Laboratory Animal Science Association

NC3Rs

National Centre for the Replacement, Refinement and Reduction of Animals in Research

Guidance on dose level selection for regulatory general toxicology studies for pharmaceuticals

The 3Rs:

Replacement refers to methods that replace or avoid the use of animals in areas where animals would have otherwise been used

Reduction refers to methods which minimise 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

Refinement refers to improvements to husbandry and procedures that minimise actual or potential pain, suffering, distress or lasting harm and/or improve animal welfare in situations where the use of animals is unavoidable

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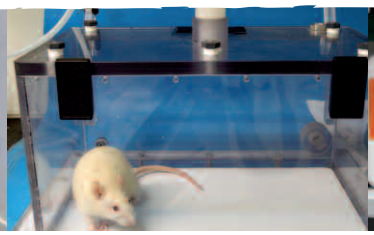
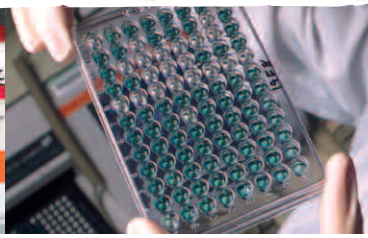
1 Background



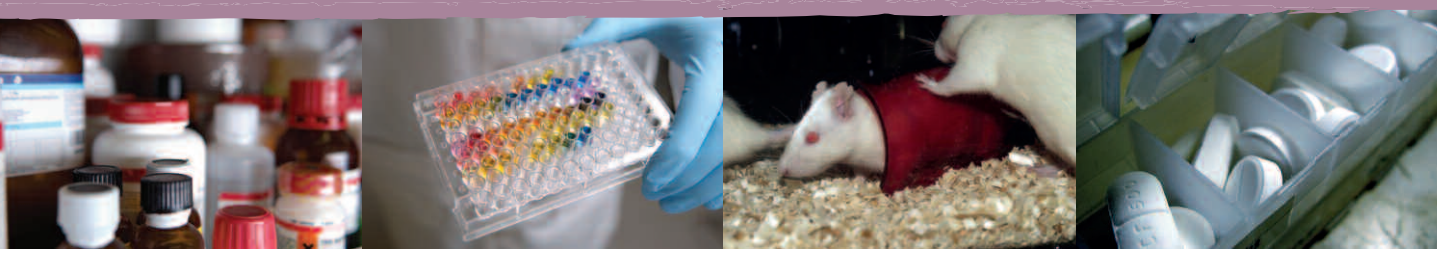


The National Centre for the Replacement, Refinement and Reduction of Animals in Research (NC3Rs) and the Laboratory Animal Science Association (LASA) share the common goal of promoting the principles of the 3Rs – the replacement, refinement and reduction of animal use in research [1,2]. Together, the organisations have formed an expert working group that comprises toxicologists from the UK's major pharmaceutical companies and contract research organisations. The group aims to facilitate good practice in the design and conduct of regulatory toxicology studies by sharing information on practices across the industry.

It is a scientific, ethical and regulatory requirement that before any potential new medicine can be administered to man its safety must be investigated in animals in order to define safe human doses. These animal tests are intended to reveal potential toxicity associated with the prospective medicine. While this requirement remains, it is essential to minimise the number of animals used and the adverse effects they may experience. Since the objective of toxicology studies in animals is to identify potential toxic effects in humans, some of the animals used will suffer adverse effects. However, careful consideration and design of studies can reduce the impact on animals without compromising scientific goals or human safety and may indeed improve the quality of scientific data [3].

2 Objectives and scope

A key objective of the NC3Rs/LASA collaboration is to provide practical guidance for Study Directors and other toxicologists working in the field of regulatory toxicology. This document is aimed specifically at scientists that are new to, or training in, the role of Study Director. Drawing on the knowledge and experience of the working group members, the guidance document is intended to supplement the process of training and mentoring of Study Directors to improve the scientific outcome of regulatory general toxicology studies (see Figure 1) and to promote the application of the 3Rs. This is achieved by a series of recommendations that are considered good practice by the group. The guidance provided in this document has the potential to make substantial progress in reducing and refining animal use in this area, through avoiding unnecessary exposure of animals to marked adverse effects thereby reducing inadvertent morbidity and mortality and avoiding potential repetition of toxicology studies (see concluding comments).



Safety assessment studies in animals span several disciplines (safety pharmacology, general toxicology, genetic toxicology, reproductive toxicology, immunotoxicology). The dose selection guidance in this document focuses on general toxicology studies but many of the principles apply to studies in other disciplines.

This document focuses on dose selection for:

- Preliminary short duration studies (e.g. dose range finding)
- Regulatory toxicology studies that are required for first clinical trials in humans (e.g. good laboratory practice [GLP] two week or one month studies or cyclical dosing regimens for anti-cancer drugs)
- Longer term studies (e.g. three and six month studies)

Written within the context of the UK regulatory system for the use of animals, the Animals (Scientific Procedures) Act 1986 (ASPA) [4], the principles outlined in this document are applicable to studies submitted to regulatory authorities worldwide. The recommendations are for standard routes of administration such as oral and intravenous. Specialised routes including inhalation and continuous infusion are not covered. Information on these routes can be obtained from relevant textbooks [5, 6]. Dose selection for biopharmaceuticals is also out of the scope of this document.

New clinical tools to conduct limited evaluations of potential new medicines in humans are available e.g. clinical trials for exploratory investigational new drugs (eINDs) [7] and microdosing. These approaches enable human data to be obtained earlier in the drug development process and provide the opportunity to reduce the number of potential new medicines requiring traditional safety and toxicology testing in animals. The studies are used to gather information on certain properties (e.g. pharmacokinetics) that, if inappropriate for humans, could prevent further development of the potential new medicine and therefore further animal use. As the initial investigations in humans are limited, full characterisation of the toxic potential is not always necessary prior to these trials, therefore dose selection for eINDs and microdosing is not covered in this document. Medicines with appropriate characteristics will require the standard preclinical safety assessment package prior to further clinical trials.

