Part I
Transversal Issues Concerning Animal Models in Drug Discovery

In Vivo *Models for Drug Discovery*, First Edition. Edited by José M. Vela, Rafael Maldonado, and Michel Hamon.

1
The 3Ns of Preclinical Animal Models in Biomedical Research

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In vivo experimentation has played a central role in biomedical research in the past, and it has also been a hot issue of public, scientific, and even philosophical discussion for centuries [1–3]. At present, a paradigm regarding needs, usefulness, and ethical treatment of animals in research has evolved, but discussion is open [3].

It is a matter of fact that the use of animal experimentation is a genuine need for the primary purposes of target validation and estimation of multiple parameters of new therapeutic drugs, including efficacy, margin of safety, and metabolism and pharmacokinetics. It is also obvious that current unmet needs regarding experimentation with animals primarily focus on the development of better animal models, with improved translation to humans, as well as on further advancements of replacement alternatives, minimization of number and suffering of animals used, and continuous improvement of the well-being of laboratory animals [4].

Accordingly, there are three major needs (3Ns) regarding nonhuman animal models in biomedical research: (i) the need for use, (ii) the need for better, and (iii) the need for three Rs (3Rs) – replace, reduce, and refine – guiding principles. Justification of one of these needs does not justify the neglect of or insufficient perseverance with the other two.

1.1
First N: The Need for Use of Animal Models

The use of animal models in biomedical research has been making great contributions to the medical advancements, and it is likely to remain an integral part of research in the foreseeable future.

Animal models (in most cases, rodent models) are well-established tools for both fundamental and applied biomedical research, and thus the main instrument for drug discovery, validation, preclinical, and toxicological studies. They are widely used due to the deep knowledge obtained (e.g., the mouse became the second and the rat the third mammal, after humans, to have its whole genome sequenced), the possibility of genetic (e.g., inbred strains) and environmental standardization, the access to a broad spectrum of strains, genetic modifications (transgenic and gene

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knockout models available), and pharmacological interventions adapted to address specific scientific problems, and their general – although sometimes controversial – acceptance by the scientific community, patent regulatory bodies, health regulatory authorities, and ultimately a society with unmet medical needs that demands better and safer medicines.

Scientists involved in biomedical research rely on animal models as an important means of generating knowledge and obtaining information on the potential relevance and therapeutic application of their discoveries. Indeed, most biomedical researches, including those grounded in molecular studies, need at some point validation of their findings in a suitable cell, tissue, organ, or preferably whole animal model reproducing or mimicking as much as possible the physiology or behavior under study.

Regulatory authorities require evidence for both efficacy and safety of novel compounds in appropriate animal models. The need for more effective medicines and the emphasis on risk avoidance in our society have resulted in a broad range of regulations intended to guarantee efficacy and safety of new pharmaceutical products. Many of these regulations rely on animal tests. In fact, animal testing is a key element of the product assessment legislative and regulatory procedures: animals used for regulatory requirements for the production and quality control of products and devices for human and veterinary medicine and to satisfy regulatory toxicological and other safety requirements accounted for at least 23% of the total number of animals used for experimental purposes in the European Union in 2008 [1].

From the intellectual property viewpoint, patents are granted for inventions that are novel, involve an inventive step (nonobviousness) with regard to the state of the art, and are useful for or susceptible to application. In order to encourage innovation, the subject matter claimed in a patent application must not be already known or be part of the prior art, and for this reason it is essential to file a priority application before any public disclosure or use of an invention. Waiting too long to file an application threatens the novelty of the invention and inventors may lose forever the chance to obtain a patent if the subject of the invention is revealed prior to the filing date. Accordingly, patent applications for new drugs are usually filed early, during the drug discovery or preclinical development program, before clinical trials would eventually demonstrate safety and efficacy in humans. Experiments in appropriate animal models are thus a main source of data to meet the substantive conditions of patentability and support the claims of the patent application [4].

Finally, the market and ultimately the society demand better medicines based on the differentiation of novel compounds from those already on the market, and potential advantages of new drugs in terms of efficacy and/or safety are usually demonstrated early on during the drug discovery program using appropriate, as much as possible translatable to humans, animal models.

Independent of its acceptance, justification of animal experimentation seems reasonably clear based on the benefits that research relying on animal models has conferred and still confers upon humans. The benefits involved here are understood to include such things as advances in knowledge as well as things

more commonly regarded as tangible benefits, such as improvements in disease diagnosis and treatment. There are thousands of evidences showing how valuable data obtained from animal experimentation underlie or have allowed key discoveries and improvements with positive impact on human health. It is important to note that for the purpose of this chapter, it is assumed that either in the short or in the long term (or both), the benefits of research using animal models are substantive, an assumption that is compatible with the possibility that alleged benefits of some research could be considered spurious or that benefit arguments could be debatable [2]. In any case, this is not the place to undertake an analysis of the balance between the costs and benefits of the myriad experimental uses of animals in biomedicine or to philosophically debate over moral quandaries regarding animal experimentation.

Even so, animal models need to be improved as findings arising from current preclinical animal models often poorly translate to human disease and clinical practice. In addition, animal models are not the only source of valuable data supporting new discoveries, and more and better alternative models need to be developed to replace and/or reduce the number of animals used, while increasing their well-being.

1.2 Second N: The Need for Better Animal Models

The translation of novel discoveries from basic research to clinical application is a long and often inefficient and costly course. This goal has resulted over the years in phrases such as "from bench to bedside," "from mouse to man," "from laboratory findings to clinical practice," or "today's science; tomorrow's medicine." The rather recent terms "translational research," "translational pharmacology," and "translational medicine" also highlight this goal, emphasizing the distinctive scientific processes that have to be done to move (or translate) basic research into a finally approved therapeutic agent [5]. Translational research has become a top priority in national and international road maps to human health research.

