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Guidelines

Guidelines for the welfare and use of animals in cancer research

Abstract: Animal experiments remain essential to understand the fundamental mechanisms underpinning malignancy and to discover improved methods to prevent, diagnose and treat cancer. Excellent standards of animal care are fully consistent with the conduct of high quality cancer research. Here we provide updated guidelines on the welfare and use of animals in cancer research. All experiments should incorporate the 3Rs: replacement, reduction and refinement. Focusing on animal welfare, we present recommendations on all aspects of cancer research, including: study design, statistics and pilot studies; choice of tumour models (e.g., genetically engineered, orthotopic and metastatic); therapy (including drugs and radiation); imaging (covering techniques, anaesthesia and restraint); humane endpoints (including tumour burden and site); and publication; best practice.

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Keywords: animal welfare; cancer research; fundamental and translational research; replacement, reduction and refinement (3Rs); pilot studies; tumour models; genetically engineered mouse models; human tumour xenografts; orthotopic models; metastatic models; therapy; imaging; pharmacokinetic, pharmacodynamic and efficacy studies; drugs; radiation therapy; imaging techniques; anaesthesia; restraint; humane endpoints; tumour burden; clinical signs; publication; best practice.

Lay Summary

In order for scientists to understand how cancers develop and spread throughout the body and to discover new and more effective ways to diagnose and treat cancer, it is necessary to carry out research on live animals. Animal studies (over 95% of which are conducted in mice) are essential to understand the complexities of the fundamental processes that underpin cancer within living organisms. They are also required by regulatory authorities before any trials of new drugs can be tested in humans. Animal studies are only performed after every feasible test has been conducted on cancer cells in the laboratory and where no alternative exists. Adverse effects on the animals are minimised as far as possible. However, it is a source of concern for society and research scientists alike that, as we cannot replace all animal experiments in the immediate future, the highest standards of welfare are upheld. This publication builds on two previous sets of guidelines to provide updated and enhanced recommendations for the care and use of animals in cancer research; to develop procedures that reduce, replace or refine animal studies; and to communicate best practice throughout the world. In all cases, however, experimental designs and procedures should be tailored to the needs of the specific studies.
BACKGROUND AND SCOPE

Over the last decade there has been an extraordinary increase in our knowledge of the fundamental molecular processes that are involved in the development of cancer and its response to treatment (Hanahan and Weinberg, 2000; Vogelstein and Kinzler, 2004; Stratton et al., 2009). The public rightfully expect this explosion in basic research understanding to be translated into rapid improvements in prevention, diagnosis and treatment, particularly for the more common cancers and indeed for any malignant disease where there is still clearly an unmet need for more effective therapies. In recent years the identification of the genes and pathways that give rise to cancer dependencies and vulnerabilities has taken us further towards the development of individualised, molecularly targeted therapies (Sawyers, 2004; Collins and Workman, 2006; Workman and de Bono, 2008).

Along with growth in fundamental knowledge and greater translational insight has come the development of new in vitro and ex vivo methodologies and research techniques that should further extend our still incomplete genetic, molecular and holistic understanding of cancer, and in addition should help to ensure that improved methods for diagnosis, therapy and prevention will be developed more effectively for patient benefit. Nevertheless, we are still some way from the point where all of the necessary information that is required to introduce a new drug into the clinic in terms of safety and efficacy could be gained without the use of animals in research. Moreover, animals remain essential to extend our understanding of the mechanisms responsible for cancer and to identify, for example, new targets and biomarkers.

It is clearly important that the welfare of animals in cancer research is protected, both from an ethical point of view and also because it is widely acknowledged to be entirely consistent with good science (Osborne et al., 2009). Under the earlier sponsorship of the former United Kingdom Coordinating Committee for Cancer Research (UKCCCR), two sets of guidelines have been published previously (Workman, 1988; Workman et al., 1998). Although these guidelines were well received, and are still widely used and cited, it is over 10 years since they were last revised, in which time the science has moved on appreciably. The main aim of this article is to provide new guidelines for the cancer research community concerning the use of experimental animals in oncology, with a major emphasis on their welfare. We focus on rodents as these are predominantly used for cancer research: in 2008, for example, the UK government Home Office statistics showed that 96.8% of animals used in cancer research were mice (http://scienceandresearch.homeoffice.gov.uk/animal-research/publications-and-reference/statistics/index.html).

While development of medicines may require testing in other species, use of animals in regulatory toxicology is outside the scope of this review.

The present guidelines should be applied to studies focused on all aspects of cancer research, including experiments aimed at understanding fundamental cancer biology as well as translational work, and should be used in conjunction with more general guidelines for the care and welfare of animals (see examples below and Additional information). It is expected that animal housing will be maintained according to the highest standards, including environmental enrichment (Tsai et al., 2006), and that local ethical review will precede any experimental animal studies. In addition, these guidelines should be used in conjunction with appropriate national legislation: UK Animals (Scientific Procedures) Act 1986; USA Institute for Laboratory Animal Research (ILAR) Guide for the Care and Use of Laboratory Animals (http://dels.nas.edu/Laboratory/); EU webpage on laboratory animals (http://ec.europa.eu/environment/chemicals/lab_animals/home_en.htm); Public Health Service Policy on Humane Care and Use of Laboratory Animals (Office of Laboratory Animal Welfare, National Institutes of Health, 2002); http://grants.nih.gov/grants/olaw/references/psbpol.htm. A complementary key recent publication, coordinated by the UK’s National Centre for the Replacement, Refinement and Reduction of Animals in Research (NC3R), is also recommended (Biotechnology and Biological Sciences Research Council; Department for Environment, Food and Rural Affairs; Medical Research Council; Natural Centre for the Replacement, Refinement and Reduction of Animals in Research; Natural Environment Research Council; Wellcome Trust, 2008). We also feel it is important that the public is made fully aware of the current justification for the use of animals in cancer research and the genuine concern for their welfare by researchers involved with their use. To help with this, a lay summary of the guidelines is also provided on page 1555.

A glossary of terms can be found at the end of this article. Finally, a lay summary of the guidelines is also provided on page 1555. A lay summary of the guidelines is also provided on page 1555.
of human (xenogeneic) tumour cells can be achieved using immunodeficient (e.g., nude or SCID) mice to prevent rejection (Table 1A). Most transplantable tumours are established subcutaneously. These subcutaneous (s.c.) tumours are simple to initiate but may lack relevance in terms of stromal/vascular interactions and metastasis. More complex models may involve orthotopic transplantation at appropriate primary sites, or inoculation of tumour cells through routes which maximise the chance of metastatic spread (Table 1B). There is an increasing trend to establish xenograft tumours directly from human cancers, to avoid artificial selection of cells in tissue culture and changes in gene expression and phenotype, which this may induce. Such transplants may better model the principal facets of clinical cancer, for example, maintenance of tumour architecture, heterogeneity, expression of certain targets and response to therapy (Dong et al, 2010), but can be less reproducible (especially as primary grafts) and slower growing than well-established models (Neale et al, 2008; Rubio-Viqueira and Hidalgo, 2009). Detailed molecular and genetic characterisation, facilitated by modern high-throughput technologies (e.g., see http://www.sanger.ac.uk/genetics/CGP), is now available for human cancer cell lines used for xenografts (Masters et al, 2001; Park et al, 2010) and is important to understand the biology of these models and to select the most appropriate for each study.

**Box 1** THE 3RS: REPLACEMENT, REDUCTION AND REFINEMENT

**Replacement**

Absolute replacement techniques avoid the use of animals; relative replacement techniques include substituting non-vertebrate species

1. Investigate the potential of novel and existing alternative approaches to animals
2. Use in silico and/or in vitro pre-screens before commencing animal studies

**Reduction**

Minimise the number of animals used to achieve specific scientific objectives

1. Ensure that all studies are scientifically robust and apply appropriate statistical methods to experimental design
2. Reduce experimental variability by conducting studies of animals of defined health status and, wherever possible, using inbred strains
3. Minimise surplus breeding by avoiding unnecessarily narrow specifications for animal sex, age and weight
4. Freeze rodent embryos, sperm and cancer cell lines not immediately required for scientific studies
5. Prevent duplication by making specific strains and genetically modified lines available throughout the research community
6. Consider use of serial sampling or longitudinal imaging in which each animal acts as its own control to reduce study group sizes

**Refinement**

Continual review of improvements in experimental design, techniques and husbandry to minimise adverse effects and improve welfare

