Home Office

NON-TECHNICAL SUMMARY

Mechanisms of fungal infection and drug resistance

Project duration

5 years 0 months

Project purpose

- (a) Basic research
- (b) Translational or applied research with one of the following aims:
  - (i) Avoidance, prevention, diagnosis or treatment of disease, ill-health or abnormality, or their effects, in man, animals or plants
  - (ii) Assessment, detection, regulation or modification of physiological conditions in man, animals or plants

Key words

Infectious disease, Aspergillosis, Drug targets, Resistance

Animal types	Life stages
Mice	adult

Retrospective assessment

> The Secretary of State has determined that a retrospective assessment of this licence is not required.

Objectives and benefits

Description of the projects objectives, for example the scientific unknowns or clinical or scientific needs it's addressing.

What's the aim of this project?

Aspergillus fumigatus is a fungal pathogen that causes significant morbidity and mortality in humans and animals. This project aims to further our understanding of how and why *A. fumigatus* is pathogenic, how resistance to our current antifungals emerges and how infection can be overcome.

Potential benefits likely to derive from the project, for example how science might be advanced or how humans, animals or the environment might benefit - these could be short-term benefits within the duration of the project or long-term benefits that accrue after the project has finished.

Why is it important to undertake this work?

Infection by Aspergillus causes more deaths globally than any other fungal disease. Aspergillus fumigatus alone is responsible for over 400,000 deaths every year, placing it among the leading causes of infection related mortality globally behind TB and alongside malaria. Voriconazole is the first line therapeutic for the treatment of all forms of aspergillosis. However, since we first demonstrated the emergence of azole resistance in A. fumigatus in 1991, we have documented a worrying increase in its frequency, now posing an imminent global public health crisis that requires immediate action. We only have a limited understanding of how and why A. fumigatus is such a successful pathogen, while other Aspergillus species are not. Gaining a greater understanding of the nature of pathogenicity in this organism will allow us to develop novel methods of treatment that can overcome the emergence of resistance. By addressing this challenge, we have a potential to have an immediate impact on the lives of millions of people afflicted with these diseases.

What outputs do you think you will see at the end of this project?

This project will yield a number of critical outputs:

1. We will generate an understanding of how and why A. fumigatus is such a successful pathogen. This knowledge can be used to develop novel antifungal drugs.

2. We will explore if strains that are resistant to antifungals are still pathogenic. This will help us understand which drugs or combinations of drugs may work best to suppress resistance, and how to develop tests to develop resistance rapidly in the clinic.

3. Improved methods to reduce the use of animals in infection studies will be developed.

Our work will be published in peer reviewed journals, and at the earliest opportunity will be presented at scientific conferences. We also undertake outreach activities with key stakeholders to inform them of the critical role infection models play in improving patient outcome.

Who or what will benefit from these outputs, and how?

Beneficiaries will include:

1. Immediate: Scientists, clinicians and industries pursuing novel anti-fungal therapies and looking to understand the pathogenic impacts of drug resistance mechanisms. We are already working with drug discovery companies who have compounds in different clinical phases of development, clinicians who wish to optimise treatment regimes, and groups involved in diagnostic development who are keen to understand what mechanisms of resistance may appear clinically.

2. Medium-term: Pharmaceutical companies seeking novel antifungal agents will be able to exploit our outputs to progress novel drugs through pre-clinical trials. I have significant experience in this area of translational science and there is currently a significant interest to fund ongoing preclinical antifungal studies.

3. Long-term: Individuals suffering, or at risk from, Aspergillus-related disease: The health burden of diseases caused by fungi is considerable. Over 300,000 people are thought to get invasive aspergillosis every year. Around 150,000 of these patients will die. In addition, around 3 million suffer from chronic pulmonary aspergillosis, mortality rates are around 15% per year. Improvement in treatments will reduce the burden of this disease.

How will you look to maximise the outputs of this work?

We have a long standing track record of rapidly publishing our scientific outputs in peer reviewed journals. Prior to publication, we will seek to present our work at both national and international conferences.

Many of our research programs are collaborative and we frequently exchange staff between labs. Our best practice methods, which will seek to implement the 3Rs at every stage, will be shared with our collaborators.

