Identifying and validating novel targets with *in vivo* disease models: Guidelines for study design

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In vivo **studies are an important tool for the identification and validation of novel drug targets in medicine; however, the interpretation of submitted and published data is often compromised by inadequate study design. Different study protocols, including the number of control groups and group size calculations, differ in target identification and validation studies. Furthermore, animal studies require that the selected target or compound meets the requirements for species specificity and target specificity; thus, providing the rationale for the selection of a particular species, strain, gender and age of the animals is necessary. Altogether, the presentation of target validation studies should meet defined criteria similar to those used in human trials.**

Introduction

Basic research continues to identify potential drug targets for human applications. Despite major developments in computed model systems, *in vitro* models and *ex vivo* models, *in vivo* animal testing is still a necessary part of modern drug development. Animal studies remain necessary to determine pharmacokinetics and distinguishing the effects on tissue remodeling and systemic effects that are related to the specific molecular target from those that are not. Furthermore, the optimal timing for therapeutic intervention can only be assessed in appropriate animal models of human disease states. Interestingly, the interpretation of data obtained from animal studies often ignores the limitation of such studies. In fact, data interpretation is restricted by the experimental design, which varies depending on the aim of the study. For example, technologies that generate mice or other small vertebrates with deletions or overexpression of single genes represent a way to study experimentally the functional role of a specific target *in vivo*; thus, such technologies can be classified as *in vivo* target identification studies. Validating novel targets for therapy of disease usually requires that the disease has developed before the specific target is modulated.

Conventional mutant animals with permanent deletion ('knockout') or overexpression ('transgene') of single genes might not represent the most appropriate model system for *in vivo* target validation studies because single genes can have multiple functions during the different phases of a disease. Thus, predicting the outcome of therapeutic intervention in humans in an advanced stage of the disease usually requires a different experimental approach, such as conditional manipulation of the target gene or specific agonistic and/or antagonistic compounds that modulate the function of the gene product (Boxes 1 and 2). Furthermore, agonists and antagonists usually have biological effects beyond the specific target and drug development programs generate compounds with yet unknown target specificities. Thus, *in vivo* drug validation studies might cover additional aspects that are independent of a single target.

The intention of the study defines how its design will differ for *in vivo* target identification versus drug and/or target validation. However, the intention of published *in vivo* studies is either often not clearly stated or the proposed intention does match with the reported study design, thus compromising data interpretation. Here, we discuss general requirements for the design of *in vivo* studies, with a focus on the criteria that define studies that either intend to identify or to validate novel targets.

Identifying novel therapeutic targets

The identification of new therapeutic targets covers large areas of biomedical research. For example, targets can be identified by data mining *in silico* (i.e. searching ever-expanding databases) [1–3]. However, molecular biology remains essential for identifying

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1359-6446/06/$ - see front matter doi:10.1016/j.drudis.2007.04.001

BOX 1

In vivo target identification

Novel targets can be identified through numerous different technologies, including gene transcription profiling ('transcriptomics'), protein expression profiling ('proteomics'), metabolic pathways ('metabolomics'), protein glycosylation ('glycosilomics'), protein–protein interactions ('interactomics') and systems biology *in silico*.

Novel targets can also be identified *in vivo* using genetic engineering, somatic mutagenesis or conditional mutagenesis using RNAi technologies, However, identifying the significance of the target for the specific disease process usually includes *in vivo* studies; for example, comparing the spontaneous phenotype of transgene animals with their wild-type or by comparing their phenotypes after inducing a disease model.

BOX 2

In vivo target validation

Validating targets for therapy usually requires the target to be modulated after the disease has established. Ways in which this can be achieved, include conditional target gene knockout technology, specific agonists and/or antagonists that modulate the target.

Agonists and/or antagonists can act at the transcriptional level (e.g. anti-sense oligonucleotides), the posttranscriptional level (e.g. RNAi) or the protein level (e.g. receptor antagonists, antibodies and aptamers). Before agonists or antagonists can be used *in vivo*, *in vitro* studies are required with cells overexpressing the target (e.g. receptor binding or ligand displacement studies, receptor activation studies, enzyme activity studies, etc.). For interventional *in vivo* studies, an appropriate disease model must be selected in a (mammal) species. Mice are most appropriate but rats are preferred for models of solid organ transplantation as surgical procedures are often difficult to perform at the small scale of mice. Control groups for all manipulations including the vehicle are necessary. The antagonist should be started only after the disease has established to mimic a therapeutic approach and to exclude functions of the target that contribute to organ development or the induction of the disease model.

putative targets in biological systems and molecular biologists have developed RNAi as a tool for large-scale and efficient drug target discovery; for example, the recent generation of RNAi-based constitutive or conditional transgenic mice has become a feasible, fast and cost-effective approach to studying mammalian gene function *in vivo* [4,5]. A detailed discussion of established and evolving tool for target identification is beyond the scope of this article, and the reader is referred to recent reviews for further information [6,7].