1. Apply all available knowledge to predict adverse effects and ensure that appropriate humane endpoints are developed and specialist care is provided, especially when using genetically modified animals (e.g., immune deficient or tumour-prone strains)
2. Provide animals with an appropriate environment (e.g., nesting material, shelter for rodents), including sufficient space and complexity to satisfy their normal species-typical behaviours
3. Undertake pilot studies of unfamiliar tumour cell lines or novel procedures to establish experimental and humane endpoints
4. Perform post-mortem examinations as a routine part of all pilot studies and to investigate any unexpected deaths
5. Include appropriate controls to understand individual and combined effects of tumours and treatments
6. Use anaesthesia and analgesia whenever appropriate. This should be regularly reviewed by a vet to ensure that contemporary best practice is followed
7. Consider imaging methods to monitor non-superficial tumour burden and to aid the timely implementation of humane endpoints
8. Do not allow animals to become moribund: death as an intentional endpoint is unacceptable
9. Maintain and share detailed information, on all experimental procedures, including behaviour of the tumour and host animals under various conditions
10. Include principal details relating to the use of animals, such as study design, adverse effects, specialist care and the 3 Rs, in all scientific publications

**Box 2** IMPLEMENTATION AND MONITORING

For assurance that best practice is implemented and rigorously applied, practical guidelines should be clear and readily available, with staff fully engaged and educated in their use

1. Establish a clear chain of responsibility to ensure that prompt action is taken where necessary; for example, if the condition of an animal deteriorates unexpectedly
2. Establish humane endpoints, specialist care, monitoring methods and criteria for intervention between researchers, veterinary and animal care staff before initiating studies
3. Ensure that all staff involved in the use and care of animals are aware of their personal and legal responsibilities, and are trained to recognise adverse effects and apply humane endpoints
4. Seek additional external expertise where unfamiliar models or techniques are being introduced
5. Ensure adequate staffing levels are available for the duration of the study, particularly during crucial periods where additional observations are needed
6. Inspect animals at a frequency determined by the known biology of the tumour, the effects of any interventions and the clinical status of the animals
7. Monitor adverse effects, including general signs of welfare and more detailed indicators appropriate to the specific model and procedures
8. Give all staff appropriate training and professional development opportunities
9. Provide appropriate supervision of staff and confirm, review and document competence on a regular basis

Autochthonous tumour models

There are two broad categories: those arising in outbred or inbred rodents (Table 2A), or those from animals harbouring genetic changes that alter tumour susceptibility (Table 2B). Certain mouse or rat strains are susceptible to spontaneous development of tumours. More commonly, tumours are induced by chemical carcinogens, radiation, viruses or bacteria. Such models may mimic some of the aetiological events in human cancer development; exposure to such agents may induce systemic effects that are difficult to replicate in genetically engineered models.

Major advances have been made in the development of sophisticated mouse models of cancer that mimic many of the genetic and biological characteristics of human malignancies, although the host genetic background may affect tumour incidence and/or malignant potential (Lifsted et al, 1998; Winter and Hunter, 2008). A range of technologies now allows the inducible expression of oncogenes or inactivation of tumour-suppressor genes in vivo in a precisely controlled manner in virtually any tissue or cell type. (Chen et al, 2004; Christophorou et al, 2006; Sharpless and DePinho, 2006). Such genetically engineered mouse models...
(GEMMs) provide excellent experimental systems to develop a deeper understanding of cancer biology in vivo and are increasingly being used for preclinical testing of molecularly targeted therapies, as they depend on or are ‘addicted’ to the specific molecular abnormalities and biochemical pathways engineered to drive the malignant process.

Routine use of GEMMs for preclinical testing of anticancer therapies can be hampered by variable tumour latency, incomplete penetration and complicated breeding schemes. The full potential of such mouse models is yet to be realised and further work is required to derive maximum benefit for cancer patients from these initiatives (Frese and Tuveson, 2007). Newer models (e.g., exploiting double or multiple genetic abnormalities) have resulted in enhanced tumorigenicity and metastatic capacity, and some studies have shown that mouse cancer models with relevant human gene mutations respond to appropriate targeted therapies (Politi et al, 2006), and also may develop common secondary mutations associated with acquired resistance (Politi et al, 2010). As an example of target validation, reversible, systemic expression of a dominant-negative mutant Myc oncogene in transgenic Ras-induced lung carcinoma model caused the tumours to regress, whereas effects on normal regenerating tissue were well tolerated and reversible (Soucek et al, 2008). To overcome heterogeneity issues, transplantation of transgenic tumours can provide higher throughput models, for example, for testing therapeutics (Varticovski et al, 2007). Commonly used GEMMs include mammary carcinomas induced by the viral oncogene polyoma virus middle T (Guy et al, 1992; Fluck and Haslam, 1996; Marcotte and Muller, 2008) or by the human or rat Her2/neu oncogene (Chan et al, 1999; Quaglino et al, 2008), or colon adenomas and carcinomas induced by inactivation of the adenosomatis polyposis coli (APC) tumour-suppressor gene (Taketo, 2006). Space constraint does not allow a full description or listing of the many more sophisticated, patient-like models now available, examples of which are shown in Table 2B. The reader is referred to the more complete information available at http://emice.nci.nih.gov/mouse_models.

A key question that continues to be debated is whether human cancer xenografts or murine transgenic models best reflect the human disease in terms of biology and predictions of efficacy of therapeutic agents (Becher et al, 2006; Dennis, 2006; Garber, 2006; Sausville et al, 2006; Sharpless and DePinho, 2006). Some GEMMs have shown patterns of sensitivity to chemotherapeutic agents and development of resistance that are similar to their human tumour counterparts (Rottenberg and Jonkers, 2008). The predictive value of neither type of model has been fully established; however, there is agreement that molecular characterisation of all tumours is required to underpin the choice of model.

### Selection and optimisation of experimental systems

As mentioned, selection of tumour models should be based on both molecular characteristics, for example, expression or mutation of a target of interest or other relevant molecular pathway, either endogenously or through transfection/transgenic technology, together with desired properties such as the rate and reproducibility of growth, metastatic potential and chemosensitivity.

### Cell line verification and molecular characterisation

Given the frequency of misidentification and cross-contamination (Nardone, 2007; Lacroix, 2008) it is essential that all cell lines are rigorously checked for their provenance and genetic identity (Parodi et al, 2002; Yoshino et al, 2006). It is also important that cell lines are free from contamination with infectious agents such as mycoplasma, which can influence their biological behaviour and present a risk to handlers and animals (Ishikawa et al, 2006; Sung et al, 2006; Harlin and Gajewski, 2008). Regardless of origin, detailed characterisation of tumours should be performed and checked periodically to ensure that desired properties are maintained and are commensurate with the molecular pathology of the corresponding human malignancy (Santarius et al, 2010). A thorough literature review should establish their reported tumorigenic and immunogenic properties, with special attention paid to the selection of the correct host animal strain and sub-strain. Residual immune responses to xenografted tumours in nude/SCID mice may occur and the sex of the host should be

### Table 1A Transplantable tumour models

| Examples of models | Advantages | Disadvantages |
|--------------------|------------|--------------|
| **Syngeneic**      |            |              |
| MC26 colon cancer in BALB/c mice (Alsheikhly et al, 2004) | Conventional rodents and normal housing | Tend to be aggressive and grow over a short time frame |
| B16 melanoma in C57Bl mice (Rusciano et al, 1994) | Covers wide range of tumour types | Not applicable if investigating human-specific parameters |
| 4T1 mammary carcinoma in BALB/c mice (Kim et al, 2009) | Models immune and stromal interactions | The genetics and histology of tumours may not reflect the human situation |
| **Xenogeneic**     |            |              |
| HCT116 colon cancer in athymic mice (Huxham et al, 2004) | Allows direct investigation of human cells | Requires genetically immunodeficient (nu/nu or SCID) animals |
| PC3 prostate cancer in athymic mice (Patel et al, 2002) | Human cancer cell lines are increasingly being characterised by genetic and other molecular techniques (Ihle et al, 2009; Bignell et al, 2010) | Requires sterile isolation and ACDP containment level-II |
| Systemic leukemias in irradiated NOD/SCID mice (Ili et al, 2004; Lock et al, 2005) | Can be an established cell line or human primary tissue (Neale et al, 2008; Rubio-Viqueira and Hidalgo, 2009) | Genetically modified cells will require ACGM containment |
| GFP transgenic mice to enhance visualisation of tumour—host interactions (Yang et al, 2004) | Amenable for immune reconstitution | May not be suitable for use with agents modifying the immune system or where cellular interactions are being investigated |
| Luciferase-expressing cancer cells for bioluminescent imaging (Dickson et al, 2007; Comstock et al, 2009; Shibata et al, 2009) | Can be grown s.c. or orthotopically | |