Translational research is a paradigm for research, an alternative to the dichotomy of basic (or fundamental) and applied research. It is actually a distinct research approach seeking to make findings from basic science useful for practical applications enhancing human health and well-being. It is necessarily a much more multidisciplinary style of research, with low and permeable barriers and much interaction between academic research and industry practice.

Translation almost always involves animal models of disease in order to evaluate the possible therapeutic use of a compound. Appropriate animal models for the evaluation of efficacy and safety of new drugs or therapeutic concepts are thus critical for the success of translational research. Unfortunately, although testing in animal models is a key step, animal models do not always reflect the clinical situation. In fact, translational research frequently fails to replicate in the clinic what has been demonstrated in the laboratory.

Despite great advances in basic knowledge, the improved understanding has not yet led to the proportional introduction of truly novel pharmacological treatment approaches. Transgenic and knockout techniques have revolutionized manipulation of rodents and other species to get greater insights into human disease pathogenesis, but we are far from generating ideal animal models of most human disease states [6]. In addition, rapid advances in modern omic sciences coupled with the high-speed synthetic and high-throughput screening capabilities should provide new targets, new insights into efficacy and risk factors, shortened drug discovery cycle times, and better drug candidates. But this is not (always) the case. Drugs fail at a higher rate in phase II trials, the point at which researchers first test efficacy in humans, and a reason for the high attrition in the clinic has been suggested to be the poor predictive power of animal models for efficacy in humans [7–9]. Indeed, the US Food and Drug Administration (FDA) in its Critical Path Initiative report points to the limited predictive value of currently available animal models as one of the reasons for the recent slowdown, instead of the expected acceleration, in innovative medical therapies reaching patients, and states that better predictive nonclinical screening methods are urgently needed [4]. Altogether, with the increased emphasis on translational medicine, the use of high-quality, predictive, *in vivo* animal models has been recognized as an essential component of modern drug discovery if late-stage failure for lack of clinical efficacy is to be avoided.

Two fundamental reasons for this "lost in translation" problem have been suggested: the "butterfly effect" (intrinsically related to the behavior of many animal models) and the "two cultures" problem (differences between the methodologies for preclinical and clinical research) [10].

It is clear that modeling has intrinsic limitations. An animal model is defined as any experimental preparation developed in an animal for the purpose of studying a human condition, and thus, as implied by the term "modeling," no perfect animal model exists for any disorder [7]. The cross-species predictability is always an issue as the animal response to the pharmacological manipulation may engage different mechanisms/pathways and thus confound the actual human response to pharmacological interventions. The imprecise diagnostic criteria for some illnesses also inevitably lead to problems when trying to model the condition. In addition, the complex nature of human conditions makes it difficult/impossible to reproduce human behaviors and deficits [11]. For example, language deficit plays a major role in autistic spectrum disorders, but rodents do not have language so it is not possible to develop a language-impaired "autistic" mouse. Going further, how predictive specific knockout models are for the effects of acute or chronic pharmacological intervention in patients? How well does locomotor responsiveness to the administration of psychostimulants or altered water maze learning predict the antipsychotic and cognition-enhancing effects of novel compounds in patients? [7].

But not always the failure of apparently promising interventions to translate to the clinic may be caused by inadequate animal data and overoptimistic conclusions about efficacy drawn from methodologically flawed animal studies. The decision to conduct clinical trials is not always supported by reliable evidence of efficacy in animal models [12], and in the clinical setting, improved patient classification,

more homogenous patient cohorts in clinical trials, standardized treatment strategies, improved drug delivery systems, and monitoring of target drug levels and drug effects are warranted [13]. Clinical trials should also adopt more practices from basic science and show greater responsiveness to conditions of clinical practice [14]. The disparity between the results of animal models and clinical trials may be explained in some cases by shortcomings of the clinical trials. For instance, these may have insufficient statistical power to detect a true benefit of the treatment under study or allow therapy at later time points, when the window of opportunity has passed [15]. In addition, both positive and negative results contribute to knowledge but, in contrast to many clinical studies, negative studies obtained with animal models are usually not reported. Negative results are often considered by investigators and journal referees and editors as unsuccessful or with low scientific value and attractiveness to be published, although such information is vital [8]. As neutral or negative animal studies are more likely to remain unpublished than negative clinical trials, the impression is that the former are more often positive than the latter, which overstates the disparity between the results of animal models and clinical trials [15].

Unfortunately, the difficulties in developing new compounds, particularly those working through novel mechanisms, are currently leading to a lack of confidence (as many pharmaceutical companies are terminating in-house research, more often in complex conditions such as neurological and psychiatric disorders) and a state of skepticism regarding the usefulness of animal models (will their use only allow discovery of more "me-too" compounds?) [5]. To address this problem, it is not enough to investigate and bring about new models. Changing the way academic researchers, drug developers, and regulatory agencies operate is advised. Instead of moving progressively from simple cultured cell models to imperfect animal models and then into clinical trials [9], future efforts should be focused more on the underlying mechanisms at work in a disease and finding drugs to affect one or the other mechanism. More intensive clinical and preclinical interactions are needed to ensure that basic science knowledge gained from animal models and information from the clinical/human domain converge to develop truly translational measures in both preclinical and clinical testing. Information must flow in both directions from humans to nonhumans and then back again so that it is not lost in translation [16]. In addition, the research should not be stalled at the animal model stage, but instead the clinical trials need to be focused, safe, and ethical, backed up by a robust, translationally relevant preclinical research strategy [17]. This new translational approach combined with the evolving focus on the identification of reliable biomarkers that correlate with clinical and functional endpoints provides a fresh and optimistic framework.