Figure 1: Potential new medicine: cascade of general toxicology studies

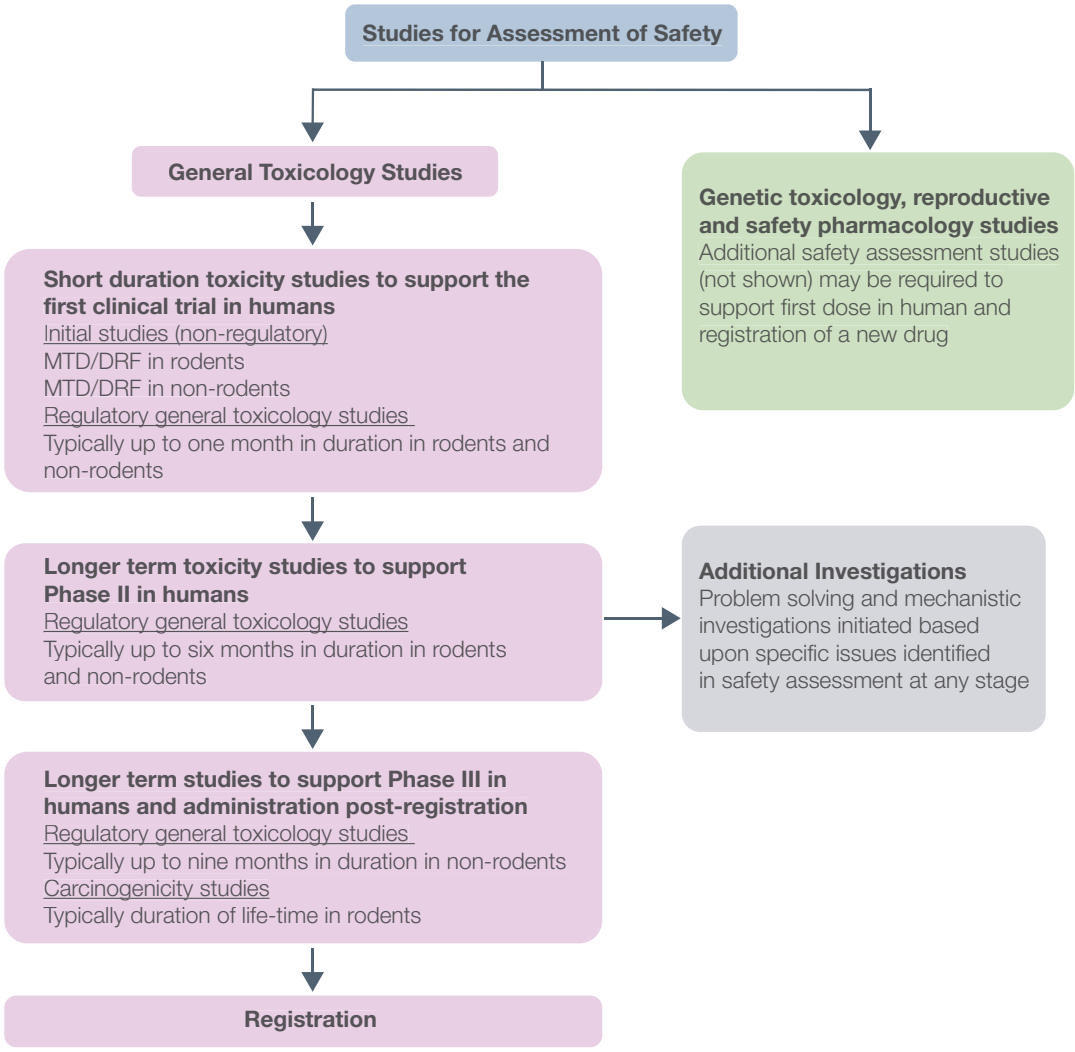


Figure 1 shows a sequence of regulatory general toxicology studies (in pink) required for the development of a new medicine.

3 Introduction to dose selection

3.1 Animal use in preclinical safety studies

The ethical imperative to conduct preclinical safety testing of new pharmaceutical products before administration to humans is embodied in the Declaration of Helsinki [8]. Regulatory approval is required before the first clinical trial and this is contingent on a demonstration of the intrinsic hazard of the test item and an assessment of the potential risk that such hazard may pose in humans. Currently this necessitates the use of animals.

Information from safety studies in animals is used to characterise:

- Target organ toxicity
- Relationship between dose or exposure and response
- Potential reversibility of any observed effects
- Relevance of any findings to man
- Potential parameters for monitoring adverse effects in clinical studies

Preclinical safety studies are conducted to provide greater understanding of the potential intrinsic hazard of the test item and to estimate safety margins. Safety margins are used to determine an initial safe starting dose for clinical trials, a safe dose for continued use in humans through longer clinical trials, and ultimately to achieve successful review of registration dossiers to support marketing approval and use of new medicines within the wider population.

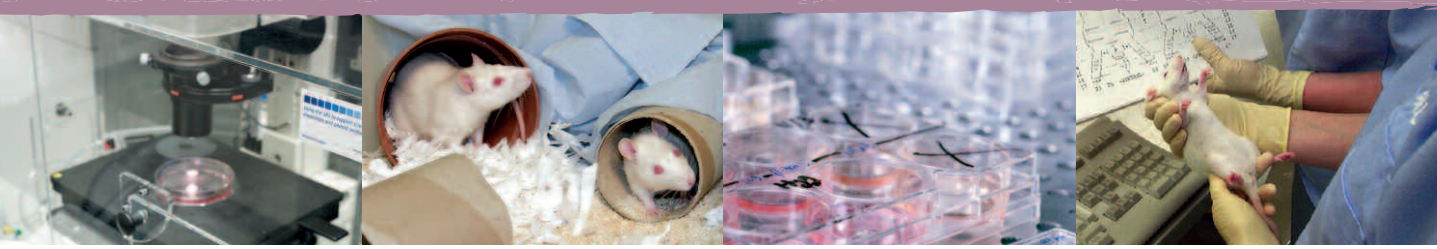
3.2 Regulatory requirements to determine toxicity and dose response

Careful consideration of the doses used in preclinical studies is necessary to fulfil the scientific needs of safety assessment and to satisfy regulatory authorities. The current Committee for Proprietary Medicinal Products (CPMP) note for guidance on repeated dose toxicity studies indicates that doses should be selected to establish a dose or exposure response to treatment [9]. This can generally be achieved by the use of three groups of animals receiving the test item, at low, intermediate and high doses, plus a control group which receives vehicle alone. Experience has shown that three doses will usually cover the span between no effect and adverse effects, although there are exceptions (see Section 7.4). The CPMP guidance also indicates that the high dose should be selected to enable identification of target organ toxicity, or other non-specific toxicity, or until limited by volume or limit dose. In addition to establishing toxicity, it is necessary from a scientific perspective to establish the No Observed Effect Level (NOEL) and/or the No Observed Adverse Effect Level (NOAEL) that may be used along with other information, such as the pharmacologically active dose, to determine the first dose in human studies.

3.3 Establishing toxicity and managing adverse effects

Toxicology studies are conducted as part of a tiered programme as shown in Figure 1. The first studies conducted are referred to as pilot studies, preliminary studies, sighting studies or dose range finding studies (from herein, dose range finding [DRF]) and these are used to select doses for subsequent regulatory studies of up to one month. The one month studies are in turn used to inform dose selection for longer term regulatory studies (e.g. three and/or six months).

The main objective of regulatory toxicology studies is to establish the potential hazards associated with the test item by identifying potential organ toxicity. To achieve this objective and fulfil regulatory requirements, evidence of toxicity in animals is required. Appropriate dose selection is critical to establishing toxicity. The primary parameter used in dose selection is the tolerability of the test item in animals. Tolerability can be determined by observations such as clinical signs, reductions in body weight or a decrease in food consumption. Key parameters such as systemic exposure (measured as the concentration of test item [bound and unbound] in plasma/serum or blood) and histopathology may also be used to support dose selection. Depending upon the nature of the test item, other specific parameters may also be used, for example, changes in



haematology parameters for certain anti-cancer drugs or blood pressure or electrocardiogram (ECG) effects for compounds targeting the cardiovascular system. The clinical condition of an animal usually gives an initial indication that the test item is causing systemic toxicity. Due to the risk of unexpected or marked adverse clinical signs, the management of animal welfare issues is paramount in toxicology studies. This includes appropriate monitoring of clinical and other parameters and the use of humane endpoints.

For many clinical signs, for example, emesis, subdued behaviour and pilo-erection, there are no premonitory observations that would allow early intervention. However, once these signs, have been observed in one animal they should be used to intervene earlier wherever possible (i.e. before the observations become as marked) in the remaining animals. A specific example of the use of a humane endpoint is described in **Example 1**.

Careful consideration should be given to dose selection so that the impact on the animal can be minimised while still achieving the scientific objective of the study. Determining an appropriate dose requires relevant experience and judgement as it is often influenced by the nature of the test item, its target pharmacology and its intended therapeutic use in humans. Selecting a dose that is too high or selecting a dose that does not produce toxicity may risk repetition of the study thus requiring the use of additional animals. It may also prevent identification of target organs and early

indicators that can be used to monitor potential effects in human studies. There is an inherent risk that selecting doses from initial studies using small numbers of animals or short dosing duration may not predict what happens when larger numbers of animals or longer dosing durations are used in the subsequent regulatory studies. Application of the recommendations in this document reduces the risk of a study which does not meet its scientific objectives by providing information to allow thorough investigation of appropriate doses in the early studies.