In the process of target identification, a detailed understanding of the species-specific expression and function of the drug target is vital. For example, Toll-like receptor-11 was identified as a novel pattern recognition receptor by searching EST databases. However, whereas the murine version of this receptor mediates innate immunity in response to the recognition of uropathogenic bacteria [8], the gene encoding the human form carries a stop codon. Thus, this receptor is an inappropriate drug target in humans. Therefore, to be of potential therapeutic value, data mining for potential targets must focus initially on the human system (Box 1). The process of target identification thus requires stringent selection of a few targets out of a large number of potential targets that appear to be most applicable for further characterization [9].

The compound

To further test the functional significance of the selected group of potential targets, specific agonists or antagonists are required to modulate the function of the targets *in vitro* and *in vivo*. Potential compounds might be specifically designed or selected by high-throughput screening against human target proteins. Further *in vitro* testing of candidate compounds will reduce the number of suitable candidates to one or a few lead compounds, which will be further characterized using *in vivo* disease models.

Species specificity

Before using lead compounds in animal systems, detailed information about the crossreactivity of the compounds with the target in the species of interest are necessary. For example, lower binding affinity in mice requires higher dosing which, in turn, might compromise target specificity. For example, BX471, a small molecule antagonist to the human chemokine receptor CCR1 blocks murine CCR1, albeit at 200-fold higher concentrations but remains still target specific [10,11]. By contrast, the viral chemokine vMIP2 blocks several human and rat but not murine chemokine receptors. Thus, *in vivo* testing of this compound is restricted to the rat.

Target specificity

In vitro testing must ensure that the compound does not interact with related (and unrelated) proteins, often family members of the intended drug target. For example, in the case of receptor antagonists, human embryonic kidney cells are usually transfected with several related receptors that should not be affected by the compound. If they are, the specificity of the compound is uncertain and effects observed *in vivo* might not relate to the proposed target itself.

Solubility

Many compounds are poorly soluble in water and must be dissolved in organic solutions. Such vehicles might cause considerable toxicity and systemic side effects depending on the dose and the frequency of application. Thus, controls groups, treated with the vehicle only, are necessary.

Pharmacodynamics and pharmacokinetics

The rationale for dosing the animal must be evident from appropriate pharmacodynamic and pharmacokinetic assays. Sufficient compound levels at the organ site of interest depend on a variety of factors, including adsorption, distribution, metabolism and excretion of the compound. Such ADME profiles are required for studies with animal models. In renal disease models, for example, one has to be aware that renal dysfunction can significantly alter the pharmacokinetics of the compound, which might require pharmacokinetic studies at various stages of renal dysfunction.

Administration

The route of administration is important not only because of animal welfare, but also for future use of the compound in humans, where the oral route is the desired route of administration. Hence, oral dosing is preferable whenever possible. Frequent

subcutaneous, intraperitoneal or intravenous injections lead to considerable stress in the animals and could influence treatment outcome. Assistant application devices, such as minipumps, should be avoided wherever possible, as they require surgery, additional control groups and can cause technical complications, particularly with viscous solutions.

The animal

For both target identification and target validation studies, small rodents remain the first choice. Rats are preferred for most solid-organ transplantation models because of technical reasons related to surgical procedures. Some disease models, such as anti-Thy1.1 nephropathy or Heymann nephritis, are restricted to rats because mice lack the disease-specific antigens [12,13].

However, mice are the preferred species when gene knockout technology is in use. A poor description of the used strain often limits the reproducibility and interpretation of data generated in mice. Many institutes breed mice in-house but only regular refreshment of this colony with mice from a genetically defined commercially available colony will ensure that the mice maintain the genetic characteristics of the source colony that is accessible to other researchers. Genetic drift can cause a change in the phenotype of a colony of a distinct mouse strain over time. For example, the MRLlpr mice bred at the Jackson Laboratories (http://www.jax.org) have lost their lymphoproliferative phenotype during the past decade, which could be restored by breeding a new colony from material frozen decades ago.