Abbreviations: ACDP = Advisory Committee on Dangerous Pathogens; ACGM = Advisory Committee on Genetic Modification; GFP = green fluorescent protein; NOD = non-obese diabetic; s.c. = subcutaneous; SCID = severe combined immunodeficient.
## Table 1B  Transplantation models used for therapeutic studies

| Transplantation site | Notes and examples of models | Advantages | Disadvantages |
|----------------------|------------------------------|------------|--------------|
| **Subcutaneous tumour transplantation** | A high % of established lines will grow at this site | Simplest model for routine evaluation of anticancer agents Uses cancer cells or tissue Simple administration with anaesthetic only required for tissue transplantation Allows continuous monitoring Facilitates the use of bilateral transplantation after pilot studies | Ectopic site for most tumours Metastases rarely develop from s.c. tumour xenografts May be poorly vascularised with necrotic centres |
| **Tumour cells within hollow fibres** | Amenable for use with a wide range of cell lines (Decker et al, 2004) | Allows multiple cell lines to be implanted for simultaneous assessment Useful for human cell lines that fail to grow as xenografts Short-term assay requiring ex vivo end-stage analysis Provides a quick assessment of drug efficacy and may predict xenograft response | Careful scoring system required Lack of vasculature may miss ‘indirect’ effects or access issues Inflammation around fibres can affect responses Variation in properties of fibres Possibility of paracrine effects if multiple cell lines implanted in same animal |
| **Experimental metastasis (site of spread)** | | | |
| Intravenous (primarily lung) | 3DC13B lymphoma (Golay et al, 2006) M24met melanoma (Becker et al, 1996) A549 NSCLC (Kennel et al, 1999) Lewis lung 3LL (Li et al, 2001) | Most cells injected i.v. colonise lung Some (notably lymphoid tumours) may colonise other organs Models end-stage of metastasis in a controlled manner Simple model to establish Cells may be tagged for imaging | Bolus injection of cells does not mimic natural dissemination and the ‘conditioning’ effect of primary tumour growth Does not model the full spectrum of metastatic cascade Difficult to monitor and quantify accurately without imaging Pilot studies required to establish incidence and growth kinetics |
| Intra-peritoneal–(ascites and peritoneal/omentum-associated tumours) | GW-39 colon (Sharkey et al, 1991) MGLVA1 gastric (Watson et al, 1999b) OVCA3 ovarian (Zavaleta et al, 2008) | Mimics late-stage ovarian cancer and carcinomatosis associated with some abdominal cancers | Difficult to quantify |
| Intra-tibial* (bone) | ARCaP prostate (Zhu et al, 2000) | Mimics bone tumour growth seen with myeloma or prostate and breast cancer metastases | Does not mimic natural tumour dissemination Risk of infection and pain Requires imaging |
| Intra-cardiac* (bone) | MDA MB 231 breast (Serganova et al, 2009) A375 melanoma (Nakai et al, 1992) Mat Ly-lu prostate (Blouin et al, 2008) | To some extent mimics the dissemination phase of metastasis | Requires advanced surgical technique Risky procedure requiring particular care Only suitable for a limited number of cell lines |
| Intra-portal vein (liver, nodes) | LS174T colon (Mahteme et al, 1998) | Localises tumour cells to liver Appropriate for colon, pancreatic and breast cancers | Requires advanced surgical technique to avoid cell leakage into peritoneal cavity |
| Intrasplenic (liver) | WiDR colon (Miyazaki et al, 1999) | Easier procedure than mesenteric vein or intraportal cell inoculation | Local growth in spleen may complicate the interpretation of results unless splenectomy is performed |
| **Orthotopic and spontaneous metastasis models (sites of metastasis)** | | | |
| Intra-caecal/lymphoid follicle (liver) | TK-3,-4,9 colon (Tanaka et al, 1995) | Relevant stromal/vascular cell interactions Models the full metastatic cascade Certain models may involve lymph node spread Cells may be genetically tagged to allow real-time imaging Chemosensitivity more relevant | Most require delicate surgical procedures Risk of local tumour spread Risk of infection Pilot studies must be used to determine the time frame of disease events |
| Intra-dermal (lung/nodes/liver) | NC65 renal (Nakatugawa et al, 1999) A-O7/melanoma (Graff et al, 2005) | | |
| Mammary fat pad (lungs/nodes/brain) | MDA-MB-231 breast (Serganova et al, 2009) 4T1 breast (murine) (Mitra et al, 2006) | | |
considered, particularly for hormone-responsive tumours such as breast and prostate.

**Pilot studies and optimisation**

Pilot tumour growth studies using small numbers of animals (5–10) are recommended to establish that patterns of local and metastatic growth are reproducible. They also show any adverse effects associated with tumour progression and enable humane endpoints to be identified. The data derived should feed into group numbers used for definitive studies (e.g., therapy experiments) in order for experimental time frames and statistically significant endpoints to be established. Use of a relevant positive control treatment may be useful at this stage to ensure that tumour growth/progression is as expected. This can be dictated by a variety of factors, including the site of growth. Subcutaneous tumours may grow rapidly and some are prone to developing haemorrhagic areas, which can cause rapid expansion and ulceration (e.g., human A2780 ovarian carcinoma and AR42J pancreatic carcinoma xenografts).

For tumours growing as a suspension in the peritoneal cavity, it is important to establish clear criteria to ensure that studies are terminated before animal welfare is compromised. This site is only appropriate for models where ascites is a feature of the natural progression of the human cancer (e.g., ovarian carcinoma, peritoneal mesothelioma, gastrointestinal tumour carcinomatosis). Similar criteria apply to other sensitive specialised sites such as muscle or brain. For metastatic models, pilot experiments should define the extent and time course of dissemination to internal organs.

Pilot studies should include sequential analysis of animals to determine the time course required to achieve scientific goals. Termination of studies at the earliest possible point will minimise adverse effects on the animal. Where possible, use of biomarkers (e.g., serum levels of prostate-specific antigen, PSA) and real-time imaging are highly recommended. It is also possible to measure circulating tumour cells using fluorescence and PCR-based techniques (Glinskii et al, 2003; Komatsubara et al, 2005). For spontaneously arising tumours, including those in transgenic animals, particular attention should be paid to the time course of tumour development and issues relating to the development of multiple tumours. Progression may be unpredictable and involve rapid dissemination and subsequent deterioration in clinical condition, in which case careful and frequent monitoring is required.

**Refinement and welfare issues**

Subcutaneous implantation of tumour material should use a trochar or surgical formation of a small s.c. pocket. Appropriate anaesthetics must be used and post-implantation analgesia is also strongly recommended. Veterinary advice should be sought to ensure that the agents selected reflect contemporary best practice. Anaesthesia/analgesia is also required for implantation of ‘hollow fibres’ or slow-release devices such as osmotic mini-pumps. Hormone pellets (oestrogen/testosterone) may be required to support hormone-dependent tumours, but first-time use in a particular strain will require pilot experiments with different doses/exposures to assess tolerance, especially with oestrogen pellets where urinary tract side effects may be encountered (Pearse et al, 2009).

For injection of cell suspensions, the minimum number of cells in the smallest volume should be used, consistent with the properties of the tumour. For s.c. sites, 1–5 million cells in 100 μl is typical. For orthotopic sites, this should be reduced to avoid excessive tissue damage or leakage (e.g., 50 000 cells in 30 μl into the prostate, or 10–50 000 cells in 5 μl into the brain). Intramuscular tumours in the leg can affect mobility, and this site should only be used if there is special justification (e.g., for tumours which naturally develop in this tissue). Similarly, footpad
injection, which has been traditionally used to potentiate lymphatic dissemination, is unacceptable without exceptional scientific justification and should then only involve a single paw.

Surgical removal of a primary tumour may be justified, for example, from s.c. sites, mammary fat pad or removal of the spleen following intrasplenic injection, to allow time for outgrowth of any secondary deposits. Surgery must be performed using sterile techniques with appropriate post-operative monitoring and control of any pain and inflammation/infection.

Cell lines should be checked regularly for contaminating microorganisms to avoid infection of host animals. This is especially important if tumours are routinely passaged between animals, which may be justified for those that are difficult to establish from cell cultures. Asymptomatic infection of experimental animals may affect tumour properties, for example, metastasis (Rodríguez-Cuesta et al., 2005). Procedures can be used to improve tumour take rate. For example, moderate doses of whole-body irradiation may further enhance engraftment of tumour cells in athymic mice (Baersch et al., 1997; Nijmeijer et al., 2006), although the added stress and risk to the animal must be considered. Co-administration of human tumour cells with allogeneic bone marrow transplantation may reduce graft-vs-host activity but preserve graft-vs-tumour effects in allogeneic leukaemia models (Prigozhina et al., 2002; Giver et al., 2004).