Species and numbers of animals expected to be used

- Mice: 2400

Predicted harms

Typical procedures done to animals, for example injections or surgical procedures, including duration of the experiment and number of procedures.

Explain why you are using these types of animals and your choice of life stages.

To establish an infection in a human lung Aspergillus fumigatus must bypass a patient’s immune system. To appropriately study the mechanisms of disease a similar environment is required. Rodent models and specifically mouse models of invasive aspergillosis have been established for many years.

Over 700 independent studies using mouse models have been published and these works have been critical in identifying high quality drug targets, novel antifungals, and host and pathogen factors that contribute to immune recognition. While there have been significant advances in the development of non-mammalian models over the last 20 years, the vast majority of the aforementioned discoveries would not have been possible using the alternative models.

We will use adult mice with completely developed respiratory systems, to model the disease setting of the adult human lung. Similarly aged mice will be used throughout the lifetime of the licence to ensure our data are reproducible and informative.

Typically, what will be done to an animal used in your project?

In most cases, infections cannot be established in a mouse without first suppressing the immune system. Licenced drugs are therefore used to suppress the immune system of the mice, administered most frequently via subcutaneous or intraperitoneal injection. Infections are established via intranasal administration of a set dose of fungal spores. To achieve this in a way to reduce stress to the animals, the procedure is performed under gaseous anaesthetic. For studies measuring fungal burden and fungal strain fitness, animals will be humanely culled at a set time point, usually 2 to 5 days after infection. For studies assessing fungal virulence, animals will be humanely culled prior to exceeding 20% body weight loss, relative to start of experiment, or as they begin to exhibit physical symptoms of infection, defined in our health monitoring procedures, that indicate mice are unlikely to recover. All animals will be culled 14 days after the day of infection.

What are the expected impacts and/or adverse effects for the animals during your project?

Animals that are infected with A. fumigatus would normally exhibit signs of lung infection. Initial signs of disease include body weight loss and decline in general body condition associated with a lack of grooming. Animals may become less responsive to external stimuli and exhibit laboured or rapid breathing at which point the animal will be humanely culled. Rarely (<1%) Aspergillus has caused signs of neurological damage when it has spread to the CNS e.g. ataxia, muscle tremors or seizures. Immunosuppression might lead to anaemia, blood in urine or diarrhoea. Some animals might develop renal impairment, which leads to yellow staining around the anal region and/or blood in urine. A change in body posture with the head held to one side or staggering might be observed in up to 5% of mice. Animals exhibiting neurological or renal involvement will be immediately culled.

Expected severity categories and the proportion of animals in each category, per species.

What are the expected severities and the proportion of animals in each category (per animal type)?

All mice included in these studies would be expected to experience at least mild severity procedures linked to the administration of immunosupression, anaesthesia and A. fumigatus spores.

For studies measuring fungal burden and fungal fitness of strains exhibiting typical levels of pathogenicity, severity would be expected to be mild for most mice (60%). These mice would experience weight loss of no more than 10% and would not exhibit other symptoms. For the remaining

mice (40%) a moderate severity would be expected; these mice would experience weight loss of between 10% and 20%, coupled with one or more of the other symptoms described above.

For virulence studies 90% of mice infected with strains exhibiting typical pathogenicity would be expected to experience moderate severity symptoms as defined above. The remaining 10% would experience mild severity symptoms limited to reduced weight loss of no more than 10%. For strains with reduced virulence, severity would be reduced. It is exceptionally rare that strains are identified with increased virulence. In these cases it would be expected that the severity of symptoms would be no greater than that for strains with typical levels of pathogenicity however the onset of symptoms would be faster.

What will happen to animals at the end of this project?

- Killed

Replacement

State what non-animal alternatives are available in this field, which alternatives you have considered and why they cannot be used for this purpose.

Why do you need to use animals to achieve the aim of your project?

Aspergillus infections are typically localised to the lungs and have to overcome challenges posed by the host immune system. Models that mimic the complexity of the host cannot yet be captured in in vitro or non-mammalian systems. This is because we have little understanding of how immune cells combine with each other and the lung environment (levels of oxygen, nutrient levels, fever) to eliminate an infection. These stressors and other as yet undefined host factors that contribute to the clearance of fungal infections need to be assessed in combination in order to validate specific fungal targets and therapeutic strategies. The only way to do this currently is to perform experiments in mammalian hosts.

