The Institute of Cancer Research

PHD STUDENTSHIP PROJECT PROPOSAL

FUNDER DETAILS	
Studentship funded by:	Medical Research Council industrial Collaborative Awards in Science and Engineering (MRC iCASE)
Funder specific requirements:	All MRC iCASE students will attend taught courses one day a week for the first nine months of the PhD. This training will cover computational and thematic science training as well as core and transferable skills. Students will spend the remainder of the four years on their PhD project full time with monthly cohort activities. In addition, students must spend a cumulative period of no less than three months working in the facilities of the industrial collaborator. This 3 month period can be at any point during the studentship and may consist of a number of shorter visits if appropriate.
Estimated amount and distribution of time spent with industrial partner:	• Placement of 3-6 months • Regular communication with industrial supervisor
PROJECT DETAILS	
Project Title:	**Developing innovative chemical tools to drive lytic cancer cell death**
SUPERVISORY TEAM	
Primary Supervisor:	Professor Swen Hoelder
Associate Supervisor:	Dr Ben Bellenie
Secondary Supervisor:	Professor Pascal Meier
Industry Supervisor:	AstraZeneca – To be identified
Lead contact person for the project:	Dr Ben Bellenie
DIVISIONAL AFFILIATION	
Primary Division:	Division of Cancer Therapeutics
Primary Team:	Medicinal Chemistry 4
Other Division (if applicable):	Breast Cancer Research
Other Team (if applicable):	Cell Death and Immunity

SHORT ABSTRACT

Necroptosis is a highly immunogenic form of cell death, and is triggered by phosphorylation and oligomerisation of the pseudokinase MLKL.[1] Activating this process makes tumours more responsive to treatment with immune checkpoint inhibitors,[2] but currently there are no known small molecule agents that can mediate this effect.

This multidisciplinary project will apply synthetic organic and medicinal chemistry, computational design and biological testing to design and make bifunctional compounds capable of activating MLKL. These compounds will be useful as tools to study the necroptosis pathway, and could enable new treatment combinations with broad applicability in cancer.

BACKGROUND TO THE PROJECT

Necroptosis is a form of programmed cell death that occurs downstream of the receptor-interacting protein kinases RIPK1 and RIPK3, which assemble into an oligomeric complex termed the "necrosome" or "ripoptosome".[4] Necroptotic cells undergo rapid membrane permeabilization leading to the release of intracellular contents. This has been shown to activate innate immune pattern recognition receptors (PRRs), making necroptosis a highly immunogenic form of cell death.[5] Triggering necroptosis would therefore provide a method for converting "immune cold" tumours to "hot", enabling combination with immune checkpoint inhibitors.

Necroptotic cell death is mediated by the pseudokinase MLKL. **Phosphorylation** of MLKL by RIPK3 is believed to be both necessary and sufficient to trigger oligomerisation and membrane translocation of MLKL, forming pores which disrupt membrane integrity leading to cell death.[6] **Dimerisation** of the kinase-like domain is an indispensable step in this mechanism.[7] Interestingly, **ubiquitylation** of MLKL has also been shown by the Meier group to positively regulate this process.[8]

Mediating gain-of-function events such as these therapeutically remains an unsolved problem. However, recent work has demonstrated that inducing proximity with heterobifunctional compounds represents a viable approach to switching on biological function.[3] Induction of ubiquitination (via PROTAC) is now well validated, and early examples have progressed into the clinic. Activation of signalling by homodimerisation has been reported,[3] and, perhaps most relevant here, induction of phosphorylation of specific targets has now also been demonstrated both by Choudhary[9] and within our own team.

Therefore, compounds which mediate phosphorylation, dimerisation or ubiquitylation of MLKL have potential to trigger necroptotic cell death, providing a pathway to a novel therapeutic approach for cancer immunotherapy.

PROJECT AIMS

This is a multidisciplinary research project applying chemical synthesis, in silico design and biological testing to discover new molecules which can modulate necroptosis. Objectives:

i) Design and synthesise improved, non-inhibitory ligands for the pseudokinase MLKL
ii) Using these new ligands, design and synthesise induced-proximity chemical tools to mediate phosphorylation, dimerisation, or ubiquitination of MLKL
iii) Investigate the effect of these compounds on the necroptosis pathway.

