

Mechanism of Action of Two Small Molecule Necroptosis Inhibitors

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Abstract

Necroptosis is a form of programmed cell death that is controlled by a defined set of protein effectors, despite displaying the morphological characteristics of unregulated lytic cell death (necrosis). Recently, there has been increasing interest in this type of cell death, ignited by studies demonstrating that necroptosis is involved in the pathophysiology of various diseases – including inflammatory conditions, degenerative conditions, infectious diseases and cancers – and further kindled by the progression of small molecule necroptosis inhibitors into clinical trials. The best studied form of necroptosis is driven by the tumour necrosis factor (TNF) signalling pathway, which is initiated by TNF binding to its cell surface receptor TNFR1. Importantly, TNF-induced necroptosis is regulated by three key proteins: the kinases RIPK1 and RIPK3, and the pseudokinase MLKL, which acts as the cell death executioner.

To identify novel inhibitors of necroptotic cell death, several small molecule screens were performed at WEHI. This PhD thesis details the investigation into the mechanism of action of two small molecule necroptosis inhibitors identified from these screens. I employed a suite of chemical biology, biochemistry and cell biology approaches to deduce the cellular targets of these small molecules and investigate their anti-necroptotic activity.

Chapter 2 examines the identification and mechanism of action of Compound **2**, a more potent necroptosis inhibitor than its parent compound, Compound **1**, which was identified from a small molecule screen against MLKL. I determined that Compound **2** targets all three necroptotic effector proteins – MLKL, RIPK1 and RIPK3 – *in vitro* and in cells, to potently block necroptosis in human and murine cells at nanomolar concentrations. Moreover, this study

highlights that necroptosis can be potently inhibited by targeting multiple effectors, suggesting that targeting multiple proteins in the pathway may be an ideal strategy for inhibiting necroptosis in a therapeutic context.

Chapter 3 explores the cellular activity and cellular targets of ABT-869, an inhibitor of necroptosis identified from a phenotypic screen using a cell line expressing a constitutively active MLKL mutant. I determined that ABT-869 blocks necroptotic cell death by targeting RIPK1 and possibly RIPK3, although whether ABT-869 targets RIPK3 directly or indirectly, as a result of RIPK1 inhibition, remains to be elucidated. Furthermore, this study raises some interesting questions regarding the involvement of RIPK1 downstream of MLKL activation, which could contribute to an improved understanding of how necroptosis is regulated at the molecular level.

Together, these two novel inhibitors of necroptosis identified from small molecule screens were found to block necroptotic cell death by targeting known components of the TNF-induced necroptosis pathway. This research provides insight into how small molecules can modulate necroptotic signalling by interacting with key necroptotic proteins to inhibit cell death.

Declaration

This is to certify that:

- i) this thesis comprises only my original work towards the degree of Doctor of Philosophy, except where indicated in the Preface;
- ii) due acknowledgment has been made in the text to all other material used;
- iii) this thesis is less than 100,000 words in length, exclusive of tables, maps, bibliographies and appendices.

Catia Lucchesi Pierotti

March 2020

Preface

This thesis comprises my own original work and contribution to the field, except where indicated below. I was responsible for planning and writing the initial draft of each chapter, including the figures and tables, presented in this thesis. My supervisors Professor Guillaume Lessene, Associate Professor James Murphy and Dr Mark van Delft provided feedback regarding the content and style of each chapter, as well as providing editorial assistance. I performed the editing, formatting and compiling of all chapters to produce the final thesis. The work presented in the results chapters (Chapter 2 and Chapter 3) would not have been possible without the generous contribution and assistance of other scientists. I was fortunate to have collaborated with a diverse range of scientists whose contribution greatly enriched the work detailed in the results chapters. I gratefully acknowledge the contribution of others below.

Chapter 2: Potent Inhibition of Necroptosis by Simultaneously Targeting Multiple Effectors of the Pathway

This chapter consists of a primary research article that was published in the journal *ACS Chemical Biology* on 9th September 2020. The article along with the supporting information are included in their entirety in this chapter. I am the first author on this publication and I was responsible for planning, executing and preparing the work for publication. I wrote the initial draft of the manuscript and I performed all subsequent editing and revising in response to co-author feedback, reviewer comments and editor reviews. I contributed greater than 50% of the content in the publication. The contributions of the co-authors are specified in the article (see *Author Contributions*) and also detailed below:

- I performed the chemical synthesis of Compound **1**, Compound **2** and Compound **3** prior to the commencement of my PhD
- Dr Pooja Sharma and Wilco Kersten optimised the protocol and conditions for small molecule SPR with hMLKL
- Dr Pooja Sharma performed the SPR with hMLKL pseudokinase domain
- SPR with full-length hMLKL was performed in conjunction with Annette Jacobsen
- Associate Professor Isabelle Lucet and Lung-Yu Liang performed the TSA
- Associate Professor Peter Czabotar, Associate Professor James Murphy and Dr Angus Cowan performed the X-ray crystallography and solved the crystal structure
- Sarah Garnish and Dr Anne Hempel optimised the CETSA protocol
- Sarah Garnish and Dr Anne Hempel determined the CETSA melting temperature and temperature range for hMLKL in U937 cells and mMLKL in MDF cells
- Dr Maria Tanzer generated the U937 knockout cell lines
- Dr Maria Tanzer, Annette Jacobsen and Meng-Xiao Luo performed the cell death assays
- Dr Joanne Hildebrand and Dr Maria Tanzer performed the *in vivo* TNF-induced SIRS murine model

Chapter 3: Investigating ABT-869 as a Novel Inhibitor of Necroptosis

This chapter consists of unpublished work not submitted for publication. I was responsible for planning, optimising and performing the experiments as well as analysing the data. The contributions of others are acknowledged throughout this chapter in the main text, figure legends and materials and methods, as appropriate, and also detailed below:

- Dr Kym Lowes, Dr Mark van Delft and Professor David Huang designed, optimised and performed the MLKL(Q343A) cell-based phenotypic screen
- Dr Mark van Delft, Dr Joanne Hildebrand and Dr Maria Tanzer performed the initial validation studies testing ABT-869 in preliminary cell death assays
- Dr Christoph Grohmann performed the chemical synthesis of ABT-869 (Compound 4), Compound 5, Compound 6, ABT-869 Sepharose probes and Compound 5 Sepharose probes
- Dr Jarrod Sandow performed the mass spectrometry and analysed the proteomics data
- Associate Professor James Murphy and Sam Young performed the following cloning: modifying the pFastBac vector to include an N-terminal SBP-tag, preparing the plasmid encoding SBP-mRIPK3, and preparing the bacmids encoding SBP-hMLKL, SBP-hRIPK3(2-356) and SBP-hRIPK3(2-311)
- Annette Jacobsen expressed the SBP-mMLKL and SBP-mRIPK1 proteins
- other members of the Murphy Lab expressed the SBP-hMLKL and SBP-hRIPK3(2-356) proteins
- Annette Jacobsen purified the SBP-mMLKL protein
- all other cloning, expression and purification of recombinant proteins was performed in conjunction with Annette Jacobsen
- Wilco Kersten optimised the protocol and conditions for small molecule SPR with SBP-mMLKL and SBP-hMLKL
- all SPR was performed in conjunction with Annette Jacobsen
- Sarah Garnish and Dr Anne Hempel optimised the CETSA protocol

- Sarah Garnish and Dr Anne Hempel determined the CETSA melting temperature and temperature range for mMLKL in MDF cells and hMLKL in U937 cells

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I would like to acknowledge and thank my PhD committee Associate Professor Jeff Babon, Dr Gemma Kelly and Dr Brad Sleebs for their advice, suggestions and guidance. I always found our robust discussions in my committee meetings very insightful, whether they were about guiding the direction of my research projects or my scientific career. I am grateful for their encouragement and enthusiasm throughout my PhD.

It has been an immense privilege to complete my PhD at WEHI and to be part of both the Lessene Lab and Murphy Lab. My journey at WEHI started as an undergraduate student in the Lessene Lab in the Chemical Biology Division. I would like to thank Professor Guillaume Lessene and Dr Jean-Marc Garnier for taking me on as an undergraduate student, introducing me to the world of scientific research and guiding me through Honours. I would like to thank all past and present members of the Lessene Lab and Chemical Biology Division for their assistance, input and advice over the years. Thank you especially to Dr Jean-Marc Garnier for

taking me under his wing and teaching me the practical fundamentals of organic chemistry, Dr Pat Sharp for his infectious enthusiasm for science and for being a constant source of inspiration and Dr Martin Brzozowski for his guidance with chemistry and insightful career advice.

Throughout my PhD I have also been part of the Murphy Lab, previously in the Cell Signalling and Cell Death Division, and now in the Inflammation Division. I would like to thank Associate Professor James Murphy for giving me the opportunity to join his lab, for his incredible support as I began to delve into the world of biology and for his brilliant sense of humour. I thoroughly enjoyed being a member of the Murphy Lab and having the opportunity to work with such a motivated group of scientists. I would like to thank all past and present members of the Murphy Lab, Cell Signalling and Cell Death Division and Inflammation Division for their ideas, contributions and suggestions. Thank you especially to Dr Maria Tanzer for teaching me basic biology from scratch and Annette Jacobsen for being a close collaborator and good friend ever since Honours. I really enjoyed collaborating with Annette on our respective PhD projects and working closely together over the last few years. Thank you to Annette for many interesting and insightful discussions relating to our projects, for answering my never-ending questions and for being my go-to person for anything relating to biology.

I would next like to acknowledge and thank the many people at WEHI, past and present, who I had the privilege of working with throughout the course of my PhD. I feel very fortunate to have had the opportunity to work alongside and collaborate with such a diverse range of scientists across the Institute. I am incredibly grateful for every single person at WEHI who

shared their knowledge, expertise and wisdom with me. Thank you all for your generosity, kindness and patience.

I thoroughly enjoyed being part of the vibrant WEHI community and I am grateful for the many opportunities that were available at the Institute. I would like to thank my postdoc mentor Dr Tim Johanson for such interesting and engaging discussions about science and careers, as well as for his insight and advice. I would like to acknowledge the various committees that I have been part of at WEHI: the Gender Equality Committee, AthenaSWAN SAT, WESA 2018, Education Committee and Reconciliation Committee. This has been an invaluable experience as it enabled me to contribute to the WEHI community, meet different people from around the Institute and work on my skills outside the lab. A special thanks to the WESA 2018 committee members, many of whom have become my good friends, for their teamwork and dedication. I have also been a member of the WEHI footy team ("The Wombats") and the WEHI band ("The Inflammationes"), both of which have been highlights of the last few years.

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List of Abbreviations

ADMET	absorption, distribution, metabolism, excretion, toxicity
ALS	amyotrophic lateral sclerosis
AML	acute myeloid leukaemia
AP-1	activator protein 1
ATP	adenosine triphosphate
AurKB	Aurora kinase B
BMS	Bristol Myers Squibb
CDC48	cell division cycle-associated protein 8
CETSA	Cellular Thermal Shift Assay
cFLIP	cellular FLICE-like inhibitory protein
cIAP	cellular inhibitor of apoptosis protein
CK1	casein kinase 1
CLL	chronic lymphocytic leukaemia
CLP	cecal ligation and puncture
ConA	concanavalin A
Cpdm	chronic proliferative dermatitis mutation
CRMO	chronic recurrent multifocal osteomyelitis
DAI	DNA-dependent activator of IFN-regulatory factors
DAMP	damage-associated molecular pattern
DARTS	Drug Affinity Responsive Target Stability
DDR2	discoidin domain-containing receptor 2
DIABLO	direct IAP-binding protein with low pI
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DSF	Differential Scanning Fluorimetry
dsRNA	double-stranded RNA
DUB	deubiquitinase
EAE	experimental autoimmune encephalomyelitis
EphB	Ephrin type-B receptor
ESCRT	endosomal sorting complex required for transport

FACS	fluorescence-activated cell sorting
FADD	FAS-associated death domain protein
FasL	Fas ligand
FasR	Fas receptor
FDA	Food and Drug Administration
FP	fluorescence polarisation
GBA	glucocerebrosidase
GFP	green fluorescent protein
GSK	GlaxoSmithKline
GST	glutathione S-transferase
His	histidine
hMLKL	human MLKL
HOIL-1	heme-oxidized IRP2 ubiquitin ligase 1
HOIP	HOIL-1 interacting protein
hRIPK1	human RIPK1
hRIPK3	human RIPK3
HSP	heat shock protein
HSV	herpes simplex virus
HTRF	homogeneous time-resolved fluorescence
IAP	inhibitor of apoptosis protein
IDO	indoleamine 2,3-dioxygenase
I κ B	inhibitor of nuclear factor kappa B
IKK	I κ B kinase
INCENP	inner centromere protein
IRBP	interphotoreceptor retinoid-binding protein
JNK	c-Jun N-terminal kinase
k_a	association rate constant
k_d	dissociation rate constant
K_D	binding affinity
LMP1	latent membrane protein 1
LPS	lipopolysaccharide
LUBAC	linear ubiquitin chain assembly complex

MAPK	mitogen-activated protein kinase
MAPKK	mitogen-activated protein kinase kinase
MDF	murine dermal fibroblast
MDR1	multidrug resistance protein 1
MEF	murine embryonic fibroblast
MLKL	mixed lineage kinase domain-like protein
mMLKL	murine MLKL
mRIPK1	murine RIPK1
mRIPK3	murine RIPK3
MTH-Trp	methyl-thiohydantoin-tryptophan
NASH	non-alcoholic steatohepatitis
NEMO	nuclear factor kappa B essential modulator
NF- κ B	nuclear factor kappa B
NMR	Nuclear Magnetic Resonance
NP	nucleoprotein
NPC	Niemann-Pick disease type C
NQO2	N-ribosyldihydronicotinamide:quinone oxidoreductase 2
OPTN	optineurin
PAGE	polyacrylamide gel electrophoresis
PAMP	pathogen-associated molecular pattern
PCR	polymerase chain reaction
PDAC	pancreatic ductal adenocarcinoma
PDGFR	platelet-derived growth factor receptor
PEG	polyethylene glycol
PERK	protein kinase RNA-like endoplasmic reticulum kinase
P-gp	P-glycoprotein
PI	propidium iodide
poly(I:C)	polyinosinic:polycytidylic acid
PRR	pattern recognition receptor
RFU	relative fluorescence units
RHIM	RIP homotypic interaction motif
RIPK1	receptor-interacting serine/threonine-protein kinase 1

RIPK3	receptor-interacting serine/threonine-protein kinase 3
RNA	ribonucleic acid
RU	resonance units
SAR	structure-activity relationship
SBP	Streptavidin-Binding Peptide
SD	standard deviation
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEC	size exclusion chromatography
SEM	standard error of the mean
sgRNA	single guide RNA
SHARPIN	SHANK-associated RH domain-interacting protein
SIRS	systemic inflammatory response syndrome
SJS	Stevens-Johnson syndrome
Smac	second mitochondria-derived activator of caspases
SOD1	superoxide dismutase 1
SPR	Surface Plasmon Resonance
T3SS	type III secretion system
TAB	TAK1-binding protein
TAK1	transforming growth factor beta-activated kinase 1
TAM	Tyro3, Axl, Mer
tBid	truncated Bid
TEN	toxic epidermal necrolysis
TEV	Tobacco Etch Virus
TLCS	tauroithocholic acid-3-sulfate
TLR	toll-like receptors
T _m	melting temperature
TNF	tumour necrosis factor
TNFR	tumour necrosis factor receptor
TRADD	TNFR1-associated death domain protein
TRAF2	TNFR-associated factor 2
TRAIL	TNF-related apoptosis-inducing ligand
TRAILR	TRAIL receptor

TR-FRET	time-resolved fluorescence resonance energy transfer
TRIF	TIR domain-containing adapter-inducing interferon beta
TSA	Thermal Shift Assay
TSI	TNF, Smac-mimetic, IDN-6556
TSQ	TNF, Smac-mimetic, Q-VD-OPh
TSZ	TNF, Smac-mimetic, z-VAD-fmk
UT	untreated
VEGFR	vascular endothelial growth factor receptor
vIRA	viral inhibitor of RIP activation
WEHI	Walter and Eliza Hall Institute of Medical Research
WT	wild-type
XIAP	X-linked inhibitor of apoptosis protein
ZBP1	Z-DNA-binding protein 1

Chapter 1: Introduction

Overview of Necroptosis

Cell death is an essential process in all forms of life. There are two main types of cell death in multicellular organisms: apoptosis, a form of programmed cell death, and necrosis, originally described as an unregulated form of cell death. Necroptosis, a term first coined by Degterev *et al.* in 2005 (Degterev *et al.*, 2005), is a type of programmed necrosis and has been recently described as an alternative cell death pathway that complements or functions instead of apoptosis, the classical programmed cell death pathway. Hence, the term necroptosis is the portmanteau word combining necrosis, referring to the morphology of this type of cell death, and apoptosis, referring to the well-orchestrated and highly regulated nature of this cell death process.

Necroptotic cell death displays the morphological hallmarks of necrosis, which include cell swelling, plasma membrane rupture, spillage of the cellular contents into the extracellular environment, release of damage-associated molecular patterns (DAMPs), initiation of an inflammatory response and activation of the immune system (**Figure 1.1**) (Laster *et al.*, 1988). However, unlike necrosis, which is unregulated cell death that occurs as a result of trauma, necroptosis is a tightly controlled process that is initiated by various stimuli and regulated by specific protein effectors (Grootjans *et al.*, 2017). In contrast, apoptosis displays morphological characteristics that include cell shrinkage, chromatin condensation, membrane blebbing, formation of apoptotic bodies and phagocytosis by neighbouring cells (**Figure 1.1**) (Kerr *et al.*, 1972). As such, apoptosis is classically considered to be immunologically silent. Apoptosis is classified as either intrinsic, where internal signals

activate the mitochondrial pathway, or extrinsic, where external signals activate the death receptor pathway (Green and Reed, 1998, Adams and Cory, 1998, Nagata, 1997, Ashkenazi and Dixit, 1998). Apoptotic cell death is executed by caspases, a family of cysteine proteases that cleave a multitude of cellular substrates to efficiently dismantle the cell (Thornberry and Lazebnik, 1998, Salvesen, 2002, Timmer and Salvesen, 2007). In contrast to apoptosis, necroptosis is a lytic, caspase-independent form of cell death that is executed by the killer pseudokinase, the mixed lineage kinase domain-like protein (MLKL) (Murphy and Silke, 2014).

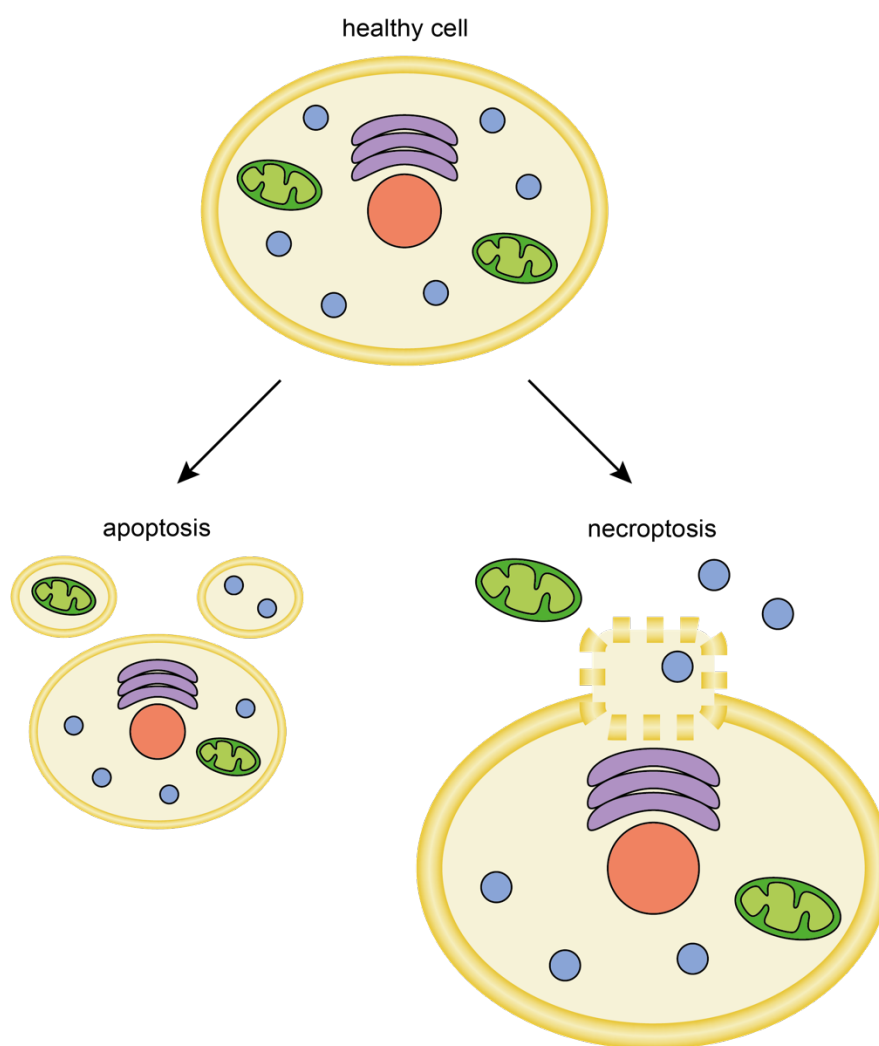


Figure 1.1 Comparison between apoptosis and necroptosis, two forms of programmed cell death.

Apoptotic cell death is a process of cellular dismantling characterised by cell shrinkage, membrane blebbing and formation of apoptotic bodies. Necroptotic cell death is a process of cell lysis characterised by cell swelling, plasma membrane rupture and spillage of intracellular contents.

Necroptotic proteins are not restricted to animals and are present in almost all forms of life including fungi and plants, which suggests that necroptosis is an ancient form of cell death that has been evolutionarily conserved across kingdoms (Kajava et al., 2014, Daskalov et al., 2016, Mahdi et al., 2020, Newton and Manning, 2016, Hofmann, 2020). Furthermore, there are a range of viral and bacterial proteins that target key components of the necroptosis pathway to thwart cell death, suggesting that necroptosis has evolved alongside microbial pathogens as an innate immune mechanism to combat pathogenic infections (Upton et al., 2010, Upton et al., 2012, Guo et al., 2015, Pearson et al., 2017, Liu et al., 2018, Petrie et al., 2019, Nailwal and Chan, 2019). There is growing evidence that necroptotic cell death has important roles in human physiology and diseases, and that dysregulated necroptosis is linked to various pathologies (Khoury et al., 2020).

Initiation and Execution Mechanisms

Necroptosis can be triggered by a variety of extracellular or intracellular stimuli that are recognised by specific receptors or sensors (**Figure 1.2**) (Grootjans et al., 2017). Extracellular necroptotic stimuli can activate the tumour necrosis factor receptors (TNFRs), through TNF binding to TNFR1, Fas ligand (FasL) binding to the Fas receptor (FasR) and TNF-related apoptosis-inducing ligand (TRAIL) binding to the TRAIL receptor (TRAILR) (Vercammen et al., 1998a, Vercammen et al., 1998b, Holler et al., 2000, He et al., 2009). Extracellular necroptotic stimuli can also result in activation of the toll-like receptors (TLRs), through double-stranded RNA (dsRNA) binding to TLR3 and lipopolysaccharide (LPS) binding to TLR4 (He et al., 2011, Kaiser et al., 2013). Intracellular necroptotic stimuli include viral or bacterial nucleic acids, which activate DNA-dependent activator of IFN-regulatory factors (DAI), also known as Z-DNA-binding protein 1 (ZBP1) (Upton et al., 2012, Maelfait et al., 2017).

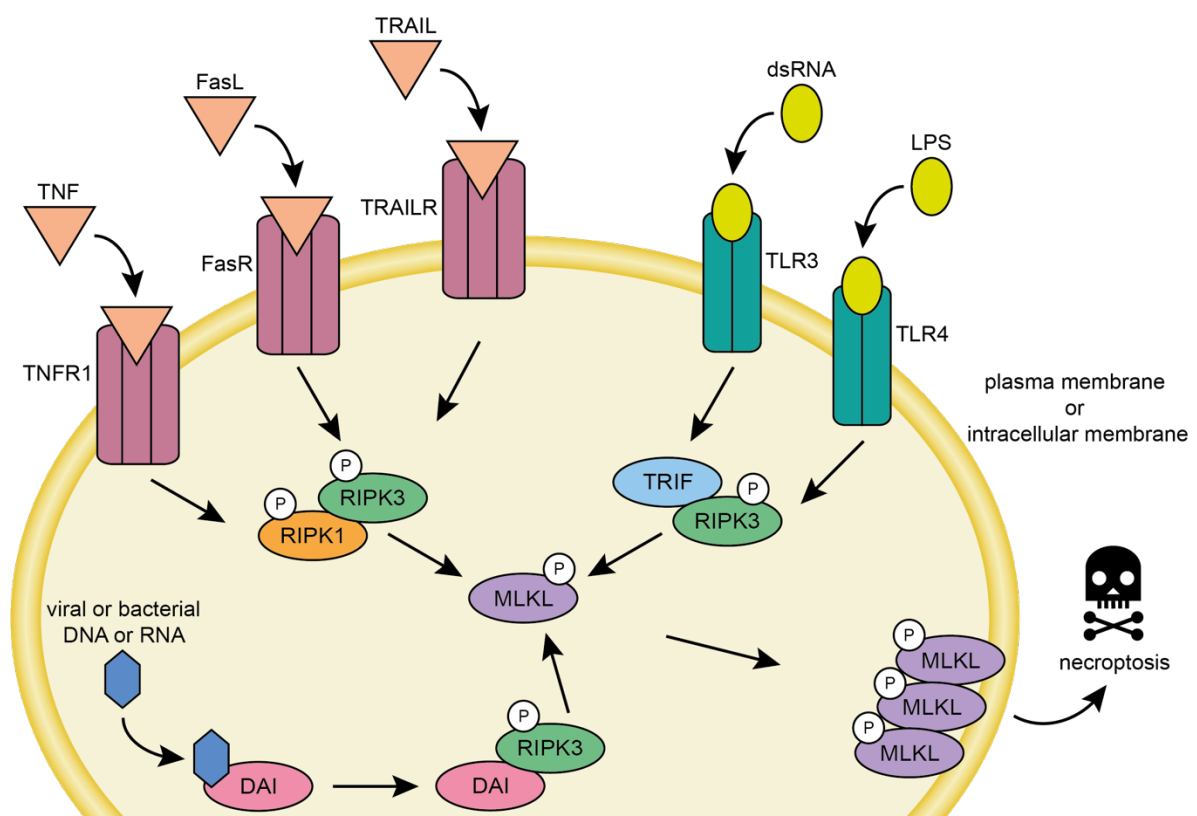


Figure 1.2 Necroptosis can be initiated by a variety of extracellular or intracellular stimuli.

Extracellular necroptotic stimuli include TNF, FasL and TRAIL, which are recognised by their respective death receptors TNFR1, FasR and TRAILR to activate RIPK1 and RIPK3. Other extracellular necroptotic stimuli include dsRNA and LPS, which activate the toll-like receptors TLR3 and TLR4, respectively, to signal via TRIF. Intracellular necroptotic stimuli include viral or bacterial DNA or RNA, which are recognised by DAI.

The TNFRs are a superfamily of cytokine transmembrane receptors that have a diverse range of functions, including important roles in inflammatory gene transcription and cell death (Locksley et al., 2001). A subset of the TNFRs are known as the death receptors, which include TNFR1, FasR and TRAILR, that each contain a cytoplasmic death domain and can be activated to trigger necroptosis (Wilson et al., 2009, Holler et al., 2000, He et al., 2009). Death receptor signalling via TNF binding to TNFR1, FasL binding to FasR and TRAIL binding to TRAILR can all induce necroptosis via receptor-interacting serine/threonine-protein kinase 1 and 3 (RIPK1 and RIPK3) (Vercammen et al., 1998a, Vercammen et al., 1998b, Holler et al., 2000, He et al., 2009, Cho et al., 2009, Zhang et al., 2009). TNF-driven necroptosis is the best studied necroptotic signalling pathway and as this form of necroptotic cell death is the subject of this thesis, it will be discussed in more detail in the following section.

The TLRs are membrane-bound pattern recognition receptors (PRRs) located on either the plasma membrane or intracellular membranes, which have important roles in the innate and adaptive immune systems by recognising invading pathogens through the detection of viral and bacterial pathogen-associated molecular patterns (PAMPs) and by initiating immune responses (Kawai and Akira, 2010). Activation of certain TLRs, such as TLR3 and TLR4, can trigger necroptosis (He et al., 2011, Kaiser et al., 2013). TLR3 is located on intracellular membranes, such as endosomes and lysosomes, and recognises viral dsRNA or its synthetic

analogue poly(I:C) (Alexopoulou et al., 2001, Matsumoto et al., 2002, Kawai and Akira, 2010). TLR4 is located on the plasma membrane and recognises bacterial LPS (Hoshino et al., 1999, Takeuchi et al., 1999, Kawai and Akira, 2010). Activation of TLR3 and TLR4 by their respective PAMP ligands results in signalling via the adaptor protein TIR domain-containing adapter-inducing interferon beta (TRIF), where TRIF and RIPK3 bind via their respective RIP homotypic interaction motif (RHIM) domains to promote RIPK3 autophosphorylation and RIPK3-mediated phosphorylation of MLKL, which drives necroptotic cell death (He et al., 2011, Kaiser et al., 2013).

DAI is an intracellular cytosolic PRR that recognises viral or bacterial nucleic acids and consists of two Z-DNA binding domains and three RHIM domains (Takaoka et al., 2007, Wang et al., 2008b, Kaiser et al., 2008, Upton et al., 2012, Maelfait et al., 2017). DAI becomes activated upon binding viral or bacterial DNA or RNA, enabling DAI and RIPK3 to interact via their respective RHIM domains, resulting in RIPK3 activation that promotes RIPK3 autophosphorylation and phosphorylation of MLKL to drive necroptosis (Upton et al., 2012, Maelfait et al., 2017). Under normal conditions, the RIPK1 RHIM domain functions as a brake that prevents DAI from interacting with RIPK3, which prevents MLKL activation and necroptotic cell death (Lin et al., 2016, Newton et al., 2016b, Ingram et al., 2019). In the absence of a functional RIPK1 RHIM domain, DAI can interact with and activate RIPK3 to trigger MLKL-dependent necroptosis, which causes perinatal lethality in *Ripk1^{RHIM/RHIM}* mice and *Ripk1^{-/-}* mice (Lin et al., 2016, Newton et al., 2016b, Ingram et al., 2019).

TNF Signalling Pathway

NF- κ B and MAPK Activation

The default TNF signalling pathway results in activation of nuclear factor kappa B (NF- κ B) and mitogen-activated protein kinase (MAPK) that promote inflammation, proliferation and cell survival (**Figure 1.3**). This signalling cascade is initiated by the binding of TNF, a pleiotropic cytokine produced by many cell types, to its receptor TNFR1, which is expressed on the surface of most cells (Wajant et al., 2003). This results in the formation of a receptor-associated multi-protein complex, termed Complex I (Micheau and Tschopp, 2003). TNF forms a homotrimer that induces trimerisation of TNFR1, enabling the association of the intracellular death domains of the receptor and the subsequent recruitment of death domain containing proteins, such as TNFR1-associated death domain protein (TRADD) and RIPK1 (Hsu et al., 1995, Kelliher et al., 1998, Micheau and Tschopp, 2003). The adaptor protein TRADD is then able to recruit other signalling proteins, including TNFR-associated factor 2 (TRAF2), which in turn recruits the cellular inhibitor of apoptosis proteins (cIAPs) (Hsu et al., 1996, Micheau and Tschopp, 2003, Mahoney et al., 2008, Varfolomeev et al., 2008, Vince et al., 2009).

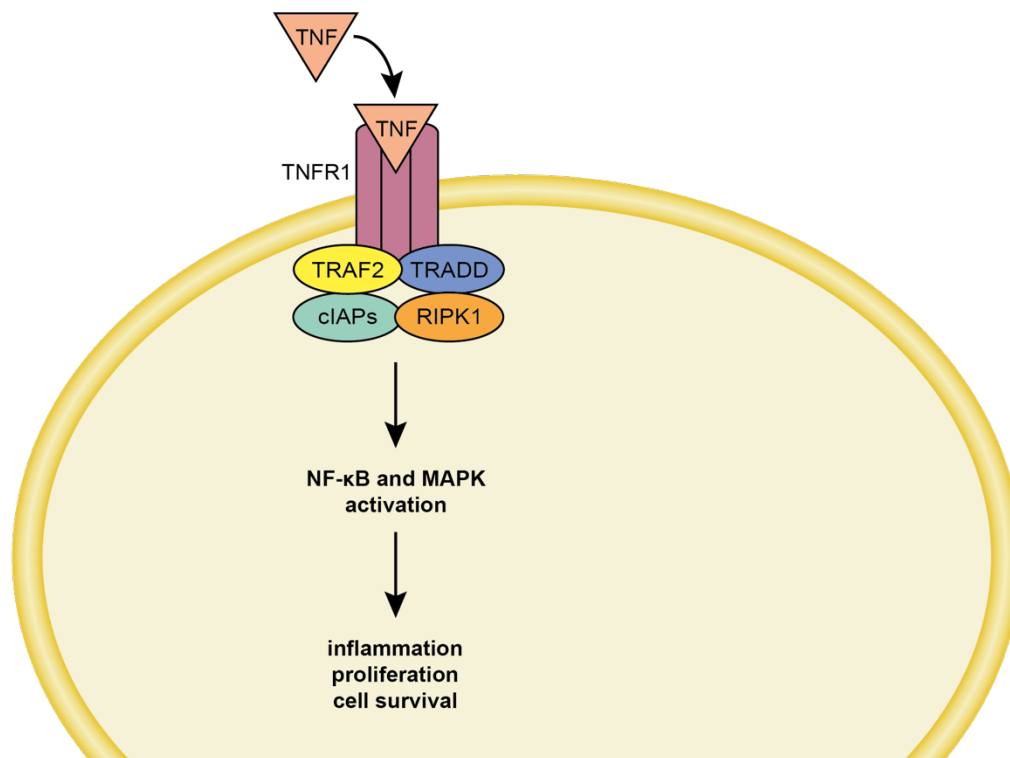


Figure 1.3 TNF signalling pathway results in activation of NF-κB and MAPK that promote inflammation, proliferation and cell survival.