Emphasis is given to selection of the high dose where welfare concerns may be greatest. Worked examples providing practical information on dose selection are provided in Section 7. Since the outcome of a toxicology study is unknown beforehand, it is difficult to give definitive or prescriptive rules about dose selection. Choosing the right doses depends on the combined skill and experience of the toxicologists, the discovery scientists who understand the target and the pharmacokinetics, pharmacodynamics and metabolism of the compound and the clinicians who understand the intended patient population. The worked examples can help new Study Directors ask the right questions and consider the most important issues.

Example 1: The use of humane endpoints

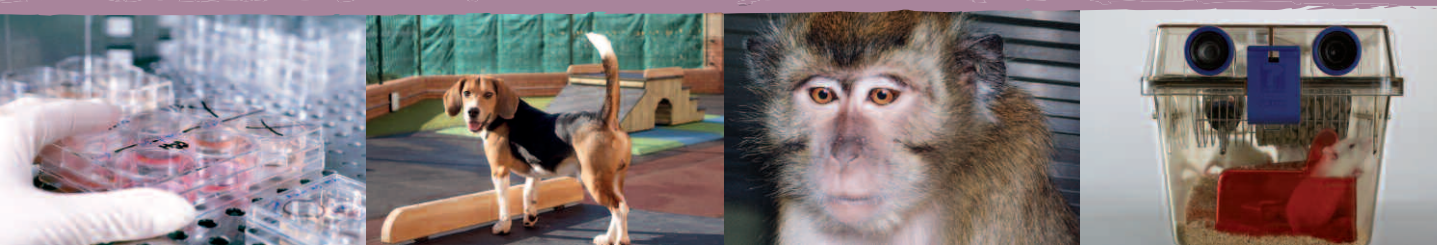
In a one month study, one animal showed adverse effects that required the animal to be euthanased on Day 15. These included effects on body weight (body weight loss > 10%), reduced food consumption and subdued behaviour. A blood sample was taken for haematology and clinical chemistry assessment. The results indicated elevated levels of several enzymes and components indicative of abnormal liver function (e.g. alanine aminotransferase, glutamate dehydrogenase and bilirubin) and at necropsy the liver appeared abnormal. Therefore, additional blood sampling time points were incorporated into the remainder of the study to monitor the markers detected in the euthanased animal. Evidence of elevation of the markers was used in conjunction with less marked effects on body weight, food consumption and clinical signs as a humane endpoint for the remaining animals on the study.

4 Selection of the high dose

There are five general criteria for defining the high dose in a toxicology study. These are (i) maximum tolerated dose, (ii) limit dose, (iii) top dose based on saturation of exposure, (iv) maximum feasible/practical dose, or (v) dose providing a 50-fold margin of exposure. For a full description of the options for selecting the high dose in general toxicity studies see ICH guidance M3 (R2) [10].

4.1 Maximum Tolerated Dose

An important part of a toxicology study is to identify a dose where target organ toxicity is likely to be observed but where the dose is not so high that the study is jeopardised by morbidity or mortality. This dose is referred to as the maximum tolerated dose (MTD) because the animal receiving the test item would not tolerate adverse effects that may occur at higher doses. The MTD is defined as the highest dose that will be tolerated for the study duration.



Typically, duration of dosing has an influence on the MTD because the animal may be less able to tolerate consecutive high doses over long periods of time. For example, the MTD for a single administration is likely to be higher than the MTD for three or more days of dosing. The MTD for seven days dosing may be greater than that for 28 days dosing which may in turn, be greater than for 90 days dosing. Generally, for studies longer than 90 days, the MTD will remain unchanged. Defining the MTD in the studies of shortest duration informs dose setting in subsequent studies and is crucially important in application of the 3Rs since this reduces the chances of larger numbers of animals that are used in regulatory studies being exposed to unanticipated suffering.

The MTD is usually determined by parameters such as clinical signs and reductions in body weight and food consumption. A review of clinical signs and endpoints for adverse effects associated with the MTD in short term toxicology studies is currently being carried out [11]. However, it is possible to identify an appropriate MTD using clinical signs of moderate severity as described in the report of the FELASA working group on pain and distress [12].

Table 1 contains examples of clinical signs of moderate severity in rodents taken from the FELASA working group report. Table 2 contains examples of clinical signs of moderate severity adapted from this report for use in dogs. It is important to note that the descriptions in the moderate column in Tables 1 and 2 represent the upper limit of severity for identifying the MTD. Utmost importance should be given to determining humane endpoints, without reaching these levels of severity.

The framework outlined in the FELASA report does not define either the intensity or duration of clinical signs and therefore variation exists across the industry in the interpretation of clinical signs indicative of the MTD. There is a need for clear updated guidance based on objective measures. This is particularly relevant given recent work questioning the value of acute toxicity tests and, in the absence of lethality as an endpoint, a clear definition of MTD is essential [13]. Other data such as clinical pathology findings or pharmacological parameters such as ECG may also help determine an MTD. For example, the hematopoietic system is often a target for compounds being developed to treat certain cancers and therefore haematology changes may be used, in combination with clinical signs, to define the MTD in early studies. Similarly, changes in clinical chemistry parameters measured in blood and/or urine may act as early indicators of toxicity. For example, increases in alanine aminotransferase may indicate the development of liver toxicity.

Tables 1 and 2 in this document provide examples of general guidance for endpoint assessment. Specific guidance within your establishment should be referred to. Each animal must be assessed individually with input of experienced personnel (such as a veterinary surgeon) when appropriate.

Clinical signs should be evaluated with consideration of the biological and pharmacological properties of the test item, the time of occurrence relative to dosing, and their duration and severity. Incorporating clinical observations into a formal scoring system has been found to be artificially rigid, and in some cases, may encourage working up to the limit of severity, rather than early intervention.

Guidance tables are normally used with the following recommendations:

- Any deviations from normal should result in careful observation to identify any deterioration in the animals' condition
- Clinical signs or conditions associated with the test item will normally be determined as being of moderate severity rather than mild where they are prolonged in nature, of a severity that has the potential to impact on the animals ability to eat, drink, rest or sleep, or have the ability to cause the animal harm
- Observation of a combination of signs of moderate severity should result in immediate action (such as cessation of dosing, amelioration of the signs by treatment, euthanasia)
- Observation of a single clinical sign from the substantial severity limit should result in immediate action (usually euthanasia)

Table 1: Endpoint guidance for mild, moderate and substantial severity in rodents

Mild	Moderate	Substantial
Reduced weight gain	Weight loss of up to 20%	Weight loss greater than 25%
Food and water consumption 40-75% of normal for 72 hours	Food and water consumption less than 40% of normal for 72 hours	Food and water consumption less than 40% for 7 days, or anorexia (total inappetence) for 72 hours
Partial piloerection	Staring coat – marked piloerection	Staring coat – marked piloerection – with other signs of dehydration such as skin tenting
Subdued but responsive, animal shows normal provoked patterns of behaviour	Subdued animal shows subdued behaviour patterns even when provoked	Unresponsive to extraneous activity and provocation
Interacts with peers	Little peer interaction	
Hunched transiently especially after dosing	Hunched intermittently	Hunched persistently ('frozen')
Transient vocalisation	Intermittent – vocalisation when provoked	'Distressed' – vocalisation unprovoked
Oculo-nasal discharge transient (typically signs of chromorhinodacryorrhoea in rodents)	Oculo-nasal discharge persistent	Oculo-nasal discharge – persistent and copious
Normal respiration	Intermittent abnormal breathing pattern	Laboured respiration
Transient tremors	Intermittent tremors	Persistent tremors
No convulsions	Intermittent convulsions	Persistent convulsions
No prostration	Transient prostration (less than 1 hour)	Prolonged prostration (more than 1 hour)
No self-mutilation	No self-mutilation	Self-mutilation

Table 1 lists possible clinical signs in rodents and classifies the signs as mild, moderate and substantial according to the FELASA working group report[12].