### Table 2A Primary tumour models

| Model type                              | Examples of models                                                                 | Advantages                                                                 | Disadvantages                                                                 |
|-----------------------------------------|-----------------------------------------------------------------------------------|---------------------------------------------------------------------------|--------------------------------------------------------------------------------|
| Chemically-induced tumours              | Dimethyl hydrazine – gastric cancer (Watanabe et al., 1999)                       | Model the full spectrum of carcinogenic events                            | Low incidence and heterogeneous tumour development                           |
|                                         | Azoxymethane – colon cancer (Hirose et al., 2004)                                | Useful in chemoprevention studies                                         | Safety aspects associated with use of carcinogens – may need to house animals in isolator |
|                                         | Diethyl nitrosamine – hepatocellular carcinoma (Ha et al., 2001)                  |                                                                           | Long time frame for tumour development                                       |
|                                         | Dimethyl benzanthracene – breast cancer (Hawarian and Stanslas, 1998)             |                                                                           | Continuous monitoring not feasible                                           |
|                                         | N-acetylcysteine – squamous oesophageal carcinoma (Balansky et al., 2002)        |                                                                           | Often highly immunogenic                                                      |
|                                         | Dimethylbenzanthracene/ tetracyano/ phorhol acetate (TPA) – skin cancer (Johansen et al., 2009) |                                                                           |                                                                                  |
| Radiation-induced tumours               | Ultraviolet light (Ahsan et al., 2005; De Fabo, 2006; El-Abaseri and Hansen, 2007) | Models non-melanoma (using UVA) and melanoma (UVB) skin cancer            | Requires hairless mice                                                       |
| Inflammation-induced tumours            | Helicobacter pylori-induced gastric cancer in gerbils (Zheng et al., 2004)       | Use of conventional rodents to facilitate the involvement of the full spectrum of immune mediators | Limited availability of models                                               |
|                                         |                                                                                   | Models malignant progression and amenable for use of chemopreventive agents | Long time frame and variability in tumour development                       |
| Surgically-induced tumours              | Oesophago-gastroduodenal anastomosis model of oesophageal carcinogenesis (Chen et al., 1999) | Can model malignant progression or metastatic spread                     | High level of skill required for initiation                                 |
|                                         |                                                                                   |                                                                           | Incidence may not be 100%                                                    |
|                                         |                                                                                   |                                                                           | Accurate quantification can be difficult unless using real-time imaging     |
| Spontaneous tumours, sometimes with viral/genetic component | T138 mice and mammary carcinoma (Wood et al., 1992; Nordmark et al., 1996) | Develop cancer without any intervention                                  | Limited tumour types and strains                                             |
|                                         | Cotton rats and neuroendocrine gastrointestinal tumours (Martinsen et al., 2003) | Conventional rodents, therefore fully immunocompetent                     | Variability in the time frame of tumour development                         |
|                                         | Eker rat model of tuberous sclerosis (Kenerson et al., 2005)                     |                                                                           |                                                                                  |

**THERAPY**

**Preclinical discovery and development of therapeutics**

There is a concerted effort to identify and develop small-molecule drugs or biopharmaceuticals (e.g., antibodies, protein therapeutics,
vaccines, gene therapy) targeted against cancer cells or associated host cells (Sawyers, 2004; Collins and Workman, 2006; Workman and de Bono, 2008). A representative ‘test cascade’ for discovering new small-molecule inhibitors of cancer targets is shown in Figure 2. As a consequence of extensive in vitro testing, comparatively small numbers of prioritised compounds progress to examination in vivo (Collins and Workman, 2006). In vivo studies use sequential, discriminatory tests to prioritise compounds at each stage. Different tests may need to be applied to biopharmaceuticals, such as antibodies and vaccines, as they may work by recruiting host effectors (e.g., cytotoxic leukocytes). Epitope specificity can also require the development of an antibody or vaccine initially using anti-rodent reagents (before switching to the clinical form) or use of a genetically modified mouse model. In addition, agents directed

| Examples of models | Spectrum of tumours in humans | Spectrum of tumours in modified models | References |
|--------------------|------------------------------|--------------------------------------|------------|
| Rb                 | Brain, pituitary             | Retinoblastoma, osteosarcoma, medulloblastoma | Additional loss of p107, p130 | (Robanus-Maandag et al, 1998) |
| Trp53              | Osteosarcoma, lymphoma, soft-tissue sarcoma, germ cell tumours | Additional loss of Terc Trp53(ER)TAM | Breast and other carcinomas, germ-cell tumours | (Artandi et al, 2000) (Christophorou et al, 2005) |
| Apc (ApcMIN, ApcD716, ApcD580) | Multiple polyps in small intestine | Polyps in colon progressing to carcinomas | Polyplis in colon | (Shibata et al, 1997) |
| Ink4a              | Fibrosarcoma, lymphoma, squamous-cell carcinoma | Familial melanoma, sporadic pancreatic, brain tumours | Crossed with Arf+/− mice | (Krimpenfort et al, 2001) |
| Brca1              | No tumour susceptibility     | Mammmary tumours                      | (Xu et al, 1999) |
| Brca2              | No tumour susceptibility     | Conditional mammary-specific inactivation of Brca1 | (Jonkers et al, 2001) |
| Nf1                | Pheochromocytoma, myeloid leukaemia | Neuro-crest-derived benign neurofibroma and malignant fibrosarcoma | Additional loss of Trp53 | (Reilly et al, 2000) |
| Nf2                | Osteosarcoma, fibrosarcoma, lung adenocarcinoma, neuroblastoma, pancreatic adenocarcinoma | Schwannomas | Schwann-cell precursor-specific ablation of Nf2 | (Giovannini et al, 2000) |
| K-rasG12D          | Lung adenoma, adenocarcinoma, PanIN | NA | Mutant Trp53 Pancreas-specific K-rasG12D−/−IKKα/IKKβ−/− deficiency or combined with Trp53R172H | Metastatic lung cancer Metastatic pancreatic cancer | (Johnson et al, 2001) (Agurrie et al, 2003) (Hingorani et al, 2005) |
| AML/ETO or MLL fusion proteins | AML                          | AML |                          | (Zuber et al, 2009) |
| MYCN               | Neuroblastoma, Rhabdomyosarcoma, medulloblastoma | Neuroblastoma, Rhabdomyosarcoma, medulloblastoma | TH-driven overexpression in neural crest | (Weiss et al, 1997) |
| MYCC               | Pancreatic adenoma, melanoma, lymphoma, AML, breast | Pancreatic adenoma, melanoma, lymphoma, AML, breast | Bcl-XL crosses Lck overexpression Eu-Tat-c-myc MMV-c-myc | (Pelengaris et al, 1999) (Felsher and Bishop, 1999) (Sinn et al, 1987) |
| Ptc1               | Medulloblastoma              | Trp53-knockout crosses                 |                         | (Wetmore et al, 2001) |
| T-antigen          | Pancreatic adenocarcinoma    | RIPI-Tag2 driven overexpression in pancreatic islets |                         | (Bergers et al, 1999) |
| PTEN               | Breast, endometrial, glioblastoma, prostate, and thyroid carcinoma, Cowden syndrome, hamartomas, urothelial tumours of renal pelvis | Breast, endometrial, glioblastoma, prostate, and thyroid carcinoma, Cowden syndrome, hamartomas, urothelial tumours of renal pelvis |                         | (Stambolic, 2000; Qian, 2009) |

Abbreviations: AML = acute myeloid leukaemia; NA = not available; PanIN, pancreatic intraepithelial neoplasia.
**STAGE 1:** TUMOUR TYPE
- Defined by molecular status
- Defined by clinical tumour type
- Defined by in vitro studies

**STAGE 2:** ANIMAL HOST
- Transplanted
  - Human tumour xenograft
  - Immunodeprived animal
- Autochthonous
  - Animal tumour
  - Immunocompetent syngeneic host
  - Specific genetic modification
  - Transgenic/engineered host

**STAGE 3:** TUMOUR MICROENVIRONMENT
- Relevant
  - Orthotopic site
  - Stromal/host cell co-inoculation
- Irrelevant
  - Subcutaneous transplant

**STAGE 4:** TUMOUR STATUS
- Pre-cancerous lesion
- Early stage tumour
- Established or invasive tumour
- Metastasis
- Spontaneous
- Experimental

**STAGE 5:** TUMOUR EVALUATION
- Internal tumours
  - Imaging techniques
  - Blood/serum biomarkers
  - Palpation, clinical signs
  - Superficial tumours
  - Caliper measurements

**STAGE 6:** DOSING REGIMEN
- Based on clinical schedules
- Based on existing appropriate animal data
- Based on mechanism of action, PK, PD, tolerability

**STAGE 7:** ENDPOINT ANALYSES
- Tumour volume and weight
  - Ex vivo clonogenicity
  - Efficacy biomarkers
  - Body weight, condition, clinical signs
- Regrowth delay, time to progression
- Body fluids, tumour, normal tissues
- Toxicity biomarkers

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**Figure 1** An illustrative process for tumour model selection and use. This representative schema provides an illustration of factors to be considered when designing an animal study. In this particular example, all the factors listed at a given stage (and potentially others) should be considered before moving down, stepwise, to the next stage. Here, an initial consideration is that the choice of model may be based on the relevant molecular status, clinical tumour type or in vitro studies. At the next stage, the animal host will be dictated by the need for, say, a human tumour xenograft versus a genetically engineered mouse model, which have advantages discussed in the text. Considerations of tumour environment and site then follow, after which, in therapy studies, are dosing and endpoint aspects. Note that this schema is illustrative and not prescriptive and that each study must be tailored to the specific scientific question and experimental objectives, with appropriate humane endpoints always applied and pilot studies carried out as needed.
against the tumour microenvironment (e.g., angiogenesis, tumour-promoting stromal or inflammatory cells) will require appropriate specialised assays. A range of technical platforms are used preclinically to define responses to therapy, the most informative of which are adopted for use in patients (Figure 3). Careful assessment of a therapy’s safety profile (outside the scope of this review) is also required for regulatory submission.