Which non-animal alternatives did you consider for use in this project?

We have worked extensively in recent years with in vitro and tissue culture models and wherever possible, and scientifically justified, we replace in vivo studies with in vitro alternatives. In addition we have made extensive use, and pioneered the use of insect (*Galleria mellonella*) models of infection. In some circumstances, these studies can be used to effectively replace the number of experimental procedures that ultimately require the use of animals. Unfortunately these models are unable to replicate the complexity of a murine system in its entirety. We have work ongoing in the wider group that seeks to provide alternative models to replace the use of animals (e.g. the use of human-induced pluripotent stem cell derived lung tissue organoids; single-cell models to explore immune recognition of fungal cells) and when validated they are likely to replace some of the animal experiments described in this project.

Why were they not suitable?

The Galleria mellonella model of infection can be useful in screening strains for pathogenicity studies however there are many significant differences between insect models and mammalian models that are not adequately replicated. The immune system and the nutrient composition of larvae is distinct from that of the murine lung. Our prior data evaluating the fitness of multiple strains of *A. fumigatus* in parallel has revealed that the insect model is a poor alternative for murine infection. In vitro models can be exceptionally useful in defining specific aspects of pathogenic behaviour but cannot replicate the complex nature of the host environment.

Reduction

Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce animal numbers, and principles used to design studies. Describe practices that are used throughout the project to minimise numbers consistent with scientific objectives, if any. These may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.

How have you estimated the numbers of animals you will use?

To ensure that rigorous experimental design is employed in the conduct of all experiments expert biostatistical advice has been sought and the suggestions implemented. Continued biostatistical support will be accessible for the lifetime of the project. Required sample sizes have been estimated based on understanding of treatment effect size and variability in response measures. Where there is limited or no previous relevant information for a specific experimental design or endpoint a pilot study will be used to generate information for subsequent statistically powered studies. Data analysis will be conducted according to a pre-specified statistical analysis plan drawn up in conjunction with bio-statisticians. Important experimental results will be repeated and validated via an independent follow-up experiment to minimise the likelihood of spurious non-replicable results. Overall numbers of animals required are based on initial sample size estimates. These numbers will be updated as more recent and relevant data becomes available.

What steps did you take during the experimental design phase to reduce the number of animals being used in this project?

We have worked for many years to develop models which use the fewest animals to achieve accurate and statistically significant outcomes.

For studies that involve the identification of possible drug targets, prior to performing any comparative virulence model (which requires the use of more mice than any of our other models) significant evidence must exist that virulence or antifungal drug sensitivity could be affected. This would involve first screening strains through our suite of phenotypic assays, host cell assays, fungal burden models or our recently developed competitive fitness models of infection.

The competitive fitness model is a highly sensitive measure of fungal disease progression and permits us to test (in a single animal) many different fungal mutants simultaneously, thereby massively reducing the numbers of animals required for infection studies. For example, a study of 25 strains in

parallel would require the use of 561 mice. In our competitive study all strains are pooled in a single infection and a strong indication of virulence defects for most strains in the pool can be obtained from only 5 mice. Unfortunately it is not possible to assess the virulence of all strains using this model as the defect in virulence from some strains can be overcome by cross feeding by other strains within the pool.

What measures, apart from good experimental design, will you use to optimise the number of animals you plan to use in your project?

As previously mentioned, our studies will be staged so only strains that are likely to have a virulence defect, or have been shown to have altered drug resistance will be used in our animal models. Where possible we will perform our experiments in parallel as wild-type isolate controls can serve as comparators across several experiments.

Refinement

Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms in place to take up emerging refinement techniques during the lifetime of the project.

Which animal models and methods will you use during this project? Explain why these models and methods cause the least pain, suffering, distress, or lasting harm to the animals.