The remaining task of animal model validation is of such magnitude that no single pharmaceutical company or academic center can effectively address the issues relevant even to a specific disease. Consortia from industry and academia (e.g., Innovative Medicines Initiative and Horizon 2020 in Europe) to tackle some of the issues related to preclinical discovery approaches on a precompetitive level (indeed, specific work packages on animal models improvement are included in

most disease-focused project IMI consortia) together with the development of mechanisms for data sharing are essential, and both industrial and academic researchers must contribute. This requires a change of mindset of all the stakeholders involved: industry being willing to share more data, resources, and compounds; academia being prepared for some more practically oriented groundwork rather than cutting-edge scientific experimentation leading to high-impact publications; and governments and regulatory authorities promoting such joint initiatives and innovative approaches to improve translation of novel discoveries from basic research to clinical application [7,18].

While recognizing the difficulty of predicting efficacy in patients based on results from preclinical studies, animal models could contribute most effectively to translational medicine and drug discovery. In the following sections, some comments regarding key features and obstacles of animal modeling are depicted, with the aim to encourage preclinical–clinical translation in drug discovery and eventually improve the translational value of animal models and/or enrich the information they provide. This issue has been evaluated expertly and critically previously by other authors [5,7,15,19,20] and key data included herein have been obtained from the information in these excellent review articles.

1.2.1 Unbiased Design

Adequate internal validity of an animal experiment implies that the differences observed between groups of animals allocated to different interventions may, apart from random error, be attributed to the treatment under investigation. The internal validity may be reduced by different types of bias through which differences between treatment groups are introduced. Blinding of the experimenter to the drug administration, randomization of animal subjects, control of variables that may affect outcome and lead to erroneous conclusions, predefined (not determined on a *post hoc* basis) eligibility criteria if animals are excluded (e.g., inadvertent blood loss during surgery or weight loss), control of study conduct, and accurate statistic analysis of the results are always mandatory [5,15].

1.2.2 Comprehensive Reporting

Inadequate or incomplete reporting raises ethical as well as scientific concerns as it reduces the value gained from animal experiments, which can result in unnecessary additional studies, and might hinder the translation of experimental findings to humans by restricting the potential use of systematic reviews and meta-analyses to assess preclinical evidence. In order to maximize the output of research that uses animals, initiatives such as the ARRIVE (Animal Research: Reporting of *In Vivo* Experiments) guidelines have been developed collaboratively by scientists, statisticians, journal editors, and research funders. They consist of a checklist of 20 items with the essential information that should be included in publications

reporting animal research to describe a study in a comprehensive and transparent manner, and make recommendations on the reporting of the study design, experimental procedures, animal characteristics, housing and husbandry, and statistical analysis. The ARRIVE guidelines were simultaneously published in a number of bioscience journals in 2010 and, since then, more than 300 journals have adopted them (the full list can be found on the NC3Rs web site at www.nc3rs.org.uk/ARRIVE). Applying guidelines carefully may represent an opportunity to improve standards of reporting and ensure that the data from animal experiments can be fully scrutinized and utilized [12,21].

1.2.3 Selection of the Animal Model Based on Its Validity Attributes

Even if the design and conduct of an animal study are sound and eliminate the possibility of bias, the translation of its results to the clinic may fail because of disparities between the model and the clinical trials testing the treatment strategy. Common causes of such reduced external validity not only are limited to differences between animals and humans in the pathophysiology of disease, which are largely determined by disease-specific factors, but also include differences in comorbidities, the use of comedication, timing of the administration and dosing of the study treatment, and the selection of outcome measures [15].

A primary concern to scientists, working either in academic world or in the industry, is the selection of the most appropriate animal model to achieve the intended research goals. Quite often researchers are confronted with the choice among models that just reproduce the pharmacological effect of treatments on the expression of a specific but sometimes unconnected symptom, models that reproduce cardinal pathological features of the disorders caused by mechanisms that may not necessarily occur in the patients, versus models that are based on known etiological mechanisms but do not reproduce all clinical features.

Traditionally, animal models of human diseases are selected based on three main attributes: (1) the similarity to the specific symptoms of the human phenomena (i.e., face validity); (2) the similarity in response to pharmacological treatment (i.e., predictive validity); and (3) the degree to which a model supports a mechanistic theory between the human disorder and the model itself (i.e., construct validity). Reliability, on the other hand, requires that the outputs of the model are robust and reproducible between laboratories.

Animal models, in addition to being reliable, should ideally exhibit full validity attributes, but in practice this does not happen and every model has its own attributes that determine the purpose it can serve. Accordingly, the criteria each model fulfills to demonstrate its validity are, for practical purposes, largely determined by the objective of the model and its intended use. In this way, animal models commonly used in screening processes during drug discovery tend to be simple and rely on partial face validity (tendency to be biased due to its focus on one or few specific symptoms) and predictive validity (tendency to be biased based on the positive response to known treatments) as principal features, although they

should rely not only on the effectiveness of the compounds belonging to the drug class it is being investigated but, more importantly, on the ineffectiveness of drugs known to be devoid of any therapeutic potential in the disease as well [20]. These predictive validity-based experimental models are useful in exploring the effect of pharmacological treatments on the expression of a specific symptom (that although concurrent with the disease may be of poor clinical relevance or unrelated to the underlying pathophysiology) and are valuable to accomplish the generation of "me-too" compounds, but the discovery of new, first-in-class drugs with groundbreaking mechanisms of action requires enhanced understanding that can be better attained via construct validity, by focusing more on the underlying mechanism of the disease to find new drugs neutralizing that mechanism [20]. Hence, construct validity-based models offer better alternatives for target identification and validation as well as for drug candidate profiling (but not for screening of a large number of compounds as these models are normally costly and time consuming). In turn, care should be taken because construct validity-based models may be dependent upon uncertain etiological assumptions and inferences, which could result in taking wrong compounds into clinical trials, particularly when trying to model complex diseases with poor understanding of their etiology.