It is possible to identify an appropriate MTD using clinical signs of moderate severity as described above. The clinical signs listed represent the upper limit of severity in each category. It may be possible to identify humane endpoints and achieve the study objectives without reaching this limit.

Table 2: An example of endpoint guidance for moderate severity in dogs

	Moderate LIMIT
Body weight	■ Weight loss up to 20% ■ Food consumption less than 40% of normal for 72 hours
Clinical signs	■ Vomiting or diarrhea ■ Subdued ■ Mild dehydration ■ Abnormal vocalisation ■ Ataxia or transient convulsions

Table 2 is a modified version of the FELASA working group table for moderate severity adapted for use in dogs and represents the upper limit of severity.

4.2 Limit dose

The limit dose defines the highest dose that should be used in the absence of a demonstratable MTD. The limit dose is generally accepted as 1000mg/kg in both rodents and non-rodents. For exceptions to the 1000mg/kg and an informative decision tree see ICH M3 (R2) guidance [10].

4.3 Toxicokinetics and saturation of exposure

The primary objective of toxicokinetic assessment is to describe the systemic exposure achieved in animals and its relationship to the dose, sex, species and the time course of the toxicity study (see Section 7.2.5). In order to achieve this, blood samples are taken from animals on toxicity studies and the plasma/serum or blood concentrations of the test item or its metabolites are measured. Toxicokinetic information enhances the value of the toxicity studies by relating (i) the exposure to the test item to any toxicological findings and (ii) the exposure in animals to clinical data as part of the assessment of risk and safety in humans. Additionally, toxicokinetic data can be used to inform dose selection for a subsequent study. For example, where toxicokinetic data indicate that absorption limits exposure to the test item or its metabolites, the lowest dose which achieves maximum exposure should be used as the high dose in the absence of other dose limiting constraints. This is often referred to as saturation of exposure. For a full description of the assessment of systemic exposure in toxicity studies see ICH guidance S3A [14].

4.4 Maximum Feasible Dose

Practicalities, such as formulation, can mean the high dose is limited by what is technically feasible based upon the maximum possible concentration of the test item in the formulation and the dose volume that can be administered over the study duration (see Section 7.2.6). Volumes no greater, and ideally lower than, those described in Diehl *et al* [15] should be used.

4.5 Doses providing a 50-fold margin of exposure

Doses providing a 50-fold margin of clinical exposure, usually based on the group mean of the area under the curve (AUC) values of the test item, are also generally acceptable as the maximum dose for acute and repeated dose toxicity studies in any species [10].

5 Principles of good practice in regulatory general toxicology studies

The principles outlined below provide a framework for minimising animal use and suffering in the conduct of regulatory toxicology studies.

5.1 Rodent to non-rodent test cascade

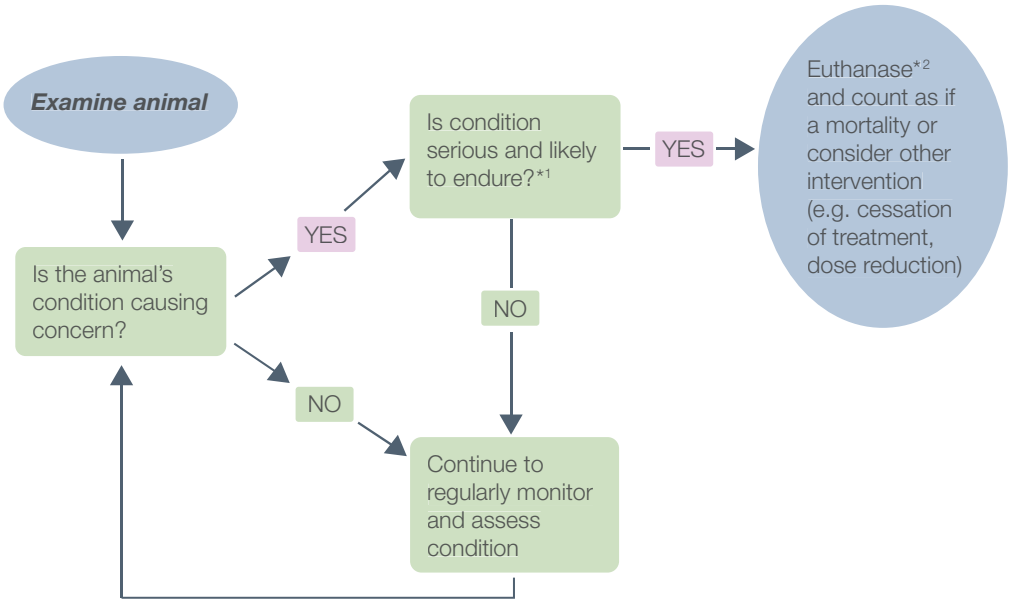
The first *in vivo* toxicity study for a new test item is usually a dose range finding (DRF) study in rodents (Section 6). For both scientific and welfare reasons, it is common practice to explore adverse effects in rodent species prior to non-rodent species. This increases the amount of information available for the design of the non-rodent studies, for example, data from the initial rodent study can be used to help set the starting dose, or to allow for specific monitoring of adverse effects in non-rodents. Additionally, toxicity may be identified in the rodent studies that prevents further development of the test item and therefore helps avoid further animal studies.

Clear scientific justification, agreed within your establishment, should be provided if a non-rodent is to be used prior to a rodent.

5.2 Anticipating potential adverse effects

It is often not possible to predict all of the adverse effects of a test item until it is administered to animals and therefore measures should be in place to manage unexpected effects. For example, in early studies (e.g. DRF) it is important to observe and monitor animals very closely for an appropriate time after dosing (typically at least until the anticipated C_{max}) and thereafter regularly throughout the rest of the day. In some cases, possible adverse effects can be anticipated based upon knowledge of the drug target. This includes information from the scientific literature, efficacy or pharmacokinetic studies, toxicological properties of closely related compounds or the target pharmacology. For example, effects such as decreased activity and/or subdued behaviour may be anticipated for a compound targeting receptors in the central nervous system (CNS). As much knowledge as possible should be gathered prior to commencing studies to allow adverse effects to be predicted and managed. The information collected in early toxicology studies should be used to set earlier humane endpoints where possible for subsequent studies. Figure 2 illustrates a flow chart for the management of adverse effects.

Figure 2: Flow diagram to show management of adverse effects



*1 At this stage it may be appropriate to discuss the animals condition in the context of the objectives of the study, i.e. has the primary study objective been met? Where possible discussion with the Study Director and Named Animal Care and Welfare Officer (NACWO) or a veterinary surgeon is encouraged. However, ultimate accountability for individual animal welfare lies with the personal licensee.

It may be of value to administer some adjunctive treatment(s) prior to, at the time of, and/or following administration of the test item, in order to minimise the potential for expected adverse effects on the animal.

*2 If an animal needs to be euthanased use the information collected to set earlier humane endpoints for subsequent animals where possible.

5.3 Staged or staggered approaches to dosing

Staged or staggered approaches to dosing allow scientific objectives to be met whilst minimising the number of animals at risk of experiencing suffering. For example, the first time the test item is administered to animals, it is good practice to employ a study design where one group of animals is dosed at a time. The observations are then noted and used to inform the next dose, which may be higher or lower depending upon the nature of the observations seen. The time between dosing each group is determined by a number of factors, such as knowledge of previous compounds in the class, the dose route and predicted pharmacokinetics. Typically at least 24 hours should be left between dosing groups for test items administered orally. The number of animals dosed at each level should be small in initial studies (e.g. one or two of each sex) with group sizes being increased once a dose response is established.