ICR The Institute of Cancer Research

UKRI Medical Research Council

RESEARCH PROPOSAL

Aim and overview

Lytic forms of cell death such as **necroptosis,** that leads to the release of intracellular content into the microenvironment, are particularly potent in driving an immune response. Inducing necroptosis could therefore provide a method for converting "immune cold tumours" to "hot tumours", enabling effective treatment combinations with immune checkpoint inhibitors.[5]

MLKL is the key mediator of necroptosis. Upon activation through phosphorylation at T357 and S358 in the pseudokinase domain, an unfolding event leads to oligomerisation of the N-terminal executioner domain and integration into the membrane, forming pores leading to membrane rupture and cell death.[1]

We propose to **design, synthesise and validate bifunctional chemical compounds** which interact with MLKL and induce this oligomerisation, leading to immunogenic cell death through necroptosis.

Design and synthesis of MLKL binding ligands

In the first part of the project we will focus on designing and synthesising improved ligands which can bind to the ATP site of MLKL. As MLKL is a pseudokinase, this site is not catalytically active, and type I ATP site binders such as "cpd **4**" (DiscoverX, 160nM) do not inhibit necroptosis.[10] An X-ray co-crystal structure is reported (Figure 1)

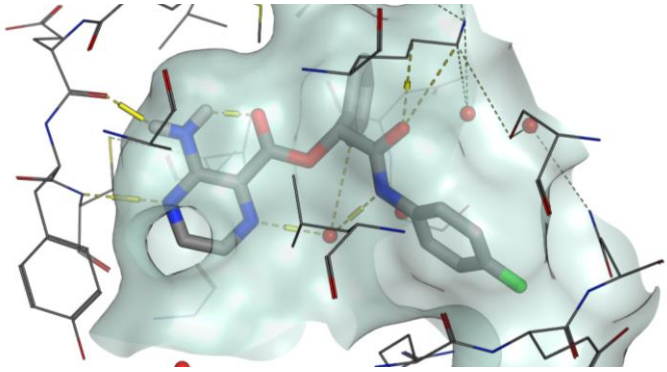

Figure 1: "cpd 4" bound to pseudokinase domain of MLKL (PDB:5KO1)

By preparing analogues of this compound, we demonstrated that linkers can be attached without eliminating MLKL binding affinity; however the ester group of "cpd 4" was unstable. Alternative starting points or replacements for the ester group are therefore required to enable the synthesis of bifunctional compounds.

Through screening of a small set of marketed kinase drugs, we have identified further compounds which can bind to MLKL. With this diverse collection of starting points in hand, we will use in silico methods and published structural information to design and synthesise improved ATP-site binders of MLKL. We will identify compounds which can bind in the 100nM range, are chemically stable, and can permeate into cells. These compounds will be tested in cellular assays to confirm that they do not inhibit necroptosis, before proceeding to further elaborate these into bifunctional molecules.

Design and synthesis of click-chemistry libraries

The design of heterobifunctional molecules requires three components: a **target-binding warhead** (in our case, MLKL) attached by a **linker** to an **effector warhead**. For example, in PROTAC, the effector is an E3 ligase such as VHL. For phosphorylation, we have validated effector warheads which bind to and activate the ubiquitous kinase AMPK.[9] As for PROTAC, the length of linker appears to be critical in enabling phosphorylation. It is therefore important that synthetic methods are sufficiently reliable to prepare libraries of multiple compounds to explore a range of **different linker lengths**. For initial work, we will use click chemistry: we have established experience in the group of using this method for formation of bifunctional compounds.

Based on our structural model we will identify appropriate **attachment points** on our MLKL linkers which we expect to be solvent exposed. This will be validated by attaching a short alkyne-capped chain to each of these positions.

Compounds will be tested to confirm binding affinity is retained. We will then prepare a library of alkynes with varying chain length and composition (PEG, alkyl). This can then be combined with azide-tagged effector groups to prepare **bifunctional libraries** using **click chemistry**. Initially, AMPK warheads (previously prepared in house) will be used to make bifunctional compounds with potential to mediate phosphorylation. An example library design is shown in Figure 2 based on "cpd 4", although this would be replaced with the improved MLKL ligands discovered in the first part of the project.