Animal models need to be optimally selected and data derived from each need to be interpreted and applied most appropriately and effectively to the drug discovery and decision-making processes. A possible strategy could be to establish a sophisticated, construct validity-based model early on for target validation, using already existing compounds that target the novel mechanism of action or other methods (e.g., RNA interfering and knockout technologies) to get information on the sensitivity and specificity of the model. Once the model has been selected and a significant correlation between *in vitro* activity at the target and *in vivo* activity in the model has been demonstrated, advanced lead compounds arising from the discovery program need be tested in the model. If wisely chosen, the preclinical model can aid the design of the clinical trial needed for human studies. Therefore, animal models closely modeling the clinical pathology, although normally sophisticated and time consuming, can be used to increase the confidence in the functional significance of a target (target validation) and determine later on the pathway for further drug development to facilitate the "win or kill" decision-making process. Especially in cases where the predictive validity of a model is relatively unknown because of the absence of clinically active reference drugs, it is critical to avoid using behavioral assays that have limited construct validity simply because they happen to be faster. Such an approach using rapid predictive validity-based models is, when appropriate, complementary and helpful for screening purposes during the intermediate drug discovery process, but not for target validation (at the very beginning) and preclinical candidate selection (at the end of the discovery process) as they would provide for more rapid but wrong decision making. Furthermore, one should exclude models that lead to false positives (effectiveness of drugs known to be clinically inefficacious) and be innovative but cautious when relying on novel but yet untested models in terms of their translational value and potential for a significant clinical outcome.

Current drugs for treating some diseases are mostly variations on a theme that was started several decades ago. Sadly, clinical efficacy has not improved substantially over the years in some areas probably because both clinical and preclinical researchers have focused too much on a specific symptom, which is only one of the hallmarks of the disease [22]. Many strategies employed in the design of animal experiments began with attempts to detect the effect of serendipitously discovered drugs already in clinics. In a simplistic view, this initial backtranslational pharmacological strategy requires the identification of just one symptom (and not necessarily the most relevant) sensitive to the modulation by both the reference drug and the new "me-too" compounds being developed, and thus heavily relies on face and predictive validities. However, the power of these strategies to predict an effective new treatment is unclear. The complexity of the clinical condition inevitably means that even the best animal models are inadequate representations of the condition they seek to mimic. Therefore, to attempt to model complex human disorders where validity is often limited to superficial similarities (referred to as face validity) that often reflect quite different underlying phenomena from the clinical situation is probably overambitious [11]. More information is needed on disrupted mechanisms underlying the pathology. Uncovering these mechanisms is necessary for these models to significantly advance discovery of new prevention or therapeutic strategies. Along this line of thinking, the predictive power of animal models can be increased by improving our ability to (1) systematically and selectively measure disease-relevant processes in rodent models, (2) identify mechanisms underlying these processes, and (3) model putative etiologic or pathogenic mechanisms that lead to these abnormalities [19].

Numerous studies are still focused on better treatment for the symptoms rather than on the causal mechanisms to prevent the development of the disorder [5]. However, when approaching therapeutic indications where there are still great unmet medical needs, we need to shift the focus from overreliance on predictive validity to the reliance on construct/etiological validity. This is certainly a high-risk/high-benefit approach that needs to be viewed as a much-needed long-term investment in the development of the field of translational research that will eventually increase the success rates. In addition, models with good construct/etiological validity could also be used for further target identification and thus provide additional opportunities for drug discovery [7].

1.2.4 Appropriate Time and Dosing

An indication of the timing and progression of the disease is needed so that the treatment may be applied at the appropriate time/age of the animal. In many cases, the progression of the disease is so rapid in the animal model compared with the clinical condition that the narrow time window for intervention decreases the probability of selecting the optimum time for treatment. Similarly, therapeutic changes exerted by drug treatments can be detected very soon following acute administrations in animals, but require much more time and chronic treatments in

patients. It is also not easy to estimate whether the time that a biochemical change takes to translate into a measurable behavioral change in the animal model is similar to the time taken to get a clinically relevant change in patients. However, all this information needs to be obtained from the animal model and compared with those in clinics to select the most appropriate time and duration of treatments.

When drugs are administered to the animal before or at the time of the disease model inductor (either drugs or experimental manipulations such as injury or surgery), data obtained best translate into a preventive therapeutic approach, which is relevant in some conditions when the disorder is expected or scheduled (e.g., prevention of nausea before cancer chemotherapy or pain before surgery). However, for regular "curative" approaches, the effect of drugs (usually restoration of normal baseline values) should be assayed in appropriate animal models once the disease has clearly developed, not at the time or soon before or after the inducing insult. Examining the effect of a compound in young (sometimes healthy) animals rather than in old animals with comorbid conditions is also a dangerous simplification when approaching therapeutic effects commonly affecting elderly people.

Finally, numerous drugs have been developed using an acute response measurement, but they are administered to patients requiring long-term treatment. It seems reasonable that if chronic treatments are required in clinical practice (as it is actually the case in most chronic conditions), subchronic/chronic treatments should be assayed in chronic models of the disease. Tachyphylaxis, desensitization, tolerance, and even addiction phenomena need to be anticipated. The system may also need time to react to the drug–receptor interaction to establish a good pharmacological response, and thus the compound needs to be given repeatedly. This is even clearer when therapeutic effects in patients have been reported following repeated but not acute treatments (e.g., antidepressants). In these cases, preclinical data relying heavily on acute behavioral tests (e.g., forced swimming test for serotonin reuptake inhibitor antidepressants) should only be considered of predictive value for "me-too" compounds based on the previous demonstration of the therapeutic effect of the drug class. Otherwise, this approach could hardly provide valuable information leading to the discovery of new first-in-class drugs.

1.2.5
Use of Biomarkers

The translational value (i.e., predictability) attained using animal models of disorders in which the molecular basis of the disease is better understood and disease biomarkers are known is potentially higher. In particular, when suitable quantitative imaging biomarkers or biochemical biomarkers from easily accessible biofluids (e.g., blood and urine) or tissues are available, the same measures used in experimental animals are feasible in patients. This approach is attractive because it potentially relies on the mechanistic action of the drug, and thus allows decisions to be made on the basis of quantitative data in experimental animals that can be later on confirmed in humans. Unfortunately, reliable biomarkers are not available for many complex diseases with unmet therapeutic needs.