In a regulatory toxicology study, dosing of the treatment and control groups will usually be performed in parallel because information on the likely adverse effects will be available from the initial studies (e.g. DRF). However, where there is uncertainty about the degree of severity of adverse effects or the selected doses, the start of treatment should be phased. Treatment groups, commencing with the lowest dose, should be dosed separately. If recovery or satellite groups are to be used, they can be started separately from the main treatment groups, minimising the risk to study groups with larger numbers of animals.

5.4 Study management

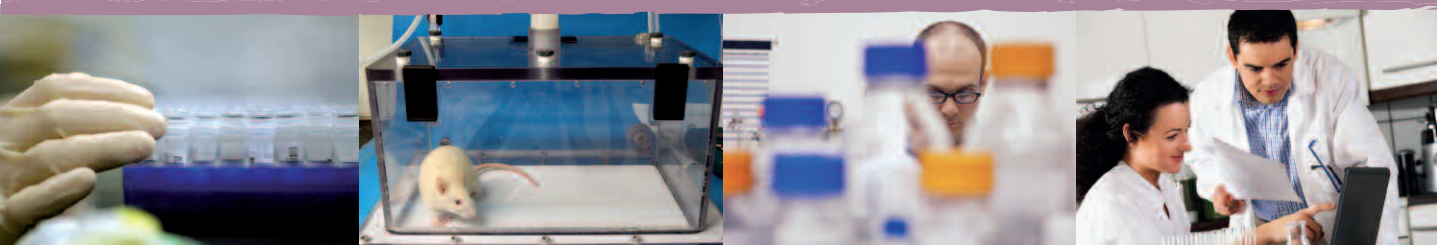
A well designed and implemented toxicology study involves understanding of the basic science, for example, the pharmacological target, the likely toxicological findings and the clinical application of the test item. The accountability for delivering a well designed toxicology study lies with the Study Director. However, input should be sought from all relevant disciplines, for example, pharmacologists, clinical pathologists, pathologists, veterinary and animal care staff and clinicians. Primary responsibility for the welfare of animals undergoing regulated procedures under the ASPA resides with the personal licence holder. Interactive study management is essential to ensure the scientific objectives are achieved whilst minimising the impact of any harmful effects on the animals. Regular monitoring of clinical signs and other data (e.g. body weight, food/water consumption) allows greater understanding of the adverse effects and adjustments to be made, if appropriate, to doses and humane endpoints. For example, early observation of inappetance or body weight loss can allow appropriate intervention, including:

- Allowing food to be available for longer periods of time
- Modifying the food or the way it is presented to the animals, to encourage appetite (e.g. moistening dry diet or offering supplements)
- Reducing the dose or stopping dosing

6 Dose range finding

The primary objective of a DRF study is to establish a dose response and provide the data to enable appropriate dose selection for subsequent regulatory toxicology studies. Generally, DRF studies are initially carried out in rodents, progressing to non-rodents when the adverse effects in rodents are understood. Guidance on industry recommendations for MTD/DRF study design in dogs has been published elsewhere [16]. The DRF studies are not normally classed as regulatory studies and provide essential information on:

- Doses for subsequent regulatory studies
- Adverse effects associated with the test item and dose, which can be used to devise a strategy to reduce the adverse effects in longer term studies, for example, application of humane endpoints
- Intrinsic hazards associated with the test item. This may be used to identify test items not suitable for future development thus avoiding subsequent animal use



Dose selection for the first regulatory studies, generally up to one month in duration, is usually based on DRF studies of seven to 14 days. The toxic effects associated with a test item and the MTD (see Section 4.1) should preferably be determined in the initial DRF studies when small numbers of animals are exposed to the highest severity of effects. Early identification of the MTD can also help reduce subsequent use of animals later in development to meet regulatory requirements, for example, by avoiding having to perform additional studies to support the high dose selection. In addition, MTD data can substitute for data formerly obtained from single dose acute toxicity studies [13].

6.1 Guidance for dose range finding study design

6.1.1 There are no regulatory guidelines for DRF studies. A DRF study may be divided into two phases, (i) a dose escalation phase consisting of a single or small number of repeated administrations to establish an MTD for short term duration and (ii) a fixed dose repeat dose phase typically of seven to 14 days. The number of dose groups required to establish a dose response should be based on previous experience, data from initial *in vivo* studies (e.g. pharmacokinetic studies), the nature of the compound and knowledge of the target.

6.1.2 The starting dose in DRF studies is selected by using information described in Section 5.2, for example, the scientific literature, efficacy models, pharmacokinetic studies and/or toxicological properties of closely related compounds. The starting dose is not intended to be overtly toxic and is often a dose (or small multiple of the dose) previously tested in other study types (e.g. drug metabolism and pharmacokinetics [DMPK] studies) with no effects. Information generated in rodents can help guide both the starting dose and the interval between doses in non-rodents, for example, if a steep dose response is observed in rodents this should be taken into account when considering dose intervals in the non-rodent.

6.1.3 The interval between doses in DRF studies (or in the dose escalation phase of the study) should be determined on a case by case basis. Frequently, half log intervals are employed (e.g. 1, 3, 10, 30, 100, 300, 1000mg/kg etc), but the interval will depend on factors such as the expected linearity and slope of the dose response, and the exposure response curve. It is not essential for the dose intervals to be evenly spaced, they should be selected to maximise the utility of the information obtained. In addition, it may be necessary to investigate doses between half log intervals depending upon the observed adverse effects and the exposure to the test item. For example, if adverse effects that would prevent dosing at a longer duration are noted

at 1000mg/kg and a dose of 300mg/kg produces no effects, a dose between 300 and 1000mg/kg should be investigated in the DRF study.

6.1.4 Analysis of test item formulation is usually conducted on regulatory toxicology studies to confirm the identity, stability and dose of the test item administered to the animals. It is recommended that this analysis is also conducted on DRF studies to resolve any problems with stability or dose prior to starting regulatory studies with larger groups of animals. In order to achieve this, an analytical method should be available to measure samples from the formulations prepared for the DRF study.

6.1.5 The formulation of the test item used in the DRF study should ideally be the same as the formulation proposed for the subsequent regulatory toxicology study in order to avoid unexpected effects that may put the regulatory study at risk. When formulation changes are predicted to have an impact on systemic exposure or local tolerance, then a bridging study should be considered before selecting doses for the regulatory study. Changes in formulation characteristics with the potential to affect exposure or tolerance include alterations in formulation excipients, alterations in the salt form of the compound and changes in particle size distribution of test item (for suspension formulations).

6.1.6 Tolerability of the animals to co-solvents, which are used to increase solubility, stability or absorption of the test item may limit the volume that can be administered. When novel vehicles or co-solvents are used it is essential to establish prior to dosing whether there is any information on the tolerability. This information may be available in the scientific literature, via internal company databases or from informal information exchange between companies [17].

6.1.7 The strain, age and source of animals used in the regulatory toxicology study should be the same as those used in the DRF study. If this is not possible, for example, when studies are conducted at different locations and supply to meet specific requirements is limited, consideration should be given to performing a bridging study before commencing the regulatory study to ensure similar tolerance and exposure of the test article between strains, sources or ages. Information should be sought from test items in the same class or from other animal studies of the same test item (e.g. safety pharmacology). The potential risk of observing a more severe adverse effect in the regulatory study should be balanced against the need to do an additional bridging study that uses more animals.