TNF binding to its receptor TNFR1 induces the formation of a receptor-associated protein complex containing TRADD, RIPK1, TRAF2 and the cIAPs that results in downstream NF-κB and MAPK activation.

cIAP1 and cIAP2 are E3 ubiquitin ligases that ubiquitylate various members of Complex I, including themselves and RIPK1 (Bertrand et al., 2008, Mahoney et al., 2008, Varfolomeev et al., 2008). The K63-linked ubiquitin chains on RIPK1 serve as a scaffold to recruit transforming growth factor beta-activated kinase 1 (TAK1) via its two polyubiquitin binding partners, TAK1-binding protein 2 and 3 (TAB2 and TAB3) (Wang et al., 2001, Takaesu et al., 2003, Kanayama et al., 2004). This platform also recruits the inhibitor of nuclear factor kappa B kinase (IκB kinase; IKK) complex, which is composed of the two catalytic subunits IKKα and IKKβ, along with the regulatory subunit nuclear factor kappa B essential modulator (NEMO; also known as IKKγ), via NEMO binding to the polyubiquitin chains on RIPK1 (Wu et al., 2006, Ea et al., 2006). The K63-linked ubiquitin chains on cIAP1 and cIAP2 function as a scaffold to recruit the

linear ubiquitin chain assembly complex (LUBAC), a ubiquitin ligase complex consisting of heme-oxidized IRP2 ubiquitin ligase 1 (HOIL-1), HOIL-1 interacting protein (HOIP) and SHANK-associated RH domain-interacting protein (SHARPIN) (Tokunaga et al., 2009, Haas et al., 2009, Gerlach et al., 2011, Tokunaga et al., 2011, Ikeda et al., 2011). HOIL-1 has a diverse substrate repertoire, including non-lysine substrates (Kelsall et al., 2019), although the functional role of these modifications remains to be established. LUBAC conjugates linear ubiquitin chains onto several components of Complex I, including itself and RIPK1, which serve as a platform to further recruit and stabilise the IKK complex (Tokunaga et al., 2009, Haas et al., 2009, Gerlach et al., 2011, Tokunaga et al., 2011, Ikeda et al., 2011).

TAK1 phosphorylates IKK β within the IKK complex, which then phosphorylates inhibitor of nuclear factor kappa B alpha (I κ B α), resulting in the K48-linked ubiquitylation of I κ B α and its subsequent proteasomal degradation (Traenckner et al., 1995, Scherer et al., 1995, DiDonato et al., 1997, Takaesu et al., 2003). This enables the release of the NF- κ B heterodimers RelA (also called p65) and p50, which are then able to translocate to the nucleus where they induce gene transcription (Zhang et al., 2017a). TAK1 also activates the mitogen-activated protein kinase kinases (MAPKKs), which activate the c-Jun N-terminal kinase (JNK) and p38 pathways to promote gene transcription via the activator protein 1 (AP-1) transcription factor (Devin et al., 2003, Wang et al., 2001, Chen et al., 2015). Together, activation of the canonical NF- κ B and MAPK pathways result in the upregulation of NF- κ B and MAPK dependent genes encoding proteins involved in inflammation, proliferation and cell survival. These proteins include various pro-inflammatory cytokines such as TNF, which can amplify Complex I signalling in a positive feedback loop; pro-survival proteins such as cellular FLICE-like inhibitory protein (cFLIP), which inhibits caspase-8 to block apoptosis; and deubiquitinases (DUBs) such as A20,

which removes K63-linked ubiquitin chains from RIPK1 to attenuate Complex I signalling in a negative feedback loop (Shakhov et al., 1990, Collart et al., 1990, Irmeler et al., 1997, Kreuz et al., 2001, Micheau et al., 2001, Lee et al., 2000, Tokunaga et al., 2012, Wertz et al., 2015).

Apoptosis Induction

TNF signalling can also lead to apoptotic cell death resulting from the formation of a cytosolic protein complex, termed Complex IIa, consisting of TRADD, FAS-associated death domain protein (FADD), RIPK1 and caspase-8 (**Figure 1.4**) (Micheau and Tschopp, 2003). However, the formation of Complex IIa and subsequent apoptosis is normally prevented by the cIAP-mediated ubiquitylation of RIPK1, which keeps RIPK1 associated with Complex I at the membrane, and by the expression of cFLIP, which heterodimerises with caspase-8 to inhibit the proteolytic activity of caspase-8 (Annibaldi et al., 2018, Van Antwerp et al., 1996, Kreuz et al., 2001, Micheau et al., 2001).

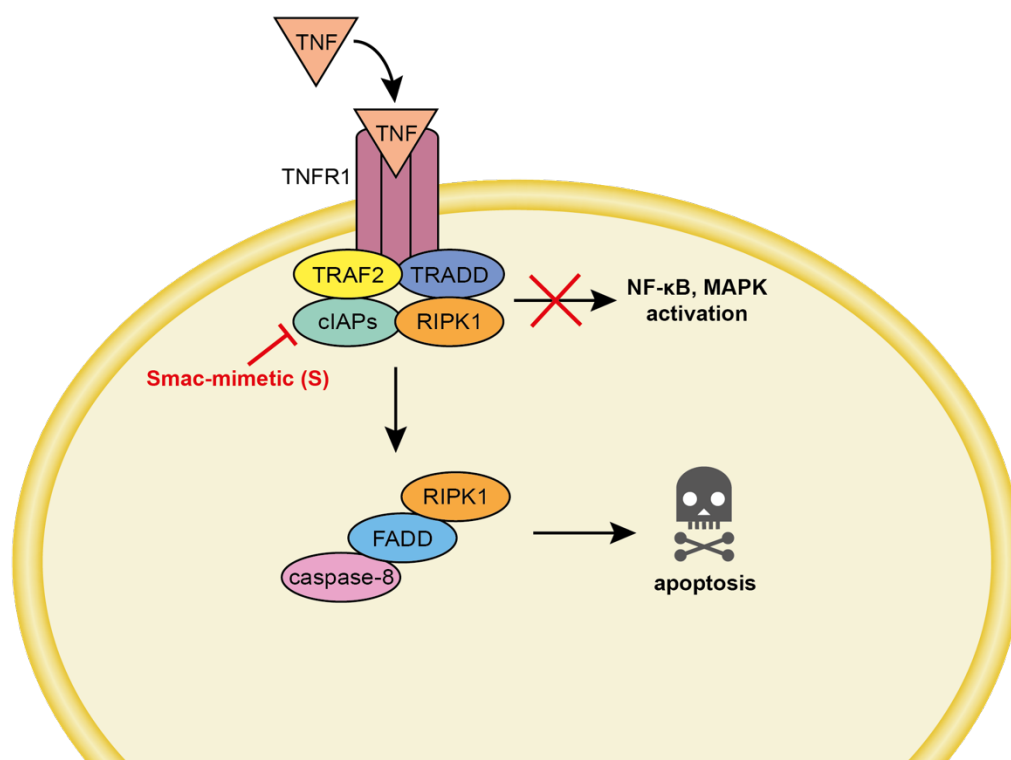


Figure 1.4 TNF signalling pathway can lead to apoptosis via activation of caspase-8.

When cIAP activity is impaired, TNF binding to TNFR1 results in the formation of a cytosolic protein complex containing RIPK1, FADD and caspase-8 that activates caspase-8 to drive apoptosis.

Endogenous antagonists of the IAPs, such as the second mitochondria-derived activator of caspases (Smac; also called DIABLO), can induce the auto-ubiquitylation and proteasomal degradation of cIAP1 and cIAP2 (Du et al., 2000, Verhagen et al., 2000, Yang and Du, 2004, Vince et al., 2007). Smac contains an N-terminal IAP-binding motif, consisting of the 4 residues Ala-Val-Pro-Ile, which mediates its ability to bind to the IAPs (Chai et al., 2000, Liu et al., 2000, Wu et al., 2000, Srinivasula et al., 2001). By inducing the degradation of cIAP1 and cIAP2, Smac prevents the cIAP-mediated ubiquitylation of RIPK1 and other components of Complex I. This impedes NF- κ B and MAPK activation, which reduces cFLIP expression and enables the formation of Complex IIa to promote apoptotic cell death (Micheau and Tschopp, 2003). In addition, Smac prevents X-linked inhibitor of apoptosis protein (XIAP) from binding and inhibiting caspase-3, caspase-7 and caspase-9 by directly competing with these caspases for binding to XIAP (Deveraux et al., 1997, Deveraux et al., 1998, Du et al., 2000, Verhagen et al., 2000, Ekert et al., 2001, Srinivasula et al., 2001). However, Smac does not promote XIAP auto-ubiquitylation and proteasomal degradation, unlike cIAP1 and cIAP2 (Yang and Du, 2004, Silke et al., 2004).

In addition, exogenous antagonists can promote Complex IIa formation and apoptosis. These include inhibition of cIAP activity by Smac-mimetics, impairment of NF- κ B signalling by IKK β inhibitors or TAK1 inhibitors and blockage of transcription or translation by inhibitors such as actinomycin D or cycloheximide (Li et al., 2004, Varfolomeev et al., 2007, Vince et al., 2007, Bertrand et al., 2008, Yang et al., 2006, Ninomiya-Tsuji et al., 2003, Wu et al., 2013b, Kleeff et al., 2000, Wang et al., 2008a). Of particular relevance to this thesis, Smac-mimetics are small molecule inhibitors of the IAPs, so called as they mimic the action of the Smac protein (Fulda and Vucic, 2012). Smac-mimetics imitate the N-terminal tetrapeptide Ala-Val-Pro-Ile of Smac,

which enable them to bind to the IAPs and induce the dimerisation, auto-ubiquitylation and proteasomal degradation of cIAP1 and cIAP2 (Li et al., 2004, Varfolomeev et al., 2007, Vince et al., 2007, Bertrand et al., 2008, Wang et al., 2008a, Feltham et al., 2011). In addition, similar to the effect of Smac on XIAP, Smac-mimetics prevent XIAP from binding and inhibiting caspase-3, caspase-7 and caspase-9, but do not induce XIAP ubiquitylation and degradation (Gao et al., 2007, Varfolomeev et al., 2007, Vince et al., 2007, Bertrand et al., 2008, Wang et al., 2008a).

The presence or absence of ubiquitylation on RIPK1 is a key determining factor for whether cells undergo pro-survival inflammatory gene transcription or apoptotic cell death following TNF ligation to TNFR1. When the cIAPs are degraded or their activity is impaired, this enables RIPK1 to dissociate from the receptor and interact with FADD via their respective death domains (Micheau and Tschopp, 2003, Wang et al., 2008a, Annibaldi et al., 2018). FADD recruits pro-caspase-8, which undergoes homodimerisation and autocatalytic cleavage to form the activated caspase-8 (Micheau and Tschopp, 2003, Oberst et al., 2010). Provided cFLIP levels are low, activated caspase-8 then cleaves pro-caspase-3 into caspase-3 or Bid into truncated Bid (tBid) to execute apoptosis (Stennicke et al., 1998, Luo et al., 1998, Li et al., 1998).

Necroptosis Induction

TNF signalling can also result in the assembly of an alternative cytosolic protein complex, termed Complex IIb or the necrosome, which consists of RIPK1, RIPK3 and MLKL, and triggers necroptotic cell death (**Figure 1.5**) (Holler et al., 2000, He et al., 2009, Cho et al., 2009, Zhang et al., 2009, Sun et al., 2012, Zhao et al., 2012). However, the assembly of the necrosome and subsequent necroptosis is usually suppressed by caspase-8, in particular, in the form of caspase-8/cFLIP_L heterodimers, which cleave RIPK1 to prevent RIPK1 oligomerisation and autophosphorylation (Lin et al., 1999, Pop et al., 2011, Oberst et al., 2011, Lalaoui et al., 2020). There is some evidence to suggest that RIPK3 is also cleaved, however this is implied from overexpression studies and its physiological role is yet to be fully characterised (Feng et al., 2007). cFLIP is expressed as a long isoform, cFLIP_L, and a short isoform, cFLIP_S, and can form heterodimers with caspase-8 to regulate the proteolytic activity of caspase-8 (Irmeler et al., 1997, Boatright et al., 2004, Feoktistova et al., 2011). cFLIP_L in the caspase-8/cFLIP_L heterodimers limits caspase-8 to low level (non-apoptotic) activity, which allows caspase-8 to cleave only a subset of its substrates, including RIPK1 to prevent necroptosis (Lin et al., 1999, Pop et al., 2011, Oberst et al., 2011). In contrast, cFLIP_S in the caspase-8/cFLIP_S heterodimers completely blocks caspase-8 activity, allowing the formation of the necrosome (Feoktistova et al., 2011).

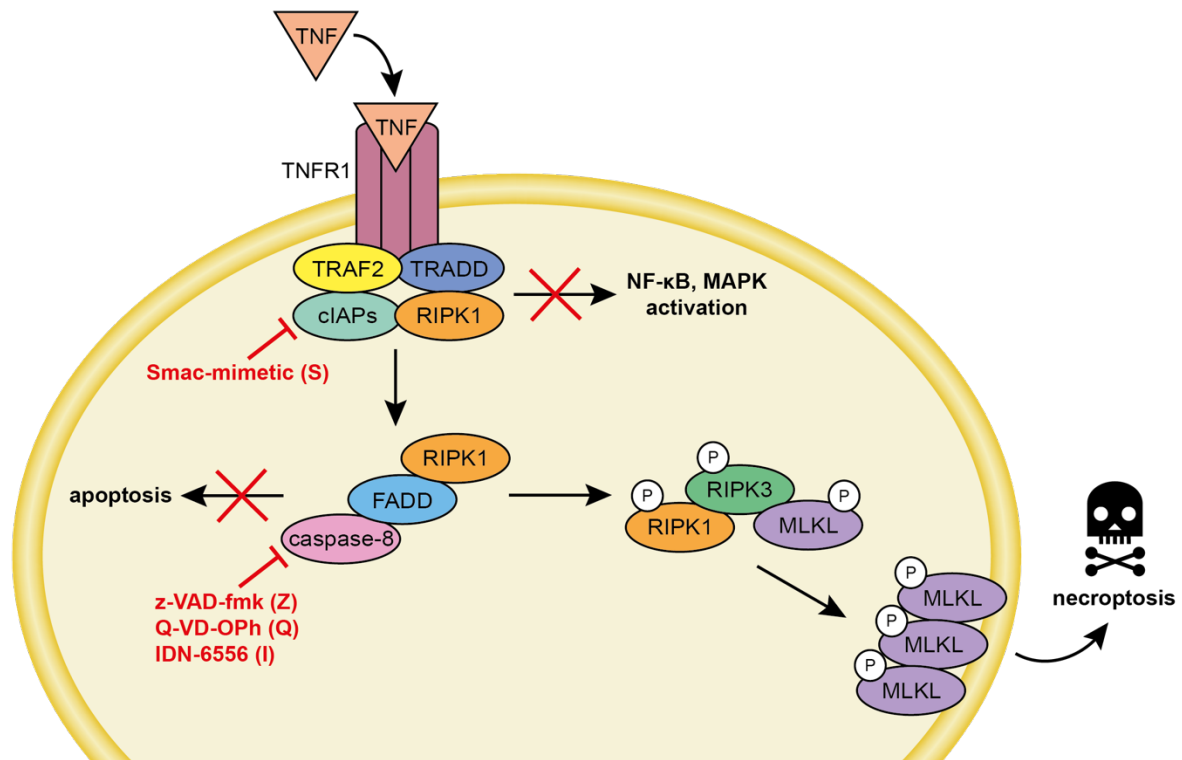


Figure 1.5 TNF signalling pathway can trigger necroptosis via activation of MLKL.

When both cIAP activity and caspase activity is suppressed, TNF ligation to TNFR1 results in the assembly of an alternative cytosolic protein complex containing RIPK1, RIPK3 and MLKL that drives MLKL activation and subsequent necroptosis.

The caspase-8 brake on necroptosis can be released by certain endogenous inhibitors of caspases. Several viruses encode proteins that antagonise caspases to suppress apoptosis, which can result in necroptosis (Nailwal and Chan, 2019). In addition, pharmacological inhibition of caspases can promote necroptosis. A range of synthetic caspase inhibitors have been described in the literature, however the three most widely used to stimulate necroptosis are z-VAD-fmk, Q-VD-OPh and IDN-6556 (also known as emricasan) (Callus and Vaux, 2007). All three of these compounds are peptide-based, irreversible, pan-caspase inhibitors (Callus and Vaux, 2007). However, IDN-6556 is more efficient at inhibiting the caspase-8/cFLIP_L heterodimers than z-VAD-fmk, which is more efficient than Q-VD-OPh (Brumatti et al., 2016). In addition, Q-VD-OPh is reported to be more effective at preventing apoptosis than z-VAD-

fmk (Caserta et al., 2003). IDN-6556 was the first irreversible pan-caspase inhibitor to enter clinical trials (Linton et al., 2005, Valentino et al., 2003).

The activity of the caspases, in particular caspase-8, is a key determining factor for whether cells die via apoptosis or necroptosis following TNF ligation to TNFR1. Impaired caspase-8 activity enables the formation of the necrosome complex, where RIPK1 recruits RIPK3 via their respective RHIM domains to form functional amyloid structures (Sun et al., 2002, Holler et al., 2000, He et al., 2009, Cho et al., 2009, Zhang et al., 2009, Li et al., 2012, Mompean et al., 2018). Within the necrosome, RIPK1 and RIPK3 are activated by autophosphorylation and their kinase activities are indispensable for necroptosis (He et al., 2009, Cho et al., 2009, Chen et al., 2013, Cook et al., 2014, Wu et al., 2014, Laurien et al., 2020). Activated RIPK3 then interacts with MLKL via their respective kinase and pseudokinase domains, leading to RIPK3 phosphorylation of MLKL, which is a key step required for MLKL activation to execute necroptosis (Sun et al., 2012, Zhao et al., 2012, Chen et al., 2013, Murphy et al., 2013, Xie et al., 2013b, Petrie et al., 2019).

It has been established that MLKL is the ultimate necroptosis executioner, however the mechanism by which MLKL executes cell death is not entirely understood. The RIPK3-mediated activation of MLKL is caused by RIPK3 phosphorylation of the MLKL activation loop at residues Thr357 and Ser358 in human MLKL and residues Ser345, Ser347 and Thr349 in murine MLKL (Sun et al., 2012, Chen et al., 2013, Murphy et al., 2013, Xie et al., 2013b, Wang et al., 2014a, Rodriguez et al., 2016). This phosphorylation of the activation loop causes structural perturbations in the pseudokinase domain, which cause a conformational change in MLKL that unleashes the 4-helix bundle domain from the constraint of the pseudokinase

domain, mediated by the brace helices (Murphy et al., 2013, Hildebrand et al., 2014, Davies et al., 2018, Petrie et al., 2018, Davies et al., 2020). This functions as a molecular switch that transforms MLKL into an active state and promotes MLKL oligomerisation and subsequent translocation from the cytoplasm to the plasma membrane (Murphy et al., 2013, Hildebrand et al., 2014, Wang et al., 2014a, Davies et al., 2018, Petrie et al., 2018, Petrie et al., 2020, Samson et al., 2020). At the membrane, phosphorylated MLKL forms high molecular weight complexes that interact with the membrane via the 4-helix bundle domain (Hildebrand et al., 2014, Wang et al., 2014a, Petrie et al., 2018, Petrie et al., 2020, Samson et al., 2020). MLKL then compromises membrane integrity, causing the cellular contents to spill into the extracellular space, the release of DAMPs and necrotic cell death (Hildebrand et al., 2014, Wang et al., 2014a, Petrie et al., 2018, Petrie et al., 2020, Samson et al., 2020).

The precise mechanism of how MLKL causes plasma membrane disruption remains to be fully defined. Various studies and groups have proposed a variety of mechanisms for MLKL membrane permeabilisation. These include the involvement of ion influx and ion/membrane channels (Cai et al., 2014, Chen et al., 2014, Xia et al., 2016, Ousingsawat et al., 2017, Douanne et al., 2019), phosphatidylinositol phosphates (Wang et al., 2014a, Dondelinger et al., 2014, Quarato et al., 2016), cardiolipin (Wang et al., 2014a, Su et al., 2014), lipid rafts (Chen et al., 2014, Fan et al., 2019) and membrane pores (Ros et al., 2017). Furthermore, a number of other cellular components have been reported as interactors or modulators in necroptosis signalling. These include heat shock protein 90 and 70 (HSP90 and HSP70) (Li et al., 2015, Jacobsen et al., 2016, Zhao et al., 2016, Bigenzahn et al., 2016, Johnston et al., 2020), endosomal sorting complex required for transport (ESCRT) proteins (Gong et al., 2017, Yoon et al., 2017, Fan et al., 2019), flotillins (Yoon et al., 2017, Fan et al., 2019), inositol phosphate

kinases (Dovey et al., 2018, McNamara et al., 2019), Tyro3, Axl and Mer (TAM) kinases (Najafov et al., 2019) and casein kinase 1 (CK1) proteins (Lee et al., 2019, Hanna-Addams et al., 2020). However, there are conflicting reports regarding the way MLKL compromises membrane integrity depending on the cell type. Additionally, some of the auxiliary proteins reported can vary depending on the cellular system and many of these studies have only been performed in either the human or murine context. In contrast, RIPK1, RIPK3 and MLKL are the three essential effectors of necroptosis in the context of TNF signalling, irrespective of the cell type or species studied.

It is becoming increasingly apparent that there are marked differences between humans and mice in the regulation of necroptosis and mechanistic differences in MLKL activation. Firstly, this is demonstrated by the species specificity of RIPK3 and MLKL; human RIPK3 does not interact with murine MLKL and murine RIPK3 does not interact with human MLKL, and therefore, human MLKL cannot reconstitute necroptosis in murine cells and murine MLKL cannot reconstitute necroptosis in human cells (Sun et al., 2012, Chen et al., 2013, Tanzer et al., 2016, Davies et al., 2018, Davies et al., 2020). Secondly, in the human system, RIPK3 and MLKL are already associated under basal conditions and this interaction is enhanced upon induction of necroptosis (Sun et al., 2012). Indeed, the stable interaction between human RIPK3 and MLKL is required for MLKL activation, as phosphorylation of MLKL occurs after its stable recruitment to the necrosome by RIPK3 (Petrie et al., 2018, Petrie et al., 2019). In contrast, RIPK3 and MLKL have a more transient interaction in the murine system, where phosphorylation of MLKL by RIPK3 occurs in a “kiss and run” mechanism (Murphy et al., 2013, Hildebrand et al., 2014). Thirdly, certain murine MLKL mutants or truncation constructs are able to trigger necroptotic cell death upon inducible expression alone, independent of

external necroptotic stimuli (Murphy et al., 2013, Hildebrand et al., 2014, Tanzer et al., 2016). In contrast, the equivalent human MLKL mutants or truncation constructs are not able to trigger necroptotic cell death in this fashion (Tanzer et al., 2016, Petrie et al., 2018).

There are varying and conflicting reports regarding the stoichiometry of the MLKL oligomers formed following phosphorylation by RIPK3. Human MLKL oligomers have been described as trimers (Cai et al., 2014), tetramers (Liu et al., 2017, Petrie et al., 2018), hexamers (Wang et al., 2014a), octamers (Huang et al., 2017) and large amyloid polymers (Liu et al., 2017, Reynoso et al., 2017). Murine MLKL oligomers have been reported as trimers (Cai et al., 2014, Hildebrand et al., 2014, Davies et al., 2018), tetramers (Chen et al., 2014, Liu et al., 2017), hexamers (Wang et al., 2014a), octamers (Huang et al., 2017) and large amyloid polymers (Liu et al., 2017). These discrepancies in the literature regarding the MLKL oligomer stoichiometry are most likely due to different techniques being used in different studies, and some studies using human and murine MLKL interchangeably. Work from our group has demonstrated that human and murine MLKL differ in the oligomer stoichiometry and that human MLKL forms tetramers (Petrie et al., 2018), while murine MLKL forms trimers (Hildebrand et al., 2014, Davies et al., 2018). This is yet another point of difference between human and murine necroptosis. Moreover, it is plausible that larger cytoplasmic MLKL assemblies occur, which likely represent the physiological MLKL species in cells. The sizes of these MLKL clusters are not definitive, but are capped in necrosomes, while the plasma membrane forms of MLKL are likely heterogeneous and represent a threshold amount for cell lysis (Samson et al., 2020).

In an experimental setting, necroptosis can be induced by treating cells with a cocktail of three components: TNF (T), which initiates the signalling cascade by binding to TNFR1; Smac-

mimetic (S), which prevents the cIAP-mediated activation of the NF- κ B and MAPK pathways; and a caspase inhibitor (z-VAD-fmk (Z), Q-VD-OPh (Q), IDN-6556/emricasan (I)), which blocks caspase-8-mediated apoptosis. This necroptosis-inducing cocktail is frequently referred to as either TSZ, TSQ or TSI depending on which caspase inhibitor is used (Brumatti et al., 2016).

The Key Necroptotic Effectors

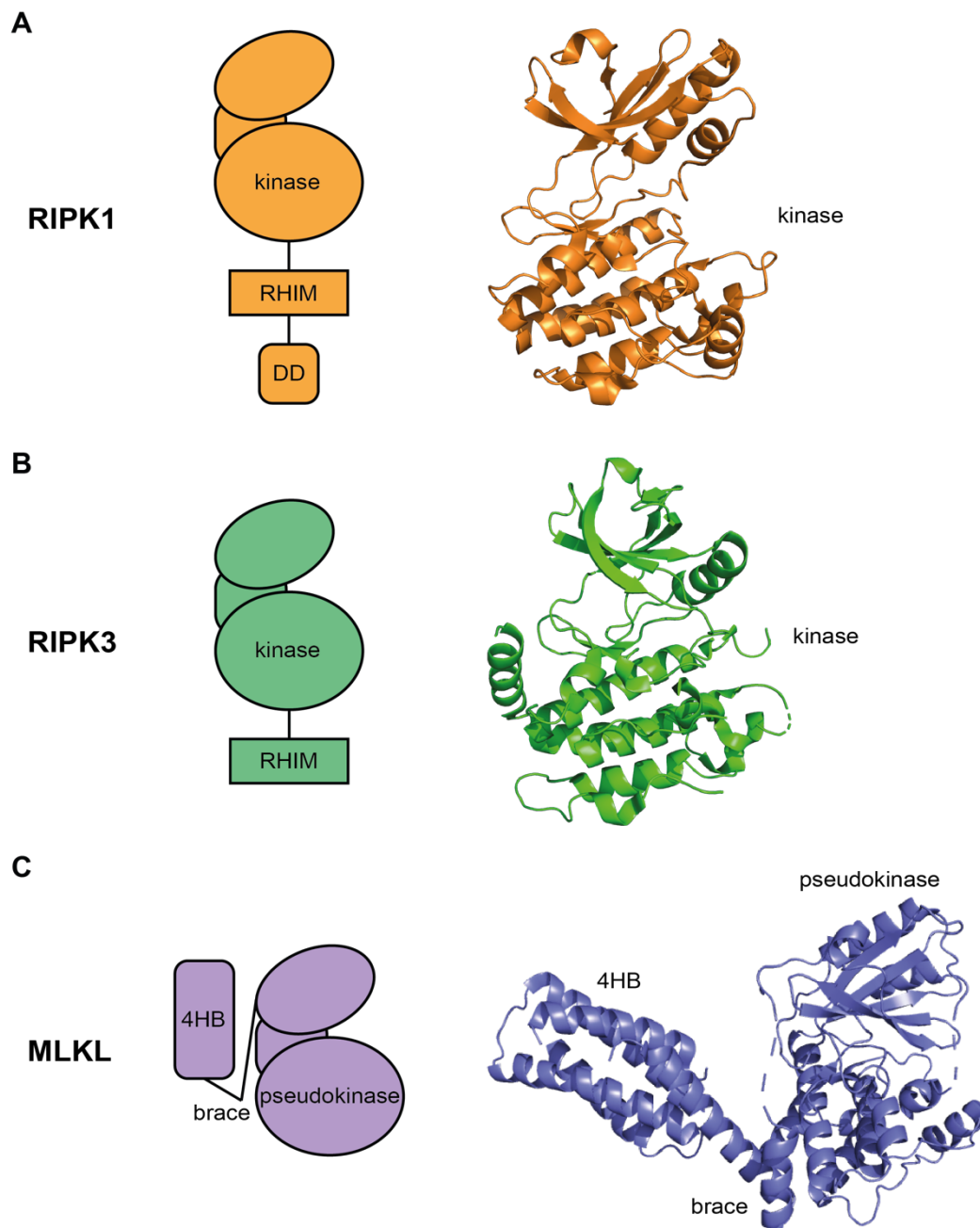


Figure 1.6 The key effectors of necroptosis are the kinases RIPK1 and RIPK3, and the pseudokinase MLKL.

(A) Schematic of the RIPK1 structure, which consists of a kinase domain, RHIM domain and death domain (DD), and crystal structure of the human RIPK1 kinase domain (PDB: 6NYH).

(B) Schematic of the RIPK3 structure, which consists of a kinase domain and RHIM domain, and crystal structure of the murine RIPK3 kinase domain (PDB: 4M66).

(C) Schematic of the MLKL structure, which consists of a 4-helix bundle (4HB) domain, brace helices and pseudokinase domain, and crystal structure of full-length murine MLKL (PDB: 4BTF).

RIPK1

RIPK1 has a pleiotropic function in TNF signalling with important roles in all three arms of the TNF pathway: NF- κ B and MAPK activation, apoptosis and necroptosis. Of particular relevance to this thesis, RIPK1 is one of the three key effectors of TNF-driven necroptosis. RIPK1 was first reported to be involved in necrosis in 2000, when Holler *et al.* found that RIPK1 was essential for necrotic cell death in Jurkat cells treated with TNF, TRAIL or FasL along with z-VAD-fmk (Holler et al., 2000). In 2005, the first small molecule inhibitor of necroptosis, necrostatin-1 (Nec-1), was reported and a follow up study by the same group in 2008 identified RIPK1 as the target of Nec-1 (Degterev et al., 2005, Degterev et al., 2008). The essential role of RIPK1 is highlighted by the perinatal lethality of *Ripk1*^{-/-} mice, which die at birth due to systemic inflammation and cell death (Kelliher et al., 1998, Rickard et al., 2014, Dillon et al., 2014, Kaiser et al., 2014). The additional deletion of *Casp8* or *Fadd* along with *Ripk3* rescues the perinatal lethality of *Ripk1*^{-/-} mice, by ablating the lethal consequences of apoptosis and necroptosis caused by RIPK1 deficiency, and *Ripk1*^{-/-}*Ripk3*^{-/-}*Casp8*^{-/-} mice or *Ripk1*^{-/-}*Ripk3*^{-/-}*Fadd*^{-/-} mice survive past weaning and develop normally to adulthood (Rickard et al., 2014, Dillon et al., 2014, Kaiser et al., 2014). However, these triple knockout mice develop lymphoproliferative syndrome as adults owing to *Casp8* or *Fadd* deletion (Rickard et al., 2014, Dillon et al., 2014, Kaiser et al., 2014).

RIPK1 is a serine/threonine kinase that structurally consists of an N-terminal kinase domain, a RHIM domain and a C-terminal death domain (**Figure 1.6A**) (Stanger et al., 1995, Sun et al., 2002, Xie et al., 2013a). There are several X-ray crystal structures of the human RIPK1 kinase domain in complex with various small molecules (Xie et al., 2013a, Harris et al., 2013, Harris et al., 2016, Harris et al., 2017, Yoshikawa et al., 2018, Wang et al., 2018, Harris et al., 2019a,

Harris et al., 2019b, Patel et al., 2020). Crystallisation of the full-length protein has proved difficult thus far, presumably due to the RHIM domain mediating polymerisation, which results in heterogeneity and insoluble protein. During necroptosis, both human and murine RIPK1 undergo autophosphorylation at Ser166, which is a critical autophosphorylation site for RIPK1 activation and the subsequent activation of its partner kinase RIPK3 (Degterev et al., 2008, Laurien et al., 2020). Several additional autophosphorylation sites have been identified, including Ser14/15, Ser20 and Ser161 in human RIPK1 (Degterev et al., 2008), as well as Ser161 and Thr169 in murine RIPK1 (Zhang et al., 2017b, Dondelinger et al., 2019, Laurien et al., 2020). However, the functional role of these additional autophosphorylation sites remains to be investigated.

RIPK3

RIPK3 has important roles in both apoptosis and necroptosis driven by TNF signalling. RIPK3 is one of the three key effectors of TNF-driven necroptosis and was first reported to be involved in necroptotic cell death by three separate groups in 2009 (He et al., 2009, Cho et al., 2009, Zhang et al., 2009). He *et al.* identified RIPK3 from a genome-wide siRNA screen in HT29 cells treated with TNF, Smac-mimetic and z-VAD-fmk to identify genes that, when knocked down, led to protection against necroptosis (He et al., 2009). Cho *et al.* similarly identified RIPK3 from an siRNA screen, but in FADD-deficient Jurkat cells treated with TNF (Cho et al., 2009). Zhang *et al.* identified RIPK3 using a different approach, where microarray analysis was performed to determine changes in gene expression between two NIH 3T3 cell lines that were susceptible to either apoptosis or necrosis (Zhang et al., 2009). *Ripk3*^{-/-} mice are viable and ostensibly normal (Newton et al., 2004), and cells lacking RIPK3 are resistant to necroptotic cell death (He et al., 2009, Cho et al., 2009, Zhang et al., 2009).

Similar to its partner kinase RIPK1, RIPK3 is also a serine/threonine kinase that structurally consists of an N-terminal kinase domain followed by an intermediate region of unknown function and a C-terminal RHIM domain, however RIPK3 lacks the C-terminal death domain present in RIPK1 (**Figure 1.6B**) (Yu et al., 1999, Sun et al., 1999, Pazdernik et al., 1999, Xie et al., 2013b). X-ray crystal structures of murine RIPK3 have been solved, including the kinase domain alone (Xie et al., 2013b), the kinase domain in complex with a small molecule inhibitor (Hart et al., 2020) and the cocrystal structure of the murine RIPK3 kinase and MLKL pseudokinase complex (Xie et al., 2013b). To date there is no crystal structure of the human RIPK3 kinase domain, presumably due to the inherent instability of this protein (as identified in Chapter 3 of this thesis). Furthermore, similar to RIPK1, crystallisation of full-length RIPK3

has been hampered by the formation of insoluble protein caused by RHIM-driven polymerisation. Following the induction of necroptosis, RIPK3 becomes activated by RIPK1 and undergoes autophosphorylation at Ser227 in human RIPK3 and Thr231 and Ser232 in murine RIPK3 (Sun et al., 2012, Chen et al., 2013, Wu et al., 2014, Petrie et al., 2019). This autophosphorylation event is crucial for the ability of RIPK3 to bind MLKL and promote necroptotic cell death (Sun et al., 2012, Chen et al., 2013, Wu et al., 2014, Petrie et al., 2019).