Although the ultimate aim of biomarker investigations is to find biomarkers that will most accurately predict disease outcome, the reality is that it is difficult to extrapolate the findings and most proposed candidate biomarkers are not consistent enough to be considered for measurement in routine practice. In fact, changes in the expression of the proposed biomarker in disease states could reflect compensatory or secondary (not causally related) adaptations, far away from the relevant mechanisms underlying the pathology. It is important to note that a biomarker-based approach is not based on "patient outcomes" (i.e., reduction in clinical symptoms or improvement in quality of life), but rather on "improved" hypothesized biomarkers that may be considered more readily amenable to translational work than functional outcome. Here is the risk. For example, if overexpression of protein X in plasma is known to correlate with the severity of a disease, a drug declining plasma protein X levels in a purported animal model of such disease could encourage further development of the molecule. However, this is not black or white as possible outcomes include confirmation of the biochemical hypothesis but no effect of the drug treatment on the behavioral measures, or alternatively, the behavioral measures improve in response to treatment but protein X expression does not change. Some may argue that behavioral measures are less sensitive because they are not measuring the relevant behavioral symptoms or they are measuring responses unrelated to the actual underlying pathology. Alternatively, behavioral measures may be considered more sensitive and more meaningful than the biochemical parameters because the concentration of protein X poorly reflects the molecular and cellular events in the specific pathway that underlies the behavioral deficits. Furthermore, often unknown is to what degree a biomarker should change to allow for reliable predictions of clinical efficacy [7]. Thus, if possible, measurement of reliable clinically recognized biomarkers, more preferably multiple reliable biomarkers identifying different pathophysiological alterations, and most preferably multiple reliable biomarkers together with (behavioral) measurement of clinically meaningful symptoms, is advised.

1.2.6 Use of Various Animal Models

No single animal model can account for the entire disease syndrome it purports to represent and every model has its strengths and weaknesses that should be taken into consideration for determining its applicability. Therefore, given the heterogeneity and etiological complexity of most diseases, the findings emerging from the combined use of different models may ensure replication of findings, provide insight into the various aspects and etiology of the disorder, and lead to better new treatments. Comparison of these findings might also elucidate genuine therapeutic effects rather than effects limited to a specific model that is not necessarily related to the disease. A full suite of animal behavioral tests allows for a comprehensive assessment of the spectrum of symptoms relevant to the disease. The use of multiple experimental manipulations and experimental designs also allows modeling several different inducing conditions and/or engaging several dependent

measures. In addition, moving a compound forward to clinical trials represents a considerable investment that many are reluctant to initiate on the basis of the outcome of a single preclinical experiment, however well designed [7].

However, if the predictions from the animal models are mixed (some positive and some negative), what is the global prediction from the aggregate of the preclinical animal data? A priori decisions regarding how many models would be required to show a convincing positive therapeutic response and acceptance of the path to be taken according to the potential outcome of each model would be most appropriate to support moving forward the molecule. This will avoid the feed-forward loop that tends to move ahead lead compounds based on the keenness for some to not give up even when substantial negative outcomes arise from testing in key animal models [7].

1.2.7
Quantitative, Multiple, and Cross-Predictive Measurements

Qualitative assessments of behavior are often subjective. This would lead the investigator to observe what they want to observe, and to render conclusions in line with their expectations. It is thus clear that quantitative assessments based on objective measurements should replace qualitative scores when possible. When not possible, independent assessment by different individuals is required.

It is also clear that multiple readouts are better than single readouts. The use of a multifactorial approach employing several dependent measures, such as imaging, electrophysiological recordings, biochemical and/or neurochemical measurements, and immunohistochemistry, along with different behavioral measures invariably results in an enrichment of the data coming from animal models. A high degree of coherence between multiple dependent variables lends support to the hypothesis, either by indicating the involvement or recruitment of the pathway hypothesized to underlie the disorder or by better defining the active dose range [7].

The predictions from the animal models on the human condition can be only as good as the correspondence between the measures in humans and those in experimental animals. Thus, it is always preferable if the parameter analyzed can be readily measurable in both animals and humans. Identical measures in humans and experimental animals are likely to be analogous or even homologous (in the sense of being mediated by the same substrates) and thus greatly facilitate translation [7]. In particular, if testing approaches applied in human research studies are chosen, measures can have clear conceptual and methodological links to tasks currently in use for nonhuman animal studies and thus have the potential for translation to animal research [23]. Such measures are highly desirable and cross-predictive, but unfortunately not always feasible to design and assess in one or the other population (i.e., experimental animals, healthy human volunteers, and patients). As a caveat, such homologous measures do not necessarily represent clinical trial endpoints as defined in guidelines by health authorities, which adds another level of complexity. In many cases, one may be limited to analogous measures assessing the same/similar process in both experimental animals and

humans. Accordingly, current animal models may be very predictive of specific measures and constructs in humans, but unfortunately such measures are not what are currently assessed in the various phases of most clinical trials [7].

An appealing strategy is thus to design tests in animal models as close as possible to those used in humans or, alternatively, to develop human tests more "rodent-like." Not only should the disease or injury itself reflect the condition in humans as much as possible, but age, sex, and comorbidities should also be modeled where possible. The investigators should justify their selection of the model and outcome measures. In turn, human clinical trials should be designed to replicate, as far as possible, the circumstances under which efficacy has been observed in animals [15]. However, it would be unrealistic to expect that animal model tests could be totally aligned to human ones because of dissimilarities between species, differences regarding feasibility and practicability (some measures do not represent clinical endpoints as defined in guidelines by health authorities or are unviable in humans, whereas others rely on elaborated responses that cannot be measured in animals).