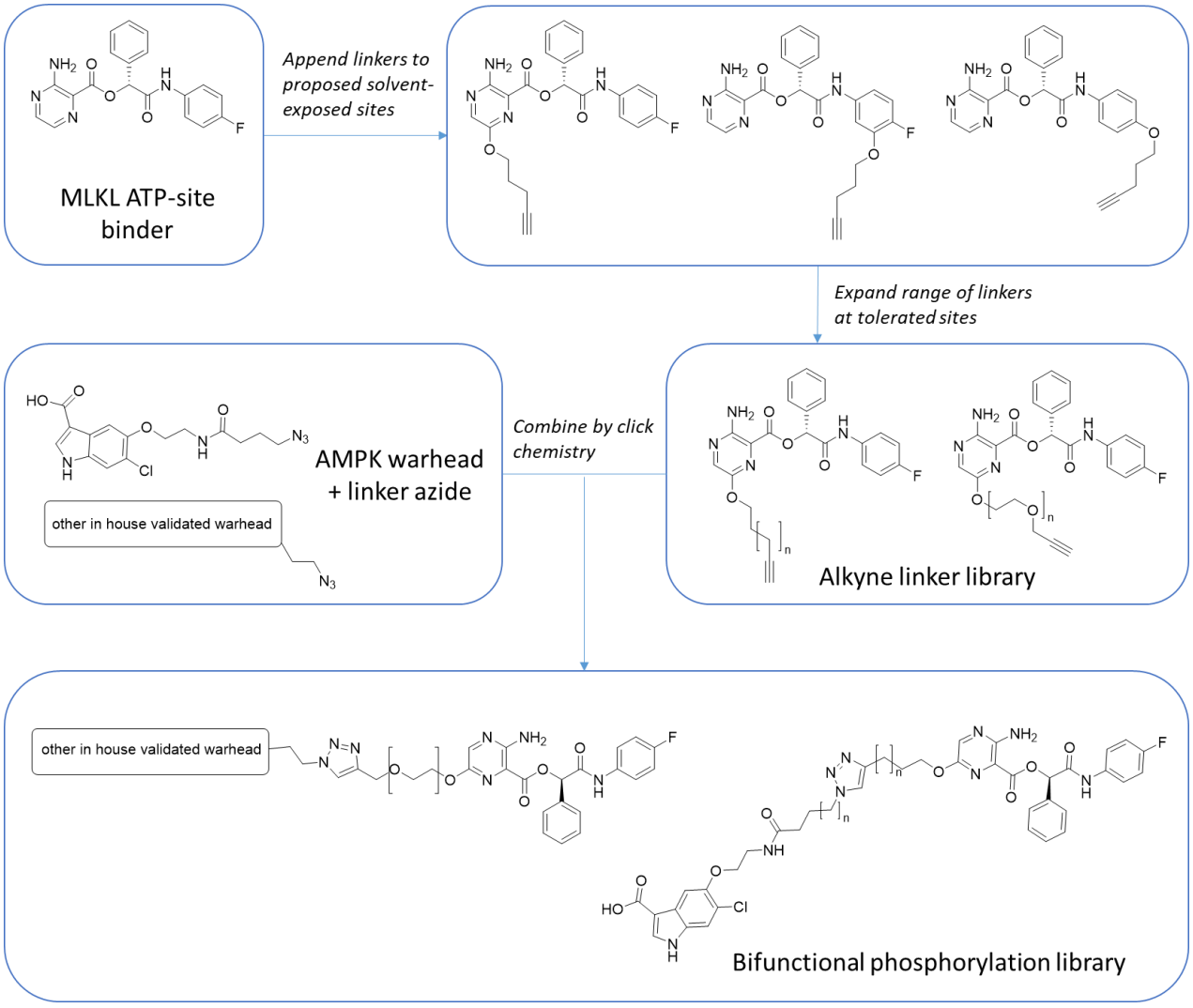

Figure 2: Example library design and synthesis plan

Alternative sets of bifunctional molecules could also be made from the same alkyne-linker library – combining with other effector kinase warheads to trigger phosphorylation, with a duplicate MLKL ligand to make potential dimerisers, or with E3 ligase ligands to make compounds which can ubiquitylate MLKL.