MLKL

MLKL is the most downstream essential effector and the terminal cell death executioner of necroptosis. MLKL was identified by two separate groups in 2012 (Sun et al., 2012, Zhao et al., 2012). Sun *et al.* identified MLKL as the cellular target of a small molecule inhibitor of necroptosis, necrosulfonamide, and a protein that coimmunoprecipitated with RIPK3 (Sun et al., 2012). Zhao *et al.* identified MLKL from screening an shRNA library in HT29 cells to identify genes that, when knocked down, provided protection from necroptosis induced by TNF, Smac-mimetic and z-VAD-fmk (Zhao et al., 2012). *Mlkl*^{-/-} mice are viable and display no abnormalities (Murphy et al., 2013), and cells lacking MLKL are resistant to necroptotic cell death (Sun et al., 2012, Zhao et al., 2012, Murphy et al., 2013).

Unlike RIPK1 and RIPK3, which are kinases, MLKL is a pseudokinase and lacks catalytic activity (Murphy et al., 2013). Structurally, MLKL consists of an N-terminal 4-helix bundle domain connected via a 2-helix linker, also known as the brace helices, to a C-terminal pseudokinase domain (**Figure 1.6C**) (Murphy et al., 2013). The pseudokinase domain of MLKL adopts a typical kinase-like fold and shares sequence homology with bona fide protein kinases, however MLKL lacks two of the three conserved residues, Asp of the HRD motif and Asp of the DFG motif, required to catalyse phosphoryl transfer (Murphy et al., 2013, Murphy et al., 2014). The Asp of the HRD motif from the catalytic loop acts as the catalytic residue in kinases, whereas this motif is present as HGK in human MLKL and HRN in murine MLKL (Murphy et al., 2014). The Asp of the DFG motif from the activation loop binds Mg²⁺ in a typical protein kinase, whereas this motif is highly conserved as GFE in MLKL orthologues of chordate species (Murphy et al., 2014). As a result, in contrast to bona fide protein kinases, MLKL is catalytically inactive and unable to catalyse phosphorylation (Murphy et al., 2013, Murphy et al., 2014).

Interestingly, the Lys of the VAIK motif that interacts with the α and β phosphates of ATP to position ATP, is generally conserved in MLKL orthologues (Murphy et al., 2014). Accordingly, the MLKL pseudokinase domain retains the ability to bind ATP, but only in the absence of Mg^{2+} (or Mn^{2+}), and as ATP binding is not required for necroptosis, the biological relevance of this interaction is not well understood (Murphy et al., 2013, Murphy et al., 2014, Petrie et al., 2018).

The X-ray crystal structure of full-length murine MLKL was solved by our group in 2013 (Murphy et al., 2013). Crystal structures of the murine MLKL pseudokinase domain alone and in complex with the murine RIPK3 kinase domain have also been reported (Xie et al., 2013b). To date there is no X-ray crystal structure of full-length human MLKL, however a recent model of this protein has been generated using molecular modelling based on crosslinking mass spectrometry experiments (Petrie et al., 2018), building on a previous model of full-length human MLKL (Arnez et al., 2015). The solution Nuclear Magnetic Resonance (NMR) structure of the human MLKL N-terminal domain has been reported (Su et al., 2014), as well as crystal structures of the human MLKL pseudokinase domain or N-terminal domain. These include the pseudokinase domain alone (Xie et al., 2013b, Murphy et al., 2014), the pseudokinase domain in complex with small molecule binders (Ma et al., 2016), the T357E/S358E pseudokinase domain mutant (Petrie et al., 2018) and the N-terminal region encompassing the 4-helix bundle and the first brace helix in complex with a monobody, a synthetic binding protein (Petrie et al., 2020).

The phosphorylation of MLKL by RIPK3 enables the transformation of MLKL from a cytoplasmic monomer to a membrane-associated oligomer. Following a necroptotic stimulus,

activated RIPK3 phosphorylates human MLKL at Thr357 and Ser358 (Sun et al., 2012, Wang et al., 2014a) and murine MLKL at Ser345, Ser347 and Thr349 (Murphy et al., 2013, Xie et al., 2013b, Rodriguez et al., 2016). In murine MLKL, the phosphomimetic S345D mutant results in a constitutively active form of MLKL, which causes necroptotic cell death in the absence of necroptotic stimuli (Murphy et al., 2013, Tanzer et al., 2015). The phosphoablating S345A mutant is resistant to necroptosis induced by TNF and z-VAD-fmk, further confirming Ser345 as a critical phosphorylation site (Rodriguez et al., 2016). Phosphoablating mutants of the other two murine MLKL phosphorylation sites, S347A and T349A, had no effect on necroptotic cell death, suggesting that they have minor or negligible roles (Rodriguez et al., 2016). Other additional phosphorylation sites have been identified, including Ser124, Ser158, Ser228, Ser248 and Ser352, some of which have been shown to regulate the activity of MLKL (Xie et al., 2013b, Tanzer et al., 2015).

There are conflicting studies regarding the effect of the human MLKL phosphomimetic mutants. Some groups reported that, similar to the murine phosphomimetic S345D mutant, the equivalent human phosphomimetic S358D mutant, or T357E/S358D double mutant, are constitutively active and exist as an oligomer (Wang et al., 2014a, Fauster et al., 2015, Bigenzahn et al., 2016, Yoon et al., 2016, Yoon et al., 2017). Other groups reported that the T357E/S358D double mutant behaves similar to wild-type MLKL and results in cell death upon application of a necroptotic stimulus (Sun et al., 2012). However, these findings have been directly challenged by our own studies, which demonstrated that the T357E/S358D and T357E/S358E double mutants are not able to trigger necroptotic cell death, even upon treatment with necroptotic stimuli (Petrie et al., 2018). These contradictory findings are possibly due to the use of different cell lines, genetic backgrounds, expression vectors and

expression methods. Importantly, these studies are in agreement regarding the behaviour of the phosphoablating T357A/S358A double mutant, which exists as a monomer and is unable to reconstitute necroptosis in cells even in the presence of a necroptotic stimulus, demonstrating the importance of these phosphorylation sites for the ability of MLKL to execute cell death (Sun et al., 2012, Wang et al., 2014a, Yoon et al., 2016, Yoon et al., 2017, Petrie et al., 2018).

Necroptosis and Disease

There is increasing evidence in the literature that implicates necroptosis in the pathophysiology of a variety of human diseases (**Figure 1.7**). Many of these studies have used murine models to investigate the role of necroptosis in a particular disease model and have demonstrated that the genetic deletion of *Ripk3* or *MLkl*, kinase dead alleles of *Ripk1* or *Ripk3*, or chemical inhibition of RIPK1 or RIPK3 afforded protection against the disease phenotype. Some studies have investigated the role of necroptosis in a human disease context using patient samples and have shown that the expression level or phosphorylation level of RIPK3 or MLKL correlated with the disease phenotype or disease severity.

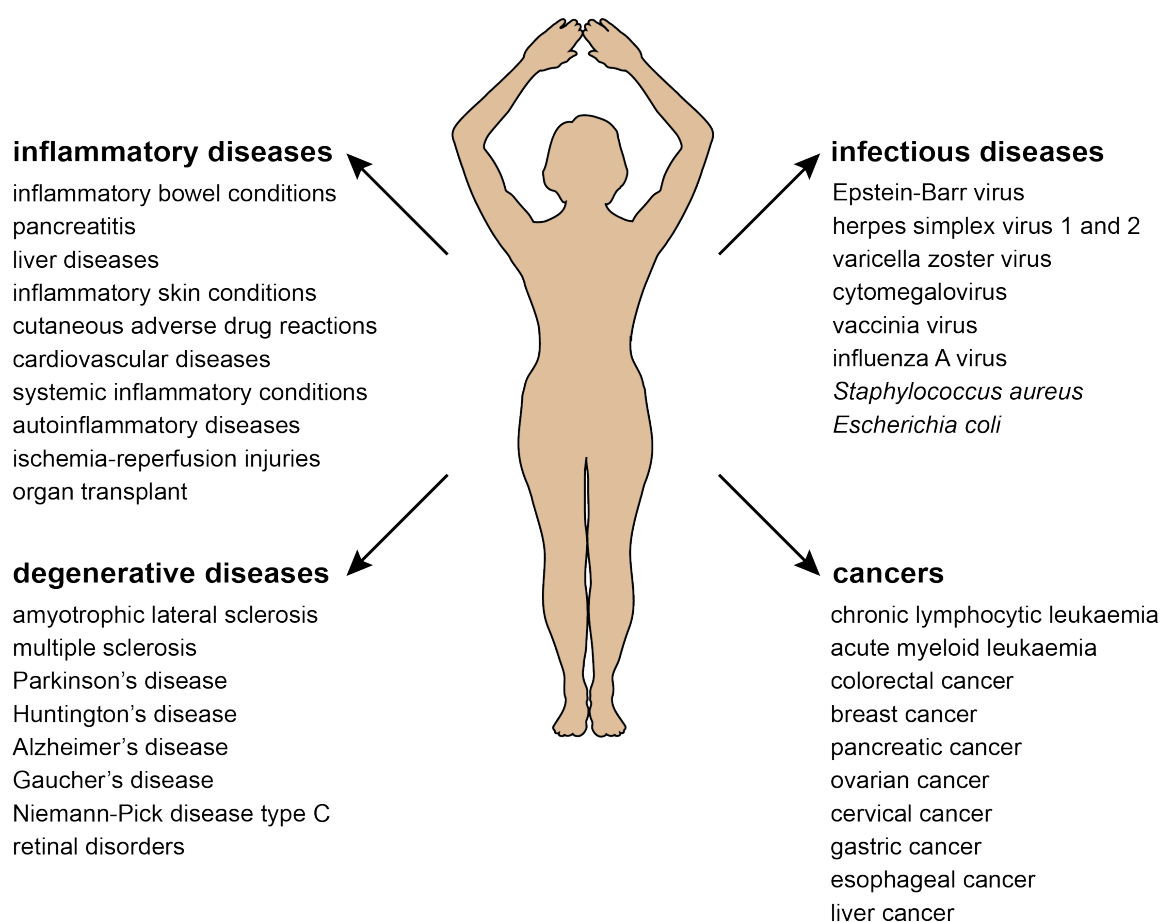


Figure 1.7 Necroptosis is implicated in the pathophysiology of a variety of human diseases.

Necroptotic cell death contributes to a repertoire of inflammatory diseases, degenerative diseases, infectious diseases and cancers.

Inflammatory Diseases

As necroptosis is an inflammatory form of cell death, the role of necroptosis in human diseases has been best described in the context of inflammatory diseases. An increasing number of studies have demonstrated that necroptotic cell death is involved in a wide range of inflammatory conditions. However, several of these studies have later been challenged by follow-up research and conflicting results have been reported for certain murine models. It is plausible that differences in microbiomes and microflora between mouse facilities, mismatch of mouse strains and comparisons of mice that are not age- and sex-matched littermates may contribute to the discrepant results in many studies. Other factors including the non-necroptotic roles of RIPK1 and RIPK3, involvement of other forms of cell death, involvement of other cell types, off-target activity of small molecules and specificity of antibodies and other reagents could also contribute to the reported disparities.

Necroptosis has been reported to be involved in the pathology of inflammatory bowel conditions (Welz et al., 2011, Gunther et al., 2011, Pierdomenico et al., 2014, Wu et al., 2019). Welz *et al.* demonstrated that the intestinal epithelial cell specific deletion of *Fadd* in mice resulted in chronic intestinal inflammation, characteristic of colitis and enteritis, which was triggered by necroptosis and prevented by the genetic deletion of *Ripk3* (Welz et al., 2011). This was supported by studies using patient samples, which identified increased necroptotic cell death and high levels of RIPK3 in patients with Crohn's disease (Gunther et al., 2011), increased levels of both RIPK3 and MLKL in the ileum and colon of children with Crohn's disease and ulcerative colitis (Pierdomenico et al., 2014), and upregulation of both RIPK3 and MLKL in the inflamed colon tissue of ulcerative colitis patients (Wu et al., 2019).

The cell death and inflammation characteristic of acute pancreatitis has been linked to necroptosis (He et al., 2009, Zhang et al., 2009, Louhimo et al., 2016, Wu et al., 2013a). He *et al.* demonstrated that RIPK3 was upregulated in the cerulein-induced acute pancreatitis model, which resulted in necroptotic cell death that correlated with the extent of tissue damage and was absent in *Ripk3*^{-/-} mice (He et al., 2009). Zhang *et al.* used the same model and reported significantly less necrosis and inflammation in *Ripk3*^{-/-} mice compared to wild-type mice (Zhang et al., 2009). Similarly, Louhimo *et al.* found that cerulein or tauroolithocholic acid-3-sulfate (TLCS) treatment to induce acute pancreatitis was reduced by either RIPK1 inhibition using Nec-1 or genetic deletion of *Ripk3* (Louhimo et al., 2016). These finding were confirmed by Wu *et al.* who reported that *Mlkl*^{-/-} mice were likewise protected from cerulein-induced acute pancreatitis (Wu et al., 2013a). However, the role of necroptosis in this acute pancreatitis model was challenged by a study from Genentech, which reported that *Ripk3*^{-/-} mice as well as *Ripk1*^{KD/KD} mice (expressing the kinase-dead RIPK1 D138N mutant) were not protected from cerulein-induced pancreatitis (Newton et al., 2016a).

Necroptotic cell death has been identified in various liver diseases including ethanol-induced liver injury, non-alcoholic steatohepatitis (NASH), drug-induced liver injury and autoimmune hepatitis (Roychowdhury et al., 2013, Wang et al., 2016, Gautheron et al., 2014, Ramachandran et al., 2013, Wang et al., 2014a, Gunther et al., 2016). Two groups reported that RIPK3 expression was elevated in liver biopsies of patients with alcoholic liver disease and that *Ripk3*^{-/-} mice were protected from liver injury induced by ethanol treatment (Roychowdhury et al., 2013, Wang et al., 2016). Similarly, Gautheron *et al.* found that RIPK3 was strongly upregulated in liver samples of NASH patients (Gautheron et al., 2014). In the context of acetaminophen-induced liver injury, Ramachandran *et al.* reported that RIPK3

mediates the hepatotoxicity in mice caused by acetaminophen treatment (Ramachandran et al., 2013). Wang *et al.* detected phosphorylated MLKL in liver biopsy samples from patients with drug-induced liver injury, which correlated with diseased tissue (Wang et al., 2014a). However, the role of necroptosis in acetaminophen-induced hepatotoxicity was challenged by Dara *et al.*, demonstrating that neither *Ripk3*^{-/-} mice nor *Mlkl*^{-/-} mice were protected from the disease phenotype (Dara et al., 2015). Dara *et al.* instead concluded that the acetaminophen-induced liver injury was mediated by RIPK1 independent of necroptosis and the necrosome (Dara et al., 2015). However, this was directly challenged by Schneider *et al.*, as the conditional deletion of *Ripk1* in liver parenchymal cells had no effect on hepatotoxicity triggered by acetaminophen (Schneider et al., 2016). In the context of autoimmune hepatitis, Günther *et al.* identified the upregulation of *MLKL* and increased levels of phosphorylated MLKL in patient liver biopsies and in the concanavalin A (ConA) murine model, and that MLKL drives hepatocyte cell death independent of RIPK3 in this model (Gunther et al., 2016). However, a recent study by Hamon *et al.* directly challenged these findings, as the conditional deletion of *Mlkl* in liver parenchymal cells had no effect on hepatitis induced by ConA treatment (Hamon et al., 2020).

Necroptosis has been associated with skin pathologies such as inflammatory skin conditions (Bonnet et al., 2011, Dannappel et al., 2014, Duan et al., 2020) and cutaneous adverse drug reactions (Saito et al., 2014). Bonnet *et al.* reported that the epidermal keratinocyte specific deletion of *Fadd* in mice resulted in severe inflammatory skin lesions, which were triggered by necroptosis and prevented by *Ripk3* deletion (Bonnet et al., 2011). Dannappel *et al.* found that the epidermal keratinocyte specific deletion of *Ripk1* in mice resulted in skin inflammation and keratinocyte necroptosis, which was prevented by deletion of *Ripk3* or *Mlkl*

(Dannappel et al., 2014). More recently, Duan *et al.* identified the increased expression of all three necroptotic effectors RIPK1, RIPK3 and MLKL in human psoriasis lesions (Duan et al., 2020). In the context of cutaneous adverse drug reactions, Saito *et al.* investigated keratinocyte cell death in Stevens-Johnson syndrome (SJS) and toxic epidermal necrolysis (TEN), demonstrating that the siRNA knockdown of *Ripk3* or inhibition of RIPK1 with Nec-1 blocked keratinocyte death in patient samples with these conditions (Saito et al., 2014).

Cardiovascular diseases, such as atherosclerosis and aortic aneurysm, have been linked to necroptosis (Lin et al., 2013, Karunakaran et al., 2016, Wang et al., 2015, Wang et al., 2017). Lin *et al.* showed that macrophage necroptosis promoted the development of advanced atherosclerosis in mice, which was reduced by *Ripk3* deletion (Lin et al., 2013). Furthermore, Karunakaran *et al.* detected phosphorylated MLKL in the atherosclerotic plaques of patients, as well as increased expression of RIPK3 and MLKL (Karunakaran et al., 2016). Wang *et al.* found that RIPK1 and RIPK3 protein levels were elevated in human aortic aneurysm tissue (Wang et al., 2015) and subsequently demonstrated that inhibition of RIPK1 using Nec-1s reduced aneurysm formation and progression in an elastase-induced abdominal aortic aneurysm murine model (Wang et al., 2017).

Necroptotic cell death has been implicated in systemic inflammatory conditions (Duprez et al., 2011, Newton et al., 2014, Newton et al., 2016a, Rickard et al., 2014, Dillon et al., 2014, Kaiser et al., 2014, Hildebrand et al., 2020) and autoinflammatory diseases (Hildebrand et al., 2020). Duprez *et al.* demonstrated that deletion of *Ripk3* or inhibition of RIPK1 using Nec-1 protected mice against the lethal TNF-induced systemic inflammatory response syndrome (SIRS), a murine model of sterile sepsis (Duprez et al., 2011). The protective effect of *Ripk3*

deficiency was also observed in the cecal ligation and puncture (CLP) model, a clinically more relevant murine model of sepsis (Duprez et al., 2011). These findings were expanded upon by studies from Genentech who reported that *Ripk1^{KD/KD}* mice, *Ripk3^{-/-}Casp8^{-/-}* mice and *MLK1^{-/-}Casp8^{-/-}* mice were completely protected from TNF-induced SIRS, suggesting that the lethal hypothermia was a consequence of both MLKL-dependent necroptosis and caspase-8-dependent apoptosis (Newton et al., 2014, Newton et al., 2016a). Furthermore, three separate studies identified that *Ripk1^{-/-}* mice die at birth from systemic inflammation and excessive cell death driven by both necroptosis and apoptosis (Rickard et al., 2014, Dillon et al., 2014, Kaiser et al., 2014). A recent study by Hildebrand *et al.* characterised the constitutively active murine MLKL D139V mutant, which causes lethal perinatal inflammation (Hildebrand et al., 2020). This study also identified *MLKL* polymorphisms found at higher frequency in patients with the autoinflammatory disease, chronic recurrent multifocal osteomyelitis (CRMO) (Hildebrand et al., 2020).

Cell death in ischemia-reperfusion injuries and solid organ transplant has been linked to necroptosis. Several studies have described that necroptosis contributes to organ damage in ischemia-reperfusion injuries in the kidney (Linkermann et al., 2012, Linkermann et al., 2013, Muller et al., 2017), steatotic liver (Ni et al., 2019), brain (Degterev et al., 2005, Yin et al., 2015) and heart (Oerlemans et al., 2012, Luedde et al., 2014, Koshinuma et al., 2014, Zhang et al., 2016). However, the role of necroptosis in ischemic brain injury has been challenged by several recent studies, which showed that loss of *Ripk3* had no effect on brain injury in two murine models of stroke (Newton et al., 2016a) and, moreover, neither MLKL nor RIPK3 were expressed in the brain or spinal cord of wild-type mice (Dermentzaki et al., 2019, Wang et al., 2020, Dominguez et al., 2020). Other studies have reported that necroptosis promotes graft

rejection and reduces allograft survival in kidney transplant (Lau et al., 2013) and heart transplant (Pavlosky et al., 2014, Kwok et al., 2017).

Degenerative Diseases

A range of studies have suggested that necroptotic cell death contributes to various degenerative disorders including neurodegenerative diseases, lysosomal storage diseases and degenerative eye conditions. In particular, necroptosis has been linked to neuronal cell death in several neurodegenerative diseases including amyotrophic lateral sclerosis (ALS) (Re et al., 2014, Ito et al., 2016), multiple sclerosis (Ofengeim et al., 2015), Parkinson's disease (Iannielli et al., 2018), Huntington's disease (Zhu et al., 2011) and Alzheimer's disease (Yang et al., 2017b, Caccamo et al., 2017).

In ALS studies, Re *et al.* established a coculture system of human adult primary ALS astrocytes and human embryonic stem cell derived motor neurons, and found that the patient-derived ALS astrocytes induced necroptotic cell death of the motor neurons (Re et al., 2014). Subsequent work by Ito *et al.* reported that the loss of optineurin (*Optn*) in mice, a gene implicated in ALS via loss of function mutations, caused myelination defects and axonal degeneration driven by necroptosis, which was further supported by elevated RIPK1, RIPK3 and MLKL levels observed in human ALS pathological samples (Ito et al., 2016). However, the role of necroptosis in ALS has been directly challenged by several recent studies showing that the genetic deletion of *Mkl1* (Wang et al., 2020), genetic deletion of *Ripk3* (Dermentzaki et al., 2019) or genetic inactivation of *Ripk1* (Dominguez et al., 2020) had no effect on disease onset, progression or survival in the superoxide dismutase 1 (SOD1) G93A mutant murine model of ALS. Indeed, strikingly, neither MLKL nor RIPK3 protein expression was detected in central nervous system tissues of wild-type mice, which argues against necroptosis contributing to neurological disorders (Dermentzaki et al., 2019, Wang et al., 2020, Dominguez et al., 2020).

In studies of other neurodegenerative diseases, Ofengeim *et al.* observed elevated cFLIP levels and reduced caspase-8 activation along with increased activation of RIPK1, RIPK3 and MLKL in the cortical lesions of multiple sclerosis patients (Ofengeim *et al.*, 2015). Iannielli *et al.* reported that Nec-1s protected dopaminergic neurons from cell death in Parkinson's disease, both in cultured neural cells derived from patients and in a murine model (Iannielli *et al.*, 2018). In a murine model of Huntington's disease, Zhu *et al.* found that Nec-1 improved motor function and delayed disease onset, however only modestly increased survival (Zhu *et al.*, 2011). Similarly, in a murine model of Alzheimer's disease, Yang *et al.* found that Nec-1 directly targeted tau hyperphosphorylation and amyloid- β aggregation, which reduced neuronal cell death and improved cognitive impairment (Yang *et al.*, 2017b). Furthermore, Caccamo *et al.* identified that necroptosis was activated in postmortem human brains with sporadic Alzheimer's disease, which correlated with decreased brain weight and cognitive function (Caccamo *et al.*, 2017). However, given the recent studies demonstrating that neither RIPK3 nor MLKL were expressed in the brain or spinal cord of wild-type mice, any potential role for necroptosis in these neurodegenerative diseases requires further examination (Dermentzaki *et al.*, 2019, Wang *et al.*, 2020, Dominguez *et al.*, 2020).

Lysosomal storage diseases, rare inherited metabolic disorders, have been reported to involve necroptotic cell death. Gaucher's disease is the most common lysosomal storage disease and is caused by an autosomal recessive mutation in the glucocerebrosidase (*GBA*) gene (Rosenbloom and Weinreb, 2013). Vitner *et al.* demonstrated that RIPK1 and RIPK3 levels were elevated in a murine model of Gaucher's disease and that *Ripk3* deficiency improved clinical symptoms such as motor coordination and lifespan in this model (Vitner *et al.*, 2014). Niemann-Pick disease type C (NPC) is another lysosomal storage disease and is

caused by an autosomal recessive mutation in either the *NPC1* or *NPC2* gene (Vanier, 2010). Cougnoux *et al.* identified that RIPK1 and RIPK3 protein levels were increased in fibroblasts and cerebellar tissue of NPC1 patients as well as *Npc1*^{-/-} mice, and that inhibition of RIPK1 using Nec-1 delayed disease progression and increased survival of the *Npc1*^{-/-} mice (Cougnoux *et al.*, 2016).

Necroptosis has been linked to degenerative eye conditions, where studies have shown that necroptotic cell death plays a role in murine models of various retinal disorders. Trichonas *et al.* reported increased expression of RIPK1 and RIPK3 following retinal detachment in mice and that the resultant photoreceptor death was reduced in *Ripk3*^{-/-} mice (Trichonas *et al.*, 2010). Sato *et al.* identified that cone and rod photoreceptor degeneration in mice lacking interphotoreceptor retinoid-binding protein (IRBP) was driven by necroptosis through upregulation of RIPK1 and RIPK3 (Sato *et al.*, 2013). Using a different model, Murakami *et al.* demonstrated that cone, but not rod, photoreceptor death was caused by necroptosis in mice carrying a mutation in a rod-specific gene (Murakami *et al.*, 2012). In a model of retinal degeneration induced by dsRNA/poly(I:C), the same group showed that *Ripk3*^{-/-} mice were protected from inflammation and retinal cell loss (Murakami *et al.*, 2014).

Infectious Diseases

There is increasing evidence to suggest that necroptosis evolved as an innate immune mechanism to combat microbial infections. A variety of studies have demonstrated that necroptosis is a cell death response involved in various infectious diseases including viral and bacterial infections. In some cases, the pathogen promotes necroptosis to aid its dissemination, while in other cases, the pathogen blocks necroptosis to avoid triggering an immune response.

Viruses have evolved a variety of strategies to modulate the host necroptotic machinery and host response to infection (Nailwal and Chan, 2019). Some viruses suppress necroptotic cell death, often by encoding protein inhibitors of the host necroptotic effectors. Such viruses include the Epstein-Barr virus (Liu et al., 2018), herpes simplex virus 1 and 2 (HSV-1 and HSV-2) in human cells (Guo et al., 2015, Huang et al., 2015) and varicella zoster virus (Steain et al., 2020), as well as the murine cytomegalovirus (Upton et al., 2008, Upton et al., 2010, Upton et al., 2012) and murine vaccinia virus (Koehler et al., 2017). The Epstein-Barr virus encodes latent membrane protein 1 (LMP1) that interacts with RIPK1 and RIPK3 to modulate their ubiquitylation and suppress necroptosis (Liu et al., 2018). HSV-1 and HSV-2 encode the viral proteins ICP6 and ICP10, respectively, which contain RHIM domains that disrupt the interaction between RIPK1 and RIPK3 by competing for their binding (Guo et al., 2015, Huang et al., 2015). Similarly, the murine cytomegalovirus encodes the viral inhibitor of RIP activation (vIRA) that has a RHIM domain, enabling it to disrupt the RIPK1-RIPK3 RHIM and RIPK3-DAI RHIM interactions (Upton et al., 2008, Upton et al., 2010, Upton et al., 2012). The murine vaccinia virus encodes the innate immune evasion protein E3, which contains a Z-DNA binding domain that competes with DAI to prevent RIPK3 activation (Koehler et al., 2017).

Interestingly, while the varicella zoster virus inhibits necroptosis and encodes the RHIM-containing protein ORF20, the ORF20 RHIM itself is not responsible for this necroptosis inhibition, but instead forms amyloid complexes with DAI to inhibit DAI-driven apoptosis (Steain et al., 2020).

In contrast, some viruses promote necroptosis by engaging and activating the host necroptotic effectors, such as the influenza A virus (Thapa et al., 2016, Kuriakose et al., 2016, Zhang et al., 2020, Shubina et al., 2020) and HSV-1 in murine cells (Wang et al., 2014b, Huang et al., 2015). A study of influenza A virus reported that DAI senses viral RNA, which promotes DAI to associate with and activate RIPK3, resulting in induction of both necroptotic and apoptotic cell death (Thapa et al., 2016). A separate study of influenza A virus reported that DAI senses the viral proteins nucleoprotein (NP) and RNA polymerase subunit PB1, which triggers cell death (Kuriakose et al., 2016). More recent studies have found that influenza A virus produces Z-RNAs that are recognised by DAI and trigger DAI-driven necroptosis (Zhang et al., 2020), and that necroptosis can act as a stand-alone cell death mechanism in the absence of apoptosis in influenza A viral infection (Shubina et al., 2020). Interestingly, unlike HSV infection in human cells, HSV-1 infection in murine cells induces necroptosis through ICP6 directly interacting with RIPK3 and/or RIPK1 via their respective RHIM domains, which activates RIPK3 to promote MLKL-dependent necroptosis (Wang et al., 2014b, Huang et al., 2015).

Bacteria have also evolved mechanisms to modulate necroptosis signalling in the host response to infection, whereby some bacteria promote necroptotic cell death, while other bacteria inhibit necroptotic cell death. *Staphylococcus aureus* activates RIPK1/RIPK3/MLKL-

mediated necroptosis to promote bacterial clearance by reducing excessive pathological inflammation (Kitur et al., 2016). Enteropathogenic *Escherichia coli*, however, blocks necroptosis via its type III secretion system (T3SS) effector EspL, a bacterial cysteine protease that cleaves RHIM-containing proteins including RIPK1, RIPK3, TRIF and DAI (Pearson et al., 2017).

Cancer

Necroptosis has been linked to various human cancers, however the exact mechanistic role of necroptosis in cancer is complex and not well understood. There is no consensus in current literature on whether necroptosis promotes or impairs tumour progression and indeed the role of necroptosis appears to, at least in part, depend on the cancer type and tumour microenvironment. While some studies have reported that necroptosis has tumour promoting effects, other studies have reported that necroptosis has tumour suppressing effects.

Downregulated expression of the necroptotic effectors RIPK3 and MLKL has been reported in several cancers, suggesting a tumour suppressor role for necroptosis. Decreased RIPK3 expression has been identified in patient samples of chronic lymphocytic leukaemia (CLL) (Liu et al., 2012), acute myeloid leukaemia (AML) (Nugues et al., 2014, Hockendorf et al., 2016), colorectal cancer (Moriwaki et al., 2015, Feng et al., 2015, Bozec et al., 2016) and breast cancer (Koo et al., 2015). Furthermore, many cancer cells lines lack RIPK3, in contrast to primary cells and normal tissue, suggesting that loss of RIPK3 is important for cancer cell survival (He et al., 2009, Moriwaki et al., 2015, Koo et al., 2015, Geserick et al., 2015, Yang et al., 2017a). Low expression of MLKL has been identified in patient samples and has been associated with poor prognosis and decreased overall survival in several cancers (Hu et al., 2018) including pancreatic adenocarcinoma (Colbert et al., 2013), ovarian cancer (He et al., 2013), cervical squamous cell carcinoma (Ruan et al., 2015), AML (Hockendorf et al., 2016), colorectal cancer (Li et al., 2017b) and gastric cancer (Ertao et al., 2016, Sun et al., 2019). However, the mechanisms underlying this downregulation of RIPK3 and MLKL are still unclear.

In contrast, several studies have suggested that necroptosis promotes cancer progression. Upregulated expression of the necroptotic effectors RIPK1, RIPK3 and MLKL has been reported in patient samples of several cancers, including enriched RIPK3 expression in serous ovarian cancer (McCabe et al., 2014), high expression of RIPK1 and RIPK3 in pancreatic ductal adenocarcinoma (PDAC) (Seifert et al., 2016), increased RIPK3 and MLKL levels in breast tumours (Jiao et al., 2018) and high levels of phosphorylated MLKL in esophageal and colon cancer that correlated with decreased patient survival (Liu et al., 2016). Furthermore, necroptosis has been reported to promote metastasis due to tumour cell induction of necroptotic cell death in endothelial cells, leading to tumour cell extravasation and migration (Strilic et al., 2016). This process is negatively regulated by TAK1, which restricts RIPK3-driven necroptosis in endothelial cells (Yang et al., 2019). In addition, it was found that necroptosis underlies tumour necrosis in solid tumours and promotes metastasis as a result of tumour cell necroptosis (Jiao et al., 2018), and that a necroptosis microenvironment influences the lineage commitment of transformed hepatocytes in liver tumorigenesis (Seehawer et al., 2018). The necroptotic machinery has also been found to induce immune suppression in the tumour microenvironment to promote tumour progression in PDAC (Seifert et al., 2016), which is driven by high expression of RIPK1 in tumour-associated macrophages (Wang et al., 2018). Recent studies, however, have questioned the relevance of necroptosis in tumour progression and metastasis (Patel et al., 2020). Work from Genentech demonstrated that neither the chemical nor genetic inhibition of RIPK1 affected tumour growth in PDAC models, and that neither the genetic inactivation of *Ripk1* nor loss of *Ripk3* affected lung metastasis in a B16 melanoma model (Patel et al., 2020). This suggests that the effects observed in previously published studies were context or model specific, which warrant further examination.

Small Molecule Inhibitors of Necroptosis

RIPK1 Inhibitors

As a result of the plethora of studies demonstrating the role of necroptosis in various pathologies, there has been increasing interest in targeting the pathway using small molecules, which could lead to the development of novel therapeutics. Several small molecule inhibitors of necroptosis have been described in the literature and many of these compounds target RIPK1 (**Figure 1.8**). The first small molecule inhibitor of necroptosis, necrostatin-1 (Nec-1), was described by Degterev *et al.* in 2005 (Degterev *et al.*, 2005). Nec-1 was identified from screening a chemical library of ~15,000 compounds for small molecules that blocked necroptosis induced by TNF and z-VAD-fmk in human U937 cells (Degterev *et al.*, 2005). Nec-1 exhibited an EC₅₀ of 490 nM in FADD-deficient Jurkat cells treated with TNF to stimulate necroptosis (Degterev *et al.*, 2005). In 2008, the same group identified that RIPK1 was the cellular target of Nec-1 and that Nec-1 inhibited RIPK1 kinase activity (Degterev *et al.*, 2008). However, Nec-1 is chemically identical to methyl-thiohydantoin-tryptophan (MTH-Trp), a known inhibitor of the immunomodulatory enzyme indoleamine 2,3-dioxygenase (IDO) (Muller *et al.*, 2005), and indeed Nec-1 was shown to inhibit human IDO (Takahashi *et al.*, 2012). Nec-1 also exhibited toxicity at lower doses *in vivo* in the TNF-induced SIRS murine model, raising concerns about its specificity and off-target activity (Takahashi *et al.*, 2012). From structure-activity relationship (SAR) studies of Nec-1, an optimised analogue, 7-Cl-O-Nec-1 or Nec-1s, was identified with an EC₅₀ of 210 nM in FADD-deficient Jurkat cells treated with TNF (Teng *et al.*, 2005, Degterev *et al.*, 2005, Degterev *et al.*, 2008). Nec-1s displayed greater *in vivo* stability, did not inhibit human IDO and did not show toxicity at lower doses *in vivo*, making it the preferred necrostatin analogue to use in necroptosis studies in cells and *in vivo* (Takahashi *et al.*, 2012).