1.2.8
Pharmacokinetic–Pharmacodynamic Integration

Pharmacokinetic–pharmacodynamic (PK–PD) integration in pharmacology research is fundamental to improve interpretation of data coming from animal models for different purposes, including target validation or optimizing the development of lead compounds, and has become mandatory for regulatory bodies. The concentration–effect relationship is necessary for translational purposes [24] and it is central to drug discovery in the pharmaceutical industry, but PK–PD integration is still comparatively rare in experimental pharmacology practiced in academic laboratories (in part because drug analysis techniques are not widely available to academic scientists). Its absence diminishes the interpretative value of published experimental data and can allow the presentation of misleading information and inaccurate extrapolation to clinical use [25].

PK–PD integration, also called quantitative pharmacology, focuses on concentration–response and time–response relationships based on drug exposure measurements (drug concentrations in plasma or other compartments such as tissues or organs proposed as the site of action for the drug), plasma protein binding (unbound fraction available for target engagement), exposure–effect relationships (correlation between the time course of the effect and drug exposure), and the measurement of active metabolites. This will provide valuable information to extrapolate to clinical use: plasma levels needed to get a significant therapeutic response (e.g., 80% of effect) and levels that, when exceeded, correlate with the occurrence of adverse effects. If the effect is mediated by an active metabolite, the effect could be delayed with respect to that expected based on the exposure to the parent compound. If this is the case, is the metabolite acting through the same mechanism as the parent compound or through a different, perhaps known, failed mechanism of action? If the pharmacological effect at the same dose is increased in

subchronic/chronic versus acute treatment, is the increased effect achieved following repeated administrations due to drug accumulation (pharmacokinetic effect) or does it actually reflect a pharmacodynamic, disease-modifying effect? PK–PD integration can answer these relevant questions. Similarly, toxicokinetic (TK) information can substantially enhance the value of the data generated from toxicity testing. Use of TK information can help to ensure that studies are designed to be of most relevance to assessing potential risk in humans, and avoid the use of excessively high doses that could result in unnecessary suffering in experimental animals [26].

Target engagement is a conglomerate of the compound dose size, systemic exposure to the compound (pharmacokinetics), interaction with the target (affinity and efficacy, pharmacology), and physiological (system) reaction to the target–drug interaction. In fact, a compound may have excellent target binding affinity, but fails to engage its target due to low bioavailability or being cleared rapidly from plasma [25]. It can also be present at outstanding levels, but mostly bound to extracellular matrix proteins or fatty compartments and thus not available to interact with its intended molecular target. To get additional information, receptor occupancy studies (e.g., *ex vivo* binding experiments) can be done to assess the actual engagement of the molecular target onto which the drug supposedly binds and correlate it with the pharmacological effect exerted by the drug [27]. Imagine that 50% of maximum possible efficacy is attained when 100% of receptors are occupied by a drug with full intrinsic functionality. In this case, increasing the dose would not result in higher efficacy (but would probably increase adverse effects), and data could be better interpreted in the sense that engaging solely the selected molecular target by a high-affinity selective drug is not enough to achieve a relevant therapeutic effect.

1.2.9
Predefinition and Adherence to the Desired Product Profile

To ensure that any drug discovery project is addressing the requirements of the patients and health care providers and delivering a benefit over existing therapies, the ideal attributes of a novel drug need to be predefined by a set of criteria called a target product profile [28]. The target product profile is an important strategic planning and decision-making tool that is used to define essential attributes required for a specific drug to be clinically successful and of substantial benefit over existing therapies. The desired profile of a pharmaceutical product is thus a list of key features such as desired mechanism of action (i.e., molecular target and mechanism), efficacy (i.e., acceptable levels of efficacy), therapeutic indications (i.e., target patient population), safety (i.e., acceptable levels of safety), advantages respect to competitors, route of administration and dosing schedule, metabolism, and pharmacokinetics.

The descriptions of the animal models that are to be used in the selection of drug candidates are not necessarily included in the product profile, but efficacy and safety data supporting the attainment of the desired predefined attributes are

expected to be obtained in appropriate models of the targeted disease. It is thus important to keep focusing throughout the drug discovery process on the selection of the appropriate models and experimental designs that better translate to the clinic and better inform about the attainment (or not) of the desired predefined attributes, and rely on the data obtained in such key experiments to substantiate strategic go/no-go decisions. In other words, the target (desired profile) must be drawn first and then arrows (compounds) must be fired to try to reach the objective. Alternatively, the arrow is first fired and then the target is drawn around (Figure 1.1). This makes much easier the "attainment of the objective," but the success when moving the compound forward... This is something different (although it can provide interesting opportunities if unintended positive results are obtained by "chance" with a compound).

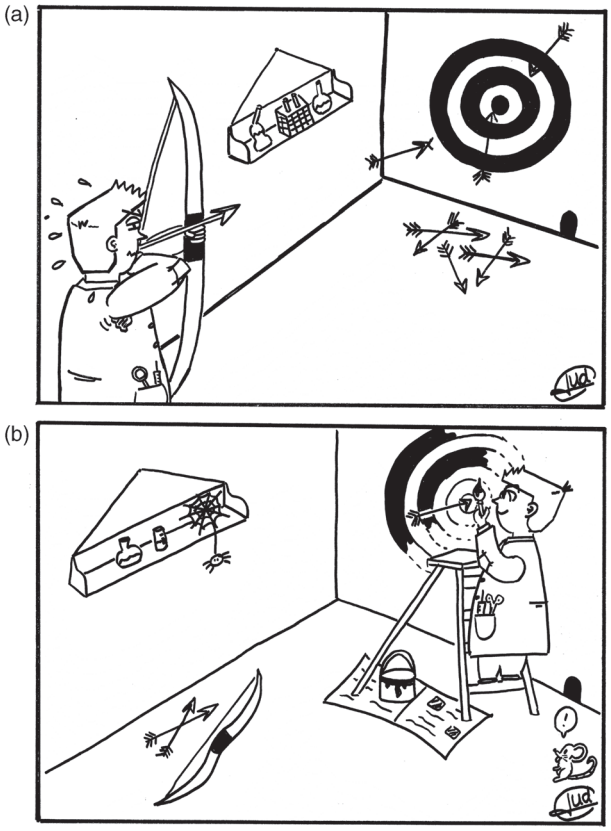

Figure 1.1 Target-driven versus arrow-driven approach for drug discovery. In the target-driven approach (a), the target (desired profile) is drawn first and then arrows (compounds) are fired to try to reach the objective. Alternatively, in the arrow-driven approach (b), the arrow is first fired and then the target is drawn around.