Thus, to assure reproducibility of data requires that the study population is clearly defined, for example, by using a common strain and even individual stock designations of each provider (accessible online via http://www.informatics.jax.org/imsr/IMSRSearchForm.jsp). This is necessary because the same toxic, metabolic, immunological or mechanical stimulus might cause a different phenotype in different colonies with their specific genetic background of the same strain. For example, collagen 4A3-deficient mice are used as a model of Alport disease [14]. In a mixed SvJ129/C57BL/6 background, these mice die from Alport disease at an average of ten weeks of age, whereas the genetic deletion crossed to a C57BL/6 background delays disease progression and prolongs survival to more than 21 weeks [14,15]. In another example, backcrossing Toll-like receptor-9-deficient C57BL/6 mice into the autoimmune strain of MRLl$^{pr/lpr}$ mice for an unacceptable single generation did not affect the onset of autoimmune tissue injury [16]. By contrast, backcrossing for six generations for 24 different known disease susceptibility loci identified a severe aggravation of autoimmune tissue injury associated with the lack of Toll-like receptor-9 [17].

It is well known that factors such as strain, colony, age, gender, diet, housing conditions, microbial status and handling can confound the outcome of interventional studies in laboratory animals. A detailed description of the 'animal characteristics' in animal studies similar to the study population selection criteria and the baseline 'patient characteristics' in human clinical trials would be desirable for any type of *in vivo* study.

The disease model

A disease model is a pathological condition resembling a human disease that either develops spontaneously or is induced by distinct manipulations. These can include surgery, injections of antigens or injection of toxins. Several criteria define the possibilities and limitations of a disease model.

Species dependency

Some disease models are restricted to certain species. For example, Heyman-(membranous glomerulo) nephritis depends on megalin expression in the glomerular basement membrane of rats [13].

Strain dependency

Many disease models depend on a defined strain of the species of interest. For example, Balb/c mice are preferred for adriamycin-induced nephrotic syndrome [18]. C57BL/6 mice are resistant to this model as they are to many other models that intend to induce proteinuria in mice [19]. Balb/c or 129Sv mice are also preferred for disease models that depend on immune responses of the Th2 type, whereas C57BL/6 mice tend to produce Th1 responses upon injury [20].

Strain dependency is particularly important in spontaneous models of disease. For example, the genetic susceptibility to systemic autoimmunity of NZM2410 mice has been traced to three gene polymorphisms, of which only the combination of all three will cause disease [21–23]. Other disease models, which rely on the genetic deletion of a single gene, are modified by the genetic background. For example, the homozygous deletion of the *lpr* gene will not cause severe lupus-like autoimmune tissue injury unless it occurs in the MRL strain of mice [24]. Obesity and sequelae of type 2 diabetes associated with the db/db genotype occurs only in the BKS strain of mice [25].

Technical feasibility

Some microsurgical manipulations are more difficult to perform in mice than in rats or in rabbits. For example, transplantation of most solid organs is technical demanding in mice and high technical failure rates are unacceptable from an ethical point of view [26]. Moreover, difficult technical procedures can add substantially to the variability of the induced disease phenotype.

Duration

Some disease models require months to develop. For example, late stages of diabetic organ damage might not occur before eight months of age in db/db mice. The benefits of such study protocols need to be balanced against the availability of the compound to be tested or the feasibility of drug administration. Subcutaneous injections three times in a day would be unacceptable for a period of months from the point of view of both the animal and the researcher. Often, additional manipulations can speed up the progression of disease model. For example, uninephrectomy-induced hyperfiltration of the remaining kidney accelerates various types of glomerular pathologies [27].

Relevance to human disease

Some disease models are frequently used in target identification studies, but might be less significant for target validation. For example, injection of a Th1 antibody induces mesangiolysis and a compensatory mesangio-proliferative glomerular lesion in rats [13]. The so-called 'anti-Th1-nephritis' lacks an equivalent human

disease and, thus, it is more suitable for identifying than for validating therapeutic targets.

Animal welfare

In choosing a disease model, animal welfare must always be given highest priority. Refining experimental disease protocols can markedly improve animal welfare [28]. 'Single shot' models should be preferred to models with multiple manipulations. Models with mostly painless procedures should always be preferred to painful procedures, such as foot pad injections, adjuvant use or intramuscular injections [29]. Pain killers given to animals after surgery or other painful manipulations do not usually interfere with the study result.