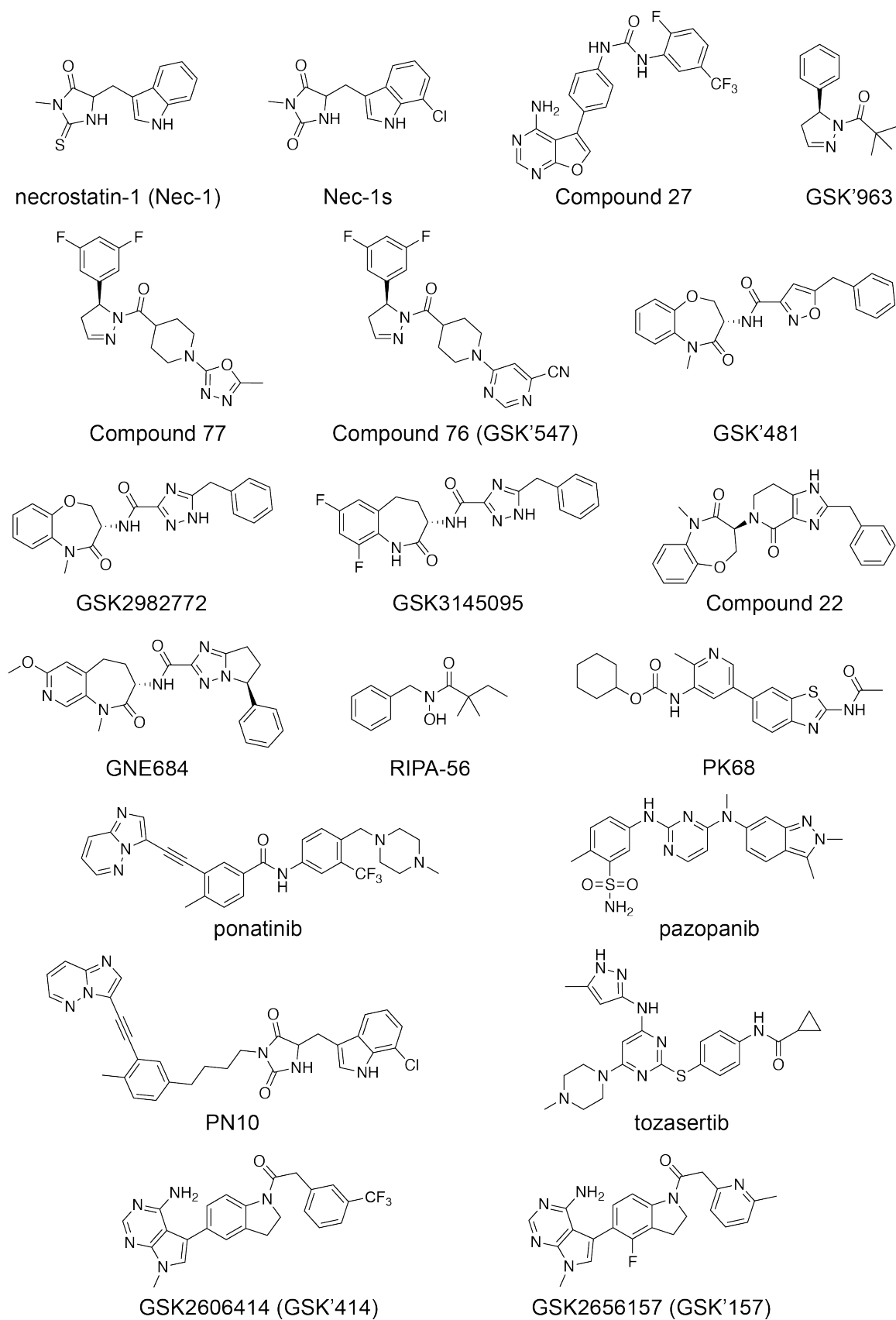


Figure 1.8 Small molecule inhibitors of necroptosis that target RIPK1.

Following on from the initial necrostatins, a team at GlaxoSmithKline (GSK) identified and developed several small molecule inhibitors of necroptosis that target RIPK1. In their initial studies, Harris *et al.* in 2013 screened their in-house GSK kinase inhibitor libraries against the human RIPK1 kinase domain using fluorescence polarisation (FP) binding assays and identified three series of RIPK1 inhibitors (Harris et al., 2013). The most promising, Compound 27, bound to RIPK1 with an IC_{50} of 63 nM in FP binding assays and inhibited RIPK1 with an IC_{50} of 13 nM in ADP-Glo kinase activity assay (Harris et al., 2013). Compound 27 blocked necroptotic cell death induced by TNF and z-VAD-fmk in U937 cells with an IC_{50} of 250 nM and was protective in the TNF-induced SIRS murine model (Harris et al., 2013).

In 2015, the same group conducted a high-throughput screen of the GSK compound collection, containing ~2 million compounds, against the human RIPK1 kinase domain using ADP-Glo assays (Berger et al., 2015). Hybridisation of two hits from the screen resulted in a small yet potent chiral RIPK1 inhibitor, GSK'963, the active *S* enantiomer, with an IC_{50} of 16 nM and 1.6 nM in FP binding assays and ADP-Glo enzymatic assays, respectively (Berger et al., 2015, Harris et al., 2019a). GSK'963 inhibited necroptosis in human and murine cells with IC_{50} values of 1-4 nM and did not inhibit human IDO *in vitro* (Berger et al., 2015). The inactive *R* enantiomer, GSK'962, did not show any RIPK1 activity in FP assays or ADP-Glo assays and displayed no or only weak inhibition of necroptosis in cellular assays (Berger et al., 2015, Harris et al., 2019a).

The team at GSK then performed lead optimisation of the dihydropyrazole series based on the GSK'963 scaffold with the aiming of improving the pharmacokinetic properties (Harris et al., 2019a). This led to the identification of Compound 77, which displayed a favourable oral

PK profile in multiple species and showed good inhibition of human RIPK1, with an IC₅₀ of 20 nM in ADP-Glo assays, as well as good cellular activity, with an IC₅₀ of 63 nM in U937 cells (Harris et al., 2019a). They also identified Compound 76 (also called GSK'547) which, unlike Compound 77, displayed potent inhibition of necroptosis in murine L929 cells with an IC₅₀ of 32 nM (Harris et al., 2019a, Wang et al., 2018). Compound 76/GSK'547 was used as a tool compound *in vivo*, showing efficacy in the experimental autoimmune encephalomyelitis (EAE) murine model of human multiple sclerosis and the Rd10 murine model of human retinitis pigmentosa (Harris et al., 2019a). In addition, Compound 76/GSK'547 was found to reduce tumour burden, reduce metastases and extend survival in a murine model of PDAC (Wang et al., 2018).

In 2016, Harris and GSK colleagues described a more potent and more selective RIPK1 kinase inhibitor, GSK'481 (Harris et al., 2016). This small molecule was identified from screening the GSK DNA-encoded small molecule libraries against the human RIPK1 kinase domain, resulting in the identification of a benzoxazepinone series exemplified by GSK'481 (Harris et al., 2016). GSK'481 inhibited RIPK1 kinase activity with an IC₅₀ of 1.6 nM in ADP-Glo assays and blocked necroptosis induced by TNF and z-VAD-fmk in U937 cells with an IC₅₀ of 10 nM (Harris et al., 2016). Notably, GSK'481 displayed mono-kinase selectivity for RIPK1 when tested against 456 other kinases at 10 µM and exhibited species specificity for primate RIPK1 compared to non-primate RIPK1 (Harris et al., 2016).

As a result of these promising features, GSK'481 was selected for lead optimisation with the aim of improving the PK profile of the benzoxazepinone series, which resulted in the development of the clinical candidate, GSK2982772 (Harris et al., 2017). Compared to

GSK'481, GSK2982772 displayed improved inhibition of RIPK1 kinase activity, with an IC_{50} of 1.0 nM in ADP-Glo assays, and improved inhibition of necroptosis in U937 cells, with an IC_{50} of 6.3 nM (Harris et al., 2017). GSK2982772 retained the mono-kinase selectivity and species specificity of GSK'481, and also displayed good PK and safety profiles in rats and monkeys, with favourable physicochemical and ADMET (absorption, distribution, metabolism, excretion, toxicity) properties (Harris et al., 2017). In preclinical studies, GSK2982772 decreased the production of inflammatory cytokines in human explant tissue from ulcerative colitis patients (Harris et al., 2017). GSK2982772 was the first RIPK1 inhibitor to enter clinical trials in 2015 for the treatment of inflammatory diseases and is currently in Phase II for psoriasis (NCT02776033), rheumatoid arthritis (NCT02858492) and ulcerative colitis (NCT02903966) (Harris et al., 2017, Weisel et al., 2017).

Based on the benzoxazepinone scaffold, GSK then developed a clinical candidate for oncology indications, GSK3145095 (Harris et al., 2019b). GSK3145095 retained potent RIPK1 inhibition, with an IC_{50} of 6.3 nM in ADP-Glo assays, and potent necroptosis inhibition, with an IC_{50} of 10 nM in U937 cells, as well as mono-kinase selectivity at 10 μ M, primate RIPK1 species specificity and a good oral PK profile in rats, dogs and monkeys (Harris et al., 2019b). GSK3145095 was evaluated in human *ex vivo* tumour cultures from resected pancreatic, colorectal, gastric and breast cancer patient tumours, where treatment with GSK3145095 resulted in increased effector-memory T cells and immunogenic T cells, promoting a tumour suppressive phenotype (Harris et al., 2019b). As a result, GSK3145095 entered Phase I clinical trials for pancreatic adenocarcinoma and other solid tumours (NCT03681951) (Harris et al., 2019b).

Yoshikawa *et al.* at Takeda Pharmaceutical described a brain-penetrating RIPK1 inhibitor, Compound 22, partially based on the benzoxazepinone series (Yoshikawa et al., 2018). They first performed a high-throughput screen of their compound library against the human RIPK1 kinase domain using time-resolved fluorescence resonance energy transfer (TR-FRET) binding assays and identified a hit that overlapped with the benzyl group and 5-membered heteroaromatic ring of GSK2982772 in the RIPK1 cocrystal structure (Yoshikawa et al., 2018). Hybridisation of the hit with GSK2982772, followed by optimisation using a structure-based drug design approach, resulted in Compound 22 (Yoshikawa et al., 2018). Compound 22 inhibited TNF-mediated necroptotic cell death in human HT29 cells, with an IC_{50} of 2.0 nM, and in murine L929 cells, with an IC_{50} of 15 nM (Yoshikawa et al., 2018). Moreover, Compound 22 demonstrated reduced efflux via the drug transporter P-glycoprotein (P-gp) (also known as multidrug resistance protein 1, MDR1), sufficient brain exposure in mice following oral administration and reduced clinical symptoms in the EAE murine model of multiple sclerosis (Yoshikawa et al., 2018).

To address the limitation that most of the previously described benzoxazepinone analogues targeted primate RIPK1 more effectively than non-primate RIPK1, Patel *et al.* at Genentech developed a potent cross-species inhibitor of RIPK1, GNE684, derived from the benzoxazepinone scaffold (Patel et al., 2020). GNE684 inhibited TNF-mediated necroptosis in human, mouse and rat cell lines as well as human, monkey, pig and mouse primary cells with EC_{50} values mostly below 100 nM (Patel et al., 2020). GNE684 was then evaluated in several murine models of inflammatory diseases and cancers (Patel et al., 2020). GNE684 provided near-complete protection in the TNF-induced SIRS model and ameliorated skin inflammation in chronic proliferative dermatitis mutation (*Cpdm*) mice that are deficient in SHARPIN (Patel

et al., 2020). In addition, GNE684 reduced collagen antibody-induced arthritis, providing comparable protection to the genetic inactivation of *Ripk1* (via expression of the kinase-dead RIPK1 D138N mutant) (Patel et al., 2020). However, GNE684 had no effect on overall survival or tumour growth in PDAC models and expression of RIPK1 D138N had no effect on lung metastasis in a B16 melanoma model (Patel et al., 2020). These findings validated the role of RIPK1 in inflammatory disease models, but questioned the role of RIPK1, or at least its kinase activity, in cancer models (Patel et al., 2020).

In 2017, Ren *et al.* conducted a phenotypic screen of 200,000 compounds to identify small molecules that blocked necroptosis induced by TNF, Smac-mimetic and z-VAD-fmk (TSZ) in HT29 cells (Ren et al., 2017). Following medicinal chemistry SAR efforts to improve the potency and metabolic stability of the initial hit, RIPA-56 was identified (Ren et al., 2017). RIPA-56 inhibited TSZ-induced necroptosis in HT29 cells with an EC₅₀ of 28 nM and TZ-induced necroptosis in L929 cells with an EC₅₀ of 27 nM (Ren et al., 2017). The target of RIPA-56 was confirmed to be RIPK1, as RIPA-56 blocked RIPK1 kinase activity in ADP-Glo assays with an IC₅₀ of 13 nM, but did not block RIPK3 kinase activity at 10 µM or human IDO enzymatic activity at 200 µM (Ren et al., 2017). RIPA-56 was efficacious in the TNF-induced SIRS murine model where it protected against lethality and multiorgan damage (Ren et al., 2017). In addition, RIPA-56 was found to reduce aging of the mouse male reproductive system, a process shown to be promoted by necroptosis (Li et al., 2017a).

Similarly, Hou *et al.* in 2019 performed a phenotypic screen against TSZ-stimulated necroptosis in HT29 cells by screening 240 potential kinase inhibitors with novel scaffolds (Hou et al., 2019). Optimisation of the original hit to improve the anti-necroptotic activity and

drug-like properties led to the identification of PK68 (Hou et al., 2019). PK68 inhibited TNF-driven necroptotic cell death in HT29 cells with an EC₅₀ of 23 nM and in L929 cells with an EC₅₀ of 13 nM (Hou et al., 2019). This small molecule was found to target RIPK1 *in vitro*, with IC₅₀ values of 90 nM against human RIPK1 and 45 nM against murine RIPK1 in ADP-Glo assays, but did not inhibit RIPK3 kinase activity up to 1 μM (Hou et al., 2019). *In vivo* studies demonstrated that PK68 was highly protective in the TNF-induced SIRS murine model and prevented lung metastasis of melanoma B16-F10 cells and lung carcinoma RFP-LL/2 cells in murine cancer models (Hou et al., 2019).

Drug repurposing efforts have identified several anti-cancer compounds that target RIPK1 to inhibit necroptosis. In 2015, Fauster *et al.* identified the BCR-ABL inhibitor ponatinib and the vascular endothelial growth factor receptor (VEGFR) inhibitor pazopanib, from a phenotypic screen of 268 Food and Drug Administration (FDA)-approved drugs for their ability to inhibit TNF-induced necroptosis in FADD-deficient Jurkat cells (Fauster et al., 2015). Ponatinib and pazopanib attenuated necroptosis in FADD-deficient Jurkat cells with an EC₅₀ of 89 nM and 254 nM, respectively, and also blocked necroptosis driven by TNF, TRAIL or FasL in HT29 cells (Fauster et al., 2015). In an earlier study of kinase inhibitor selectivity, it had been reported that pazopanib binds to RIPK1 (Davis et al., 2011). A chemical proteomics approach was employed to identify the cellular targets of ponatinib, by conjugating ponatinib to a bead matrix for pulldown experiments (Fauster et al., 2015). Following validation to confirm the direct targets, it was found *in vitro* and in cells that ponatinib targets both RIPK1 and RIPK3, while pazopanib preferentially targets RIPK1 (Fauster et al., 2015).

Similar findings were published by Degterev and colleagues earlier in 2015, who identified ponatinib as a dual RIPK1 and RIPK3 inhibitor from screening a small panel of known kinase inhibitors for their ability to block necroptosis in TNF-stimulated FADD-deficient Jurkat cells (Najjar et al., 2015). Ponatinib inhibited the kinase activity of human RIPK1 and RIPK3 in ADP-Glo assays with an IC_{50} of 12 nM and 1.6 nM, respectively (Najjar et al., 2015). Subsequent modifications to the ponatinib scaffold were made to enhance the selectivity towards RIPK1 over RIPK3, before developing a Nec-1s/ponatinib hybrid inhibitor, PN10, based on structure-guided design (Najjar et al., 2015). The PN10 hybrid inhibitor, which impaired RIPK1 kinase activity with an IC_{50} of 90 nM in ADP-Glo assays, showed better activity in necroptotic cell death assays than either component inhibitor alone, with an IC_{50} of 10 nM in FADD-deficient Jurkat cells (Najjar et al., 2015).

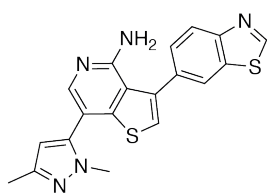
Aurora kinase inhibitors have also been reported to target RIPK1 and inhibit necroptosis. The pan-Aurora kinase inhibitor tozasertib was described by Martens *et al.* to attenuate TNF-mediated necroptosis by impairing RIPK1 kinase activity (Martens et al., 2018). They identified that while tozasertib blocked necroptotic cell death in the same concentration range (high nanomolar to low micromolar) as impairing cytokinesis, its two cellular effects were not interrelated, as other RIPK1 kinase inhibitors did not affect cytokinesis and other Aurora kinase inhibitors did not block necroptosis (Martens et al., 2018). Tozasertib inhibited human RIPK1 with an IC_{50} of 208 nM in ADP-Glo assays (Martens et al., 2018), confirming an earlier report from a kinase inhibitor selectivity study that tozasertib binds to RIPK1 with a K_D of 20 nM (Davis et al., 2011). Analogues of tozasertib from SAR studies were then generated, where some analogues displayed increased specificity for necroptosis over cytokinesis and increased selectivity towards RIPK1 over the Aurora kinases, whereas other analogues displayed the

opposite trend (Martens et al., 2018, Hofmans et al., 2018). One of the more necroptosis-selective analogues of tozasertib was subsequently found to attenuate cisplatin-induced renal damage by suppressing necroptosis, inflammation and oxidative stress in a murine model of acute kidney injury (Wang et al., 2019).

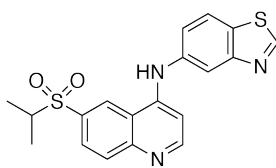
Furthermore, two protein kinase RNA-like endoplasmic reticulum kinase (PERK) inhibitors, GSK2606414 (GSK'414) and GSK2656157 (GSK'157), were identified to be potent inhibitors of RIPK1-dependent cell death (Rojas-Rivera et al., 2017). GSK'414 and GSK'157 attenuated necroptosis induced by TNF and z-VAD-fmk in murine embryonic fibroblast (MEF) cells with an IC_{50} of 7.3 nM and 1.9 nM, respectively (Rojas-Rivera et al., 2017). Indeed, GSK'157 was found to be a much more potent inhibitor of RIPK1-dependent cell death than PERK signalling in a cellular context (Rojas-Rivera et al., 2017). Their anti-necroptotic activity was independent of PERK inhibition, as other PERK inhibitors had no effect on TNF-driven death of MEF cells, and the siRNA knockdown of PERK had no effect on necroptosis or the ability of GSK'414 and GSK'157 to block necroptotic cell death (Rojas-Rivera et al., 2017). GSK'414 and GSK'157 inhibited human RIPK1 kinase activity with an IC_{50} of 18.2 nM and 69.1 nM, respectively, in ADP-Glo assays (Rojas-Rivera et al., 2017). Importantly, this study highlighted the risk of misinterpretation when using these “selective” PERK inhibitors in a cellular context to examine ER stress, cell death and inflammation (Rojas-Rivera et al., 2017).

RIPK3 Inhibitors

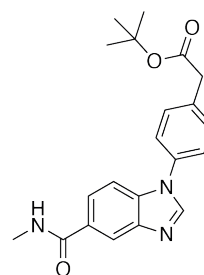
Several small molecule inhibitors of necroptosis described in the literature target RIPK3 (**Figure 1.9**). The first RIPK3 inhibitors were two compounds from GSK reported by Kaiser *et al.* in 2013, GSK'843 and GSK'872, following the optimisation of hits from a small molecule screen using FP binding assays (Kaiser *et al.*, 2013). Both GSK'843 and GSK'872 inhibited necroptosis mediated by TNF, TLR3, TLR4 or DAI in murine cells (Kaiser *et al.*, 2013). The same group in 2014 reported a more potent RIPK3 inhibitor, GSK'840, which was identified from a screen against the human RIPK3 kinase domain using a DNA-encoded library of more than 10^9 compounds and FP binding assays (Mandal *et al.*, 2014). GSK'840 interacted with human RIPK3 with an IC_{50} of 0.9 nM in these assays, compared to GSK'843 and GSK'872, with IC_{50} values of 8.6 nM and 1.8 nM, respectively (Mandal *et al.*, 2014). In ADP-Glo assays, GSK'840 inhibited RIPK3 kinase activity with an IC_{50} of 0.3 nM, compared to GSK'843 and GSK'872, with IC_{50} values of 6.5 nM and 1.3 nM, respectively (Mandal *et al.*, 2014). While GSK'840 blocked necroptosis more efficiently than either GSK'843 or GSK'872 in human cells, GSK'840 was unable to inhibit necroptosis in murine cells (Mandal *et al.*, 2014). However, all three compounds caused toxicity at higher concentrations that, unexpectedly, was due to induction of RIPK3-dependent apoptosis (Mandal *et al.*, 2014).



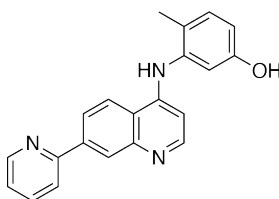
GSK'843



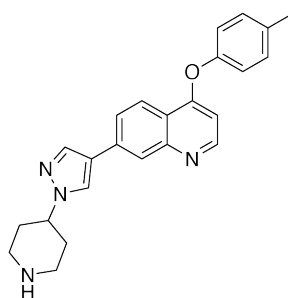
GSK'872



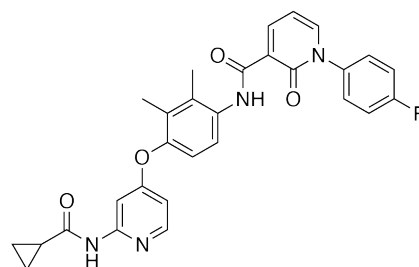
GSK'840



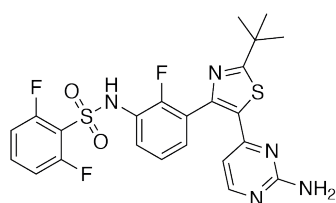
GW440139B (GW'39B)



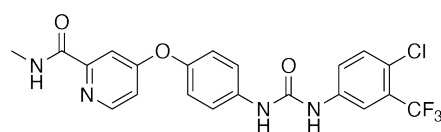
HS-1371



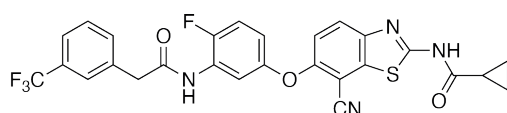
Compound 18



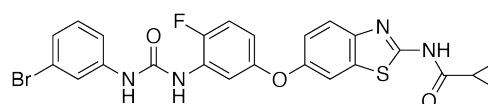
dabrafenib



sorafenib*



TAK-632



Compound 42

Figure 1.9 Small molecule inhibitors of necroptosis that target RIPK3.

*While sorafenib was reported to target RIPK3 (Li et al., 2014, Martens et al., 2017), there are conflicting results regarding its ability to inhibit necroptosis in cells. Some studies identified that sorafenib does not inhibit necroptosis (Li et al., 2014, Hildebrand et al., 2014), while another study found that sorafenib blocks necroptosis (Martens et al., 2017).

In 2016, Rodriguez *et al.* performed a screen of 8,904 bioactive compounds for their ability to inhibit necroptosis driven by dimerisable RIPK3 in murine NIH-3T3 cells and identified

GW440139B (GW'39B) with an EC₅₀ of 73.6 nM (Rodriguez et al., 2016). GW'39B blocked TNF- or TRIF-mediated necroptosis and prevented RIPK3 phosphorylation of MLKL, in a range of murine and human cell lines (Rodriguez et al., 2016). In 2018, Park *et al.* screened their kinase-targeted chemical libraries for compounds that inhibited human RIPK3 kinase activity in radiometric kinase assays and identified HS-1371 with an IC₅₀ of 20.8 nM (Park et al., 2018). HS-1371 blocked RIPK3 autophosphorylation in HT29 cells and attenuated TNF-mediated necroptosis in both human and murine cells (Park et al., 2018). More recently, Bristol Myers Squibb (BMS) described a series of RIPK3 inhibitors identified from high-throughput screening efforts against full-length human RIPK3 using homogeneous time-resolved fluorescence (HTRF) binding assays (Hart et al., 2020). A structure-based drug design approach then resulted in Compound 18, with an IC₅₀ of 9.1 nM for RIPK3 in HTRF binding assays (Hart et al., 2020). Compound 18 displayed good selectivity for RIPK3 over RIPK1, with an IC₅₀ of 5.5 μM, and RIPK2, with an IC₅₀ > 10 μM, in these assays (Hart et al., 2020).

Drug repurposing approaches have also been applied to identify small molecule inhibitors of RIPK3. Indeed, some Raf kinase inhibitors have been reported to target RIPK3 and block necroptosis. Li *et al.* found that six known Raf inhibitors impaired human RIPK3 kinase activity *in vitro*, with the most potent being the B-Raf V600E inhibitor dabrafenib (Li et al., 2014). Dabrafenib inhibited RIPK3 kinase activity with an IC₅₀ of 250 nM in luminescent assays and 2 nM in radioactive assays, and blocked necroptosis in HT29 cells triggered by TNF, TRAIL or FasL in combination with Smac-mimetic and z-VAD-fmk (Li et al., 2014). Notably, dabrafenib prevented acetaminophen-induced necrosis in human hepatocyte cells and reduced liver damage *in vivo* in an acetaminophen-overdose murine model (Li et al., 2014). Sorafenib, another B-Raf inhibitor, was found in the same study to weakly inhibit human RIPK3 kinase

activity, with an IC_{50} of 14.52 μ M in luminescent assays (Li et al., 2014). In a separate study, sorafenib was identified from two independent phenotypic screens and was reported to inhibit human RIPK1 kinase activity with an IC_{50} of 1.5 μ M in ADP-Glo assays (Martens et al., 2017). Streptavidin pulldown experiments using a biotinylated derivative of sorafenib for target identification enriched RIPK1, RIPK3 and MLKL, although sorafenib only slightly reduced RIPK3 kinase activity *in vitro* at 10 μ M and functional targeting of MLKL was excluded (Martens et al., 2017). Furthermore, there are mixed reports in the literature on the ability of sorafenib to exert an effect on necroptosis in a cellular context; some studies demonstrated that sorafenib has no effect on necroptosis (Li et al., 2014, Hildebrand et al., 2014), while another study showed that sorafenib inhibits necroptosis (Martens et al., 2017).

More recently, the pan-RAF inhibitor TAK-632 was identified as an inhibitor of necroptosis from a chemical library screen of 500 fluorine-containing compounds in TSZ-treated HT29 cells (Chen et al., 2019). TAK-632 attenuated necroptosis in a range of human and murine cell lines, albeit at micromolar concentrations (Chen et al., 2019). Through Drug Affinity Responsive Target Stability (DARTS) assays and streptavidin pulldown assays using a biotinylated derivative of TAK-632, both RIPK3 and RIPK1 were identified and validated as targets (Chen et al., 2019). SAR studies and structural optimisation of TAK-632 resulted in a more potent analogue, Compound 42, with selectivity for RIPK3 over RIPK1 (Zhang et al., 2019). In DiscoverX KINOMEscan competition binding assays, Compound 42 displayed a K_D of 81 nM for RIPK3 and did not bind to RIPK1 at 5 μ M, in comparison to TAK-632 that interacted with RIPK3 and RIPK1 with a K_D of 105 nM and 480 nM, respectively (Zhang et al., 2019). Compound 42 inhibited necroptosis with an EC_{50} of 0.44 μ M in TSZ-stimulated HT29 cells,

compared to TAK-632 with an EC_{50} of 1.44 μ M (Zhang et al., 2019). However, the kinase selectivity profile of these compounds remains an outstanding challenge (Zhang et al., 2019).

MLKL Inhibitors

In contrast to RIPK1 and RIPK3, there are far fewer small molecules targeting MLKL (**Figure 1.10**). As MLKL is a pseudokinase and therefore lacks catalytic activity, targeting MLKL with small molecules has relied on less conventional methods. In 2012, Sun *et al.* identified necrosulfonamide (NSA) as an inhibitor of necroptosis, in one of two studies that led to the discovery of MLKL, which was a previously unknown component of the pathway (Sun *et al.*, 2012). NSA was identified from medicinal chemistry efforts and SAR studies of a hit from a high-throughput screen of ~200,000 compounds for small molecules that blocked TSZ-induced necroptosis in HT29 cells (Sun *et al.*, 2012, Liao *et al.*, 2014). Interestingly, another hit from this phenotypic screen led to the discovery of the RIPK1 inhibitor RIPA-56 (Ren *et al.*, 2017), as discussed in a previous section. NSA inhibited necroptosis in HT29 cells with an IC₅₀ of 124 nM, yet was unable to block necroptotic death in murine cells (Sun *et al.*, 2012). MLKL was identified as the direct cellular target of NSA from streptavidin pulldown experiments using a biotinylated derivative of NSA (Sun *et al.*, 2012, Liao *et al.*, 2014). It was found that NSA targets human MLKL, but not murine MLKL, due its α,β -unsaturated enone moiety that covalently binds, via a Michael addition reaction, to a cysteine residue in the N-terminal domain of human MLKL that is not conserved in murine MLKL (Sun *et al.*, 2012).

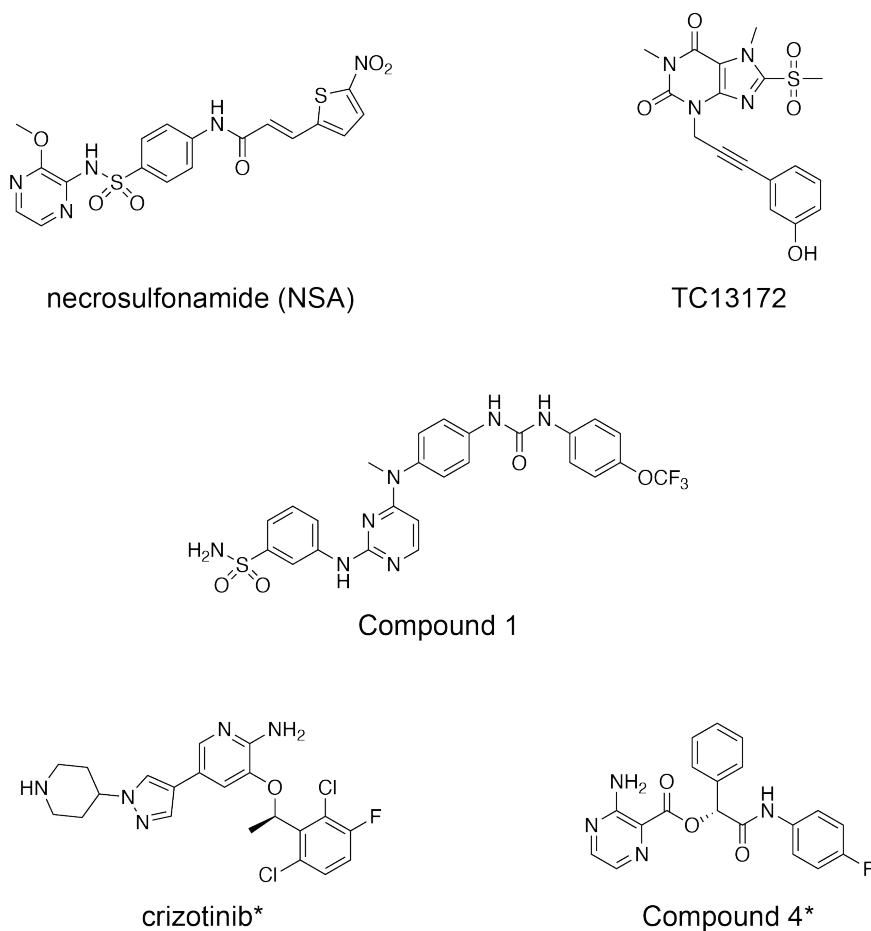


Figure 1.10 Small molecule inhibitors of necroptosis that target MLKL.

*Although crizotinib and Compound 4 were reported to bind to MLKL, they did not show any activity against necroptosis in cells (Ma et al., 2016).

The same group later investigated another hit from the same screen by undertaking SAR studies and medicinal chemistry exploration (Yan et al., 2017). The most potent analogue they identified, TC13172, inhibited TSZ-stimulated necroptosis in HT29 cells with an EC_{50} of 2 nM (Yan et al., 2017). Through the use of chemical proteomic approaches, TC13172 was found to have the same mechanism of action as NSA and was a covalent inhibitor of human MLKL that modified Cys86, the same cysteine residue covalently modified by NSA (Yan et al., 2017). Accordingly, TC13172 was unable to block necroptotic death in murine cell lines (Yan et al., 2017).

The first non-covalent inhibitor of MLKL, Compound 1, was reported in 2014 by our teams at WEHI (Hildebrand et al., 2014). Compound 1 was identified from a target-based screen against the murine MLKL pseudokinase domain, where a small molecule library of 367 kinase inhibitors (Published Kinase Inhibitor Set (Drewry et al., 2014)) was screened by Thermal Shift Assay (TSA) (Hildebrand et al., 2014). Compound 1 was validated by Surface Plasmon Resonance (SPR) to bind to the murine MLKL pseudokinase domain with a K_D of 9.3 μ M and blocked necroptosis induced by TNF, Smac-mimetic and Q-VD-OPh (TSQ) in murine dermal fibroblast (MDF) cells with an $IC_{50} < 50$ nM (Hildebrand et al., 2014). Furthermore, Compound 1 retarded endogenous MLKL translocation to the membrane in subcellular fractionation and Blue-Native PAGE experiments (Hildebrand et al., 2014).