1.2.10
Comparison with Gold Standard References

Competitive advantages of new drugs with respect to existing ones are required (and they should be predefined as key attributes in the target product profile). Animal models allow direct face-to-face comparison of the pharmacological effects of the drug being investigated with a standard best-in-class reference drug, referred to as the "gold standard." The gold standard is often a currently used medication for the targeted disorder that is perceived as being the best, or one of the best, treatment. In the absence of a marketed drug, the gold standard may be a drug acting on the same or different mechanism that has shown efficacy in human studies or is under active preclinical development by competitors for the same therapeutic indication. Differentiation with respect to the reference compound(s) can be attained not only based on improved efficacy and/or better safety profile, but also based on other attributes such as a more convenient route of administration (i.e., oral versus intravenous), dosing (i.e., once a day versus three times a day), faster onset of action, and other differences and innovative features that could be perceived as advantages for patients, physicians, or payers in the context of the particular disease [7].

1.2.11
Reverse Translation/Backtranslation (Bedside-to-Bench Approach)

There is usually a disconnection between the preclinical and clinical teams during drug development, but increasing their interactions and establishing better communication are the best ways to improve translational research. Discussion between preclinical and clinical scientists to improve consistency in preclinical and clinical study designs (e.g., methods, instruments, study groups, study duration, endpoints, and statistical analysis) is highly recommended [10].

In most cases, information flow is unidirectional. The drug discovery workflow is often a progression from *in vitro* to *in vivo* and from preclinical to clinical, with flow of animal data to the clinical domain but not vice versa. Conversely, the product profile is defined primarily clinically and commercially and is provided to guide preclinical research, but no significant preclinical/clinical cross-validation and crosstalk between both disciplines occur. Such a unidirectional flow does not allow maximizing the contribution of animal model data to the process and prevents for any pragmatic and rational modification of animal models to avoid false negatives and positives [7,29].

Emphasis is placed on the need to improve the flow of information from the clinical/human domain to the preclinical domain and the benefits of using truly translational measures in both preclinical and clinical testing. This strategy takes research from bedside to bench, focusing on results from clinical trials to stimulate basic scientific investigation [30]. Traditionally, animal models of human phenomena have been evaluated based on similarity to the human syndrome, response to appropriately corresponding medications, and the degree to which a

model supports a common mechanistic theory between the human disorder and the model itself. The "reverse translation" approach relies on patient-based findings to develop suitable animal models, emphasizing their construct validity as a starting point [31]. For example, an individual case report or a small case series in which the clinician notes an unexpected positive response to a drug used for another purpose can be employed to find new therapies. Such bedside-to-bench observations in human disease can help focus the direction of animal research, which in turn will improve the translational process because they are already known to be associated with a clinical endpoint [32]. As focusing on complex clinical phenotypes may be ineffective for the development of novel and effective treatments, new approaches have also been proposed in the form of reverse translation, which include identification and characterization of intermediate phenotypes reflecting defined, although limited, aspects of the human clinical disorder and thereby develop animal models homologous to those discrete human behavioral phenotypes in terms of psychological processes and underlying neurobiological mechanisms [11,33]. All these approaches deserve attention, although the current emphasis on specific dimensions of pathology that can be objectively assessed in both clinical populations and animal models has not yet provided significant successful preclinical–clinical translation in drug discovery [7,34].

1.3
Third N: The Need for 3Rs Guiding Principles

In 1959, the report by Russell and Burch was published as *The Principles of Humane Experimental Techniques,* the basic tenet of their report being that the humanest possible treatment of experimental animals, far from being an obstacle, is actually a prerequisite for a successful animal experiment [35]. The authors proposed the principles of replacement, reduction, and refinement (most often referred to as the 3Rs) as the key strategies to provide a systematic framework to achieve the goal of humane experimental techniques. Today, the principles of the 3Rs are embedded in legislation that governs the use of animals in science across the world.

Replacement as one of the 3Rs is defined as the substitution of conscious living higher animals by "insentient" material. There are a number of alternative methods that can be proposed to replace the use of live animals in either all or part of a project. Replacement can be absolute (techniques that do not involve animals at any point, such as computer modeling, *in vitro* methodologies, or use of human volunteers) or relative (animals are still required to provide cells or tissue, but experiments are conducted *in vitro* using tissue cultures, perfused organs, tissue slices, and cellular or subcellular fractions; alternatively, "phylogenetic reduction" can be applied using other species such as invertebrates or larval forms of amphibians and fish). These methods are well suited and can be cost effective and time saving. They can not only replace but also provide a level of knowledge that complements studies in whole animals.

The goal of reduction, the second of the 3Rs, is to reduce the number of animals used to obtain information of a given amount and precision. It includes methods that minimize animal use and enable researchers to obtain comparable levels of information from fewer animals, or to obtain more information from the same number of animals, thereby reducing future use of animals. Examples could include improved experimental design and statistical analysis, data and resource sharing, and the use of techniques such as imaging. To achieve this, designed studies need to be scientifically and statistically valid with only the minimum number of animals used and not unnecessarily repeated. In fact, improvement of the models to increase their predictive value ultimately results in a reduction of the number of animals and tests needed to reveal the effect. The principle of reduction of number of animals should not be applied at the expense of greater suffering to individual animals and the number of animals used must satisfy statistical requirements (neither too few nor too many). The reductionist approach has been encouraged by the explosion of the genomic and proteomic technologies that opened up new areas of discoveries in biomedical research. The development of early screening *in vitro* techniques, high-content analyses, novel imaging, and analytical techniques has reduced the number of animals that are necessary for an experiment, while simultaneously providing higher quality data. In addition, entirely computerized strategies that use sophisticated algorithms to simulate biology without needing animals at all are being developed.