In summary, choosing a disease model requires matching the criteria for species, strain, technical feasibility, duration and animal endurance. The possibilities and limitations of each disease model determine this choice, as well as the criteria for the design of a target validation study.

The study design

Number of groups

The nature of the study determines its design. A target validation study requires appropriate control groups in the experimental set up. If a new target or compound is to be validated *in vivo,* appropriate groups that control for all types of manipulation are necessary (Box 2). By contrast, an exploratory approach intending to identify a new target *in vivo* would only require two groups (i.e. with and without the specific intervention) [30]. The same design is used for assessing the phenotype of certain knockout mice in comparison to the respective wild-type strain. In all cases, the scientific report should indicate the nature of the study to provide a rationale for the selected study design.

Group size

The experimental design is usually a compromise between statistical needs for the detection of biological effects and constraints of animal welfare, cost and time. Defining a quantitative primary endpoint is required for group size calculations, which also requires definition of the power for detecting a difference at a given p-value between two treatment groups [31]. Detection of small differences between the groups (< 1 standard deviation) requires large group sizes. If big differences between the groups are to be expected as a result of the intervention and lesser effects can be ignored (> 1 standard deviation), small group sizes are feasible.

In target identification studies, minor effects are usually not of interest. Thus, small group sizes limiting the detection of significant differences to more than 1 standard deviation of the primary end point are appropriate. By contrast, target validation studies have to be designed to detect also smaller effects in group–group comparisons. Hence, the detection level will need to be <1 standard deviation, which will increase the appropriate group size. Furthermore, the group size calculation is based on the variability of the primary end point in the control group as determined in pilot studies or retrieved from the literature. Online sample size calculators assist (e.g. http://home.clara.net/sisa/samsize.htm) but do not substitute for a statistician's advice in designing the study protocol.

The analysis

Inclusion and exclusion criteria need to be defined in all types of animal study that involve disease models. For example, if the effect of a compound is to be tested in an inducible model of multiple sclerosis, each individual animal should meet the disease criteria before inclusion in the study. As another example, studies with spontaneous models of diabetes should exclude those animals that rarely show elevated blood glucose levels, which, for example, can affect up to 10% of db/db mice from certain providers. This is analogous to human clinical trials, in which individuals are only included that match defined diagnostic criteria. Similarly, exclusion criteria must be defined. Unexpected morbidity or mortality might occur before end points at a given time point are reached. Whether these animals will be included in the data analysis (intention-to-treat analysis) or not (on-treatment analysis) should be clearly stated and reasons given.

Blinded data analysis

Any semi-quantitative or quantitative assessment of outcome data that is not performed fully in an automated fashion must be analyzed by a blinded observer who is unaware of the group allocation of individual animals to avoid an interpretation bias.

Statistics

Discussing statistical approaches in detail is beyond the scope of this review and is described elsewhere [32]. The widely applied *t*-test and one-way analysis of variance (ANOVA) both assume that data are continuous, at least approximately normally distributed and that the variances of the different data sets are homogeneous (i.e. are equal). These assumptions can be tested by using the Kolmogorov-Smirnov test to check for normal distribution of the data and Levene's test for equality of variances. Often, small group sizes compromise homogeneous distribution of the data and Mann-Whitney U or Kruskal-Wallis testing might be appropriate. When multiple comparisons become necessary, ANOVA, with appropriate *post-hoc* correction for multiple comparisons, must be applied to control the probability of obtaining false positive results. For example, the Bonferroni correction, the least significant difference test or the Student-Newman-Keuls can control for this source of error. A detailed discussion about the use of appropriate statistics cannot be provided here and researchers should consult a statistician to obtain information not only about the sufficient group size, but also about the appropriate statistical method to be applied for data analysis.

Special aspects of target validation

The role of mutant mice

Mice genetically deficient for a single gene product are a widely used tool for identifying the functional role of a selected target *in vivo*. When so-called conventional 'knockout' mice reveal a spontaneous phenotype or a phenotype upon induction of a disease model, the functional significance of the target for the disease process has been documented, but this does not necessarily validate that target for a potential therapeutic use. In fact, lack of the gene product throughout development might affect organogenesis, cause compensatory upregulation of other genes or might affect the induction of disease models. For example, models of

antigen-specific immunity require dendritic cell activation and migration to lymph nodes in its early phase. Lack of chemokine receptor CCR7 inhibits this process and models involving antigen-specific immunity will not develop properly [33]. The inconsistent autoimmune phenotype of $MRL^{lpr/lpr}$ mice that either lack Toll-like receptor 9 or that are treated with a respective receptor antagonist is one example [17,34]. By contrast, conditional knockout mice functionally silence a single gene on demand by exposing the animal to a 'silencer drug', which inhibits the promoter activity through interactions with genetically engineered promoter inserts [35]. This technology enables a single protein or cell type in adult mice to be depleted at any stage of development or disease model [36]. This technology represents an appropriate tool for validating a target before specific antagonists become available. Thus, conditional rather than conventional knockout mice can be used for *in vivo* target validation studies.