Ma and colleagues at Biogen performed a high-throughput screen of a 5,000 diversified compound library using DiscoverX KINOMEscan competition binding assays to identify small molecules that bind to MLKL, but not to RIPK1 or RIPK3 (Ma et al., 2016). From this screen, they identified that the anti-cancer drug crizotinib and another small molecule called Compound 4 bind to full-length human MLKL with a K_D of 217 nM and 230 nM, respectively (Ma et al., 2016). However, neither crizotinib nor Compound 4 showed any activity against necroptosis in FADD-deficient Jurkat cells treated with TNF, leading the authors to suggest that compounds binding to the ATP pocket of MLKL would not be able to block necroptosis (Ma et al., 2016).

Thesis Aims and Overview

This thesis aims to study the mechanism of action of two small molecule inhibitors of necroptosis and deduce the cellular targets that underlie their anti-necroptotic activity (**Figure 1.11**).

Specifically, the two project aims of this thesis are:

1. To investigate the mechanism of action of Compound **2**, a more potent analogue of the previously reported inhibitor of necroptosis, Compound **1** (Chapter 2).
2. To identify the cellular targets of ABT-869, a novel inhibitor of necroptosis identified from a phenotypic screen (Chapter 3).

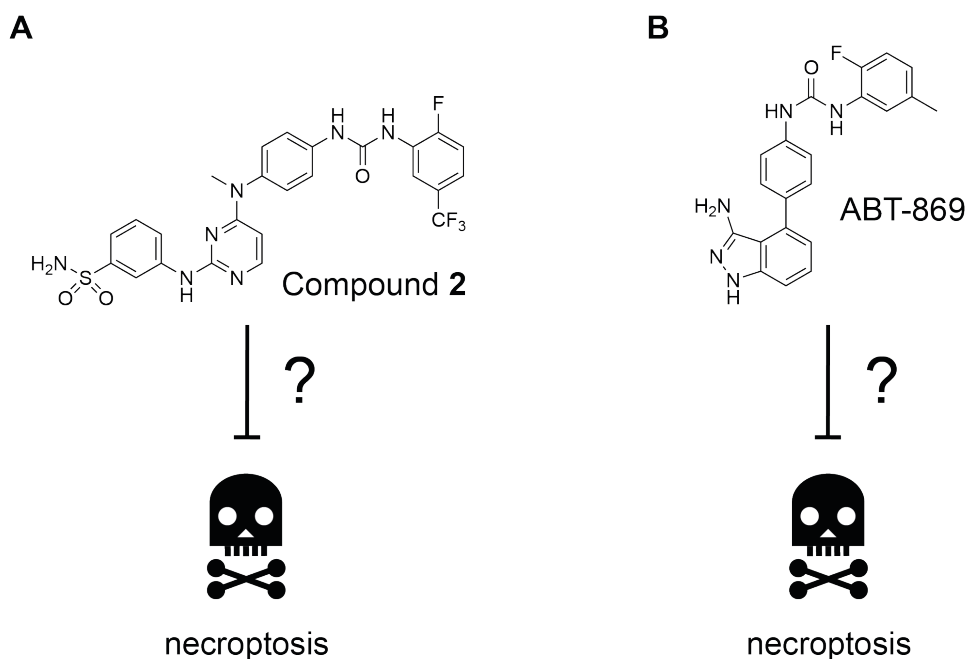


Figure 1.11 Thesis aims and overview: investigating two small molecule inhibitors of necroptosis.

(A) Chapter 2 aims to explore the mechanism of action of Compound **2**.

(B) Chapter 3 aims to identify the cellular targets of ABT-869.

Chapter 2: Potent Inhibition of Necroptosis by Simultaneously Targeting Multiple Effectors of the Pathway

Preamble

This chapter consists of a publication that was published in the journal *ACS Chemical Biology* on 9th September 2020. The article and supporting information are presented here in their entirety. The contributions of the co-authors of this publication are detailed in the Preface. The results table containing the raw data of the TREEspot interaction maps in **Figure 3A** can be found in Appendix 1.

Potent Inhibition of Necroptosis by Simultaneously Targeting Multiple Effectors of the Pathway

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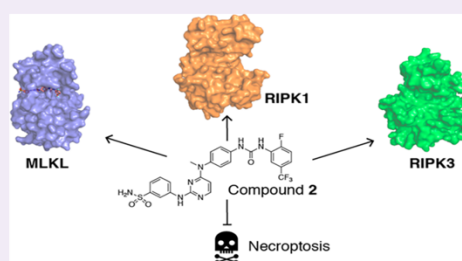
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ABSTRACT: Necroptosis is an inflammatory form of programmed cell death that has been implicated in various human diseases. Compound 2 is a more potent analogue of the published compound 1 and inhibits necroptosis in human and murine cells at nanomolar concentrations. Several target engagement strategies were employed, including cellular thermal shift assays (CETSA) and diazirine-mediated photoaffinity labeling via a bifunctional photoaffinity probe derived from compound 2. These target engagement studies demonstrate that compound 2 binds to all three necroptotic effector proteins (mixed lineage kinase domain-like protein (MLKL), receptor-interacting serine/threonine protein kinase 1 (RIPK1) and receptor-interacting serine/threonine protein kinase 3 (RIPK3)) at different levels *in vitro* and in cells. Compound 2 also shows efficacy *in vivo* in a murine model of systemic inflammatory response syndrome (SIRS).



INTRODUCTION

Necroptosis is a regulated form of cell death for which there is growing evidence of important roles in physiology and disease. Necroptosis can complement other regulated forms of cell death, such as apoptosis, to compensate when the apoptotic machinery is defective or has been disarmed.¹ In contrast to apoptosis, cell and organelle swelling, loss of plasma membrane integrity, and release of cellular contents are all hallmarks of necroptotic death. The pathway can be initiated by extracellular stimuli, such as tumor necrosis factor (TNF) superfamily members and toll-like receptor (TLR) ligands, and intracellular stimuli, such as viral DNA, which can activate DNA-dependent activator of IFN-regulatory factors (DAI)/Z-DNA-binding protein 1 (ZBP1).^{2–4}

The TNF-driven necroptotic response is currently the best understood. The interaction of TNF with its cell surface receptor, TNF receptor 1 (TNFR1), results in the formation of complex 1, a receptor-associated protein complex containing TNFR1, TNFR1-associated death domain (TRADD), TNFR1-associated factor 2 (TRAF2), cellular inhibitor of apoptosis proteins (cIAPs), and receptor-interacting serine/threonine protein kinase 1 (RIPK1).⁵ Under conditions where cIAP activity is impaired, for example, in the presence of Smac-mimetic compounds, a cytosolic protein complex forms containing FAS-associated death domain (FADD), RIPK1, and caspase-8. This complex, termed complex 2a, promotes

caspase-8-mediated apoptosis.⁵ If caspase-8 activity is also blocked, for example, in the presence of caspase inhibitors, the formation of another cytosolic protein complex, termed the necrosome, is triggered.^{6,7} Within the necrosome, RIPK1 binds to receptor-interacting serine/threonine protein kinase 3 (RIPK3) via their RIP homotypic interaction motif (RHIM) domains leading to RIPK3 phosphorylation.^{8–12} Activated RIPK3 phosphorylates and activates mixed lineage kinase domain-like protein (MLKL), which results in the execution of necroptotic cell death.^{13–15}

RIPK1, RIPK3, and MLKL have been established as the three key arbiters of TNF-mediated necroptosis. MLKL is currently regarded as the ultimate effector of the pathway, but how MLKL causes cell death is yet to be fully elucidated. Active MLKL undergoes oligomerization and translocation to cell membranes where it disrupts membrane integrity resulting in the release of cellular contents and death of the cell.^{16–20} This MLKL-driven death has been recently reported to involve

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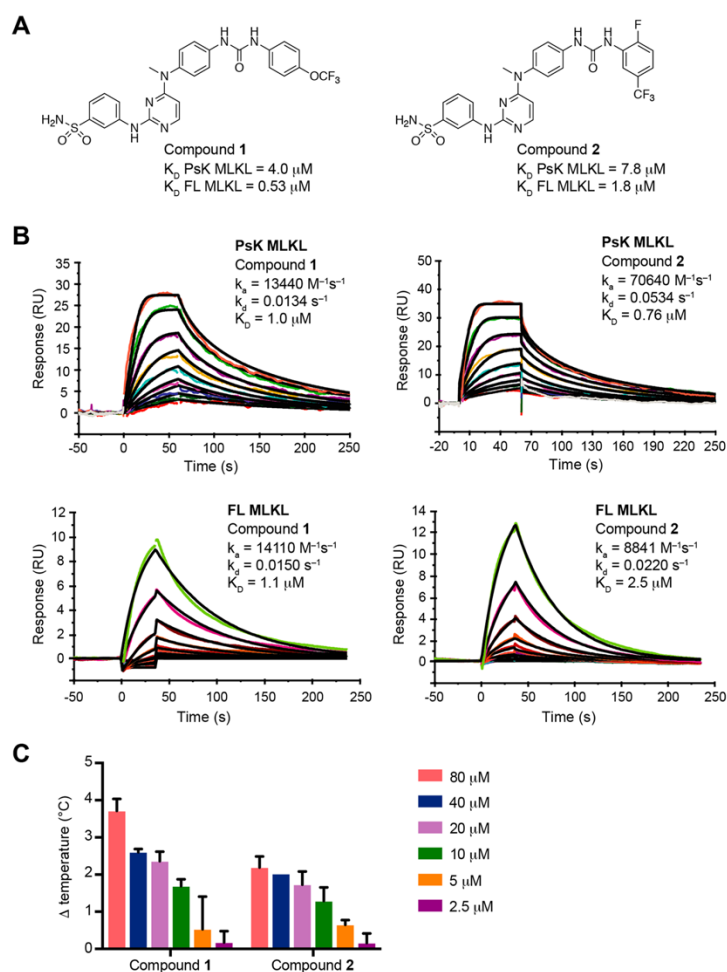


Figure 1. Compound 2 binds to human MLKL. (A) Chemical structure of compound 1 and analogue 2 with their binding affinities (K_D) for the human MLKL pseudokinase domain (PsK) and full-length (FL) proteins, obtained from the DiscoverX KINOMEScan platform using the KdELECT service. Each value is the mean of two replicates. (B) Surface plasmon resonance (SPR) kinetic sensorgrams for compounds 1 and 2 binding to the human MLKL pseudokinase domain (PsK) and full-length (FL) proteins. Increasing concentrations of compounds (0.039 μM to 10 μM for PsK and 0.0024 μM to 2.5 μM for FL) were flowed over MLKL (20 $\mu\text{g mL}^{-1}$ for PsK and 110 $\mu\text{g mL}^{-1}$ for FL) immobilized on the biosensor surface. Data (colored curves) were fitted to a 1:1 binding kinetic interaction model (black curves) to give the association rate (k_a), dissociation rate (k_d), and binding affinity (K_D). One representative result shown from $n = 2$ (PsK) or $n = 3$ (FL) independent experiments. (C) Thermal stability of the human MLKL pseudokinase domain upon binding of compounds 1 and 2 measured by thermal shift assay (TSA). Increasing concentrations of compounds (2.5 μM to 80 μM) were tested for their ability to increase the thermal denaturation temperature of MLKL (2.5 μM). Y-axis depicts the difference in melting temperature between free and ligand-bound MLKL. Data represent the mean of $n = 2$ independent experiments, and error bars represent SD. Individual thermal stability curves are shown Figure S1A,B.

a number of other cellular components including heat shock protein 90 (HSP90),^{21–23} heat shock protein 70 (HSP70),²⁴ endosomal sorting complex required for transport (ESCRT) proteins,^{25,26} and kinases involved in the inositol phosphate signaling pathway.^{27,28} The exact nature of the complexes between RIPK1, RIPK3, and MLKL,^{10,18,29–31} the differences between human, mouse, and other species,^{18,32–35} and the involvement of other proteins in the final stages of necroptosis are of outstanding interest.

Necroptosis has been implicated in the pathophysiology of a variety of diseases, which can be broadly grouped into three classes: inflammatory conditions,^{36–41} infectious diseases,^{10,42–47} and cancer.^{48,49} As a result, there has been increasing interest in developing small molecule inhibitors of necroptosis as potential therapeutics. Indeed, several small molecule inhibitors targeting RIPK1^{50–57} or RIPK3^{49,58–60} have been described. The pseudokinase MLKL, whose role in necroptosis was discovered more recently,^{13–15,61} is the

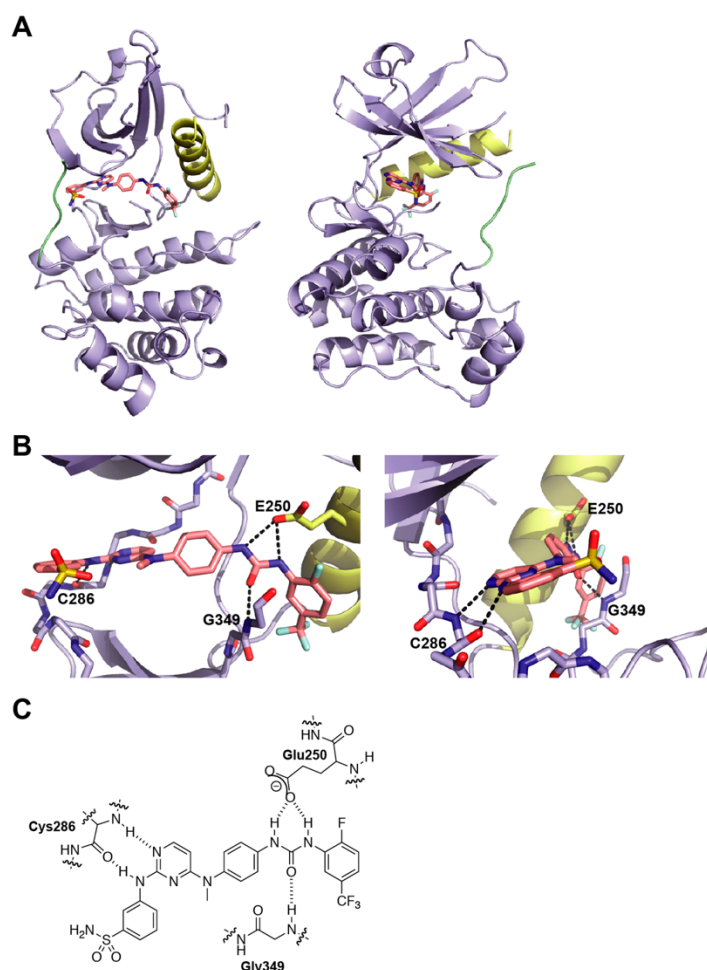


Figure 2. Compound 2 binds in the nucleotide binding pocket of MLKL. (A) X-ray crystal structure of compound 2 bound to the pseudokinase domain of human MLKL at 2.3 Å. The protein is shown in violet, the activation loop in green, the α C helix in yellow, and compound 2 in pink. X-ray crystallography data collection and refinement statistics are shown in Table S1. (B) Compound 2 makes specific interactions with MLKL in the ATP-binding site located between the N-lobe and C-lobe of the pseudokinase domain. See also Figure S1C. (C) Key residues mediating the interaction between compound 2 and MLKL in the ATP-binding pocket.

reported target of a smaller number of inhibitors, including necrosulfonamide,¹³ compound 1,¹⁷ and TC13172.⁶²

We previously reported that compound 1, a small molecule that binds the “pseudoactive” site of the murine MLKL pseudokinase domain, inhibits necroptosis in murine cells.¹⁷ In the present study, we report the identification of compound 2, a more potent necroptosis inhibitor derived from compound 1. We demonstrate that compound 2 binds to MLKL, RIPK1, and RIPK3 at different levels to potently block necroptosis in human cells at nanomolar concentrations. We also show that compound 2 provides protection in a murine model of TNF-induced systemic inflammatory response syndrome (SIRS). In the course of this study, we developed a chemical biology tool that validates this series’ engagement of MLKL, RIPK1, and RIPK3. Collectively, our data demonstrate that this pharmacophore is active in both murine and human cells and confirm

MLKL, RIPK1, and RIPK3 as targets underlying the antinecrotic activity of this series.

RESULTS AND DISCUSSION

Compound 2 Binds to Human MLKL. We previously identified an MLKL binder, compound 1, from a small molecule screen of a kinase inhibitor library against the MLKL pseudokinase domain.¹⁷ We showed that compound 1, originally described by GlaxoSmithKline (GSK) as a vascular endothelial growth factor receptor 2 inhibitor,⁶³ binds to the nucleotide binding site of the murine MLKL pseudokinase domain and inhibits necroptosis in murine cells in a dose-dependent manner.¹⁷ Using this chemical scaffold, and information from the original GSK series,⁶³ we performed structure–activity relationship studies to identify more potent

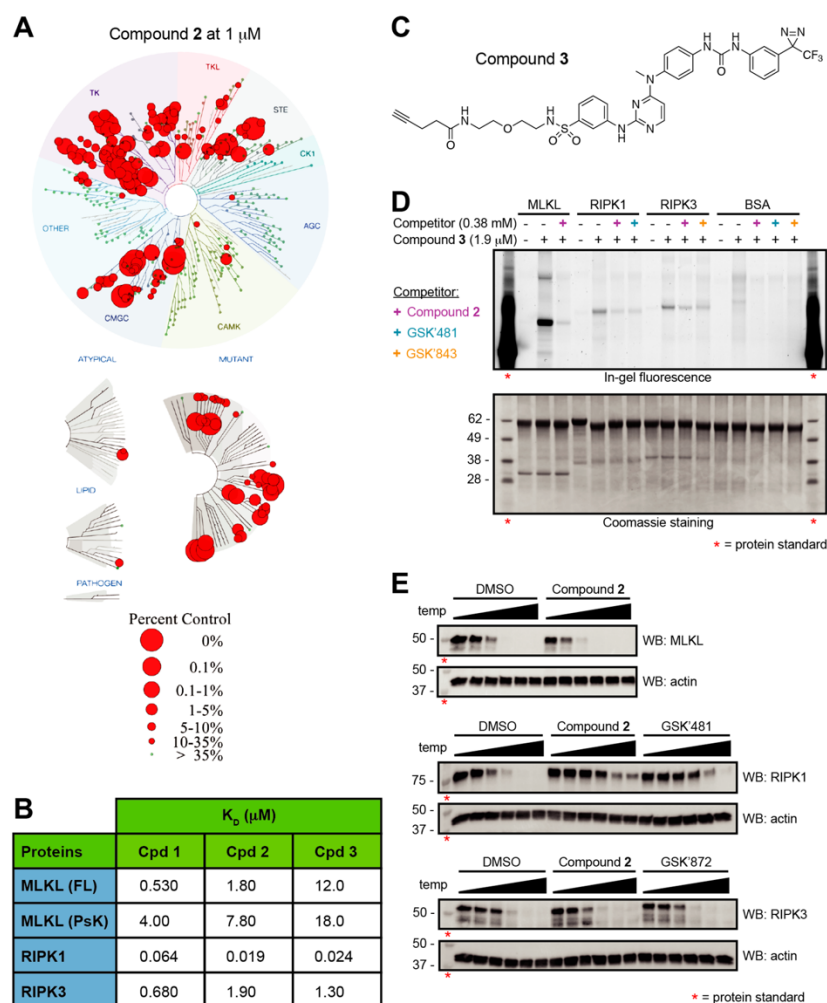


Figure 3. Compound 2 interacts with the other necroptotic effectors, RIPK1 and RIPK3, in addition to MLKL, *in vitro* and in cells. (A) TREEspot interaction maps depicting the kinome selectivity profile of compound 2, obtained from the DiscoverX KINOMEScan platform using the scanMAX service. Compound 2 was profiled against 468 kinases (including atypical, lipid, pathogen, and disease relevant mutant kinases) at 1 μM . Binding affinities are reported as percent control, represented schematically by the size of the red circles, where larger circles indicate higher affinity binding. See also Tables S2 and S3. (B) Binding affinities (K_D) of compounds (Cpd) 1, 2, and 3 for human full-length (FL) MLKL, MLKL pseudokinase domain (PsK), RIPK1 kinase domain, and RIPK3 kinase domain obtained from the DiscoverX KINOMEScan platform using the KdELECT service. Each value is the mean of two replicates. (C) Chemical structure of compound 3, a bifunctional photoaffinity probe derived from compounds 1 and 2. The diazine moiety enables covalent photo-cross-linking to target proteins, while the alkyne enables attachment of an azide-containing reporter via CuAAC click chemistry. (D) *In vitro* photoaffinity labeling with compound 3 and recombinant MLKL, RIPK1, and RIPK3. Compound 3 (1.9 μM) and the specified competitor (compound 2, RIPK1 inhibitor GSK'481, RIPK3 inhibitor GSK'843; 0.38 mM) were incubated with the MLKL pseudokinase domain, RIPK1 kinase domain, RIPK3 kinase domain or BSA (0.07 mg mL⁻¹). Samples were irradiated with UV light (1 min), labeled with TAMRA-azide, resolved by SDS-PAGE, and visualized by in-gel fluorescence scanning (upper panel) then stained with Coomassie (Brilliant Blue, lower panel). Red asterisks denote protein standards. One representative result shown from $n = 5$ independent experiments. (E) Cellular thermal shift assays (CETSA) in human U937 cells were used to perform cellular target validation. Cells treated with compound 2, RIPK1 inhibitor GSK'481, or RIPK3 inhibitor GSK'872 (all 20 μM) were subjected to an increasing temperature gradient. Following separation of soluble and insoluble proteins, the remaining soluble proteins were detected by Western Blot. Red asterisks denote protein standards. One representative result shown from $n = 2-3$ independent experiments. See also Figure S4A.

necroptosis inhibitors, leading to the identification of the 2-fluoro-5-trifluoromethyl analogue, compound 2 (Figure 1A). Using a competition binding assay from the DiscoverX

KINOMEScan platform,⁶⁴ where compound binding to a given kinase or pseudokinase is measured through competition with an active/pseudoactive site directed probe, we observed

that compound **2** bound to the human MLKL pseudokinase domain (PsK) with a binding affinity (K_D) of $7.8\ \mu\text{M}$ and to full-length human MLKL (FL) with a K_D of $1.8\ \mu\text{M}$ (Figure 1A). This was similar to compound **1**, with binding affinities of $4.0\ \mu\text{M}$ and $0.53\ \mu\text{M}$ for PsK MLKL and FL MLKL, respectively.

The ability of these small molecules to interact with human MLKL *in vitro* was confirmed by Surface Plasmon Resonance (SPR) in a direct binding assay. In these experiments, the human MLKL pseudokinase domain (PsK) or full-length (FL) protein was immobilized to the biosensor surface, and binding kinetics were assessed at increasing concentrations of each compound (Figure 1B). Compound **2**, with a K_D of $0.76\ \mu\text{M}$ for PsK MLKL and $2.5\ \mu\text{M}$ for FL MLKL, again showed similar binding affinity to the parent compound **1**, which bound PsK and FL MLKL with a K_D of $1.0\ \mu\text{M}$ and $1.1\ \mu\text{M}$, respectively. The sensorgrams of both compounds **1** and **2** displayed slow association (k_a) and dissociation (k_d) rates, suggesting that formation of the binding complex required substantial protein conformational changes but that this complex was very stable once formed. To further validate these compounds as MLKL interactors *in vitro*, we used thermal shift assays (TSAs) to assess binding to the human MLKL pseudokinase domain (Figure 1C, Figure S1A,B). Increasing concentrations of both compounds **1** and **2** resulted in an increase in the temperature difference of thermal denaturation between free and ligand-bound human MLKL. This confirmed that compounds **1** and **2** bind to the pseudokinase domain and validated these small molecules as human MLKL binders.

Compound 2 Binds in the Nucleotide Binding Pocket of MLKL. To determine the binding mode of compound **2** to human MLKL, compound **2** was cocrystallized with the human MLKL pseudokinase domain and the X-ray crystal structure was solved to $2.3\ \text{\AA}$ (Figure 2, Figure S1C, Table S1). The crystal structure of the MLKL–compound **2** complex demonstrated that the small molecule bound in the ATP-binding, “pseudoactive” site located between the N-lobe and C-lobe of the pseudokinase domain (Figure 2A,B). Compound **2** formed three key hydrogen bonding interactions with MLKL involving residues Glu250, Cys286, and Gly349 (Figure 2C). Notably, Glu250 is the αC helix glutamate that pairs with the $\beta 3$ VAIK motif ATP-binding lysine 230 in the unliganded structure, while Gly349 is part of the GFE motif present in MLKL orthologs in place of the DFG motif found at the start of the activation loop in conventional (active) protein kinases.⁶⁵

While the cocrystal structure did not provide any obvious explanations for the slow on and off rates observed by SPR (Figure 1B), both the SPR binding kinetics and the binding mode observed in the crystal structure are reminiscent of classical type II kinase inhibitors. Type II inhibitors are characterized by their ability to occupy the ATP pocket in the inactive “DFG-out” kinase conformation, in comparison to type I inhibitors that occupy the ATP pocket in the active “DFG-in” conformation. However, there are key differences between the active sites of kinases and pseudokinases. While kinases have well-defined ATP-binding pockets and catalytic residues, pseudokinases variously lack ATP binding or catalytic residues from their “pseudoactive” sites.⁶⁵ In our cocrystal structure, residues of the activation loop following Gly349 are not resolved. However, superimposition with the crystal structure of apo human MLKL (pseudokinase domain, PDB

4MWI) revealed that compound **2** and the GFE motif occupy overlapping positions (Figure S1C). This indicates that the GFE would need to adopt the “out” conformation to allow for compound **2** binding. Our findings suggest that, despite important differences, both kinases and pseudokinases are able to bind type II inhibitors in a similar fashion.

Compound 2 Interacts with the Other Necroptotic Effectors, RIPK1 and RIPK3, in Addition to MLKL, *in Vitro* and in Cells. As compound **1** was originally described as an inhibitor of VEGFR2 and other kinases,⁶³ we sought to assess the broader kinase selectivity of our compounds across the kinome. Using the DiscoverX scanMAX kinase assay panel, compound **2** was evaluated against 468 kinases including atypical, lipid, pathogen, and disease relevant mutant kinases (Figure 3A, Table S2, and Table S3). Compound **2** exhibited a similar kinase interaction repertoire to that previously reported for compound **1**.⁶⁶ Among the compound **2** targets revealed in this kinase assay panel was the necroptotic effector RIPK1. RIPK3, the other key necroptotic effector kinase, was not featured in this panel. Therefore, we investigated the binding of our compounds to RIPK1 and RIPK3 more specifically using individual binding assays from the DiscoverX KINOMEScan platform. In these assays, compound **1** was observed to bind RIPK1 with a K_D of $64\ \text{nM}$ and RIPK3 with a K_D of $0.68\ \mu\text{M}$, while compound **2** bound to RIPK1 and RIPK3 with a K_D of $19\ \text{nM}$ and $1.90\ \mu\text{M}$, respectively (Figure 3B). Together with the binding observed to MLKL (Figure 1A), these data suggest that both compounds can interact with all three key necroptotic effector proteins, MLKL, RIPK1, and RIPK3, *in vitro* and most likely compete with ATP binding in the active or pseudoactive site of these proteins.

Photoaffinity labeling has been established as a method to demonstrate target engagement and to identify protein targets of small molecules.⁶⁷ To further validate the ability of compounds **1** and **2** to bind MLKL, RIPK1, and RIPK3 and to assess the binding to these three proteins using an orthogonal assay, *in vitro* photoaffinity labeling was employed using a probe derived from the parent scaffold. The bifunctional photoaffinity probe that we designed (compound **3**) contained a diazirine moiety and a terminal alkyne (Figure 3C). The diazirine group enables covalent photo-cross-linking between the probe and its target proteins via the highly reactive carbene intermediate formed upon irradiation with UV light at $\sim 350\ \text{nm}$.⁶⁷ The terminal alkyne enables attachment of an azide-containing reporter, such as a tetramethylrhodamine (TAMRA) fluorophore, via the copper(I)-catalyzed azide–alkyne cycloaddition (CuAAC) or “click chemistry” reaction.⁶⁷ The positions of these two chemical handles on the parent scaffold were designed based on the orientation of compound **2** within the MLKL binding site in the crystal structure (Figure 2). The diazirine was installed on the terminal phenyl ring buried deep within the binding pocket to ensure specificity and efficiency of cross-linking. The terminal alkyne was installed on the aryl-sulfonamide moiety, which protruded from the active site and extended into solvent space. The DiscoverX KINOMEScan binding assays indicated that addition of these moieties did not impact the ability of compound **3** to bind RIPK1 or RIPK3, although they did somewhat impact binding to MLKL, when compared with compound **2** (Figure 3B). The slight decrease in binding affinity of compound **3** for MLKL is most likely due to the diazirine, which does not fully recapitulate the 2-fluoro-5-trifluoromethyl substituents. As the terminal alkyne would extend into solvent space, it is

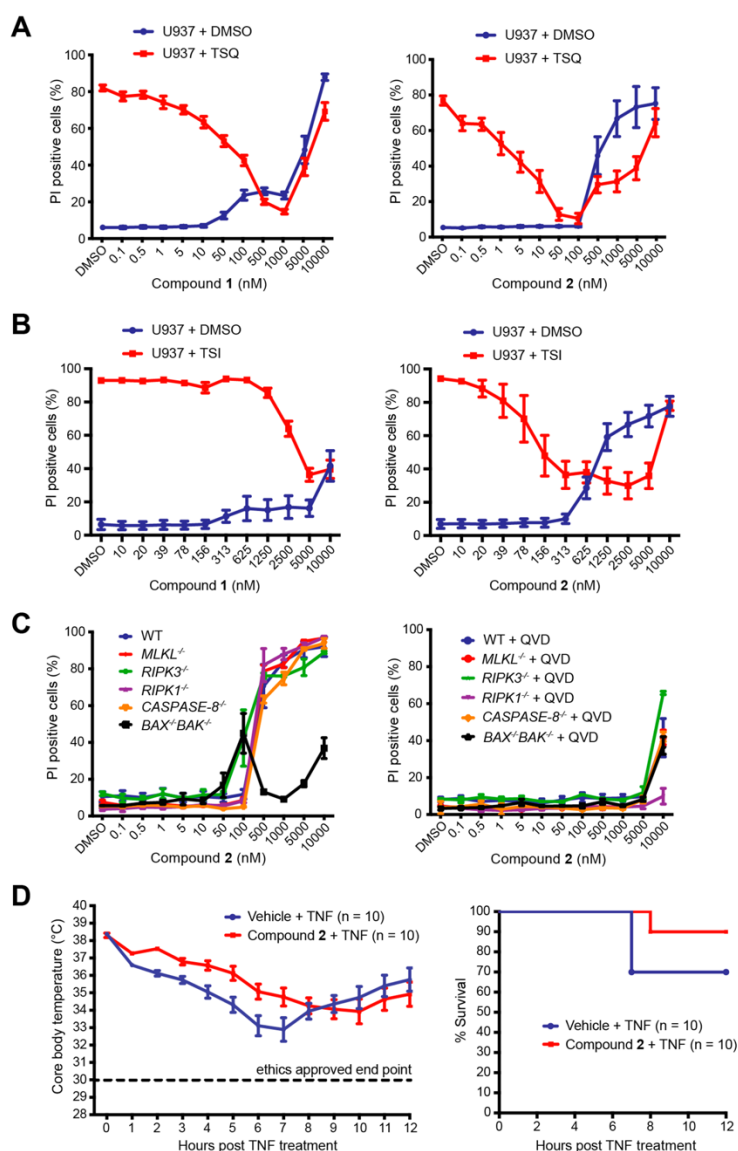


Figure 4. Compound 2 efficiently blocks necroptosis in human cells and shows modest protection *in vivo*. (A) U937 cells were stimulated with TSQ (TNF, Smac-mimetic, Q-VD-OPh) to induce necroptosis and treated with increasing concentrations of compounds 1 or 2. Cell death was quantified by propidium iodide (PI) staining using flow cytometry. Data represent the mean of $n = 10$ and $n = 4$ independent experiments for compound 1 and compound 2, respectively, and error bars represent SEM. See also Figures S2 and S3A–C. (B) U937 cells were stimulated with TSI (TNF, Smac-mimetic, IDN-6556) to induce necroptosis and treated with increasing concentrations of compound 1 or 2. Cell death was quantified by PI staining using flow cytometry. Data represent the mean of $n = 4$ independent experiments, and error bars represent SEM. See also Figures S2 and S3A–C. (C) Compound 2 was tested against a range of U937 cell lines lacking key necroptotic or apoptotic effector proteins to determine the cause of the toxicity observed at higher concentrations in panels A and B. The cell lines were treated with increasing concentrations of compound 2 in the absence or presence of the caspase inhibitor Q-VD-OPh (QVD). Cell death was quantified by PI staining using flow cytometry. Data represent the mean of $n = 3$ independent experiments, and error bars represent SEM. See also Figure S3D. (D) Compound 2 was assessed in a murine model of TNF-induced systemic inflammatory response syndrome (SIRS). C57BL/6 female mice aged 9.5 weeks were treated via IP injection with either 40 mg kg⁻¹ compound 2 or vehicle alone for 30 min, followed by 300 μg kg⁻¹ TNF via IV injection. Core body temperature was measured hourly for 12 h after TNF treatment. The experiment was performed blinded with $n = 10$ mice per group, and error bars represent SEM. See also Figure S4B–D.

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unlikely to affect binding interactions between compound 3 and MLKL.

In vitro photoaffinity labeling experiments with compound 3 were performed by incubating this photoaffinity probe with the human MLKL pseudokinase domain, RIPK1 kinase domain, RIPK3 kinase domain, and BSA (as a control) at a 1:1 ratio of probe to protein. All samples were then spiked with a 20-fold excess of BSA. Following UV irradiation, attachment of TAMRA via click chemistry, and in-gel fluorescence scanning, we found that compound 3 labeled all three necroptotic effectors, but not BSA (Figure 3D). Interestingly, the labeling was strongest for MLKL, despite having a weaker binding affinity than RIPK1 and RIPK3 in the DiscoverX KINOMEScan binding assays. This may reflect the differences between residues within the active sites of RIPK1 and RIPK3 and the pseudoactive site of MLKL, which lend themselves to photo-cross-linking more readily within MLKL. The labeling of MLKL, RIPK1, and RIPK3 by compound 3 was competed by preincubation with compound 2, confirming that the photo-cross-linking was specific. Moreover, compound 2 competed the labeling of RIPK1 and RIPK3 with similar efficiency to the known RIPK1 inhibitor GSK'481⁵⁶ and RIPK3 inhibitor GSK'843.⁵⁸ These data again demonstrate that compounds 2 and 3 bind to all three key necroptotic effectors, MLKL, RIPK1, and RIPK3, *in vitro*.