The third of the 3Rs, refinement, is any decrease in the incidence or severity of "inhumane" procedures applied to those animals that still have to be used. It includes improvements in scientific procedures and husbandry that minimize actual or potential pain, suffering, distress, or lasting harm and/or improve animal welfare in situations where the use of animals is unavoidable. There are two key issues: to assess the impact of any procedure or condition on the well-being of the animal and strategies to decrease invasiveness or eliminate or minimize that impact. Strategies to achieve the goal of refinement often need to be customized to a specific set of circumstances. Examples could include reducing stress by developing new approaches such as training animals, use of noninvasive techniques, or enrichments that improve living conditions. With increasing knowledge and experience, a number of useful guidelines have been developed to assist in minimizing the impact of particular procedures and practices. Refinement is applied to all aspects, including housing, husbandry, and care, techniques used in scientific procedures, periprocedural care, health and welfare monitoring, and experimental design [36]. However, although care is taken to prevent unnecessary suffering in animal experiments, suffering is an inherent aspect of modeling some distressful conditions (e.g., anxiety, depression, posttraumatic stress disorder, and pain), which represent an extra challenge.

This is an area where knowledge is rapidly expanding and collaboration is important to speed up the goals. For example, a European initiative including 18 companies undertook an evidence-based review of acute toxicity studies, where lethality was mentioned as an endpoint in regulatory guidelines, and assessed the value of the data generated. The conclusion of the working group was that acute

toxicity studies (the so-called LD_{50} test) are not needed prior to first clinical trials in humans. Instead, information can be obtained from other studies, which are performed at more relevant doses for humans and are already an integral part of drug development. The conclusions were discussed and agreed with representatives of regulatory bodies from the United States, Japan, and Europe, and acceptance of the recommendations effectively led to "replacement" of acute studies in guidelines [37].

These matters are expertly reviewed in different excellent review articles [16,38–48] and a number of web pages (e.g., http://www.ccac.ca/en_/education/niaut/stream/cs-3rs, www.animalethics.org.au/three-rs, http://awic.nal.usda.gov/alternatives/3rs, www.nc3rs.org.uk/page.asp?id=7, www.felasa.eu/recommendations, http://www.forschung3r.ch/en/links/, and http://3rs.ccac.ca/en/about/).

Conducting the 3Rs search is not always easy. Alternative methods are not necessarily covered in the mainstream literature, and methods that may well be relevant to one or more of the 3Rs are not always identified as such. In addition, appropriate keywords may not be used. For these reasons, specialized 3Rs-related databases (visit ecvam-dbalm.jrc.ec.europa.eu/ and www.nc3rs.org.uk/category.asp?catID=3) can be useful to allow a researcher to search in a more focused manner for specific alternative methods (e.g., *in vitro* methods that may replace the use of animals in a given protocol; appropriate anesthesia and/or analgesia to help minimize pain and distress; environmental enrichment techniques; models, simulators, computerized mannequins, and other alternatives to the use of animals for education and training purposes).

The implementation of the 3Rs in biomedical research was analyzed in 14 major biomedical journals between 1970 and 2000. During this period, the total number of articles published annually by the journals more than doubled, but the proportion of studies using animals decreased by 30%. There was also a significant increase in the proportion of animal studies using untreated euthanized animals as donors of biological materials, a gradual decrease in the number of chronic studies, and a 50% decrease in the average number of animals used per published paper. There was an improvement in the reporting of the specification of the animals' husbandry, conditions of care, and environment. Parameters of importance for the evaluation of welfare of the animals were generally poorly reported, but the proportion of papers with adequate information on most of the parameters analyzed increased between 1970 and 2000 [49]. In fact, there are many initiatives that aim to replace, reduce, or refine laboratory animal use. Such efforts are supported by academia, industry, and regulatory authorities, although there is the perception that the implementation of the 3Rs in animal research has not increased as expected [50–52].

It is now more than 25 years since both Council of Europe Convention ETS123 and EU Directive 86/609/EEC (now replaced by Directive 2010/63/EU, with effect from January 1, 2013) on the protection of animals used for scientific purposes were introduced to promote the implementation of the 3Rs in animal experimentation and to provide guidance on animal housing and care. However, full implementation of this legislation depends upon scientists' ability to understand

animal welfare issues and to accept the legitimacy of the public's interest in the conduct of science. Education and training of those involved in research and testing is fundamental, and a number of guidelines from different sources including the Federation of European Laboratory Animal Science Associations (FELASA) and the US National Research Council could serve as prototype teaching material. In fact, humane science is good science and this is best achieved by vigorous application of the 3Rs. Animal experiments using the smallest number of animals and causing the least possible pain or distress are consistent with the achievement of a justifiable scientific purpose, and alternative testing methods can have advantages over traditional animal tests, although implementing an alternative from idea to acceptance can take years.

Communication is required between stakeholders, such as regulatory authorities, industry, and academia, about 3R developments and the chances they offer. Sharing test data will help to build up experience with the specific 3R models and facilitate the process of building new experiences, rules, practices, and routines. For example, sharing data and reviewing study designs of pharmaceutical companies and contract research organizations in the United Kingdom have allowed the identification of opportunities to minimize animal use in regulatory toxicology studies [53]. At the end, such a multitude of relatively small steps can lead to a landslide in favor of the 3Rs [50].

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