Defining tolerable doses for efficacy studies

An investigational treatment should be examined at a potential therapeutic dose level and using a relevant dosing regimen that covers the longest duration anticipated. These parameters can, for example, be estimated from consideration of mechanism of action, in vitro potency, pharmacokinetics, protein binding and pharmacodynamic biomarker data. Studies typically use two mice per dose level with a doubling dose-escalation or dose-halving de-escalation design. For studies involving a single dosing event, an interval of 24h should be used before an alternative dose level is examined, to allow any acute adverse effects to be seen. For more chronic administration schedules (e.g., daily for 21–28 days) this interval should be at least 5 days. Animals should be examined at least twice daily (see humane endpoints below). Note that presence of a tumour may reduce host tolerance to therapy. Studies of mice may be used to predict dose requirements in other species through allometric scaling of pharmacokinetic parameters (Freireich et al., 1966).

Combination studies

There is a strong rationale to study combinations of agents in vivo to guide clinical studies. Relevant prior in vitro studies such as Combination Index or isobologram analyses to discriminate additive, synergistic or antagonistic interactions should be completed to guide the selection of combinations and schedules. Compounds are added to tumour cells in culture over a range of concentrations, alone or in combination, and the changes in sensitivity are observed. Compounds may also be added sequentially as the order of administration may significantly influence responses (Chou, 2006). Care needs to be taken with in vivo studies in addressing the choice of individual drug doses and scheduling, particularly if overlapping toxicities are likely. Pilot experiments must assess tolerability (see above), and pharmacokinetic data (see below) should also be generated to determine whether interpretation of efficacy data is affected by pharmacokinetic interactions (Siim et al., 2003).

Pharmacokinetic studies

In vitro and in silico methods are useful to predict absorption, distribution, metabolism and elimination (ADME) properties and to help prioritise compounds for evaluation in animals (Table 3; Singh, 2006). However, at present such methods are unable to predict accurately the full pharmacokinetic profile of an agent. Pharmacokinetic studies should use a validated and sufficiently sensitive detection method, ideally avoiding the need to pool separate blood samples, thereby minimising animal usage. Typical experiments on mice use a single dose and 5–8 time points (2–3 mice per point) over 24–48h with small molecules (usually administered p.o., i.v. or i.p. at doses of 0.5–100 mg kg

−1) and over 1–21 days with biopharmaceuticals (administered i.v., i.p. or s.c. at doses ranging from 10 to 1000 μg per mouse).

More recently, repeat sampling of small volumes of blood from a superficial vein in mice over a series of time points has been established to reduce animal numbers. This can be employed either for isolation of plasma and analysis by sensitive liquid
chromatography–mass spectrometry/mass spectrometry (LC/MS-MS or tandem MS) instrumentation (Abatan et al., 2008), or by spotting microlitre volumes of whole blood onto specialised paper cards, which are then dried and extracted before analysis (Barfield et al., 2008). In rats, a 5–8 time-point pharmacokinetic profile may be generated using 2–6 animals in total, through repeated blood sampling. ‘Cassette dosing’, which involves administration of low doses of compound mixtures, should also be considered initially as this can reduce animal usage (Watanabe et al., 2006; Smith et al., 2007). Wherever possible, computational compartmental kinetic modelling should be used to predict optimal doses or multiple dosing protocols, to facilitate more limited sampling (Rowland and Tozer, 1995). It is noteworthy that the plasma half-life of monoclonal antibodies is frequently extended in immunocompromised mice, which are deficient in IgG production (Bazin et al., 1994).

**Pharmacodynamic biomarkers**

Initial studies of investigational therapies using tumour-bearing animals should aim to determine whether the target, or an appropriate downstream pathway or phenotype, is modulated by using suitably validated pharmacodynamic biomarkers (Collins and Workman, 2006). Typically, animals are humanely killed at intervals to determine the extent and duration of
pharmacodynamic changes and to investigate biomarkers in tumour and normal tissues (e.g., blood or skin) that may be relevant to clinical development (Banerji et al, 2005). In vaccine studies, responses are assessed by changes in immune status, including evidence of tumour-infiltrating leukocytes by immunohistochemistry, and specific cellular or humoral immunity (Gajewski, 2000). It should be possible to use much smaller group sizes of 3–5 in pharmacodynamic studies in comparison to those in efficacy studies (see below). Simultaneous measurement of drug concentrations and mechanistic biomarkers is recommended to reduce animal numbers and establish a pharmacokinetic–pharmacodynamic relationship. Judicious application of such studies in a drug discovery test cascade should be used to prioritise agents before entry into efficacy studies.

Efficacy determinations

All relevant information should be used to guide the design of the tumour efficacy studies. Such studies generally involve examination of treatment effects over a 2- to 4-week period and establish how the therapeutic response relates to pharmacokinetic and pharmacodynamic parameters. Typically, with treatments delivered by an appropriate route of administration (Table 4), response is determined in 6–10 animals per study group (or a control group) either by direct twice-weekly calliper measurement of superficial tumours (Kelland, 2004), counting lung or liver metastases ex vivo, or using imaging methodologies (Edinger et al, 2002; Hoffman and Yang, 2005; Brindle, 2008; McCann et al, 2009; Yang et al, 2009). Alternatively, post-treatment excision of tumours for in vitro determination of clonogenic survival, or determination of the dose required to inhibit tumour growth by 50% (tumour control dose-TCD50) may be appropriate (see Radiation therapy section below). Methods are available to determine sample sizes for single- and combination-agent studies and to allow for incomplete data sets (Tan et al, 2005). For certain targets, alternative, surrogate in vivo efficacy models in non-tumour-bearing animals may be used, such as assessment of anti-oestrogenic activity by determining the effect on hypothalamic function (Kato et al, 1968).

Table 3 Some in vitro assays for pharmacokinetic/ADME properties

| Property                               | Assays                                                                 |
|----------------------------------------|------------------------------------------------------------------------|
| Chemical and physical properties       | In silico predictions or in vitro measurements of logP (Mannhold et al, 2009), logD (Bruneau and McElroy, 2006; Dohta et al, 2007), pKa (Lee et al, 2007) and solubility (Colclough et al, 2008; Du-Cuny et al, 2008) |
| Metabolism                             | Mouse, rat, human liver microsomes, hepatocytes, 59 incubations, UGT assays (Houston and Carlile, 1997; Riley et al, 2002) |
| Passive diffusion/cell uptake          | PAMPA (Ottaviani et al, 2006)                                          |
| Cell and gut permeability predictions  | Caco-2 cells (Artursson et al, 2001)                                    |
| Blood–brain barrier permeability predictions | hCMEC/D3 cells (Poller et al, 2008)                                   |
| Drug–drug interactions (e.g., CYP450)  | Human liver microsomes, hepatocytes, CYP enzyme screens (Masimirembwa et al, 2001) |
| Protein binding                       | Measurement by dialysis or ultrafiltration (Howard et al, 2010)          |

Table 4 Maximum volumes to be administered on each occasion

| Route                         | Mouse | Rat   |
|-------------------------------|-------|-------|
| Intravenous, bolus injection  | 10 ml kg⁻¹ | 5 ml kg⁻¹ |
| Intra-arterial, bolus injection | NA    | 0.1 ml |
| Intraperitoneal, bolus injection | 20 ml kg⁻¹ | 10 ml kg⁻¹ |
| Subcutaneous, bolus injection  | 20 ml kg⁻¹ | 10 ml kg⁻¹ |
| Intramuscular, bolus injection | 0.05 ml per site, using contralateral limbs for sequential doses or 0.1 ml on one occasion only | 0.1 ml per site, using contralateral limbs for sequential doses or 0.2 ml on one occasion only |
| Intradermal, bolus injection   | 0.05 ml per site² | 0.1 ml per site² |
| Oral, by gavage               | 20 ml kg⁻¹, or 50 ml kg⁻¹ on one occasion only | 20 ml kg⁻¹, or 30 ml kg⁻¹ on one occasion only |
| Slow intravenous/arterial injection | 0.8 ml over 2 min | 5 ml over 2 min |
| Constant intravenous infusion  | 0.04 ml per min | 0.2 ml per min² |
| Constant intra-arterial infusion | NA    | 0.1 ml per min |
| Constant intraperitoneal infusion | 0.04 ml per min³ | 0.2 ml per min³ |
| Intratracheal, bolus injection | 0.1 ml | 0.1 ml |

Abbreviations: ADME = absorption, distribution, metabolism and elimination; CYP450 = cytochrome-P450; PAMPA = parallel artificial membrane permeability assay; UGT = uridine diphospho (UDP)-glucuronosyltransferase.
Administration of experimental agents

Various sources are available for advice on well-tolerated injection volumes and recommended administration schedules. It is important to note that, from an animal welfare point of view, frequency and duration of dosing are as important as the volume and composition of the injected solution. Some commonly used examples are given in Table 4 and the following references: Diehl et al (2001); Morton et al (2001). More frequent dosing would need to be justified by pharmacokinetic or pharmacodynamic data. As the control group is involved in every comparison, i.e., to all treated groups, it is often appropriate to set the control group size to be higher in comparison with the treated groups

Experimental design including statistics

To maximise the scientific integrity of data generated while at the same time using the minimum number of animals, statistical expertise should be applied to all experimental design and analyses (Festing, 2002; Festing and Altman, 2002; Festing et al, 2002; see Boxes 3 and 4).