To determine whether these compounds bind to MLKL, RIPK1, and RIPK3 in cells, cellular thermal shift assays (CETSA)⁶⁸ were used to perform cellular target validation. U937 cells, a human lymphoma cell line known to express RIPK1, RIPK3, and MLKL and undergo necroptosis, were treated with compound 2 and subjected to a temperature gradient, focused around the melting temperature of each protein of interest. Following the separation of soluble and insoluble protein fractions by centrifugation, proteins in the soluble fraction were analyzed by Western blot (Figure 3E). Compound 2 markedly increased the thermal stability of RIPK1, to a greater extent than the known human RIPK1 inhibitor GSK'481,⁵⁶ demonstrating that compound 2 binds to human RIPK1 in cells. Interestingly, compound 2 decreased the thermal stability of MLKL and, to a lesser extent, RIPK3. Compound 2 was also observed to strongly stabilize RIPK1, destabilize MLKL, and slightly destabilize RIPK3 in murine dermal fibroblasts (MDFs) (Figure S4A). As both increases and decreases in protein thermal stability upon compound treatment are indicative of target engagement in CETSA,⁶⁹ these data collectively indicate that compound 2 binds to MLKL, RIPK1, and RIPK3 in both human and murine cells.

Compound 2 Efficiently Blocks Necroptosis in Human Cells and Shows Modest Protection *In Vivo*.

We next sought to establish whether the ability of our compounds to bind MLKL, RIPK1, and RIPK3 resulted in the inhibition of necroptosis in cellular assays. To evaluate their ability to block necroptosis in human cells, compounds 1 and 2 were tested in a series of cell death assays. U937 cells stimulated with the necroptosis-inducing cocktail TSQ (TNF, Smac-mimetic, Q-VD-OPh) were treated with increasing concentrations of either compound 1 or compound 2, and cell death was measured by propidium iodide (PI) uptake and flow cytometry (Figure 4A). Increasing concentrations of both compounds resulted in marked inhibition of necroptotic cell death. Compound 2 displayed low nanomolar inhibition of necroptosis and blocked cell death more potently than compound 1. Similarly, when necroptosis was induced by a

more potent necroptotic stimulus, TSI (TNF, Smac-mimetic, IDN-6556), in U937 cells, compound 2 inhibited cell death at nanomolar concentrations with greater potency than compound 1 (Figure 4B).

We then sought to evaluate whether these small molecules were able to inhibit necroptosis across a range of human cell lines. In HT29 cells, a human colon cancer cell line, stimulated with either TSI or TSQ to trigger necroptosis, compound 2 blocked cell death at nanomolar concentrations (Figure S2A,B). Consistent with the U937 cell death assays, compound 2 more potently inhibited necroptosis in HT29 cells than compound 1. Both compound 1 and compound 2 also blocked TSI-stimulated death in THP-1 cells (Figure S2C), a human monocytic leukemia cell line, which undergo necroptosis upon TSI but not TSQ treatment (Figure S3C). To confirm that our photoaffinity probe retained similar cellular activity to compounds 1 and 2 in human cells, compound 3 was also evaluated in necroptotic cell death assays in U937, HT29, and THP-1 cells (Figure S2D). In agreement with the *in vitro* binding assays (Figure 3B), the addition of the diazirine and terminal alkyne moieties did not abolish the antinecroptotic activity of the scaffold in these cellular assays. Together, our cellular data demonstrate that compounds 1, 2, and 3 are inhibitors of necroptotic cell death induced by TSQ and TSI in several human cell lines.

It was observed that higher concentrations of these compounds induced cell death in both the presence and absence of necroptotic stimuli (Figure 4A,B and Figure S2). To explore the type of cell death elicited by higher concentrations of compound 2, we tested U937 cell lines engineered to lack key necroptotic or apoptotic effector proteins (Figure 4C). These cell lines were subjected to increasing concentrations of compound 2 in the absence or presence of Q-VD-OPh. Concentrations of compound 2 exceeding 100 nM were found to induce cell death independently of MLKL, RIPK3, RIPK1, or caspase-8. This death was largely inhibited by Q-VD-OPh indicating that the apoptosis pathway was likely responsible. Consistent with this, such death was largely absent in BAX^{-/-}BAK^{-/-} U937 cells, apart from a transient and limited increase in cell death between 50 nM and 500 nM. The blockade of this cell death response by Q-VD-OPh suggested that it was caspase-dependent, yet BAX/BAK-independent. We further evaluated the toxicity of compound 2 in HeLa cells, which are not susceptible to necroptotic stimuli, to determine whether its toxicity profile matched that observed in our engineered U937 cell lines. HeLa cells endogenously lack RIPK3 and are unable to undergo necroptosis.⁹ When treated with increasing concentrations of compound 2, cell death was observed at similar concentrations and this death was largely inhibited by Q-VD-OPh (Figure S3D), mirroring the effect observed in the engineered U937 cell lines. These various instances of cell death independent of necroptosis stimulation could potentially result from the complex polypharmacology of compound 2, which would likely inhibit a number of kinases at these concentrations, based on the known kinase inhibition profile of compound 2 (Figure 3A). Importantly, these data indicate that the cell death triggered by higher concentrations of compound 2 is not dependent on the necroptotic effectors RIPK1, RIPK3 and MLKL. Therefore, concentrations of compound 2 up to 100 nM are optimal for use in necroptotic cell death assays, to both enable inhibition of cell death and avoid toxicity due to non-necroptotic targets.

Having established compound **2** as a nanomolar inhibitor in cells, compound **2** was next evaluated *in vivo* using a TNF-induced systemic inflammatory response syndrome (SIRS) model (Figure 4D). We first verified that compound **2** was similarly active in murine cells (Figure S4B) and then assessed its pharmacokinetic (PK) profile in female C57BL/6 mice (Figure S4C). In the TNF-SIRS murine model, TNF induces a robust inflammatory response resulting in hypothermia and lethality, which is rescued by the deletion of *Ripk3* or *Mkl1*.^{70–72} Similarly, we found that deletion of *Ripk3* provided protection and deletion of *Mkl1* provided partial protection against lethal core body temperature decline in this model (Figure S4D). Mice pretreated for 30 min with compound **2** at 40 mg kg^{−1} via intraperitoneal injection showed a delayed decrease in core body temperature compared to vehicle only treated mice (Figure 4D). This correlated with a modest delay in hypothermia (average minimum temperature for compound **2** treated group reached at *t* = 10 h compared to *t* = 7 h for the vehicle only treated group) and an increased overall 12 h survival of mice treated with compound **2** (1 death at *t* = 8 h) compared to vehicle (3 deaths at *t* = 7 h). Despite the dose-dependent toxicity of compound **2** observed *in vitro*, the toxicity *in vivo* was not an issue over the 12 h duration of the SIRS model. Initial biosafety experiments, conducted by dosing female C57BL/6 mice daily with compound **2** at 40 mg kg^{−1}, showed that this treatment was well tolerated for up to 10 days, but mice then began to exhibit weight loss and treatment could not be prolonged beyond 12 days (unpublished data). This demonstrated that the adverse effects on the mice caused by compound **2** were well beyond the time frame of the SIRS model.

A 2016 report suggested that compound **1** provides only weak protection against necroptosis in one cell line (FADD-deficient Jurkat cells) and suggested that small molecules binding to the ATP-binding pocket of MLKL cannot inhibit necroptotic cell death.⁶⁶ In contrast, our studies in murine cells¹⁷ and in multiple human cell lines (this work) demonstrate that compounds **1**, **2**, and **3** are genuine inhibitors of necroptosis. Given that these compounds interact with all three key necroptotic effectors MLKL, RIPK1, and RIPK3 *in vitro* and in cells, and that these proteins operate in the same pathway, it is difficult to ascertain the individual contribution of inhibiting each one. While the compounds exhibited higher affinity for RIPK1 than for MLKL or RIPK3 (Figure 3B), the combined inhibition of all three proteins may contribute to their nanomolar cellular activity. Our studies also leave open the possibility that compounds binding to the ATP pocket of MLKL might be effective inhibitors of necroptosis.

In conclusion, compound **2** is a nanomolar inhibitor of necroptosis. It targets all three key necroptotic effectors, MLKL, RIPK1, and RIPK3, at different levels *in vitro* and in cells. As a result, it is a potent inhibitor in a range of human cell lines and provides protection in a murine model of systemic inflammatory response syndrome (SIRS). Cellular thermal shift assays (CETSA) were used to perform target validation in human and murine cells. The photoaffinity probe compound **3** was used to confirm target engagement via diazirine-mediated photoaffinity labeling and, as such, is a useful tool to study necroptosis at the molecular level.

■ ASSOCIATED CONTENT

Supporting Information

Supplementary Tables S3, (XLS). The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acscchembio.0c00482>.

Binding of compounds **1** and **2** to MLKL, results of necroptosis evaluation in human cells for compounds **1**–**3**, response of human cell lines to necroptotic stimuli and inhibitors, compound **2** engagement of MLKL, RIPK1 and RIPK3, compound **2** PK profile, and results of *Mkl1* and *Ripk3* genetic deletion in the *in vivo* SIRS model, X-ray crystallographic data collection and refinement statistics, selectivity score results for compound **2** from kinase assay panel, and detailed methods and compound syntheses (PDF)

Complete list of 468 kinases tested in compound **2** kinome selectivity profile from DiscoverX KINOMEScan scanMAX kinase assay panel (XLSX)

Accession Codes

Coordinates of the MLKL pseudokinase domain in complex with compound **2** have been deposited in the Protein Data Bank (PDB) with the accession code 6O5Z.

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Notes

The authors declare the following competing financial interest(s): John Silke was formerly a scientific advisory board member of Tetralogic Pharmaceuticals. All authors

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Potent Inhibition of Necroptosis by Simultaneously Targeting Multiple Effectors of the Pathway

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SUPPLEMENTARY FIGURES

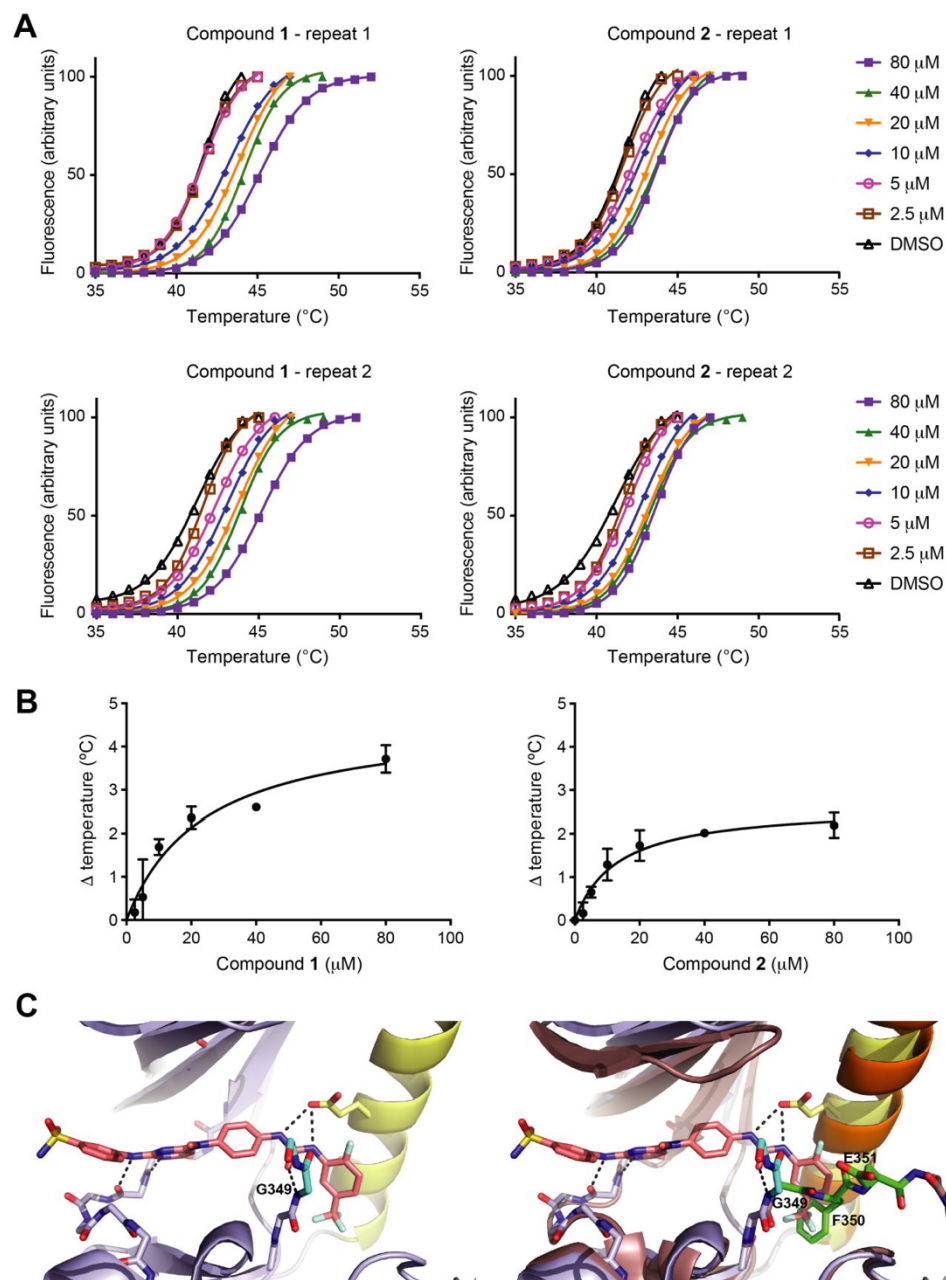


Figure S1. Compounds **1** and **2** bind to human MLKL in the nucleotide binding pocket.

(A) Increasing concentrations of Compounds **1** and **2** (2.5 μM to 80 μM) were tested for their ability to increase the thermal denaturation temperature of the human MLKL pseudokinase domain (2.5 μM) measured by Thermal Shift Assay (TSA).

(B) Difference in melting temperature between free and ligand-bound MLKL observed upon increasing concentrations of Compounds **1** and **2** compared to the DMSO control. Data represent the mean of the $n = 2$ independent experiments shown in **(A)** and error bars represent SD.

(C) X-ray crystal structure of Compound **2** (pink) bound to the human MLKL pseudokinase domain (violet with αC helix in yellow). In the region of the GFE motif, the Gly349 (cyan) main chain forms an hydrogen bond to Compound **2**; the activation loop after Gly349 is not resolved (left panel). When overlaid with the crystal structure of apo human MLKL pseudokinase domain (PDB: 4MWI,¹ mauve with αC helix in orange), Compound **2** and the GFE motif (green) occupy the same space (right panel). This indicates that the GFE would need to be in the "out" conformation to allow for Compound **2** binding.

Figures **S1A** and **S1B** are related to Figure **1C**.

Figure **S1C** is related to Figure **2**.

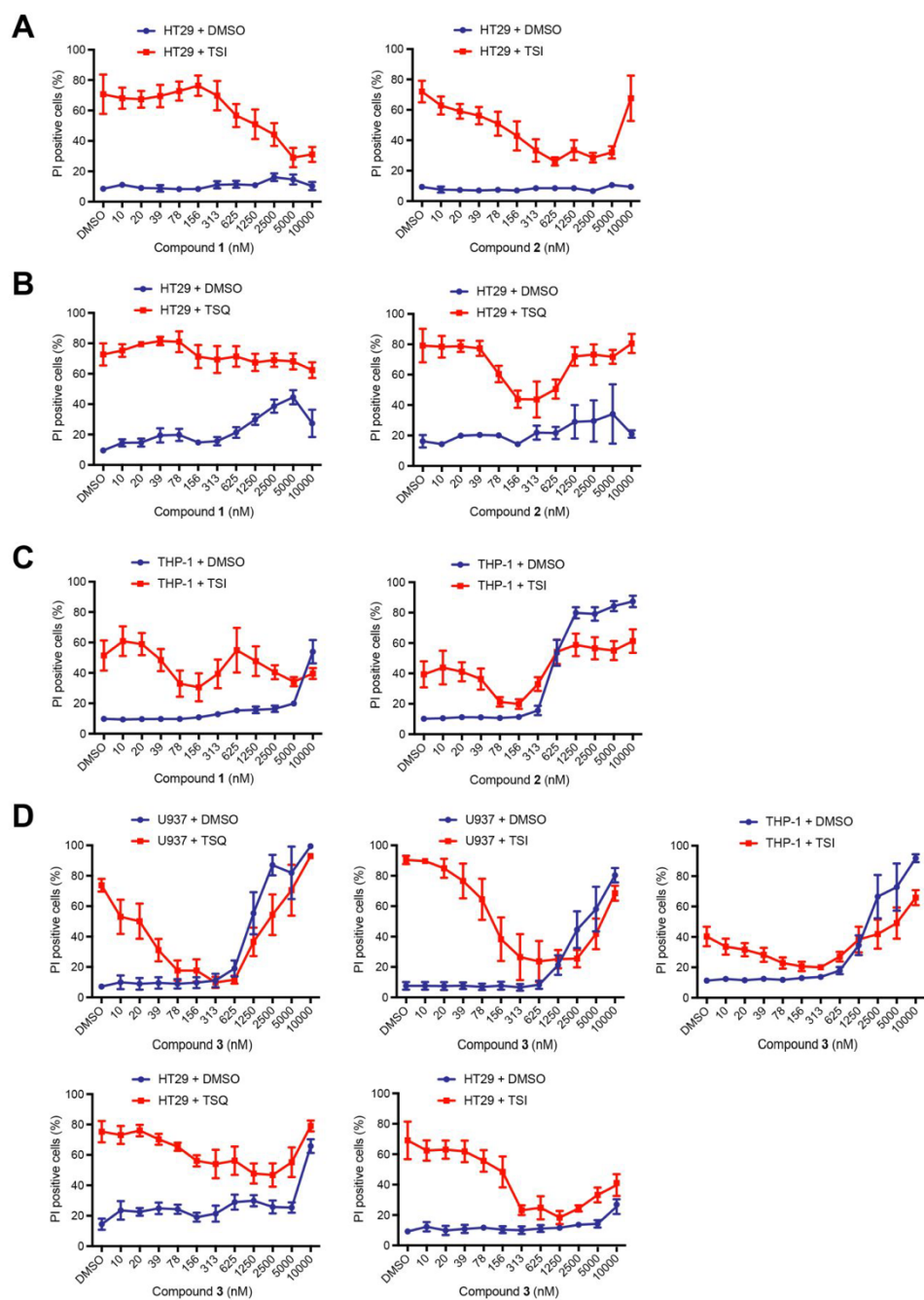


Figure S2. Compounds 1, 2 and 3 block necroptosis in human cells.

(A) HT29 cells were stimulated with TSI (TNF, Smac-mimetic, IDN-6556) to induce necroptosis and treated with increasing concentrations of Compound 1 or 2.

(B) HT29 cells were stimulated with TSQ (TNF, Smac-mimetic, Q-VD-OPh) to induce necroptosis and treated with increasing concentrations of Compound 1 or 2.

(C) THP-1 cells were stimulated with TSI to induce necroptosis and treated with increasing concentrations of Compound 1 or 2.

(D) U937, HT29 or THP-1 cells were stimulated with TSQ or TSI to induce necroptosis and treated with increasing concentrations of Compound 3.

For **(A–D)** cell death was quantified by propidium iodide (PI) staining using flow cytometry. Data represent the mean of $n = 3$ –5 independent experiments and error bars represent SEM.

Figure S2 is related to **Figures 4A** and **4B**.

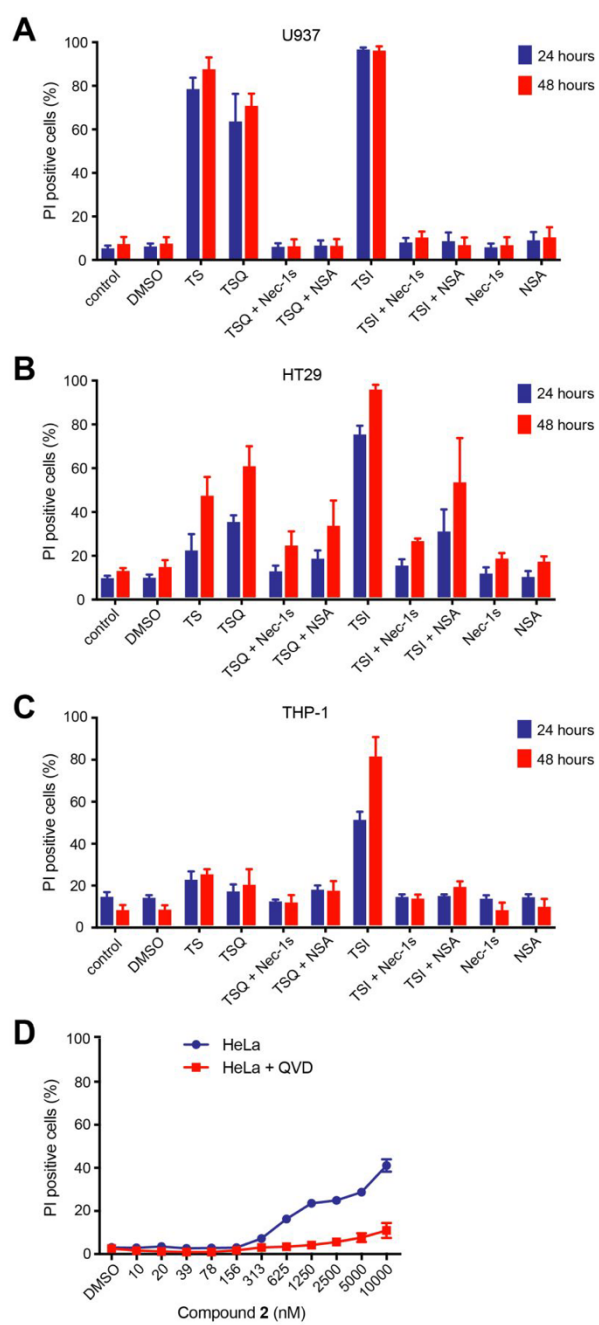


Figure S3. Response of human cell lines to necroptotic stimuli and inhibitors.

(A–C) U937 **(A)**, HT29 **(B)** and THP-1 **(C)** cells were stimulated with TSQ (TNF, Smac-mimetic, Q-VD-OPh) or TSI (TNF, Smac-mimetic, IDN-6556) to induce necroptosis and treated with the necroptosis inhibitors Nec-1s (5 μ M) and NSA (1 μ M) as controls.

(D) HeLa cells, which endogenously lack RIPK3 and are unable to undergo necroptosis,² were treated with increasing concentrations of Compound **2** in the absence or presence of the caspase inhibitor Q-VD-OPh (QVD).

For **(A–D)** cell death was quantified by propidium iodide (PI) staining using flow cytometry. Data represent the mean of $n = 3$ –5 independent experiments and error bars represent SEM.

Figures S3A–C are related to **Figures 4A** and **4B**.

Figure S3D is related to **Figure 4C**.

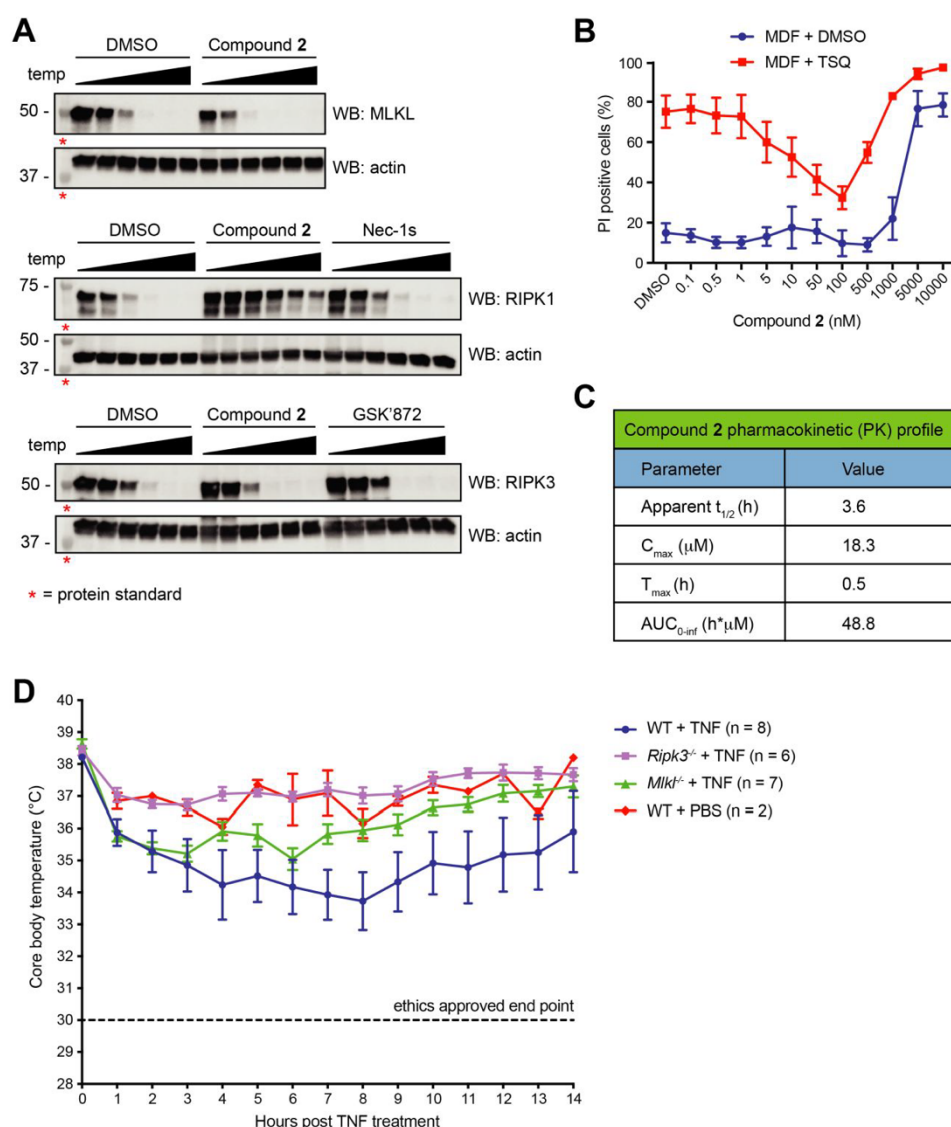


Figure S4. Compound **2** engages MLKL, RIPK1 and RIPK3 to block necroptosis in murine cells; Compound **2** *in vivo* pharmacokinetic (PK) profile; and the impact of genetic deletion of *Mlkl* and *Ripk3* in an *in vivo* model of TNF-induced SIRS.

(A) Cellular Thermal Shift Assays (CETSA) in murine dermal fibroblast (MDF) cells were used to perform cellular target validation. Cells treated with Compound **2**, RIPK1 inhibitor Nec-1s or RIPK3 inhibitor GSK'872 (all 20 μ M) were subjected to an increasing temperature gradient. Following separation of soluble and insoluble proteins, the remaining soluble proteins were detected by Western Blot. Red asterisks denote protein standards. One representative result shown from $n = 3$ independent experiments.

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(B) MDF cells were stimulated with TSQ (TNF, Smac-mimetic, Q-VD-OPh) to induce necroptosis and treated with increasing concentrations of Compound 2. Cell death was quantified by propidium iodide (PI) staining using flow cytometry. Data represent the mean of $n = 3$ independent experiments and error bars represent SEM.

(C) Pharmacokinetic studies of the systemic exposure of Compound 2 in female C57BL/6 mice following IP administration at 40 mg kg^{-1} , performed by the Centre for Drug Candidate Optimisation. Blood samples were collected over a 24 hour period and analyzed by LC-MS to determine the pharmacokinetic parameters: apparent half-life (apparent $t_{1/2}$), maximum plasma concentration (C_{max}), time at which maximum plasma concentration occurs (T_{max}) and total plasma exposure over time ($\text{AUC}_{0-\text{inf}}$).

(D) Murine model of TNF-induced systemic inflammatory response syndrome (SIRS). C57BL/6 mice on a wild type, *Mlkl*^{-/-} or *Ripk3*^{-/-} background aged 8–15 weeks were treated with either $300 \mu\text{g kg}^{-1}$ TNF or DPBS alone via IV injection. Core body temperature was measured hourly for 14 hours post TNF treatment. Experiment was performed blinded with $n = 2\text{--}8$ mice per group and error bars representing SEM.

Figure S4A is related to Figure 3E.

Figures S4B–D are related to Figure 4D.

SUPPLEMENTARY TABLES

Table S1. X-ray crystallography data collection and refinement statistics.

Table S1 is related to Figure 2 and Figure S1C.

Structure ^a	MLKL pseudokinase domain in complex with Compound 2
Wavelength	0.9537
Resolution range	47.08–2.28 (2.36–2.28)
Space group	P 1 21 1
Unit cell	50.20 118.81 52.73 90 116.78 90
Total reflections	93506 (8083)
Unique reflections	24748 (2205)
Multiplicity	3.8 (3.7)
Completeness (%)	98.84 (90.02)
Mean I/sigma(I)	10.2 (1.1)
Wilson B-factor	42.20
R-merge	0.114 (1.408)
R-meas	0.133 (1.646)
R-pim	0.068 (0.847)
CC1/2	0.997 (0.492)
CC*	0.999 (0.774)
Reflections used in refinement	24687 (2237)
Reflections used for R-free	2445 (229)
R-work	0.1827 (0.3323)
R-free	0.2312 (0.3802)
CC(work)	0.964 (0.708)
CC(free)	0.955 (0.564)
Number of non-hydrogen atoms	4452
macromolecules	4289
ligands	60
solvent	103
Protein residues	531
RMS(bonds)	0.005
RMS(angles)	1.02
Ramachandran favored (%)	96.74
Ramachandran allowed (%)	3.26
Ramachandran outliers (%)	0.00
Rotamer outliers (%)	0.63
Clashscore	11.64
Average B-factor	61.59
macromolecules	61.72
ligands	71.07
solvent	50.36
Number of TLS groups	8

^aStatistics for the highest-resolution shell are shown in parentheses.

Table S2. Selectivity Score (S-score) results table of Compound **2** from the DiscoverX KINOMEScan scanMAX Kinase assay Panel.

Table S2 is related to Figure 3A.

Selectivity Score Type ^a	Compound 2		
	S(35)	S(10)	S(1)
Number of Hits	128	95	32
Number of Non-Mutant Kinases	403	403	403
Screening Concentration (nM)	1000	1000	1000
Selectivity Score	0.318	0.236	0.079

^aSelectivity Score (S-score) is a quantitative measure of compound selectivity, which is calculated by dividing the number of kinases that the compound binds to, by the total number of distinct kinases tested (excluding mutant variants). S(35) = (number of non-mutant kinases with %Ctrl<35)/(number of non-mutant kinases tested); S(10) = (number of non-mutant kinases with %Ctrl<10)/(number of non-mutant kinases tested); S(1) = (number of non-mutant kinases with %Ctrl<1)/(number of non-mutant kinases tested).

METHODS

Competition Binding Assays. Binding affinities (K_D) of all compounds and kinome selectivity profile of Compound 2 were obtained from DiscoverX KINOMEScan, a commercial kinase inhibitor binding platform, using the *Kd*ELECT service and *scan*MAX service, respectively.³ Briefly, active/pseudoactive site competition binding assays were performed by measuring compound binding to a given kinase/pseudokinase through competition with a promiscuous probe. The ability of Compounds 1, 2 or 3 to compete with the immobilized, active/pseudoactive-site directed probe was quantitatively measured via qPCR of the DNA tagged-protein. For the binding affinities (K_D) from *Kd*ELECT, Compounds 1, 2, or 3 were tested in an 11-point 3-fold dilution series against human MLKL (both the pseudokinase domain and full-length proteins), RIPK1 kinase domain and RIPK3 kinase domain where binding affinities (K_D) represent the mean of two replicates. For the kinome selectivity profile from *scan*MAX, Compound 2 was tested at a single concentration of 1 μ M against 468 kinases (including atypical, lipid, pathogen and disease relevant mutant kinases) and is reported as percent control where DMSO, the negative control, is 100% and a positive control compound is 0%. This is represented schematically in the TREEspot Interaction Maps by the size of the red circles, where larger circles indicate higher affinity binding.

Recombinant Protein Expression and Purification. Human MLKL pseudokinase domain (residues 190–471), human RIPK1 (residues 2–324) and human RIPK3 (residues 2–356) were expressed in Sf21 insect cells essentially as previously described.^{1,4} Briefly, DNA encoding each protein was cloned into pFastBac HTB (Invitrogen) as in-frame fusions with a TEV protease-cleavable N-terminal His₆ tag. Bacmids generated using the Bac-to-Bac system (Invitrogen) following transformation of DH10MultiBac *E. coli* cells were used to transfect Sf21 insect cells to generate P1 baculovirus according to the Bac-to-Bac manual (Invitrogen) and detailed elsewhere.⁵ Following a second viral amplification step, P2 virus (10% v/v) was used to infect 0.5 L Sf21 inset cells in 2.8 L Fernbach flasks shaking at 90 rpm, 27 °C for 48 hours using an established protocol.⁶ Cell pellets were resuspended in lysis buffer (20 mM HEPES pH 7.5, 200 mM NaCl, 10% v/v glycerol, 5 mM imidazole, 0.5 mM TCEP) and cells lysed via sonication. Cell debris was separated from soluble protein via centrifugation at 45000 x g

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and lysate applied to pre-equilibrated Ni^{2+} -NTA agarose (His-Tag resin; Roche) at 4 °C for 1 hour with gentle agitation. Beads were then collected via centrifugation and washed with lysis buffer and wash buffer (20 mM HEPES pH 7.5, 200 mM NaCl, 10% v/v glycerol, 35 mM imidazole, 0.5 mM TCEP) before elution in elution buffer (20 mM HEPES pH 7.5, 200 mM NaCl, 10% v/v glycerol, 250 mM imidazole, 0.5 mM TCEP). Eluted protein was mixed with 200 μg of TEV for 2 hours at 20 °C or overnight at 4 °C before the supernatant was filtered through a 0.45 μm filter and concentrated via centrifugal ultrafiltration (10 kDa MWCO; Millipore) for loading onto, and elution from, a Superdex S200 gel filtration column (GE Healthcare) equilibrated in gel filtration buffer (20 mM HEPES pH 7.5, 200 mM NaCl, 5% v/v glycerol). Proteins were concentrated to approximately 5 mg mL^{-1} and snap frozen for storage at -80 °C. For the SPR experiments, human MLKL pseudokinase domain (residues 190–471) was made with a non-cleavable N-terminal 2xHis₆ tag inserted into pFastBac1 (Invitrogen), which was expressed and purified from Sf21 cells as detailed above, except that TEV protease treatment was omitted. For the SPR experiments, human full-length MLKL (residues 2–471) was made with a non-cleavable N-terminal SBP-tag, using methods analogous to those described for untagged full-length human MLKL.⁷

Surface Plasmon Resonance. Small molecule SPR binding experiments with double His-tagged human MLKL pseudokinase domain (residues 190–471) were performed on a Biacore 4000 instrument (GE Healthcare). 2xHis₆-hMLKL was immobilized on the biosensor surface via Ni^{2+} -NTA histidine capture followed by covalent amine coupling. Briefly, the Biacore NTA Sensor Chip (GE Healthcare), containing a dextran surface pre-immobilized with nitrilotriacetic acid (NTA), was first primed with a 120 second injection of 300 mM EDTA solution, then charged with Ni^{2+} by injection of 500 mM NiCl_2 in immobilisation buffer (20 mM HEPES pH 8.0, 200 mM NaCl) at 10 $\mu\text{L min}^{-1}$. The surface was activated by a 1:1 mixture of EDC and NHS solutions (prepared according to the manufacturer's instructions) for 6 minutes at 10 $\mu\text{L min}^{-1}$. 2xHis₆-hMLKL (20 $\mu\text{g mL}^{-1}$) and an unrelated negative control reference protein were captured on the biosensor surface by injection for 10 minutes at 10 $\mu\text{L min}^{-1}$ to achieve immobilization levels of 3000–5000 Response Units (RU), while the third flow cell was left blank as a reference surface. Non-covalently bound proteins or unbound Ni^{2+} were removed with a 30 second

injection of immobilisation buffer containing 350 mM EDTA. Unreacted succinimide esters and uncoupled primary amine residues were blocked with 1 M ethanolamine for 60 seconds. Increasing concentrations (9-point 2-fold dilution from 10 μ M) of each compound were flowed over the sensor chip in running buffer (20 mM HEPES pH 8.0, 200 mM NaCl, 0.05% v/v P20, 2% v/v DMSO) with an association time of 60 seconds and dissociation time of 200 seconds at a flow rate of 30 μ L min⁻¹.