6.1.8 The impact of husbandry and environmental conditions upon experimental results is well recognised. It is important to ensure that animals are kept in similar conditions for the DRF and subsequent regulatory toxicology studies of the same test item. It is also important to maximise opportunities for well being, providing animals with an environment that satisfies their physiological and behavioural needs. This is usually managed through social housing, provision of environmental enrichment, optimal cage design, and for larger species, provision of additional stimuli such as play opportunities and specialised diet [18]. The importance of good animal care and empathetic handling by trained animal care staff should not be underestimated. These considerations are key to good experimental design.

6.1.9 Clinical pathology and histopathology on the major organs should be performed in the DRF study to identify progressive changes which may limit the duration of dosing at the high dose of the regulatory toxicology studies. Histopathology may also identify target organ toxicities that would be sufficient to stop further development of the test item and further use of animals in toxicity testing.

6.1.10 A toxicokinetic assessment should be performed in the DRF study in order to provide information that describes the systemic exposure achieved in animals and its relationship to the dose, effects, sex, species and the time course of the study. In order to achieve this, blood samples should be taken in the DRF study and an analytical method should be available to measure test item and/or major metabolites.

7 Regulatory toxicology studies

Regulatory toxicology studies of up to one month in duration to support Phase I clinical studies are informed by, and follow on from, the initial short term DRF studies. The following section provides a framework for selecting doses for regulatory general toxicology studies.

7.1 General guidance for dose selection

7.1.1 Selection of a dose that has not been investigated in the DRF study should be avoided, particularly where there is a large multiple between no/minimal effects and the dose producing signs of toxicity. This is illustrated in **Example 2**.

7.1.2 Usually the same doses are administered to both sexes. On rare occasions the exposure or tolerance may be significantly different between the sexes such that different doses should be considered for males and females. If significant sex differences become apparent in the DRF study these should be fully investigated before progressing to further studies.

7.1.3 The principles for selecting doses are applicable to most routes of administration. Where parenteral routes (e.g. intravenous, subcutaneous or intramuscular) are used it is also necessary to consider the possibility for local adverse effects, for example at the injection site, which may limit the doses that can be used. Parenteral formulations within the pH range 4-9 are recommended as irritation may occur outside of this range [19].

7.2 Guidance for selecting the high dose

7.2.1 The high dose for a regulatory toxicology study should produce signs of toxicity that are compatible with the study duration and are tolerated by the animal. This dose is referred to as the MTD (see Section 4.1) and is not expected to cause morbidity or mortality. There should be clear signs of toxicity to ensure the potential hazard of the test item is assessed. This is usually observed as a combination of clinical signs, reductions in body weight during the duration of the study and/or decreases in food consumption. Assessment of changes in clinical pathology suggestive of abnormal function, and necropsy and histopathological observations can provide additional information to support the observations.

7.2.2 The high dose in a regulatory toxicology study should not exceed doses investigated in the DRF study, even when the increase is relatively small in magnitude. This is illustrated in **Example 3**. The high dose should match one of the doses already investigated in the DRF study.

Example 2: The importance of generating an adequate dose response in a DRF study

This example describes a seven day repeat dose DRF study with once daily oral administration of the test item. At a daily dose of 800mg/kg/day, adverse clinical signs including hunched posture and piloerection are observed in the animals and one animal was euthanased on Day 7. A second dose of 200mg/kg/day produces no adverse effects in any parameter and therefore is the NOAEL.

Question: *Is this information adequate to set doses for a one month repeat dose toxicity study with once daily oral administration of the test item? If not what should be done?*

Option 1: Use the information generated to design the regulatory study.

Considerations: The dose of 800mg/kg/day is above the MTD as one animal did not tolerate the dose and had to be euthanased. Therefore, 800mg/kg/day is not suitable for administration in studies of longer duration. The dose of 200mg/kg/day demonstrates the NOAEL over seven days. One way forward is to proceed to the one month study with a high dose between 200 and 800 mg/kg/day e.g. 400 mg/kg/day. With no prior information on the tolerability of this intermediate dose, there are two unwanted possibilities; it could become a new higher NOAEL or not be tolerated. Either way, the one month study would not achieve its purpose and it may require an additional dose or doses to be tested. If this option were chosen it would be recommended that some form of staggered or staged approach should be used for dosing animals at 400mg/kg/day in the one month study so that other doses (higher or lower) could be chosen on the basis of the tolerability at 400mg/kg/day.

Option 2: Investigate additional doses between 200 and 800mg/kg/day in the DRF study.

Considerations: This option would require the use of more animals in the DRF study but would provide an assessment of the dose response between no adverse effect and the MTD. More robust data would be generated for dose setting in the one month repeat dose study and the risk of the top dose selected being too high within the first week of the one month study, where the group size is larger, would be reduced. This is the recommended option.

Good practice: Choosing Option 2 would maximise the likelihood of conducting a successful one month study and reflects more conscientious use of animals. In general, it is good practice to establish an adequate dose response between the NOAEL and toxicity in the DRF study to aid appropriate dose selection in the regulatory study. The outcome of the regulatory study is critically dependent on good design and decision making in the DRF study.

7.2.3 The amount of test item available should not dictate the doses selected and this is particularly important when selecting the high dose for the non-rodent. The high dose criteria described in this document (see Section 4) should be met when selecting doses so that the intrinsic hazard of the test item is established. If the high dose is limited by the test item and the intrinsic hazard is not determined, then it is likely that further animals will be used at a later stage in development to establish the full intrinsic hazards of the test item.

Where the test item is limited, consideration should be given to i) reducing the duration of the safety study, for example from one month to 14 days, ii) using a margin of exposure approach (Section 4.5) or iii) only using one sex. The purpose of the Phase I clinical study and any limitations resulting from the reduced scope of the preclinical study must be fully considered before using this strategy.

7.2.4 In the absence of adverse effects to define the MTD, the high dose of a general regulatory toxicology study can be selected by the limit dose, saturation of exposure, or maximum feasible dose (Sections 4.2, 4.3 and 4.4).

Example 3: The value of a thorough investigation of the high dose to be used in regulatory toxicology studies

This example describes a seven day repeat dose DRF study with once daily oral administration of the test item. Moderate sedation was seen at a dose of 300mg/kg/day from day four. Otherwise, this dose was well tolerated with no additional clinical signs, although food intake started to reduce at day six. The sedation lasted for two to three hours each day. Therefore 300mg/kg/day was considered to be the MTD as a higher dose was likely to adversely affect the food intake of the animals further.

The initial systemic exposure data from the DRF study indicated that the dosing regimen was not appropriate to support dosing in humans as the test items appeared to be excreted far more rapidly than predicted for humans. Based on DMPK modelling, it is likely that the dosing regimen in animals will have to be altered from once to twice daily administration in order to provide adequate safety margins for the intended therapy.

Question 1: *Is the information generated so far adequate to set doses for a one month repeat dose toxicity study with twice daily oral administration of the test item?*

Option 1: Use the once daily MTD dose of 300mg/kg/day as the basis for giving 150mg/kg twice daily in the one month study on the grounds that total exposure is likely to be quite similar.

Considerations: This would not involve using additional animals in a DRF study, but would mean that the potential adverse effects and exposure associated with the twice daily regimen were not evaluated prior to exposing larger groups of animals in the regulatory study. As the proposal to move to twice daily dosing to support human studies is only based on predicted/modelled exposure, it is important to confirm this prior to commencing the regulatory study.

Option 2: Investigate the proposed dosing regimen by adding additional doses to the original DRF study or by carrying out a separate DRF study.

Considerations: This would require the use of additional animals in the DRF study, but would confirm tolerability and exposure in the new dosing regimen prior to commencing the regulatory study in larger numbers of animals. As a starting point it would be reasonable to investigate 150mg/kg given twice daily as the potential high dose as it is already known that 300mg/kg was tolerated with moderate sedation when administered once daily. This is the recommended option.