When to initiate treatment with the compound?

In a clinical setting, patients usually present with a clinical manifestation of disease before treatment is considered. Thus, to be of value in a target validation study, drug administration to the animal should not be started before the disease model has been established; in the clinical situation, treatment is often administered only at a very late stage of the disease process. Thus, the significance of data in a target validation study becomes greater when the interventions prove to be effective when initiated at a late stage of the disease process.

By contrast, early treatment might interfere with the role of the target in the induction phase of the disease model. For example, blockade of chemokine receptor CCR2 has opposite effects on collagen-induced arthritis when initiated before versus after immunization with collagen [37]. Appropriate samples should be collected at onset of treatment to define the stage of disease at this time point (e.g. blood glucose levels, urinary microalbumin, and renal histology as markers of diabetic nephropathy in db/db mice). A few exceptions exist. Diseases such as transplant rejection or radiocontrast-induced acute renal failure are suitable for preventive therapy because well defined risk factor profiles identify patients at risk. When a new drug is validated in an appropriate animal model for one of these diseases, the initiation of drug administration at the time or even before induction of the model should be appropriate.

Summary

Animal study reports often lack important information about the rationale for the study design, which can compromise data interpretation. All animal studies require that the selected target or compound meets the requirements for species specificity and target specificity. The rationale for selecting the particular species, strain, gender and age of the animals must be provided and these should be accessible to the general scientific community. Study protocols including the number of control groups and group size calculations differ in target identification or validation studies. Target validation studies used should meet defined criteria similar to those used in human trials. Hence, we propose the use of standardized check lists, which could be submitted along with the manuscript and be published online in a supplementary material format (Table 1).

TABLE 1

Requirements for target validation *in vivo*

Target
Provide rationale for modulating the target (from target identification studies)
Provide information about species-specific expression, regulation and function of target
Compound
Provide data on target specificity of the compound for the tested species
Provide data on pharmacokinetics of the compound in species used and on the route of administration as a rationale for dosing
Provide rationale for using assistant devices, if unable to do so in humans: catheters or minipumps cause additional trauma to the animal, device failure can be common and additional control groups are necessary
Animal
Provide rationale for the species, strain, gender and age of animals used
Clearly define genetic background of the animals, which should be freely accessible to the scientific community; do not use mice from own-inbred colony that is not regularly refreshed by breeders from a public provider
Backcross transgenic animals at least for six generations into a defined inbred strain; do not consider siblings as appropriate controls for inappropriately backcrossed animals
Genotype each individual transgenic animal because the backcrossing exercise is a constant source of errors; do not rely on breeding protocols even in homozygous colonies of mutant mice
Provide information about strain, colony, age, gender, housing conditions, microbial status, diet and number of animals per unit; report any treatment or handling that was not shared by animals of all groups
Study protocol
Define type of the study and its primary end point; seek help from a statistician to calculate appropriate group size based on the variability of the primary end point in the disease model
Use control groups for the vehicle and all other manipulations done to the animals
Disease model
Choose a disease model that enables a maximum conclusion for the corresponding human disorder at the lowest level of animal discomfort
Consider strain dependency of many disease models
Legal
Obtain legal permission for animal experimentation from local authorities before start of study
Analysis
Define inclusion (e.g. parameters that indicate sufficient disease induction) and exclusion criteria (e.g. parameters indicating excessive morbidity) before the study
Clearly define primary and secondary endpoints before start of study
Get data analyzed by blinded observer
Use appropriate statistics that are recommended by the statistician who approved the study design

Acknowledgements

This work was supported by grants from the Deutsche Forschungsgemeinschaft (An372/8-1, Vi 231/2-1, GRK 1202), the Fritz Thyssen Foundation, the Else Kröner Fresenius Foundation, the Wilhelm Sander-Foundation, and the EU network 'INNOCHEM' (FP6-518167).

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