Small molecule SPR binding experiments with SBP-tagged human full-length MLKL (residues 2–471) were performed on a Biacore S200 instrument (GE Healthcare). SBP-hMLKL was immobilized on a Biacore Series S Sensor Chip SA (GE Healthcare), containing a dextran surface pre-immobilized with streptavidin (SA). Briefly, the surface was first activated with a solution of 1 M NaCl in 50 mM NaOH and then SBP-hMLKL (110 μ g mL⁻¹) was injected in immobilization buffer (20 mM HEPES pH 8.0, 200 mM NaCl) to obtain immobilization levels of 4000–5000 Response Units (RU). As a control, the first flow cell was left blank as the reference surface. Increasing concentrations (11-point 2-fold dilution from 2.5 μ M) of each compound were flowed over the sensor chip in running buffer (20 mM HEPES pH 8.0, 200 mM NaCl, 0.05% v/v P20, 2% v/v DMSO) with an association time of 35 seconds and dissociation time of 200 seconds at a flow rate of 30 μ L min⁻¹. The protein-immobilized surface was regenerated with an injection of 1 M NaCl for 30 seconds at 30 μ L min⁻¹.

All immobilization and small molecule binding experiments were performed at 25 °C. The amount of compound binding to the immobilized proteins as a function of time was recorded as sensorgrams. Data were reference (FL MLKL) or double reference (PsK MLKL) subtracted by the Biacore Evaluation Software Version 1.1, fit to a 1:1 binding kinetic interaction model and the K_D determined from the k_d/k_a ratio. Data shown are one representative result from two (PsK MLKL) or three (FL MLKL) independent experiments.

Thermal Shift Assays. Thermal shift assays were performed as previously described,^{1,6,8,9} using a Rotor-Gene Q PCR (QIAGEN). Human MLKL pseudokinase domain (residues 190–471) was diluted in 20 mM Tris pH 8.0, 150 mM NaCl, 1 mM DTT to a final concentration of 2.5 μ M and assayed with the given concentration of compound

in a total reaction volume of 25 μ L. Compounds were tested at various concentrations to achieve between 80 μ M to 2.5 μ M final concentrations. SYPRO Orange (Molecular Probes) was used as the fluorescent probe and detected at 555 nm. Data shown represent the mean and SD of two independent experiments.

X-ray Crystallography. Recombinant human MLKL pseudokinase domain (residues 190–471) was expressed and purified as previously described.¹ Protein was concentrated to 9.8 mg mL⁻¹. 2 x 2 μ L aliquots of 100 mM Compound 2 in DMSO was added to 200 μ L of protein to give a final concentration of 2 mM Compound 2, 2% v/v DMSO. Samples were centrifuged to remove precipitate and the supernatant was loaded onto an S200 24 mL gel filtration column (GE Healthcare) with a running buffer of 20 mM HEPES pH 7.5, 200 mM NaCl, 5% v/v glycerol. Protein containing fractions were pooled and concentrated to an OD of 11.81 and volume of 180 μ L using a 10 kDa MWCO concentrator (Millipore). Crystals of the complex were grown in 100 mM HEPES pH 7.5, 200 mM MgCl₂, 25% v/v PEG3350 and frozen in well solution supplemented with 20% v/v ethylene glycol. Diffraction data were collected at the Australian Synchrotron (MX2) at 100 K and processed with XDS.¹⁰ The structure was solved by molecular replacement with PHASER¹¹ searching for hMLKL (4MWI), followed by several rounds of building in COOT¹² and refinement in PHENIX¹³. PDB and constraint files for Compound 2 were generated with ELBOW.¹⁴ The structure was deposited in the Protein Data Bank (PDB) with the PDB ID 6O5Z.

Photoaffinity Labeling. Human MLKL pseudokinase domain (residues 190–471), human RIPK1 (residues 2–324), human RIPK3 (residues 2–356) and BSA (Sigma-Aldrich) were prepared as 12 μ L of 0.15 mg mL⁻¹ solutions in gel filtration buffer (20 mM HEPES pH 7.5, 200 mM NaCl, 5% v/v glycerol). An additional 12 μ L of 3.0 mg mL⁻¹ BSA was added to each sample. Samples were incubated with 1 μ L of DMSO or 10 mM competitor (Compound 2, GSK'481 (supplied by Catalyst Therapeutics),¹⁵ GSK'843 (supplied by Catalyst Therapeutics)¹⁶; 0.38 mM final concentration) for 30 minutes at room temperature. Samples were treated with 1 μ L of DMSO or 50 μ M Compound 3 (1.9 μ M final concentration), at a 1:1 molar ratio of Compound 3 to protein of interest, for 30

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minutes at room temperature. Samples were individually irradiated in open microcentrifuge tubes on ice for 1 minute with UV light from a 1000 W Hg(Xe) lamp (Oriol Instruments, model no. 66926) fitted with a band-pass filter for irradiation at 355 nm (Oriol Instruments, part no. 59810) and a filter to absorb heat (Oriol Instruments, part no. FSQ-KG2). Click labeling was then performed with TAMRA-azide (Click Chemistry Tools) using the Click Chemistry Protein Reaction Buffer Kit (Click Chemistry Tools) according to the manufacturer's protocol, but scaled down by half. Briefly, each component of the kit (45 μ L of Reaction Buffer, 10 μ L of 2.5 mM TAMRA-azide in DMSO, 5 μ L of Additive 1, 5 μ L of Additive 2, 5 μ L of CuSO₄, 5 μ L of Reducing Agent) was added sequentially to the 26 μ L protein samples, vortexing briefly to mix after the addition of each component. The click reaction was incubated for 30 minutes at room temperature protected from light. The labeled proteins were precipitated by the sequential addition of 300 μ L methanol, 75 μ L chloroform and 200 μ L MilliQ water, vortexing briefly to mix in between each addition. Samples were centrifuged at 13000 x g for 5 minutes, the upper aqueous layer removed, washed with 2 x 225 μ L methanol, centrifuged to pellet the proteins and the supernatant removed. The protein pellets were air-dried for 1 hour and stored at -20 °C until resolved by SDS-PAGE. Data shown are one representative result from five independent experiments.

Gel Electrophoresis Analyses of Photoaffinity Labeling. Protein pellets from the photoaffinity labeling were resuspended in 40 μ L of gel filtration buffer and 40 μ L of NuPAGE LDS Sample Buffer (Invitrogen) containing TCEP (Sigma-Aldrich). Samples were heated at 95 °C and 20 μ L of each sample was run on a NuPAGE 4–12% Bis-Tris 1.0 mm x 20-well Midi Gel (Invitrogen) using NuPAGE MES SDS Running Buffer (Invitrogen) along with the See Blue Plus2 Prestained Standard (Invitrogen). The gel was transferred into MilliQ water and immediately scanned using the Typhoon FLA 7000 Imaging System (GE Healthcare). The gel was then fixed and stained with Coomassie solution (0.1% w/v Coomassie Brilliant Blue R-250 (Bio-Rad), 30% v/v ethanol, 10% v/v acetic acid), destained for 2 hours with destain solution (30% v/v ethanol, 10% v/v acetic acid) and washed in MilliQ water overnight. The gel was imaged using the Gel Doc EZ Imaging System (Bio-Rad) with Image Lab 3.0 Software (Bio-Rad).

Cell Culture and Cell Lines. U937 and THP-1 cells were cultured in human tonicity RPMI (in-house). HT29, HeLa and murine dermal fibroblast (MDF) cells were cultured in DMEM (in-house). All media contained penicillin and streptomycin (100x solution, 10,000–12,000 units mL⁻¹ penicillin and 10–12 mg mL⁻¹ streptomycin in 0.9% NaCl, Sigma-Aldrich) and was supplemented with 8–10% v/v Fetal Calf Serum.

U937 knockout cell lines were generated by CRISPR/Cas9 using two vectors generated by Marco Herold: the pFUCas9Cherry vector, which allowed constitutive expression of the Cas9 protein, and the pFgH1tUTG vector, which allowed doxycycline-inducible expression of different guide RNA sequences complementary to their target sequence.^{17,18} To select for the CRISPR/Cas9 cell lines, infected cells were singly sorted for GFP and mCherry into 96-well plates containing RPMI with 1 µg mL⁻¹ doxycycline to induce guide RNA expression.

Guide RNA target sequences

CASP8: exon 2, 2 clones, GCCTGGACTACATTCCGCAA

BAX/BAK: *BAX*: exon 4, 1 clone, CTGCAGGATGATTGCCGCCG; *BAK*: exon 5, 1 clone, CTTCGTGGTCGACTTCATGC

MLKL: exon 2, 2 clones, CAAGAAACAGTGCCGGCGCC

RIPK1: exon 2, 2 clones, AGTGCAGAACTGGACAGCGG

RIPK3: exon 3, 2 clones, GAATTCGTGCTGCGCCTAGA

Cellular Thermal Shift Assays. U937 or MDFs cells were seeded to obtain 1.5 x 10⁶ cells per temperature point. Cells were treated with DMSO, Compound 2 (20 µM) or control compounds (20 µM) GSK'481 (supplied by Catalyst Therapeutics),¹⁵ GSK'872 (supplied by Catalyst Therapeutics)¹⁶ or Nec-1s (Sigma-Aldrich)^{19,20} for 1 hour at 37 °C 10% CO₂. Cells were pelleted, washed with 5 mL DPBS (Gibco), resuspended in 650 µL DPBS containing cOMplete EDTA-free Protease Inhibitor Cocktail (Roche) and aliquoted into 6 x 100 µL samples per compound into PCR tubes. Live cell suspensions were heated at a temperature gradient, specific for the protein of interest, for 3 minutes in a T100 Thermal Cycler (Bio-Rad), cooled at room temperature for 3 minutes, snap frozen

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in liquid N₂ and stored at –80 °C overnight. Samples were freeze-thawed once to lyse the cells, transferred to microcentrifuge tubes and the soluble protein and precipitated protein were separated by centrifugation at 17000 x g for 30 minutes at 4 °C. Soluble protein fractions were resolved by SDS-PAGE and analyzed by Western Blot. Data shown are one representative result from two or three independent experiments.

CETSA temperature gradients (°C)

hMLKL (U937 cells): 46.6, 47.7, 49.5, 51.6, 53.2, 54.3

hRIPK1 (U937 cells): 44.0, 44.7, 45.9, 47.9, 50.2, 51.9

hRIPK3 (U937 cells): 44.7, 45.9, 47.9, 50.2, 51.9, 53.2

mMLKL (MDF cells): 45.3, 47.2, 49.4, 51.0, 52.2, 53.0

mRIPK1 (MDF cells): 43.9, 45.9, 48.2, 49.9, 51.2, 52.0

mRIPK3 (MDF cells): 48.9, 50.9, 53.2, 55.0, 56.2, 57.0

Western Blot Analyses of Cellular Thermal Shift Assays. 10 µL of each soluble protein sample from CETSA was added to 10 µL of NuPAGE LDS Sample Buffer (Invitrogen) containing TCEP (Sigma-Aldrich). Samples were heated at 95 °C for 10 minutes and 15 µL of each sample was run on either a NuPAGE 4–12% Bis-Tris 1.0 mm x 20-well Midi Gel (Invitrogen) using NuPAGE MES SDS Running Buffer (Invitrogen), or a 4–15% Criterion TGX Stain-Free 26 well 15 µL Protein Gel (Bio-Rad) using Tris/Glycine Buffer (Bio-Rad), along with the Precision Plus Kaleidoscope or All Blue Prestained Protein Standards (Bio-Rad). Proteins were transferred to a nitrocellulose membrane using the iBlot Dry Blotting System (Invitrogen). Membranes were blocked with 5% w/v skim milk powder in PBS-T (PBS + 0.1% v/v Tween-20) for 1 hour at room temperature, washed 3 x 20 minutes with PBS-T at room temperature, probed overnight at 4 °C with the primary antibody for the protein of interest in PBS + 2% w/v BSA + 0.02% w/v NaN₃, washed as above, probed for 1 hour at room temperature with the relevant secondary antibody in PBS-T + 5% w/v BSA and washed as above. Immobilon Forte Western HRP substrate (Merck Millipore) was added to the membranes and Western Blots were developed using chemiluminescence on the ChemiDoc Imaging

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System (Bio-Rad). All membranes were then washed overnight with PBS-T, reprobed with the loading control (actin-HRP) for 1 hour at room temperature, washed 3 x 20 minutes with PBS-T at room temperature and developed as described above.

Western Blot antibodies

hMLKL and mMLKL: rat anti-MLKL clone 3H1, produced in-house as previously described,⁸ available from Sigma-Aldrich cat #MABC604, 1:1000

hRIPK1: mouse anti-RIPK1, BD Transduction Laboratories, cat #610458, 1:1000

hRIPK3: rat anti-human RIPK3 clone 1H2, produced in-house as previously described,⁴ 1:1000

mRIPK1: rabbit anti-RIPK1, Cell Signaling Technology, cat #3493, 1:1000

mRIPK3: rabbit anti-RIPK3, ProSci, cat #2283, 1:1000

actin: chicken anti- β -actin-HRP, Santa Cruz Biotechnology, cat #sc-47778 HRP, 1:5000

secondary antibodies: goat anti-rat-HRP (cat #3010-05), goat anti-mouse-HRP (cat #1010-05), goat anti-rabbit-HRP (cat #4010-05) all from Southern Biotech and used at 1:5000

Cell Death Assays. Necroptosis cell death assays were carried out as previously described.^{21,22} U937 and THP-1 wild type cells were seeded at 3.5×10^4 cells per well into 96-well plates. HT29 and MDF wild type cells were seeded at 5.0×10^4 and 3.5×10^4 cells per well, respectively, into 48-well plates. Cells were allowed to settle over one (U937, THP-1, MDF) or two (HT29) nights at 37 °C 10% CO₂. Cells were then treated with either TSQ ((100 ng mL⁻¹ TNF, produced in-house²³), 500 nM Smac-mimetic (Compound A, Tetralogic), 10 μ M Q-VD-OPh (MP Biologicals)), TSI (100 ng mL⁻¹ TNF, 500 nM Smac-mimetic, 5 μ M IDN-6556/emricasan, Tetralogic) or DMSO alone, in addition to increasing concentrations of Compound 1, 2 or 3. Following treatment for 24 hours (TSI) or 48 hours (TSQ), cells were harvested and stained with 1 μ g mL⁻¹ propidium iodide (PI, Sigma-Aldrich) and PI positive cells were quantified by flow

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cytometry on a BD FACSCalibur instrument. Control cell death assays with wild type U937, HT29 and THP-1 cells were performed analogously, except that cells were stimulated with either TSQ or TSI to induce necroptosis, or TS to induce apoptosis, and then treated with known necroptosis inhibitors 5 μ M Nec-1s (Sigma-Aldrich)^{19,20} and 1 μ M NSA (Merck Millipore)²⁴. Cell death assays with U937 knockout cell lines were performed analogously, except that cells were treated with increasing concentrations of Compound 2 alone or in the presence of 10 μ M Q-VD-OPh for 24 hours. Cell death assays with HeLa cells were performed analogously to the U937 knockout cell lines, except that cells were seeded at 4.0×10^4 cells per well into 24-well plates and allowed to settle for 24 hours prior to treatment. Data shown represent the mean and SEM of at least three independent experiments.

Pharmacokinetic Studies. Systemic exposure of Compound 2 in female C57BL/6 mice was performed by the Centre for Drug Candidate Optimisation at the Monash Institute of Pharmaceutical Sciences. Briefly, Compound 2 was administered to female C57BL/6 mice via intraperitoneal (IP) injection at 40 mg kg⁻¹. Blood samples were collected at 0.25, 0.5, 1, 2, 4, 7.5 and 24 hours post-dose, with $n = 2$ mice per time point and a maximum of two blood samples obtained from each mouse, and were analyzed by LC-MS. Exposure parameters were determined via non-compartmental analysis and the apparent half-life (apparent $t_{1/2}$) was based on the last three timepoints.

***In vivo* TNF-induced SIRS.** Mouse recombinant TNF containing BSA as a carrier (R&D Systems, cat #410-MT-050, lot #CS1314053) was freshly dissolved in sterile DPBS (Gibco), Compound 2 was freshly dissolved in DMSO and captisol was freshly dissolved in sterile MilliQ water at 33% w/v. The Compound 2 formulation was prepared as a solution of 10% v/v DMSO, 30% w/v captisol and the vehicle solution was 10% v/v DMSO, 30% w/v captisol alone. For the Compound 2 experiment: female C57BL/6 wild type mice aged 9.5 weeks were treated via IP injection with either 40 mg kg⁻¹ Compound 2 as the above formulation or vehicle solution as the control. Mice were placed under heat lamps for 15–20 minutes and then treated with 300 μ g kg⁻¹ TNF via IV tail vein injection. The core body temperature of the mice was measured each hour following

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TNF treatment for a period of 12 hours, with the experimenter blinded to the Compound 2 treated vs vehicle only treated mice ($n = 10$ mice per group). For the wild type vs gene knockout experiment: wild type, *Mlkl*^{-/-} or *Ripk3*^{-/-} C57BL/6 mice (maintained as separate colonies, but housed in the same facility) aged 8–15 weeks were placed under heat lamps for 15–20 minutes. *Mlkl*^{-/-} and *Ripk3*^{-/-} mice were treated with 300 $\mu\text{g kg}^{-1}$ TNF via IV tail vein injection, while WT mice were treated with either 300 $\mu\text{g kg}^{-1}$ TNF or DPBS alone. The core body temperature of the mice was measured each hour following TNF treatment for a period of 14 hours, with the experimenter blinded to the genotype of the mice ($n = 2$ –8 mice per group). All mice were born and raised in the Walter and Eliza Hall Institute (WEHI) Bioservices facility and all experiments were performed according to protocols approved by the WEHI Animal Ethics Committee. The ethics approved end point was specified as either two consecutive temperature measurements below 31 °C or one measurement below 30 °C. Mice were euthanized within 15 minutes and the time of death was recorded on the survival curve as the time of the last temperature measurement.

COMPOUND SYNTHESIS

General Experimental. All non-aqueous reactions were performed under an atmosphere of dry nitrogen, unless otherwise specified. All solvents, including anhydrous solvents, were obtained commercially and used as supplied. All reactions were monitored by either thin-layer chromatography (TLC) or high-performance liquid chromatography (HPLC).

TLC was performed on silica gel 60 F₂₅₄ 0.5 mm aluminium-backed plates (Merck) and visualized under UV light and/or by applying vanillin stain (15 g vanillin, 250 mL EtOH, 2.5 mL concentrated H₂SO₄) followed by heating, or permanganate stain (3.0 g KMnO₄, 20 g K₂CO₃, 5 mL 5% aqueous NaOH, 300 mL H₂O) followed by heating. HPLC was performed on the Hewlett Packard Series 1100 HPLC System with a Gemini column (C18, 5 μ m, 50 x 2.0 mm, 110 Å) with a 0–100% v/v gradient of MeCN/0.1% v/v formic acid in H₂O/0.1% v/v formic acid over 6 minutes.

Purification of compounds by column chromatography was performed on the CombiFlash Rf Purification System (Teledyne Isco) with method details as specified. All liquid chromatography-mass spectrometry (LCMS) spectra were recorded on the Finnigan, Waters A or Waters B system as specified.

Finnigan: Finnigan LCQ Advantage Max with reverse phase HPLC analysis on a Gemini column (C18, 3 μ m, 20 x 4.0 mm) with a 10–100% v/v gradient of MeCN/0.1% v/v formic acid in H₂O/0.1% v/v formic acid over 10 minutes.

Waters A: Waters ZQ 3100 with reverse phase HPLC analysis on a XBridge TM column (C18, 5 μ m, 100 x 4.6 mm) with a 10–100% v/v gradient of MeCN/0.1% v/v formic acid in H₂O/0.1% v/v formic acid over 10 minutes.

Waters B: Waters ZQ 3100 with reverse phase HPLC analysis on a Kinetex TM XB column (C18, 5 μ m, 50 x 4.6 mm) with a 5–100% v/v gradient of MeCN/0.1% v/v formic acid in H₂O/0.1% v/v formic acid over 6 minutes.

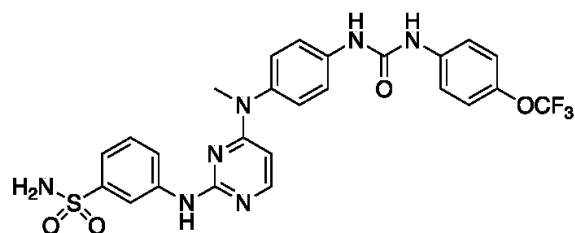
All nuclear magnetic resonance (NMR) spectra were recorded at 500 MHz or 600 MHz for ¹H NMR and 150 MHz for ¹³C NMR. Chemical shifts are reported as δ values in parts per million (ppm), referenced to the specified solvent peak (¹H NMR: 7.26 ppm for

CHCl_3 , 2.50 ppm for DMSO, 3.31 ppm for MeOH; ^{13}C NMR: 39.52 ppm for DMSO, 49.00 ppm for MeOH),²⁵ and coupling constants (J) are reported in Hz.

All HRMS spectra were recorded on an Agilent 6224 TOF LC/MS Mass Spectrometer coupled to an Agilent 1290 Infinity LC System with a RRHT column (C18, 1.8 μm , 50 x 2.1 mm) and a gradient applied over 5 minutes with a flow rate of 0.5 mL min⁻¹. Mass spectrometer conditions were as follows – ionization mode: electrospray ionization; drying gas flow: 11 L min⁻¹; nebulizer: 45 psi; drying gas temperature: 325 °C; capillary voltage: 4000 V; fragmentor: 160 V; skimmer: 65 V; OCT RFV: 750 V; scan range acquired: 100–1500 m/z .

Compound 1

3-((4-(Methyl(4-(3-(4-(trifluoromethoxy)phenyl)ureido)phenyl)amino)pyrimidin-2-yl)amino)benzenesulfonamide



The title compound was synthesized as previously reported.²⁶

LCMS (Finnigan): t_R 4.70 min, $[M+H]^+$ 574.1.

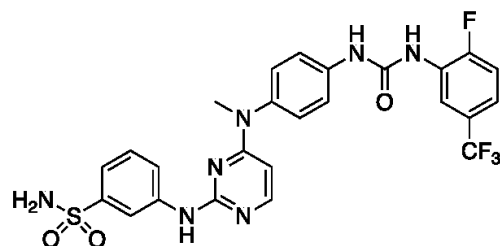
1H NMR (600 MHz, DMSO- d_6): δ 3.43 (s, 3H), 5.78 (d, J = 5.9 Hz, 1H), 7.24–7.27 (m, 4H), 7.30 (d, J = 8.6 Hz, 2H), 7.34 (d, J = 7.7 Hz, 1H), 7.39 (t, J = 7.9 Hz, 1H), 7.56–7.58 (m, 4H), 7.79 (d, J = 7.9 Hz, 1H), 7.88 (d, J = 6.0 Hz, 1H), 8.55 (s, 1H), 8.92 (s, 1H), 8.97 (s, 1H), 9.51 (s, 1H).

^{13}C NMR (150 MHz, DMSO- d_6): δ 40.1, 96.9, 115.5, 117.8, 119.69, 119.73, 120.4 (q, J = 255 Hz), 121.5, 121.9, 127.6, 129.1, 138.4, 138.5, 139.1, 141.7, 142.8, 144.4, 152.7, 156.0, 159.4, 162.6.

HRMS (ESI+) m/z $[M+H]^+$ $C_{25}H_{22}F_3N_7O_4S$ calc. 574.1479, found 574.1484.

Compound 2

3-((4-((4-(3-(2-Fluoro-5-(trifluoromethyl)phenyl)ureido)phenyl)(methyl)amino)pyrimidin-2-yl)amino)benzenesulfonamide



The title compound was synthesized as previously reported.²⁶

LCMS (Finnigan): t_R 4.60 min, $[M+H]^+$ 576.3.

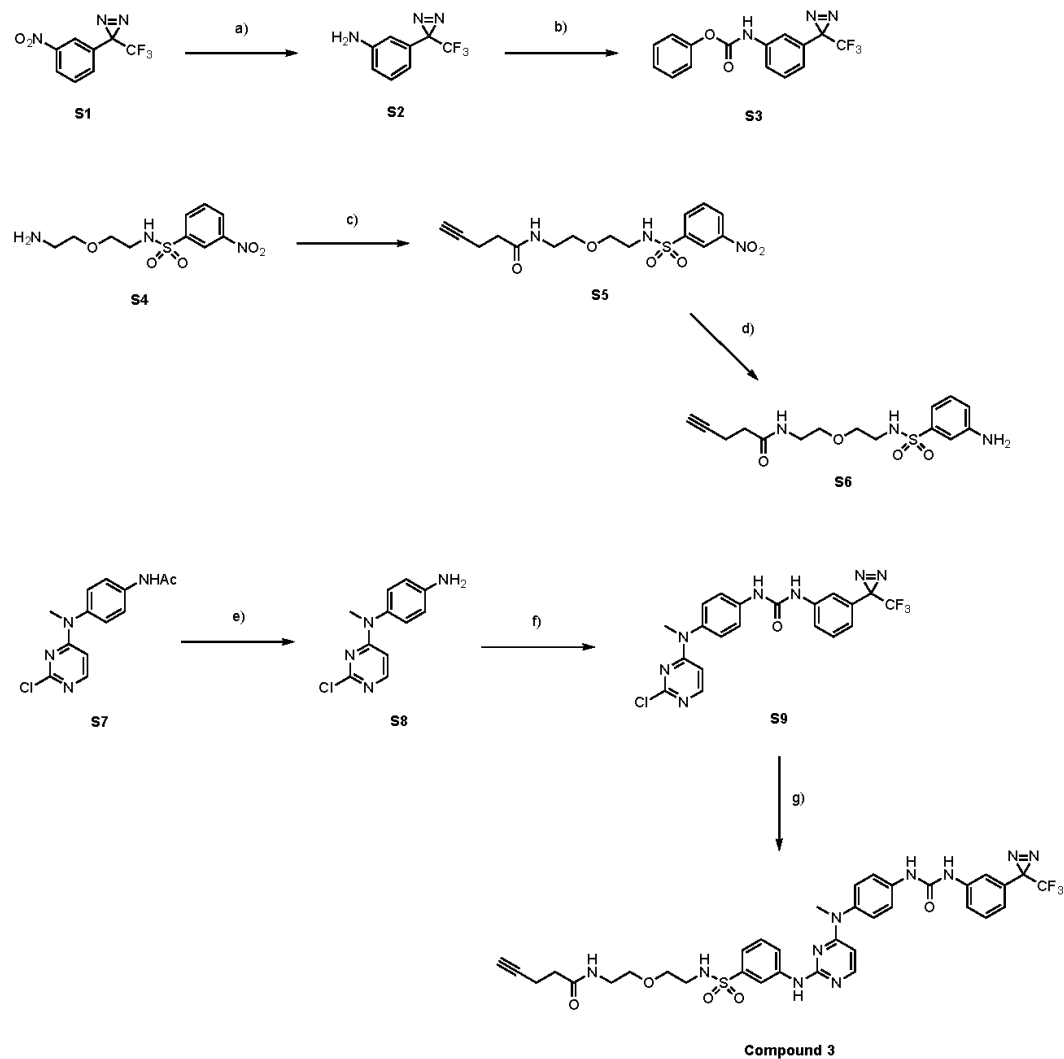
1H NMR (600 MHz, DMSO- d_6): δ 3.51 (s, 3H), 5.96 (s, 1H), 7.35 (d, J = 8.8 Hz, 2H), 7.39–7.41 (m, 3H), 7.49–7.52 (m, 1H), 7.54–7.57 (m, 2H), 7.65 (d, J = 8.8 Hz, 2H), 7.73 (d, J = 7.4 Hz, 1H), 7.91 (d, J = 7.0 Hz, 1H), 8.37 (s, 1H), 8.60 (d, J = 7.2 Hz, 1H), 9.05 (s, 1H), 9.53 (s, 1H), 10.78 (s, 1H).

^{13}C NMR (150 MHz, DMSO- d_6): δ 40.1, 97.4, 116.2 (d, J = 21 Hz), 116.8, 117.5, 119.6, 120.5, 123.4, 123.9 (q, J = 271 Hz), 125.4 (qd, J = 32, 3.0 Hz), 127.3, 128.6 (d, J = 12 Hz), 129.5, 136.8, 138.6, 139.0, 144.7, 146.1 (bs), 152.1, 152.8 (bs), 153.6 (d, J = 246 Hz), 159.1–159.8 (m), 162.4.

HRMS (ESI+) m/z $[M+H]^+$ $C_{25}H_{21}F_4N_7O_3S$ calc. 576.1435, found 576.1443.

Compound 3

The title compound was synthesized according to the following synthetic scheme.



Reagents and conditions: a) $\text{SnCl}_2 \cdot 2\text{H}_2\text{O}$, HCl, MeOH, rt, 16 h; b) phenyl chloroformate, pyridine, THF, rt, 10 min; c) 4-pentynoic acid, HOBt, EDCI·HCl, DMF, rt, 16 h; d) $\text{SnCl}_2 \cdot 2\text{H}_2\text{O}$, HCl, MeOH, rt, 16 h; e) AcCl, MeOH, rt, 16 h; f) **S3**, DIPEA, THF, 60 °C, 24 h; g) **S6**, HCl, *t*-BuOH, 50 °C, 40 h.

3-(3-Nitrophenyl)-3-(trifluoromethyl)-3*H*-diazirine (S1)

The title compound was synthesized as previously reported.²⁷

3-(3-(Trifluoromethyl)-3*H*-diazirin-3-yl)aniline (S2)

To a solution of **S1** (0.200 g, 0.865 mmol) in MeOH (6 mL) was added 2 M HCl (4 mL) and SnCl₂·2H₂O (0.781 g, 3.46 mmol). The reaction mixture was stirred in the dark at room temperature for 16 hours, concentrated under reduced pressure and brought to basic pH by the dropwise addition of 2 M NaOH. An approximately equal volume of CH₂Cl₂ was added and the solution stirred vigorously for 15 minutes then extracted into CH₂Cl₂. The organic layer was dried over MgSO₄, filtered and concentrated under reduced pressure to afford the title compound (0.148 g, 85%) as a yellow oil.

LCMS (Waters A): *t*_R 6.10 min, [M+H]⁺ 202.1.

¹H NMR (600 MHz, CDCl₃): δ 3.77 (bs, 2H), 6.46 (s, 1H), 6.53 (d, *J* = 7.8 Hz, 1H), 6.70 (d, *J* = 8.1 Hz, 1H), 7.15 (t, *J* = 7.9 Hz, 1H).

Phenyl (3-(3-(trifluoromethyl)-3*H*-diazirin-3-yl)phenyl)carbamate (S3)

To a solution of **S2** (0.130 g, 0.646 mmol) in anhydrous THF (2 mL) was added pyridine (0.172 mL, 2.13 mmol) in a dropwise manner. Phenylchloroformate (0.089 mL, 0.711 mmol) dissolved in anhydrous THF (1.5 mL) was then added in a dropwise manner. The reaction mixture was stirred at room temperature in the dark for 10 minutes, then concentrated under reduced pressure. EtOAc and H₂O were added and the solution neutralized by the dropwise addition of 2 M NaOH then extracted into EtOAc. The organic layer was washed with H₂O, dried over MgSO₄, filtered and concentrated under reduced pressure. The crude product was purified by column chromatography (5–80% v/v EtOAc in cyclohexane) to afford the title compound (0.146 g, 70%) as a pale yellow solid.

LCMS (Waters B): *t*_R 4.80 min, [M-H]⁻ 320.1.

¹H NMR (500 MHz, CDCl₃): δ 6.95 (d, *J* = 8.2 Hz, 1H), 6.97 (bs, 1H), 7.18–7.20 (m, 2H), 7.24–7.28 (m, 1H), 7.36–7.37 (m, 2H), 7.39–7.42 (m, 2H), 7.49 (d, *J* = 8.2 Hz, 1H).

