds, and thus, if that same selection of compounds is subjected to test using human-based test methods, then any LRs generated will be directly comparable with those relating to animal testing (59).

How Close Are We to the 'Single R'?

There are two aspects to this question, the first relating to technical ability and the second to industry and regulatory acceptance. These two aspects must be considered separately.

In terms of technical ability, in that small number of cases where human-based models have been formally validated (50), we have already achieved the 'single R'. In other cases that have been subjected to particular development, such as cardiotoxicity testing (60), we could probably safely abandon animal-based approaches tomorrow. In most areas, while tremendous advances have been made, it is not certain that we could justify abandoning animal-based testing at present, although in adopting this position, we have to bear in mind that we really do not know how good the current animal-based testing actually is (see above). Until we have a way of quantifying the respective performances of animal-based and human-based approaches, this remains a moot issue.

As to acceptance by industry and regulators, the position is even less clear, as it requires a sea change in thinking. While the pharmaceutical companies are increasingly aware of the problems they currently have in their reliance on non-

human testing, and of the potential value of a more-humanised testing paradigm, they remain far from convinced that a 'single R' approach is possible (61). In the case of the regulators, there is evidence that they believe that there are advantages to more human-based testing (62, 63), but it is important to understand that their role is not to dictate what methods are used, but to review data presented in drug submissions, in order to assess whether such data provide sufficient comfort regarding potential recipient patient safety. So, while it appears that they would be willing to consider favourably a move toward a more-humanised testing approach, there is still a significant way to go.

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