Chemoprevention

These studies routinely use either carcinogen-induced rat tumours (e.g., a azoxymethane-induced colorectal cancer) or mouse genetic models of carcinogenesis (e.g., ApcMin colorectal; Corpet and Pierre, 2003; Cai et al, 2009). Generally, animals receive the putative chemopreventive agent in the diet or drinking water over an extended period at innocuous doses. Tumour development is measured at the end of the study and compared with animals on a relevant control diet. Relatively large numbers of rodents (e.g., ≥14 per group; Cai et al, 2009) may be required for the observed differences between the intervention and control groups to be robust. Mechanistic and pharmacodynamic endpoints should also be included (Yang et al, 2001; Corpet and Pierre, 2003).

Radiation therapy

External beam radiotherapy is primarily used for local tumour irradiation, which requires lead shielding to minimise normal tissue exposure. Typically, s.c. tumours are used and combination treatment with a novel therapy is tested. Endpoints include local control, growth delay and in vivo–in vitro clonogenic survival (TCD50). Time to re-growth is preferred to a single time point analysis. Local tissue toxicity is usually manifest as skin erythema but should be minimised by restricting localised doses to less than 30 Gy (single dose). Exploration of better tolerated, clinically relevant fractionated doses (e.g., 2–5 Gy per fraction over 1–2 weeks) is encouraged. Should moist desquamation occur, this should not be allowed to persist for more than 24 h. Irradiated s.c. tumours can show ulceration, which may reflect tumour response. However, if there is evidence of infection and/or no signs of tissue repair the animal should be humanely killed. The acute and late effects of radiation treatment may also be examined in a relevant organ, particularly when studying new combination paradigms. A common endpoint has been the development of fibrosis in lung tissue, although more recently measurement of breathing rate has been implemented to detect symptoms before they become distressful to the animal (Jackson et al, 2010).

Radiotherapy can also be delivered in the form of targeted radionuclides (normally attached to antibodies; e.g., Martensson et al, 2005). Normal tissue toxicity will depend on antigen
expression on tissues relative to the tumour and the nature of the emitter. Whole-body irradiation can also be used to suppress the immune response of an animal, for example, or to treat disseminated disease. Selected doses should not manifest toxicity over the duration of the experiment, for example, gut toxicity within 5 days or haematological toxicity within 30 days.

**UV radiation (UVR)**

The response of mouse skin to UVR may be used, for example, to study the aetiology of non-melanoma skin cancer (van Kranen and de Gruijl, 1999; Hedelund et al, 2006). Generally, experiments are performed with hairless (Skh-hr2) mice. As mouse skin does not show signs of burning, it is important to use a biologically relevant, non-burning dose of 0.2–0.3 MED (minimal erythema dose; 50% skin thickening = 0.5 MED). Skin thickness should be measured 2–3 times weekly after increasing the dose of UVR until 20–30% thickening has occurred. If hyperplasia is maintained over 12–15 weeks skin tumours may form. A protective mouse restrainer should be used as UV radiation is damaging to eyes and ears.

**IMAGING**

**General considerations**

Imaging techniques now have a principal role in translational cancer research, enabling sequential analysis of biological endpoints in the same animal, with obvious welfare benefits. The main utility of small-animal imaging is for monitoring deep-seated tumours and metastases with or without treatment. Applications include studies of basic biological processes and of tissue pharmacokinetics and pharmacodynamic responses to treatment (Paulmurugan et al, 2002; Galbraith et al, 2003; Pillai et al, 2008; Tennant et al, 2009; Nguyen et al, 2009b). However, animal numbers may not be reduced if, for example, full endpoint analysis requires surgical intervention such as cannulation of blood vessels or when contrast agents have a long half-life. Here, sequential imaging may not be possible and alternative techniques involving tissue excision may provide more information (usually at higher spatial resolution) from the same number of animals.

There is an increasing clinical need for pharmacodynamic imaging with molecularly targeted cancer therapeutics. However, interpretation of imaging signals is often difficult and animal models have an important role in rigorous validation of new techniques. This needs to be accompanied by consideration of unique animal welfare issues. Use of external imaging techniques on small animals is not completely non-invasive as some form of anaesthesia or physical restraint is necessary and surgery or administration of contrast agents may be required.

**Imaging techniques**

The applications, advantages and disadvantages of commonly used imaging technologies are summarised in Table 5 and have also been reviewed recently (Workman et al, 2006; Brindle, 2008; Weissleder and Pittet, 2008). Whole-body optical imaging is relatively simple and cost-effective (Edinger et al, 2002). Tumour cells are genetically modified to constitutively or inducibly express a fluorescent protein (e.g., eGFP, dsRed) or an enzyme that activates an exogenously administered substrate to a bioluminescent molecule (usually luciferase for activation of a luciferin). The whole animal is imaged using sensitive optical detectors, which may or may not incorporate a tomographic facility (Figure 4). The potential influences of genetic modification and/or substrate administration on immunogenicity and response to treatment, as well as animal welfare, must be considered (Tuchin, 1993; Dennis, 2002; Condeelis and Segall, 2003; Wells et al, 2006).

Intravitral microscopy uses a wide variety of optical imaging techniques, often incorporating fluorescent or bioluminescent genetic reporters or markers, including nano-particles (Hoffman, 2005). It has particular animal welfare issues because it involves surgery to provide optical clarity and visualisation on a microscope stage or using fibre-optic light guides (Weissleder and Pittet, 2008). Some intravitral microscopy techniques (e.g., tumours growing in the intestinal mesentery) require laparotomy with deep anaesthesia, so that imaging is only possible for a few hours under terminal anaesthesia. Surgical implantation of ‘window’ chambers for tumour implantation enables imaging to be performed over days to weeks (Dewhirst et al, 1987; Lehr et al, 1993; Brown et al, 2001; Reyes-Aldasoro et al, 2008). Here, general anaesthesia is only essential for the initial surgery and imaging may be performed with restrained animals. Strict aseptic technique and good post-operative care and analgesia are essential (Richardson and Flecknell, 2005; Flecknell, 2008).

Most physical imaging techniques require use of exogenous contrast agents and only positron emission tomography (PET) and single photon emission computed tomography (SPECT) are sufficiently sensitive to allow use at true tracer levels; so possible pharmacological effects of contrast agents need to be carefully considered. The same procedures for tolerability testing should apply to imaging agents as for new drugs. Some magnetic resonance imaging (MRI) techniques use inherent properties of tissues to provide endogenous imaging contrast. For instance, BOLD (blood-oxygen-level-dependent) MRI allows assessment of tissue oxygenation. These techniques avoid the use of pharmacological agents but results may be difficult to interpret.

Contrast-enhanced CT has the highest spatial resolution of all clinically applicable imaging techniques and is amenable to rapid kinetics. However, depending on the operating parameters and scan length, this may involve considerable ionising radiation dose per scan (0.02–0.6 Gy; typically 0.1–0.3 Gy) (Boone et al, 2004; Carlsson et al, 2007; Brindle, 2008). Doses should be minimised to avoid compromising experimental results through interaction of ionising events with the biological processes of interest, as well as welfare issues; as a guide, total radiation dose >1 Gy can affect tumour growth and whole-body doses >6 Gy are generally lethal to small rodents. Users of fused PET-CT or SPECT-CT systems should note that the radiation dose from the PET or SPECT can be as large as the CT dose. In addition, iodine-based contrast agents are nephrotoxic and, if required for repeat studies, well-tolerated doses should be established.