Our practice in performing virulence assays over past 8 years has been informed by our collaborators. With them, we have used species that have the least advanced nervous system whilst retaining the ability to closely resemble human disease. Advantages for studying fungal diseases in mice include ease of use, reproducibility and availability of immunosuppression regimes which mimic host factors of human disease. Additionally murine disease models permit the use of well-established human clinical biomarkers of disease, making them highly amenable to evaluation of novel therapies and diagnostics, which will become a major focus of our work in years to come. We routinely adopt the minimum experimental duration and infectious inoculum that provides meaningful data.

In all models, intranasal inoculations are performed under anaesthesia to reduce stress to animals. We have 3 well established models in which to study invasive aspergillosis in leukopenic or corticosteroid-treated mice. Herein we describe these as our fungal burden, competitive fitness and comparative virulence models. Fungal burden and competitive fitness analysis requires a lower fungal inoculum, results in a milder disease progression and earlier end-points than comparative virulence models, therefore we will utilise the former models whenever possible.

Specifically the fungal burden and competitive fitness models use a standardised time-limited end-point (such as day 4 post-infection at which point all mice are sacrificed). For comparative virulence studies where infections progress beyond 4 days, suffering will be minimised by ensuring mice do not exceed 20% weight loss from the start of the experiment (which has been previously established as a

surrogate marker for moribundity). Close monitoring of mice will be undertaken to ensure to ensure they are humanly culled before exhibiting signs of distress that exceed our stated severity endpoint.

It is estimated that 75%-90% of mice used for comparative virulence experiments with strains with wild-type virulence characteristics will reach a moderate endpoint.

Why can't you use animals that are less sentient?

We have historically employed a larval model, using the larvae of the greater wax moth (*Galleria mellonella*), of virulence as a proxy for virulence in mice. Recently we have been able to interrogate the validity of this model with greater accuracy using our competitive fitness technology. Although there is significant overlap between strains which show decreases in virulence in mouse models of infection, this is only the case for around 60% of strains. One of our objectives over the next 5 years will be to assess why this discrepancy exists and how it can be predicted. This will enable more precise and scientifically accurate use of the Galleria model.

There are several reasons why these models may be inadequate including significant differences in the immunological and abiotic environment of the hosts, the significant dynamic changes in a murine lung that are not replicated in larval hosts.

How will you refine the procedures you're using to minimise the welfare costs (harms) for the animals?

Where possible, we will choose to conduct fungal burden experiments as opposed to virulence studies. The set endpoint for fungal burden experiments is usually before any animals would be expected to reach the humane endpoints we observe in virulence studies, thereby greatly reducing suffering.

Throughout all experiments we will adhere to a robust monitoring schedule, scoring each animal accounting for a number of visualised factors, once signs of infection are present.

Through our previous work, we transitioned from using cortisone acetate to the steroid triamcinolone in an immunocompromised mouse model, either alone (steroid model) or in combination with cyclophosphamide (leukopenic model). Triamcinolone will be administered as a single dose 1 day prior to infection, replacing cortisone acetate, which required up to 5 doses, reducing the number of subcutaneous injections required. An additional advantage to this drug is the solubility, improving the accuracy of dosing and subsequent immunosuppression over cortisone acetate.

Non-aversive handling techniques will be used to minimise the level and duration of stress, and animals will be handled as infrequently as possible. Animals will be placed in cages with sufficient environmental enrichment to minimise stress.

What published best practice guidance will you follow to ensure experiments are conducted in the most refined way?

We will adhere to PREPARE guidelines (https://norecopa.no/PREPARE) to ensure our experiments are as robust as possible, and contribute to the scientific community in the clearest possible way, removing the need for similar experiments to be conducted by other researchers in the field.

We will follow guidelines published by the Joint Working Group on refinement with regards to dosing routes and volumes, and take guidance from the wealth of NC3Rs resources available for conducting all regulated procedures, ensuring animals experience the lowest level of suffering possible.

How will you stay informed about advances in the 3Rs, and implement these advances effectively, during the project?

To maintain a broad knowledge of advancements in the 3Rs, our infection technician and other PIL holders will continue to regularly attend events organised by appropriate organisations, ensuring we have an up to date knowledge on animal research as a whole, which can then be applied to our own research.

We will monitor the literature on aspergillosis research for advances in our field of research, and attend conferences, providing the opportunity to discuss best practices with other fungal researchers. Over the lifetime of the project, we will incorporate any progression in the field into our experiments.

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