Test cascade

ATP-binding ligands, and exemplar bifunctional compounds will be tested for **binding affinity** to MLKL using a previously developed Lanthascreen assay.

For **phosphorylation**, the ADP-glo assay (also previously validated in house) can be used to show the ability of AMPK to phosphorylate a peptide substrate or target protein (MLKL). Turnover of ATP-ADP in the presence of both AMPK and MLKL suggests a phosphorylation event is taking place. We also have access via the Meier group to phospho-specific antibodies for MLKL which will be used to confirm phosphorylation at the desired site. Mass spectrometry (MS/MS) can be used to provide orthogonal confirmation, and phosphoproteomics is also available at ICR to study selectivity.

The Meier group has extensive experience in studying necroptosis, and have established a panel of cellular assays suitable for profiling compounds and studying functional consequences of their activity.

Student training

The student will gain valuable expertise in interdisciplinary research, building on their existing knowledge of organic synthetic chemistry. They will develop skills in medicinal chemistry, computational design and biological testing through hands-on experience, with one-to-one training from experienced postdoctoral scientists. They will benefit also from exposure to cancer cell biology through interactions with Prof. Meier's team, and industrial research through a placement at AstraZeneca. Supporting this, our in-house training programmes, along with seminars from AZ speakers, provide a broad understanding of drug discovery. Students will be supported to communicate their research widely, including writing up results for publication in peer-reviewed journals. As a result of their training, past students in our groups have readily found employment as scientists in academia or the pharmaceutical industry.

LITERATURE REFERENCES

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(8) Garcia, L. R.; Tenev, T.; Newman, R.; Haich, R. O.; Liccardi, G.; John, S. W.; Annibaldi, A.; Yu, L.; Pardo, M.; Young, S. N.; Fitzgibbon, C.; Fernando, W.; Guppy, N.; Kim, H.; Liang, L.-Y.; Lucet, I. S.; Kueh, A.; Roxanis, I.; Gazinska, P.; Sims, M.; Smyth, T.; Ward, G.; Bertin, J.; Beal, A. M.; Geddes, B.; Choudhary, J. S.; Murphy, J. M.; Ball, K. A.; Upton, J. W.; Meier, P. Ubiquitylation of MLKL at Lysine 219 Positively Regulates Necroptosis-Induced Tissue Injury and Pathogen Clearance. *Nat Commun* **2021**, *12* (1), 1–18. https://doi.org/10.1038/s41467-021-23474-5.

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CANDIDATE PROFILE	
Note: the ICR's standard minimum entry requirement is a relevant undergraduate Honours degree (First or 2:1)	
Pre-requisite qualifications of applicants:	• At least a 2:1 degree in a relevant scientific subject • Some experience of synthetic organic chemistry is required, either as part of their university degree or from employment.
Intended learning outcomes:	• Experience of multi-step and parallel compound synthesis • Medicinal chemistry: compound design including potency and property optimisation. • Use of in silico tools for structure visualisation, data analysis and compound design • Experience of testing compounds in biochemical and cellular assays and analysing data • Broad experience of medicinal chemistry and drug discovery, not only from this project but from exposure to other projects within the unit, and attendance of training seminars, problem sessions and workshops • Experience of working in an industrial environment through placement • Ability to present work verbally and in writing to a multidisciplinary audience, including writing and submitting of papers to high impact peer-reviewed journals
ADVERTISING DETAILS	
Project suitable for a student with a background in:	☒ Biological Sciences ☐ Physics or Engineering ☒ Chemistry ☐ Maths, Statistics or Epidemiology ☐ Computer Science ☐ Other (provide details)
Keywords:	1.Medicinal chemistry

	2.Immunogenic cell death
	3.Organic chemistry
	4.Bifunctional compounds
	5.Induced proximity
	6.Induced phosphorylation