**Anaesthesia and restraint for imaging**

Physical restraint and/or general anaesthesia are required for small-animal imaging. Both procedures can affect animal well-being and introduce experimental artefacts. Body temperature must be maintained and monitored during general anaesthesia using thermostatically controlled heating pads, microwaveable gels or warm air blowers. Light general anaesthesia using an inhalational anaesthetic such as isoflurane or a short-lived i.v. injectable such as propofol should be used for pharmacological restraint, wherever possible. Deleterious effects of physical restraint can be minimised by appropriate design of restrainers, provision of black-outs and acclimatisation (Warden et al, 2000; Narciso et al, 2003; King et al, 2005). Preferred methods will depend on the species, imaging modality and device used. Where general anaesthesia is not appropriate, sedation with use of gentle physical restraint is encouraged, taking account of veterinary advice. Acclimatisation needs to be thorough, as a short period of training can induce more stress (Warden et al, 2000; Narciso et al, 2003).
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**Table 5** Examples of imaging techniques

| Imaging method | Imaging time | Spatial resolution* | Main purpose | Advantages | Disadvantages |
|----------------|--------------|---------------------|--------------|------------|---------------|
| Optical: Bioluminescence and fluorescence | Sec – min | Organs: 50 µm Whole body; 1 – 5 mm | Monitoring tumour response to treatment in deep-seated/orthotopic sites and metastatic spread; imaging gene expression and protein–protein interactions | Relatively non-invasive (requires restraint), high sensitivity (amol – nmol); many fluorescent and near-infrared probes available; amenable to use of gene reporters; relatively cheap | In most cases, these methods require genetic modification of tumour cells for detection; quantitation relies on 2D images in current imaging systems; nude or shaved animals are required |
| Three-dimensional high-frequency ultrasonography | Sec – min | 100 µm in-plane resolution | Relative measure of tissue blood flow; three-dimensional measurement of tumour size | Relatively non-invasive (requires restraint), high sensitivity (single particle); absolute measurements are possible with suitable contrast agents, e.g., microbubbles | Specialized application |
| Magnetic resonance imaging (MRI), spectroscopy (MRS) or spectroscopy imaging (MRSI) | Min – hours | 100 µm in-plane resolution; much higher for spectroscopy without imaging | Pharmacodynamics, pathophysiology, pharmacokinetics, anatomy | Some techniques use endogenous contrast; good spatial resolution (imaging) | Poor sensitivity (mmol), so that exogenous contrast agents and drugs need to be given at high concentration |
| Standard or contrast-enhanced computed tomography (CT) | Sec – min | 100 µm | Pharmacodynamics, anatomy | Very good spatial resolution combined with relatively good sensitivity | High radiation dose (for standard CT, 2 Gy for most currently available systems), limited number of contrast agents, which may be nephrotoxic |
| Single photon emission computed tomography (SPECT) | Min | 1 – 2 mm | Pharmacodynamics, pathophysiology, pharmacokinetics | High sensitivity (pmol); Radiochemistry can be performed in nuclear medicine department | Limited radiochemicals available compared with PET, less quantitative |
| Positron emission tomography (PET) | Min | 1 – 2 mm | Pharmacodynamics, pathophysiology, pharmacokinetics | High sensitivity (pmol); allowing true tracer kinetics; unlimited range of radiochemicals making it very flexible; fully quantitative | Poor spatial resolution; full quantitation requires cannulation (for kinetic studies); requires specialized radiochemistry in most situations |
| Optical intravital microscopy | Sec | 1 – 5 µm | Real-time imaging of tumour microcirculation | Microscopic spatial resolution, good sensitivity (single cell), many fluorescent contrast agents readily available, amenable to use of gene reporters; relatively cheap | Requires surgical intervention |

*Optimum spatial resolution is usually achieved at the expense of ability to obtain fast kinetics.

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**Length of imaging sessions**

If applicable, animals should be transported to imaging facilities in suitable transport boxes, with food and water provided before imaging. The length, total number of imaging sessions and intervals between them depend on factors such as time required to acquire images, tolerance to restraint or general anaesthesia, half-life of the contrast agent and whether cannulation is required. Consideration also needs to be given to exposure of immune-deprived animals to a non-pathogen-free environment, as well as monitoring and control of animal physiology during imaging. If animals have no access to water, an imaging session should typically last no more than 2 h and total imaging time should not exceed 2 – 3 h in a 24-h period. Use of un-anaesthetised animals restrained for more than 2 h must be avoided except where there is exceptional justification, for example, for animals recovering from general anaesthesia after cannulation of superficial vessels before imaging. In this case, use of local analgesia around the cannulation site is essential. Animals anaesthetised for more than 2 h should be rehydrated if recovery is prolonged, for example, by injection of dextrose/saline. If animals need to be anaesthetised more than once per day, they must be fully recovered, eating and drinking before being re-anaesthetised. On completion of a session, animals should either be killed or kept warm until full recovery from anaesthesia or until the next analysis session. Analysis may be repeated on the same animal but typically this should not exceed five sessions within a 1- to 2-week period and typically no more than one imaging session per day.

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**HUMANE ENDPOINTS**

There are ethical, scientific and legal reasons for ensuring that adverse effects are minimised. Choice of appropriate humane endpoints provides significant opportunities for refinement, and should be developed in tandem with the requirements for a valid scientific outcome. Early endpoints reduce non-specific systemic effects and so may increase the precision of the results obtained. Pilot studies, including autopsy to determine the full extent of tumour growth, will facilitate the definition of robust and refined...
Endpoints. Endpoints for particular models must also take account of the known pathogenesis of the particular tumour model in question and should be regularly reviewed in the light of experience.

The endpoints proposed are based on animal models in widespread use (for examples see Tables 1 and 2); however, each study should be considered on its own merits. For example, tumorigenicity studies can be terminated as soon as progressive tumour growth is evident. By contrast, carcinogen-induced skin papillomas, for example, undergo malignant transformation late in their development and may require later endpoints. Imaging techniques facilitate the development of more defined endpoints for some tumour models. Every effort should be made to identify factors allowing scientific decisions to be made at the earliest stage possible, while taking into account the total burden of procedures on animal welfare. The intentional use of death as an endpoint is unacceptable and animals should not be allowed to become moribund.

The choice of site for solid tumours will influence the maximum acceptable tumour load and the appropriate humane endpoints. Sites such as the footpad, tail, eye or bone are likely to be painful or distressing and require special justification and earlier endpoints. Similarly, tumours that metastasise to sensitive sites need great care. As with all internal tumour sites, this includes clinical examination, measurement of body weight, abdominal palpation and loss of condition. Humane endpoints, specialist care and interventions should reflect best practice and be discussed and agreed between researchers, veterinarians and animal care staff before commencement of the experiment. Development and publication of appropriate experimental analyses (e.g., pharmacodynamic determinations, functional imaging) to capture detailed phenotypic information assists rational determination of endpoints.

### Figure 4

Examples of in vivo imaging in pre-clinical cancer research. (A) Optical surface bioluminescence imaging of orthotopically xenografted human PC3 prostate carcinoma cells transfected with luciferase (PC3luc2a). Mice were imaged using a Charged Coupled Device (CCD) camera, which is super-cooled to enhance detection sensitivity and image resolution. The images shown were taken after systemic administration of luciferin, with 'intensity of luminescence' shown as 'heat' maps and red as maximum intensity. The scale shows the number of photons detected. Top panel: Untreated mice at day 8–41 after transplantation; bottom panel: before and after treatment with 5 mg kg\(^{-1}\) taxotere on day 10. This technique is useful for monitoring treatment effects in deep-seated tumour sites. Light scattering through tissues makes precise quantitation difficult. (B) PET imaging of tumour cell proliferation using \(^{18}\)F-3′-fluoro-3′-deoxy-L-thymidine (FLT). Transverse and coronal (0.5 mm) images of HCT116 tumour-bearing mice 24 h before treatment and after 4 daily treatments with the histone deacetylase inhibitor LAQ842 at 25 mg kg\(^{-1}\). 30- to 60-min summed images from a dynamic scan are presented. Numerous radiotracers are available for investigating specific biochemical pathways in vivo, if specialised facilities are available. The scale shows the intensity of radiotracer uptake. (C) Intravital imaging of tumour vasculature of the P22 rat sarcoma growing in a dorsal skin flap window chamber. The image was obtained by multi-photon fluorescence microscopy after i.v. administration of 70 kDa RT-C–dextran. High spatial resolution is obtained but surgical intervention is required.
Tumour burden

Tumour burden should always be limited to the minimum required for a valid scientific outcome. For example, efficacy studies should be terminated once durable, statistically significant therapeutic effects can be shown. Therapeutic studies should be designed to avoid the need for control tumours to become excessively large. The size of any tumours should be limited when they are used simply for routine transplantation or as a source of tumour tissue. In all cases the general health and condition of an animal remains the overriding determinant. Adverse effects on the animal will depend on the biology, site, mode of growth of the tumour and any additional procedures or treatments. Despite the caveats, estimation of tumour size and burden is an important consideration in determining endpoints.

Assessment of the size of superficial tumours using callipers (usually of two diameters at right angles) is an easy and definable method. Measurement variations can be minimised by ensuring that the same well-trained technician is involved for the duration of the study. Response to therapy may be measured by changes in tumour growth rate, re-growth delay, cell survival (measured by clonogenic assay) or an appropriate surrogate marker. Excising and weighing tumours at the end of a study can provide an additional objective endpoint, which avoids errors due to variations in tumour shapes and estimations of volume or mass. For an animal carrying a single tumour, the mean diameter should not normally exceed 1.2 cm in mice or 2.5 cm in rats, or 1.5 and 2.8 cm, respectively, for therapeutic studies. Where two tumours per animal are grown, for example, in contralateral flanks, the size should be correspondingly less and should not exceed the maximum burden of a single tumour. Multiple tumours may develop in genetically modified animals (e.g., mammary tumours in polyoma virus middle T transgenic mice; Guy et al, 1992) or in the skin of animals subjected to UVR (El-Abaseri and Hansen, 2007) or chemical carcinogens (Johansen et al, 2009), for which similar limits should be observed. Exceptions to these advised size limits would require rigorous scientific justification.