Outcome: In the same DRF study, an additional group of animals was dosed to confirm tolerability and systemic exposure following twice daily administration. The dose selected was 300mg/kg/day split into two 150mg/kg doses given eight hours apart for seven days. There were some signs of subdued behaviour that were not as prolonged as those observed at a single daily dose of 300mg/kg and there was no effect on food intake.

Question 2: *The dose of 150mg/kg twice daily appeared to be better tolerated than 300mg/kg once daily and may not be the MTD. Is the information generated so far sufficient to begin a one month study?*

Option 1: Assume that 150mg/kg given twice daily is close to the MTD and increase the dose slightly to, for example, 400mg/kg/day split into two 200mg/kg doses for the one month study.

Considerations: As this is only a small increment above the previous studies it could be argued that it is not necessary to investigate this in the DRF study, and that the increase will achieve the MTD effects seen at 300mg/kg given once daily. The risk is that 200mg/kg twice daily will not be tolerated at all, and the dose will have to be reduced during the one month study.

Option 2: Add an additional group to the DRF study to establish the MTD for twice daily dosing prior to commencing the one month study.

Considerations: The assumption that the MTD is close to 150mg/kg given twice daily may be wrong and the use of 200mg/kg given twice daily may result in more marked adverse effects than expected or conversely less effects than expected. This is the recommended option.

Outcome: An additional group was added to the DRF study at 400mg/kg/day split into two 200mg/kg doses given eight hours apart for seven days. This dosing regimen produced marked signs of toxicity (prolonged subdued behaviour and marked effects on food consumption) from day five. Therefore, a high dose of 300mg/kg/day split as two 150mg/kg/day doses was selected for the one month study. The one month study was successful with the dose being tolerated with evident signs of toxicity.

Good practice: Choosing Option 2 for questions 1 and 2 will maximise the likelihood of determining an appropriate high dose for the regulatory toxicology study. From a scientific and 3Rs perspective, evaluation of potential doses and the associated adverse effects should be in the DRF study prior to the regulatory studies.

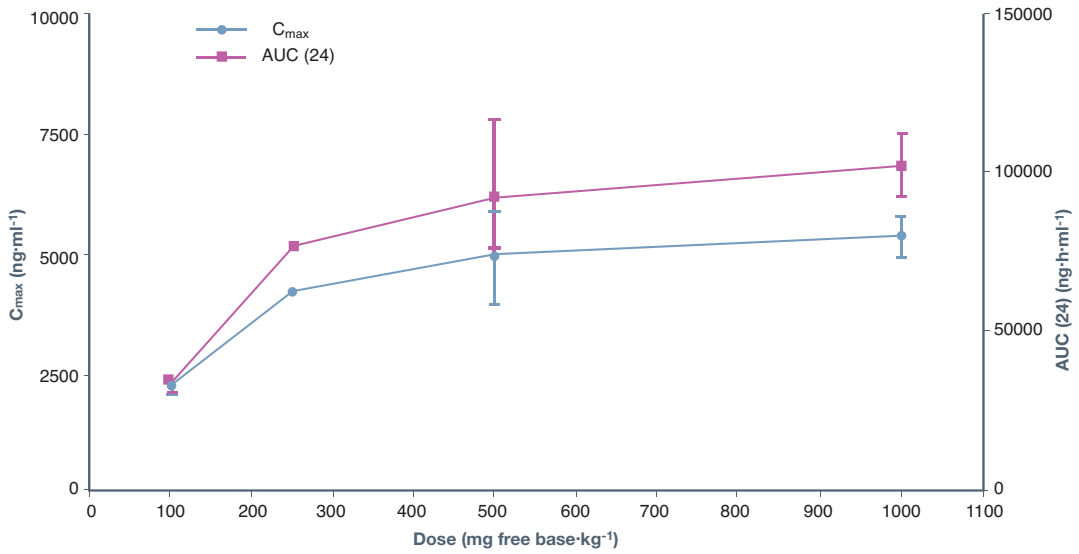
7.2.5 Saturation of systemic exposure may be used to select the high dose (see Section 4.3). Saturation can be demonstrated when measurable levels of the test item in the blood, plasma or serum no longer increase with the increase in dose. This is illustrated in Figure 3. In this case, a high dose between 500 and 1000mg/kg may be justified based upon saturation of exposure, but this would also be dependent on other factors, such as clinical signs observed in the animals. Use of saturation of exposure requires blood samples to be taken and subsequent measurement of the test item concentration in blood, plasma or serum, and analytical methods must be available at the time the DRF studies are conducted.

Care should be taken when using saturation of exposure to define the high dose. It is possible that the biological response to a test item may continue to increase in the absence of increased exposure, for example, due to high gut load or liver burden. Additionally, tissue levels of test item may differ from plasma/blood levels. In such cases it may be necessary to use a dose at the high end of the exposure plateau to maximise the chance of identifying toxicity. Further, alternative formulations or dosing regimens should be considered to optimise the systemic exposure of the test item.

7.2.6 The solubility of the formulation may limit the concentration that can be administered (see Section 4.4) as illustrated in **Example 4**. The maximum feasible/practical dose of a test item is determined by the formulation and administration characteristics. For instance, the dose may be limited by the maximum concentration that can be achieved as a solution or suspension and/or the maximum dose volume that can practically be administered to the animal.

The use of maximum feasible/practical dose is not common for standard routes such as oral gavage and every effort should be employed to ensure the formulation is optimised to allow assessment of the intrinsic hazard of the test item. Its use is more common where there are widely accepted practical issues in administration of the test item, for example, in inhalation studies where factors such as high aerosol concentrations are known to cause breathing difficulties in animals.

Figure 3: Plasma exposure vs. dose



Example 4: Maximum feasible/practical dose

In this example, several formulations of a test item have been developed. The optimal formulation, to give the highest exposure at doses required for animal studies, has an upper limit of solubility of 35mg/ml. In order to maximise systemic exposure, the test item is to be administered twice daily to rats by oral gavage in repeat dose studies of one month and longer. Using the good practice guidance on administration of substances by Diehl *et al* [10], the dose volume should not exceed 10ml/kg per administration. Using the upper limit of solubility and good practice dose volume this would equate to a maximum feasible dose of 700mg/kg/day (35mg/ml x 10ml/kg x 2).

Even in the absence of dose limiting toxicity at lower doses and/or evidence of a plateau in systemic exposure, the dose of 700mg/kg/day could be justified as the maximum feasible dose.

Good practice: The maximum dose volume varies with route and species [12]. Administration of large volumes can be difficult in practice and has the potential to cause adverse physiological effects that may compromise animal welfare. It is essential to ensure that the volume given causes minimum discomfort and does not result in physiological or pathological changes that would compromise the experiment or the animal's welfare. The lowest dose volume is recommended for the animal, this must be considered in line with delivering the study objectives.

7.2.7 With some test items, the known pharmacological activity leads to clinical signs (e.g. ataxia for certain CNS targets) or emesis that can limit the high dose in the absence of other signs of toxicity. Tolerance to these effects may develop after one to two weeks, particularly if the test item is administered at lower doses first. In these cases, dose escalation study designs can be employed to overcome the limitation, particularly for studies of one month and greater duration (see **Example 5**).

The dose is increased in a step-wise manner until the MTD is reached. With this approach, it is possible that a higher final dose can be tolerated than is possible in naive animals that have not received the test item. The final dose is maintained for the period of time required to support clinical studies. For example, if conducting a three month study it may be necessary to incrementally increase doses for two to three weeks then continue at the final doses for 12 weeks. Other doses can be started either at the same time as the incremental dose phase or once the final dose is achieved.