Determining the tumour burden of internal orthotopic cancers, systemic lymphocutaneous tumours or metastatic disease is challenging. Pilot experiments using small numbers of animals are important to allow characterisation of the kinetics and patterns of spread, to predict clinical signs and to define humane endpoints. Biomarkers or circulating cancer cells may be used as surrogates for assessing the burden of lymphomas and leukaemias, and real-time imaging is a valuable adjunct. Appropriate biochemical and pathological indicators or use of engineered reporter systems or time imaging is a valuable adjunct. Appropriate biochemical and pathological indicators or use of engineered reporter systems or time imaging is a valuable adjunct. Appropriate biochemical and pathological indicators or use of engineered reporter systems or time imaging is a valuable adjunct.

Box 5 CLINICAL SIGNS NECESSITATING IMMEDIATE INTERVENTION

1. Failure to eat or drink over a 24- to 48-h period resulting in emaciation or dehydration
2. Consistent or rapid body weight loss reaching 20% at any time or 15% maintained for 72 h compared with the pre-treatment weight of adult mice or age-matched, vehicle-treated controls. With some tumours body weight is a very poor indicator and muscle atrophy or emaciation is more useful. Body condition scoring provides a very useful indication of muscle loss (Ullman-Cullere and Foltz, 1999)
3. Persistent hypothermia
4. Bloodstained or mucopurulent discharge from any orifice
5. Laboured respiration, particularly if accompanied by nasal discharge and/or cyanosis
6. Enlarged lymph nodes or spleen
7. Hind-limb paralysis or weakness
8. Anaemia as indicated by symptoms such as pale feet, or haematological measures
9. Significant abdominal distension or where ascites burden exceeds 10% of the bodyweight of age-matched controls. Accurate determination is difficult but body girth is useful and a 20% increase should be the maximum normally allowed; similar to the appearance of a pregnant mouse
10. Incontinence or diarrhoea over a 48-h period
11. Tumours that interfere with locomotion or cause abnormal vocalisation, animal behaviour or function

Such severe symptoms are likely to occur very rarely in well-designed experimental studies, should be avoided and require immediate humane termination.

Clinical signs

In general the clinical signs shown in Box 5 are principal indicators of rare but severe symptoms of potential adverse effects, which should be avoided. Where any one sign is present the animal should immediately be humanely culled and vigilance increased for the remainder of the cohort.

With solid tumours, scoring of ulceration, distension of covering tissues and cachexia (severe body weight loss) should be incorporated into the endpoints. Ulceration is a lesion typified by necrosis of superficial tissues, which may be dry, suppurating or exudative. Necrosis resulting in skin breakdown or exudation persisting beyond 48 h is grounds for termination. Some tumours, such as those grown in sensitive sites or that develop extensive necrosis, may be painful, although objective criteria are lacking for mice. Further research is required to enable better assessment of pain and to assist in formulating the most appropriate endpoints.

Summary and concluding remarks

This set of guidelines is designed to update and enhance the second edition (Workman et al, 1998). Information is provided on the more complex, molecularly defined and biologically relevant models now available, including genetically engineered, orthotopic and metastatic tumour systems. These more ‘patient-like’ models require sophisticated methods of evaluation; hence a detailed section on the different imaging modalities that are now used has been added. Tables 1 and 2 provide examples of some widely used experimental models. Figure 1 offers an example of the type of illustrative aid that can be used to facilitate the rational choice of appropriate models in a given study. Examples of tumour models, experimental design and procedures are provided throughout. However, it is emphasised that these are intended to act as a guide only, and each study should be tailored to the specific experimental objectives. There is renewed emphasis on continuing applications of the 3Rs – replacement (of animals with alternative methods), reduction (in the numbers of animals used to achieve scientific objectives) and refinement (in experimental design, techniques and husbandry to minimise adverse effects and improve welfare). There is an expectation that the highest animal welfare standards will be demanded from grant-awarding bodies and scientific journals. It is also emphasised that there is a responsibility for researchers to publish improved models and methodology for the benefit of the research community worldwide. A comprehensive bibliography is included to cover all of the principal topics and links to other, online resources are also provided. It is to be stressed that animal welfare considerations are not only important for ethical and legal reasons, but also should be fully consistent with the highest standards of scientific investigation. It is anticipated that the appropriate use of animal models will make an important contribution to increasing further our fundamental understanding of cancer and will enhance our growing ability to diagnose, treat and prevent it.
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Translational Therapeutics
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Glossary

Allometric scaling: Calculation of doses of drugs to be administered to animals according to their relative sizes where the relationship of a biological variable to body mass is non-linear. For example, drug dosage can be linearly related to body surface area rather than to body weight.

Ascsites: Cells/fluid in the peritoneal cavity.

Autochthonous tumours: Tumours originating within the host animal, either spontaneously, or due to genetic or pharmacological intervention.

Cachexia: Severe loss of weight and muscle mass that cannot be reversed nutritionally. Can be caused by release of biologically active molecules (cytokines) from certain tumours.

Cassette dosing: Administration of multiple compounds to an individual animal followed by individual measurements in the same blood sample.

Clinically equivalent dose: A dose of a drug, which results in blood/tissue levels that reflect those that are achieved in patients.

Clonogenic assay: Measuring the effect of treatments on the ability of tumour cells to proliferate expansively. Treatment may be in vitro or initiated in vivo and the clonogenic ability of explanted cells tested in vitro.

Desquamation: Loss of skin integrity. Moist desquamation can be a consequence of exposure to ionising radiation (UV or X-rays) where the skin thins and then begins to weep tissue fluid as the epithelial cells lose their barrier function.

Distension: Stretched beyond normal dimensions.

Ectopic: Site of growth different from the tissue of origin, for example, s.c. transplantation of tumours derived from internal organs.

Erythema: Skin reddening and thickening in response to UV irradiation, as in mild sunburn.

Factorial design: Involves the inclusion of two or more variables and measuring the response to each variable and interactions between variables.

Genetically engineered mouse models (GEMMs): Animals in which the genetic material has been altered. For example, introduction of a mutation in cells of a particular organ may result in the development of benign or malignant tumours.

Hyperplasia: Refers to the proliferation of cells within an organ or tissue beyond that which is ordinarily seen. Microscopically cells resemble normal cells but are increased in numbers. It is a benign condition, unlike neoplasia, which is malignant.

Intravital microscopy: A technique, which allows direct observation of small blood vessels within the organs of anesthetised animals.

Maximum Tolerated Dose (MTD): The highest dose of a drug in which the clinical condition of the experimental animal is maintained.

Metastasis: The spread of tumour cells from a primary site to distant sites in the body, usually through the blood or lymph. The term ‘experimental metastasis’ is sometimes used to describe the colonisation of organs after injection of cells directly into the peripheral circulation.

Oncogenesis: The process of malignant transformation resulting in tumour development.

Orthotopic: Anatomically correct site (opposite of ectopic), for example, transplantation of renal tumour cells into the kidney or mammary carcinoma cells into the mammary fat pad.

Pharmacodynamics: The study of the action of and the duration of effects of agents in the body, including confirmation of mechanism of action through identification of relevant biomarkers of activity.

Pharmacokinetics: The study of the process by which agents are absorbed, distributed, metabolized and eliminated by the body, including measurement of the rate of excretion, metabolism, blood and tissue concentrations.

Syngeneic tumour models: Cells transplanted between animals of the same inbred strain.

Ulceration: An inflamed lesion on the skin or internal surface involving tissue destruction.

Xenogeneic tumour models: Cells transplanted between species (e.g., human to mouse). Requires recipients that cannot mount an immune response and reject the foreign tissue graft such as athymic mice which lack T-lymphocytes, or severe combined immunodeficient (SCID) mice.

Useful weblinks

http://scienceandresearch.homeoffice.gov.uk/animal-research/publications-and-reference/statistics/
http://www.sanger.ac.uk/genetics/CGP
http://emice.nih.gov/mouse_models
http://dels.nas.edu/ilan_n/ilarhome/reports.shtml
http://ec.europa.eu/environment/chemicals/lab_animals/home_en.htm
http://www.iasp-pain.org/AM/Template.cfm?Section=Animal_Research
http://ec.europa.eu/european_group_ethics/docs/opinion7_en.pdf
http://conventions.coe.int/treaty/en/treaties/html/123.htm
http://www.ecopa.eu/
http://caat.jhsph.edu/
http://www.imm.ki.se/soft/pdf/OECD19.pdf
http://oauo.od.nih.gov/ARAC/index.htm
http://www.research.psu.edu/arp/health/endpoints.html
http://www.nc3rs.org.uk/news.asp?id=759
http://ddgs.utu.fi/request.php4
http://www.lal.org.uk/index.php?option=com_content&view=article&id=56&Itemid
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