Example 5: Use of dose escalation phase to induce tolerance to pharmacological effects

In this example, the test item is an opioid analgesic. With this pharmacological class the doses used can potentially be limited by the adverse effects of opioid pharmacology, which include marked respiratory depression that can result in death. These adverse pharmacological effects may limit the dose required to explore target organ toxicity which is not related to the pharmacology of the test item. Identification of possible organ toxicity at doses not limited by the pharmacological effects is necessary for the safety evaluation of such test items. This can be achieved if the doses administered are increased incrementally over a period of time therefore allowing tolerance to develop. The development of tolerance to the pharmacological action of opioids is well established both in animals and humans, which means that much higher doses can be administered under certain circumstances, without impacting on animal welfare. Development of tolerance and use of increasing doses mimics what occurs in humans and the intended clinical use.

To address this situation, the one month toxicity study for this test item employed a treatment regimen involving a period of incremental dose escalation to reach the high dose tested over the one month duration. For the lower doses in the one month study, dose escalation was not necessary as any pharmacological effects were tolerated at these doses. Since abrupt withdrawal of these types of test item may also be a problem in animals and humans, a descending dose phase to avoid withdrawal symptoms was used for recovery animals before complete discontinuation of treatment during the recovery phase. This was only necessary in the high dose group of animals.

The study design is set out in the treatment regimen opposite. The test item was administered twice daily for one month in rats by intravenous injection with a 14 day recovery period. The length of the dose escalation/descending dose phases, as well as the maximum tolerated dose had been established in a preliminary DRF study.

Good practice: An escalating dose design is recommended for certain test items where tolerance to known pharmacological effects develops (e.g. some CNS targets). Use of this approach can enable administration of high enough doses to assess the intrinsic hazard. Identification of the hazard is critical to minimise the risk of attrition later in development and prevent unnecessary animal studies.

Treatment regimen (doses in mg/kg, given twice daily)							
Group	Dose escalation phase			One month treatment period	Descending dose phase*		
	Days 1 & 2	Days 3 & 4	Days 5 & 6	Days 7 to 34	Days 35 & 36	Days 37 & 38	Days 39 & 40**
1	0	0	0	0	0	0	0
2	-	-	-	2	N/A	N/A	N/A
3	-	-	-	6	N/A	N/A	N/A
4	3	6	12	20	12	6	3

* Recovery animals only
** Complete cessation of dosing from Day 41
\- Not dosed
N/A Not applicable (because assessment of recovery was restricted to high dose)

7.3 Guidance for selecting the low dose

7.3.1 The low dose in a regulatory toxicology study should ideally demonstrate the NOEL in the animal. The dose selected is usually set to achieve a small multiple of anticipated human exposure. In situations where the anticipated human therapeutic exposure has not been identified, the anticipated therapeutic exposure from an animal model may be used to set the low dose. For certain test items, particularly for anti-cancer agents, the nature of toxicity observed in animals often means that the low dose is below the therapeutic exposure level in humans.

7.3.2 In some cases there are findings identified at the low dose that are related to the procedure of administering the test item but which are deemed to be non-adverse. This is the NOAEL dose. It is important to recognise the difference between adverse and non-adverse effects for a test item [20, 21].

7.4 Guidance for selecting the intermediate dose

7.4.1 The intermediate dose in a regulatory toxicology study is required to demonstrate a dose response relationship. Understanding the intermediate dose is critical in defining the optimum pharmacological dose, which is important to establish margins of safety because dose limiting toxicity may not be readily monitored in man. Normally, dose response relationships can be achieved with a single dose between the low dose and the high dose. However, the clinician may need more information on whether exposure can be safely increased in man to get a larger efficacious effect, particularly if there is a large dose interval between the low dose and MTD. Therefore, on occasion a second intermediate dose may be required, for example:

- Where the dose range from the low dose to MTD is large (as illustrated in **Example 6**)
- To better characterise the dose response relationship to determine the threshold for adverse effects, allowing doses for subsequent studies to be selected with a greater degree of confidence
- Where the study has a dual purpose, for example, assessment of toxicity and provision of dose range finding information for a carcinogenicity study

Example 6: Use of two intermediate doses in a regulatory study

In cases where the dose range to be covered from potential no effect level to MTD is wide (e.g. 1000-fold), consideration of two intermediate doses is recommended.

In this example, the MTD of a test item was 1000mg/kg and the dose predicted to give a small multiple of human exposure was 1mg/kg. A single intermediate dose of 300mg/kg would leave a large gap between the intermediate and low doses and if there were findings at 300mg/kg but no findings at 1mg/kg then there would be insufficient information to identify where the NOAEL lies. In addition, using a NOEL/NOAEL of 1mg/kg may unnecessarily limit future clinical doses. In this case an additional intermediate dose of 30mg/kg could assist in characterising the dose response.

Good practice: Addition of a second intermediate dose group in certain circumstances may be required to fully investigate the dose response and NOEL/NOAEL. This would avoid future animal studies carried out separately to support clinical doses and regulatory requirements.

8 Concluding comments

The principles in this document provide a guide to selecting doses for general toxicology studies. By applying these principles it is possible to reduce and refine the use of animals by:

- Preventing repetition or unexpected termination of a study by:
  - Minimising variation between sequential studies
  - Applying prior knowledge and findings from database searches
  - Interactively managing studies and making timely adjustments of study conditions

- Reducing additional animal studies by:
  - Identifying the MTD to meet regulatory requirements in early studies
  - Identifying target organ toxicity early in test item development
  - Not using practical limitations to determine dose selection

- Minimising the number of animals at risk of experiencing adverse effects by:
  - Employing a staggered approach to dosing
  - Predicting adverse effects from knowledge of the target
  - Use of humane endpoints

9 Acknowledgements

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11 Abbreviations

ASPA	Animals (Scientific Procedures) Act 1986
AUC	Area under the curve
CDER	Center for Drug Evaluation and Research
C_{max}	Maximum concentration
CNS	Central nervous system
CPMP	Committee for Proprietary Medicinal Products
DMPK	Drug metabolism and pharmacokinetics
DRF	Dose range finding
ECG	Electrocardiogram
eINDs	Exploratory investigational new drugs
EMEA	European Agency for the Evaluation of Medicinal Products
FELASA	Federation of European Laboratory Animal Science Associations
FDA	Food and Drug Administration of the USA
GLP	Good Laboratory Practice
ICH	International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use
MTD	Maximum Tolerated Dose
NACWO	Named Animal Care and Welfare Officer
NOAEL	No Observed Adverse Effect Level
NOEL	No Observed Effect Level

12 Definitions

Definitions: animal welfare

Adverse effect: In the context of the ASPA this is an abnormal or harmful effect experienced by the animal that is related to the administration of the test item or the procedure involved. This may be demonstrated as a behavioural, functional, or anatomical change.

Humane endpoint: The earliest clinical endpoint that minimises animal suffering whilst also ensuring the scientific objectives of the study are reached.

Definitions: toxicology study objectives

Recovery animals: Animals that are treated for the same duration as the main study animals. After treatment, their recovery from any adverse effects observed in the main study is assessed.

Regulatory toxicology study: Any toxicology study that will be used as part of a regulatory dossier for registration purposes. This will normally be a GLP study of a defined protocol and duration of dosing, complying with guidelines such as ICH, EMEA, and CPMP Guidance.

Test item: Potential new medicine under investigation.

Vehicle: Any non-therapeutic substance mixed with a test item to facilitate the administration of the test item.

No observed effect level (NOEL): The highest experimental dose level/exposure at which there are no observed effects.

No observed adverse effect level (NOAEL): The highest experimental dose level/exposure at which there are no adverse effects with relevance to humans. The NOAEL and/or NOAEL are used to set limits for dosing in humans.

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