***N*-(2-(2-Aminoethoxy)ethyl)-3-nitrobenzenesulfonamide (S4)**

To a solution of 2,2'-oxydiethanamine (1.00 g, 9.60 mmol) in anhydrous CH₂Cl₂ (12 mL) was added Et₃N (2.68 mL, 19.2 mmol) in a dropwise manner. The solution was cooled to –50 °C and 3-nitrobenzene-1-sulfonyl chloride (1.06 g, 4.80 mmol) dissolved in anhydrous CH₂Cl₂ (12 mL) was added over 2 hours using a syringe pump. The reaction mixture was stirred at room temperature for 16 hours. H₂O was added and the crude product was extracted into CH₂Cl₂, dried over MgSO₄, filtered and concentrated under reduced pressure. The crude product was dissolved in EtOAc and purified by silica plug, washed first with EtOAc to elute the bis-addition side-product, then washed with 50% v/v MeOH in CH₂Cl₂ to elute the desired product. The solvent was removed under reduced pressure to afford the title compound (0.720 g, 52%) as a pale yellow solid.

LCMS (Waters B): *t*_R 3.11 min, [M+H]⁺ 290.1.

¹H NMR (600 MHz, CDCl₃): δ 2.89 (t, *J* = 5.1 Hz, 2H), 3.09 (bs, 2H), 3.19 (t, *J* = 5.1 Hz, 2H), 3.49 (t, *J* = 5.1 Hz, 2H), 3.57 (t, *J* = 5.1 Hz, 2H), 7.73 (t, *J* = 8.0 Hz, 1H), 8.21 (d, *J* = 7.8 Hz, 1H), 8.42 (d, *J* = 8.2 Hz, 1H), 8.72 (t, *J* = 2.1 Hz, 1H).

***N*-(2-(2-(3-Nitrophenylsulfonamido)ethoxy)ethyl)pent-4-ynamide (S5)**

To a solution of 4-pentynoic acid (0.244 g, 2.49 mmol) in anhydrous DMF (8 mL) was added HOBt (0.370 g, 2.74 mmol) and EDCI·HCl (0.525 g, 2.74 mmol) sequentially. After stirring for 1 hour at room temperature, **S4** (0.720 g, 2.49 mmol) dissolved in anhydrous DMF (4 mL) was added in a dropwise manner. The reaction mixture was stirred at room temperature for 16 hours. EtOAc and H₂O were added and the solution neutralized by the dropwise addition of 2 M NaOH then extracted into EtOAc. The organic layer was washed with H₂O, dried over MgSO₄, filtered and concentrated under reduced pressure to afford the title compound as the crude product (0.860 g) as a yellow oil, which was used directly in the next step.

LCMS (Waters B): t_R 3.72 min, $[M+H]^+$ 370.3.

1H NMR (600 MHz, CD_3OD): δ 2.25 (t, J = 2.6 Hz, 1H), 2.38 (t, J = 6.9 Hz, 2H), 2.44–2.47 (m, 2H), 3.13 (t, J = 5.3 Hz, 2H), 3.28–3.30 (m, 2H), 3.41 (t, J = 5.5 Hz, 2H), 3.46 (t, J = 5.3 Hz, 2H), 7.84 (t, J = 8.0 Hz, 1H), 8.24 (d, J = 8.2 Hz, 1H), 8.47 (d, J = 8.2 Hz, 1H), 8.66–8.67 (m, 1H).

***N*-(2-(2-(3-Aminophenylsulfonamido)ethoxy)ethyl)pent-4-ynamide (S6)**

To a solution of **S5** (0.860 g, 2.33 mmol) in MeOH (12 mL) was added 2 M HCl (8 mL) and $SnCl_2 \cdot 2H_2O$ (2.10 g, 9.31 mmol). The reaction mixture was stirred at room temperature for 16 hours, concentrated under reduced pressure and brought to basic pH by the dropwise addition of 2 M NaOH. An approximately equal volume of CH_2Cl_2 was added and the solution was stirred vigorously for 30 minutes then extracted into CH_2Cl_2 . The organic layer was dried over $MgSO_4$, filtered and concentrated under reduced pressure. The crude product was purified by column chromatography (10–100% v/v EtOAc in cyclohexane) to afford the title compound (0.050 g, 6% over two steps) as an off-white solid.

LCMS (Waters B): t_R 3.40 min, $[M+H]^+$ 340.2.

1H NMR (600 MHz, $CDCl_3$): δ 1.98 (t, J = 2.5 Hz, 1H), 2.38 (t, J = 7.2 Hz, 2H), 2.46–2.49 (m, 2H), 3.09–3.12 (m, 2H), 3.38 (t, J = 5.0 Hz, 2H), 3.41–3.44 (m, 4H), 4.06 (s, 2H), 5.56 (t, J = 5.8 Hz, 1H), 6.40 (s, 1H), 6.81 (d, J = 7.8 Hz, 1H), 7.14 (s, 1H), 7.18 (d, J = 7.8 Hz, 1H), 7.23–7.25 (m, 1H).

***N*-(4-((2-Chloropyrimidin-4-yl)amino)phenyl)acetamide (S7)**

The title compound was synthesized as previously reported.²⁶

***N*¹-(2-Chloropyrimidin-4-yl)-*N*¹-methylbenzene-1,4-diamine (S8)**

To a solution of **S7** (0.500 g, 1.81 mmol) in MeOH (10 mL) was added AcCl (1.28 mL, 18.1 mmol) in a dropwise manner. The reaction mixture was stirred at room temperature for 16 hours and then concentrated under reduced pressure to afford the title compound as the crude product (0.420 g) as a pink solid, which was used directly in the next step.

LCMS (Waters B): t_R 3.42 min, $[M+H]^+$ 235.3.

1H NMR (500 MHz, DMSO- d_6): δ 3.37 (s, 3H), 6.34 (d, J = 6.0 Hz, 1H), 7.32–7.33 (m, 2H), 7.39–7.40 (m, 2H), 8.03 (d, J = 6.0 Hz, 1H).

1-(4-((2-Chloropyrimidin-4-yl)(methyl)amino)phenyl)-3-(3-(3-(trifluoromethyl)-3H-diazirin-3-yl)phenyl)urea (S9**)**

To a solution of **S8** (0.262 g, 1.12 mmol) and **S3** (0.359 g, 1.12 mmol) in anhydrous THF (6 mL) was added anhydrous DIPEA (0.389 mL, 2.23 mmol). The reaction mixture was heated at 60 °C in the dark for 24 hours, cooled to room temperature and concentrated under reduced pressure. EtOAc and H₂O were added and the solution neutralized by the dropwise addition of 1 M HCl then extracted into EtOAc. The organic layer was dried over MgSO₄, filtered and concentrated under reduced pressure. The crude product was purified by reverse phase column chromatography (5–95% v/v MeCN/0.1% v/v formic acid in H₂O/0.1% v/v formic acid) to afford the title compound (0.180 g, 35%) as a yellow solid.

LCMS (Waters B): t_R 4.79 min, $[M+H]^+$ 462.1.

1H NMR (600 MHz, CD₃OD): δ 3.45 (s, 3H), 6.26 (d, J = 6.2 Hz, 1H), 6.90 (d, J = 7.8 Hz, 1H), 7.25 (d, J = 8.9 Hz, 2H), 7.37–7.41 (m, 1H), 7.49–7.51 (m 2H), 7.60 (d, J = 8.9 Hz, 2H), 7.86 (d, J = 6.2 Hz, 1H).

***N*-(2-(2-(3-((4-(Methyl(4-(3-(3-(3-(trifluoromethyl)-3H-diazirin-3-yl)phenyl)ureido)phenyl)amino)pyrimidin-2-yl)amino)phenyl)sulfonamido)ethoxy)ethyl)pent-4-ynamide (Compound 3)**

To a solution of **S6** (0.028 g, 0.082 mmol) and **S9** (0.038 g, 0.082 mmol) in anhydrous *t*-BuOH (2 mL) was added 3 drops of concentrated HCl and heated at 50 °C in the dark. The reaction was monitored by HPLC and additional **S6** was added as required to consume all the **S9** starting material. The reaction mixture was stirred at 50 °C in the dark for a total of 40 hours and then cooled to room temperature. EtOAc and H₂O were added and the solution neutralized by the dropwise addition of 2 M NaOH then extracted into EtOAc. The organic layer was washed with H₂O, dried over MgSO₄, filtered and concentrated under reduced pressure. The crude product was purified by column chromatography (0–10% v/v MeOH in CH₂Cl₂) to afford the title compound (0.026 g, 38%) as an off-white solid.

LCMS (Waters B): *t*_R 3.98 min, [M+H]⁺ 765.3.

¹H NMR (600 MHz, CD₃OD): δ 2.23–2.24 (m, 1H), 2.36 (t, *J* = 7.2 Hz, 2H), 2.42–2.44 (m, 2H), 3.08 (t, *J* = 4.9 Hz, 2H), 3.29–3.31 (m, 2H), 3.41 (t, *J* = 4.8 Hz, 2H), 3.44 (t, *J* = 4.8 Hz, 2H), 3.49 (s, 3H), 5.85 (d, *J* = 5.8 Hz, 1H), 6.89 (d, *J* = 7.8 Hz, 1H), 7.23 (d, *J* = 7.1 Hz, 2H), 7.37 (t, *J* = 7.7 Hz, 1H), 7.41–7.43 (m, 2H), 7.49–7.51 (m, 2H), 7.56 (d, *J* = 7.1 Hz, 2H), 7.70–7.71 (m, 1H), 7.78 (d, *J* = 5.2 Hz, 1H), 8.58 (s, 1H).

¹³C NMR (150 MHz, CD₃OD): δ 15.7, 29.5 (q, *J* = 41 Hz), 35.9, 39.1, 40.3, 44.0, 70.3, 70.5, 83.5, 98.3, 117.7, 118.3, 120.4, 121.4, 121.5, 121.7, 123.6 (q, *J* = 272 Hz), 123.7, 128.8, 130.4, 130.7, 130.8, 139.5, 140.5, 141.6, 142.1, 142.8, 154.8, 155.6, 160.3, 164.5, 174.1. *Due to overlap in the aromatic region of the spectra, one carbon peak is observed.*

HRMS (ESI+) *m/z* [M+H]⁺ C₃₅H₃₅F₃N₁₀O₅S calc. 765.2537, found 765.2543.

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Chapter 3: Investigating ABT-869 as a Novel Inhibitor of Necroptosis

Introduction and Project Background

In previous studies performed at WEHI, it was identified that certain mutations in murine MLKL result in constitutively active forms of the protein that are able to trigger necroptotic cell death in the absence of external stimuli and independent of upstream signalling (Murphy et al., 2013). These mutations involve key residues in the ATP-binding pocket or “pseudoactive” site of the pseudokinase domain. The pseudoactive site of murine MLKL features an unusual arrangement of residues where Lys K219 from the ATP-binding VAIK motif in the β 3 strand, instead of classical kinase interactions involving Glu E239 from the α C helix, interacts with Gln Q343 from the atypical activation loop helix (Murphy et al., 2013). When either Q343 or K219 are mutated to Ala or Met, respectively, and expressed in murine cells, this results in necroptotic cell death upon expression alone, even in cells that lack endogenous MLKL (Murphy et al., 2013). Accordingly, these mutants are termed the “constitutively active” MLKL mutants.

Following this discovery, a cell-based phenotypic screen was developed at WEHI with the aim of identifying small molecules that modulate necroptosis at the level or downstream of MLKL (Jacobsen et al., 2016). Given that the death-inducing phenotype of the Q343A mutant was stronger than that of the K219M mutant (Murphy et al., 2013), MLKL(Q343A) MDF cells (on a wild-type background) were chosen as the cell line for the phenotypic screen to ensure better signal to background ratio. A pilot screen was performed where 5,000 compounds from the WEHI small molecule library and 132 known kinase inhibitors were screened for their ability

to inhibit cell death in MLKL(Q343A) MDFs. From this screen, a small molecule called ABT-869 (Compound **4**, **Figure 3.1A**) was identified as a hit from within the kinase inhibitor library.

ABT-869 is a multi-targeted receptor tyrosine kinase inhibitor developed by AbbVie that targets all members of the VEGFR and PDGFR families (Dai et al., 2007). To validate this hit from the screen, preliminary cellular studies were performed at WEHI to confirm that ABT-869 was a genuine novel inhibitor of necroptosis. In these validation studies, a single concentration of 1 μ M ABT-869 attenuated necroptosis induced by expression of MLKL(Q343A) in wild-type, *Myd88*^{-/-}, and *Bax*^{-/-}*Bak*^{-/-}*Ripk3*^{-/-} murine cells (**Figure 3.1B**). ABT-869 also inhibited TSQ-stimulated necroptosis in three independently-derived wild-type MDF cell lines (WT #1, WT #2, WT #3) at this single concentration of 1 μ M (**Figure 3.1C**). Subsequently, a titration of ABT-869 was performed in wild-type MDF cells where ABT-869 displayed concentration-dependent inhibition of TSQ-stimulated necroptosis with an IC₅₀ of around 400 nM (**Figure 3.1D**). Two analogues of ABT-869, Compound **5** (**Figure 3.1A**) and Compound **6** (**Figure 3.1A**), were also evaluated in TSQ-stimulated wild-type MDF cells; Compound **5** inhibited necroptosis more weakly than ABT-869, while Compound **6** inhibited necroptosis to a similar extent as ABT-869 (**Figure 3.1D**). Furthermore, preliminary data indicated that ABT-869 blocked necroptosis more weakly in human U937 cells, compared to murine cells, with an IC₅₀ of around 2 μ M (**Figure 3.1E**).

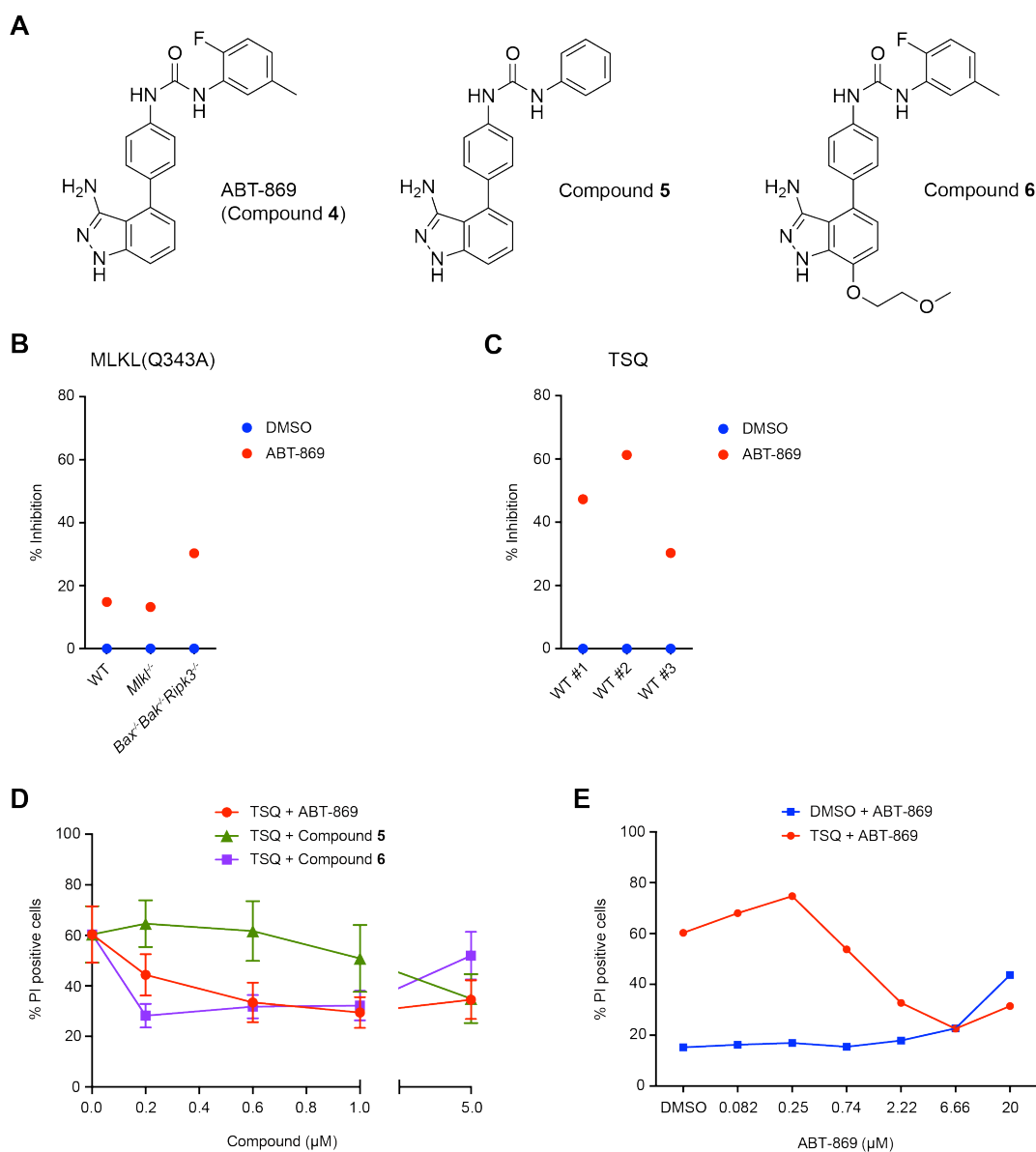


Figure 3.1 ABT-869 is a novel inhibitor of necroptosis identified from a phenotypic screen.

(A) Chemical structure of ABT-869 (Compound 4) and, its analogues, Compound 5 and Compound 6.

(B) WT MDF, *Mkl1*^{-/-} MDF or *Bax*^{-/-}*Bak*^{-/-}*Ripk3*^{-/-} MEF cells expressing MLKL(Q343A) to trigger constitutive necroptosis were treated with DMSO or ABT-869 (1 μM). Cell viability was quantified by CellTiter-Glo. Data represent the mean of duplicates. Performed by Dr Mark van Delft.

(C) Three independently-derived wild-type MDF cell lines (WT #1, WT #2, WT #3) were stimulated with TSQ (TNF, Smac-mimetic, Q-VD-OPh) to induce necroptosis and treated with DMSO or ABT-869 (1 μM). Cell death was quantified by propidium iodide (PI) staining using flow cytometry. Data shown are from n = 1 experiment. Performed by Dr Joanne Hildebrand.

(D) Wild-type MDF cells were stimulated with TSQ (TNF, Smac-mimetic, Q-VD-OPh) to induce necroptosis and treated with increasing concentrations of ABT-869, Compound 5 or Compound 6. Cell

death was quantified by propidium iodide (PI) staining using flow cytometry. Data represent the mean of n = 4 independent experiments and error bars represent SEM. Performed by Dr Joanne Hildebrand.

(E) Human U937 cells were stimulated with TSQ (TNF, Smac-mimetic, Q-VD-OPh) to induce necroptosis and treated with DMSO or increasing concentrations of ABT-869. Cell death was quantified by propidium iodide (PI) staining using flow cytometry. Data shown are from n = 1 experiment. Performed by Dr Maria Tanzer.

Results and Discussion

ABT-869 is a Novel Inhibitor of Necroptosis in Murine Cells

To further profile the activity of this compound in murine cells, I evaluated the ability of ABT-869 to inhibit cell death in a series of cellular assays where TNF and Smac-mimetic were used in combination with different caspase inhibitors to trigger necroptosis. In these assays, WT #2 MDF cells treated with ABT-869 were stimulated to induce necroptosis and cell death was measured by propidium iodide (PI) uptake analysed by flow cytometry. The ability of ABT-869 to block necroptosis was compared to negative controls (untreated and DMSO) and positive controls (RIPK3 inhibitors, GSK'872 and GSK'843) as indicated by the percentage of PI-positive cells.

When MDF cells were preincubated for 1 hour with increasing concentrations of ABT-869 and then treated with TSQ for 18-24 hours to induce necroptosis, ABT-869 blocked cell death in a concentration-dependent manner (**Figure 3.2A**). This confirmed the results from the initial validation studies following the screen, where ABT-869 inhibited TSQ-stimulated necroptosis with an IC_{50} of around 400 nM in wild-type MDFs. When the necroptotic stimulus was changed to TSZ, where z-VAD-fmk (Z) instead of Q-VD-OPh (Q) was used as the caspase inhibitor, ABT-869 also inhibited TSZ-stimulated necroptosis at 24 hours, albeit not quite as efficiently as TSQ-stimulated necroptosis (**Figure 3.2B**). Interestingly, when TSI was employed as the necroptotic stimulus, where IDN-6556 (I) was used as the caspase inhibitor, ABT-869 was unable to block necroptosis at the same time point of 24 hours (**Figure 3.2C**). The control compounds, GSK'872 and GSK'843, retained the ability to block cell death triggered by TSI at 24 hours, although 1 μ M GSK'843 was much less effective at inhibiting this cell death, compared to the same concentration in the TSQ or TSZ assays.

Furthermore, the accidental simultaneous addition of both Q-VD-OPh and IDN-6556 caspase inhibitors resulted in the “TSQI” stimulus, which was able to induce necroptosis following treatment for 18-24 hours and was efficiently inhibited by GSK’872 and GSK’843 (**Figure 3.2D**). Moreover, ABT-869 inhibited TSQI-stimulated necroptosis, with a substantial reduction in cell death at the two highest concentrations, despite not showing inhibition of TSI-stimulated death at the same time point. While the underlying mechanism for this observation is unclear, it could involve the differing proficiency of the caspase inhibitors in blocking the formation of caspase-8 homodimers and caspase-8/cFLIP_L heterodimers (Brumatti et al., 2016).

Given that TSI is a more potent necroptotic stimulus than TSQ or TSZ (Brumatti et al., 2016), I decided to try the TSI cell death assays at an earlier time point to determine if this restored the ability of ABT-869 to block TSI-induced death. When MDFs were treated with increasing concentrations of ABT-869 with (**Figure 3.2E**) or without (**Figure 3.2F**) a 1 hour preincubation, followed by TSI stimulation for 5 hours, ABT-869 inhibited cell death to similar levels as GSK’872 and GSK’843. However, these data are preliminary (n = 1) and therefore more repeats are required to confirm that these results are reproducible. Together, these data demonstrate that ABT-869 is a small molecule inhibitor of necroptosis in murine cells and blocks necroptotic cell death driven by the TNF signalling pathway.

(C) WT #2 MDF cells were treated with increasing concentrations of ABT-869, control compounds GSK'872 and GSK'843, DMSO alone or left untreated (UT) for 3 hours then stimulated with TSI (TNF, Smac-mimetic, IDN-6556) for 24 hours to induce necroptosis. Cell death was quantified by propidium iodide (PI) staining using flow cytometry. Data represent the mean of n = 2 independent experiments and error bars represent SEM.

(D) WT #2 MDF cells were treated with increasing concentrations of ABT-869, control compounds GSK'872 and GSK'843, DMSO alone or left untreated (UT) for 1 hour then stimulated with TSQI (TNF, Smac-mimetic, Q-VD-OPh, IDN-6556) for 18-24 hours to induce necroptosis. Cell death was quantified by propidium iodide (PI) staining using flow cytometry. Data represent the mean of n = 4 independent experiments and error bars represent SEM.

(E) WT #2 MDF cells were treated with increasing concentrations of ABT-869, control compounds GSK'872 and GSK'843, DMSO alone or left untreated (UT) for 1 hour then stimulated with TSI (TNF, Smac-mimetic, IDN-6556) for 5 hours to induce necroptosis. Cell death was quantified by propidium iodide (PI) staining using flow cytometry. Data shown are from n = 1 experiment.

(F) WT #2 MDF cells were treated with increasing concentrations of ABT-869, control compounds GSK'872 and GSK'843, DMSO alone or left untreated (UT), then immediately stimulated with TSI (TNF, Smac-mimetic, IDN-6556) for 5 hours to induce necroptosis. Cell death was quantified by propidium iodide (PI) staining using flow cytometry. Data shown are from n = 1 experiment.

Chemical Proteomics with ABT-869 Chemical Probes

ABT-869 was identified and established as a novel inhibitor of necroptosis in murine cells, potentially blocking the pathway at or after MLKL activation. However, the cellular targets of ABT-869 that mediate its anti-necroptotic activity were unknown. To perform target identification, I used a functionalised derivative of ABT-869 as a chemical probe in pulldown assays to enrich putative targets, enabling their subsequent identification by mass spectrometry. This chemical probe consisted of ABT-869 conjugated to Sepharose beads (**Figure 3.3A**), which was synthesised by Dr Christoph Grohmann, a Postdoctoral Researcher in the Lessene Lab at WEHI. It had been previously established by Dr Christoph Grohmann that installing a polyethylene glycol (PEG) linker on position 7 of the indazole of ABT-869, to generate the analogue Compound **6** (**Figure 3.1A**), did not abolish the anti-necroptotic activity of this series in cells (**Figure 3.1D**). Therefore, a PEG linker was installed on the ABT-869 scaffold at this position to enable conjugation to the Sepharose resin. This PEG linker was capped with a terminal amine to covalently couple ABT-869 to NHS-activated Sepharose beads via an amide bond to generate the ABT-869 Sepharose probe (**Figure 3.3A**).

Dr Christoph Grohmann also identified a less active analogue of ABT-869, Compound **5** (**Figure 3.1A**), which lacked the 2-fluoro-5-methyl disubstitution on the terminal phenyl ring and blocked necroptosis with an IC_{50} of around 2 μ M (**Figure 3.1D**). Adopting the same conjugation approach as the ABT-869 chemical probe, Compound **5** was conjugated to Sepharose beads to generate the Compound **5** Sepharose probe (**Figure 3.3A**), which was synthesised by Dr Christoph Grohmann, for use as the negative control in the pulldown assays. The use of a structurally-related negative control in affinity enrichment experiments

is important to rule out non-specific interactions with proteins that bind non-specifically to the Sepharose resin or to some other part of the chemical scaffold.

I then employed the ABT-869 and Compound **5** Sepharose probes in chemical proteomics experiments with the aim of identifying the cellular targets of ABT-869. In the pilot pulldown assays, WT #2 MDF cell lysates were pre-treated with two concentrations of ABT-869 compound or DMSO alone, then incubated with Sepharose beads to which either ABT-869 or Compound **5** had been conjugated at 2 $\mu\text{mol/mL}$ (**Figure 3.3B**). To confirm that the pilot pulldown assays were successful, samples from various steps of the protocol were analysed by SDS-PAGE (**Figure 3.3C**). The SYPRO Ruby stained gels demonstrated that the washing steps successfully removed proteins binding non-specifically, as observed from the reduction in bands in the last wash compared to the first wash. The gels confirmed that both the ABT-869 Sepharose beads and Compound **5** Sepharose beads were able to enrich proteins from MDF cell lysates.

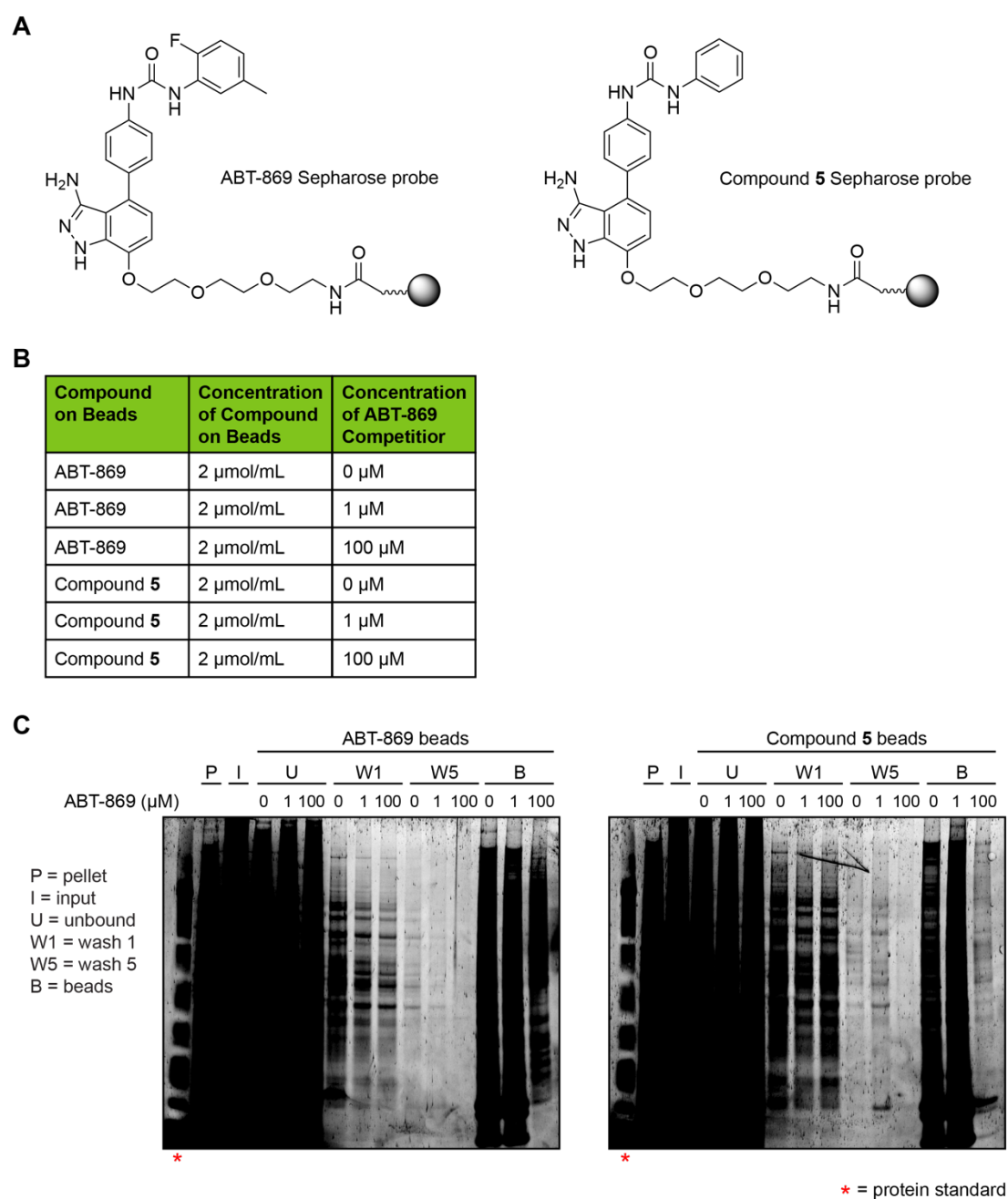


Figure 3.3 Pilot chemical proteomics with ABT-869 and Compound 5 Sepharose probes.

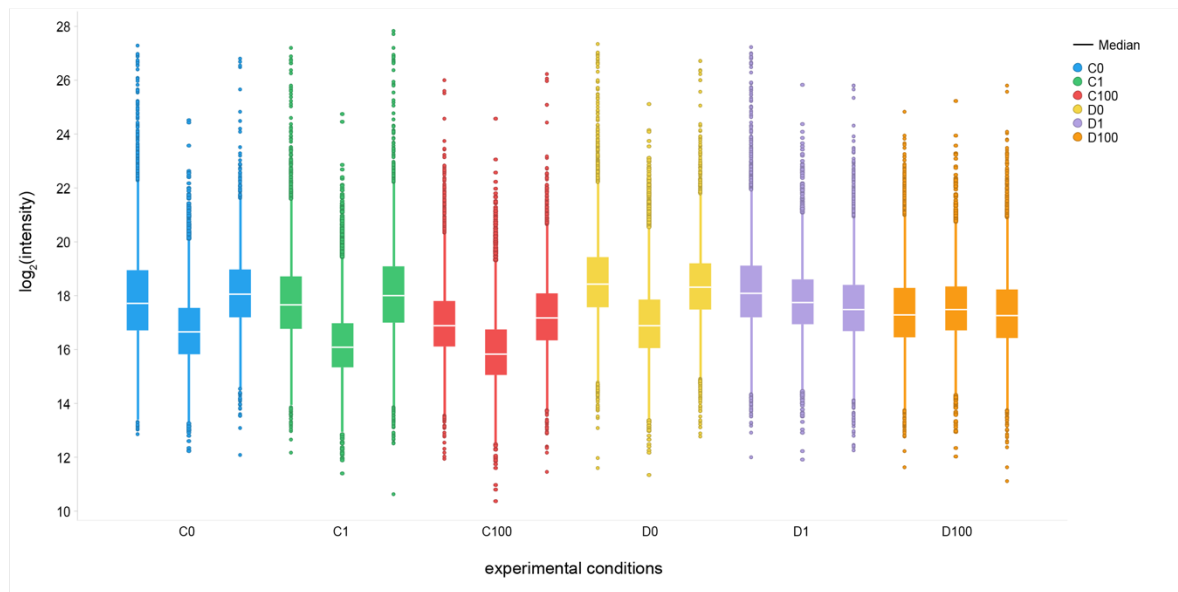
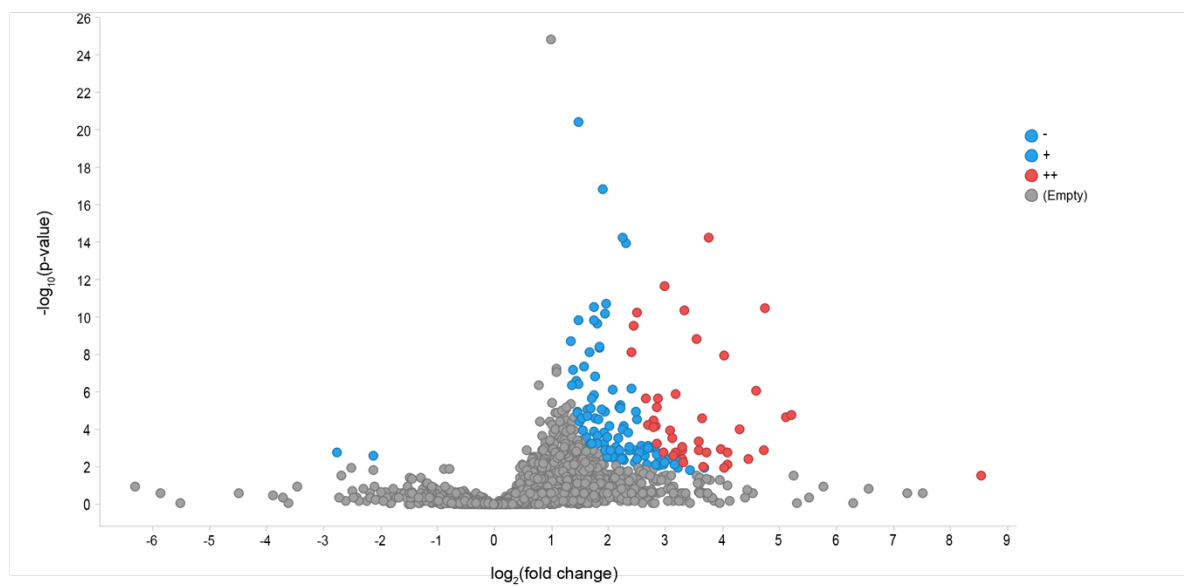
(A) Chemical structure of the ABT-869 Sepharose probe and, its negative control, the Compound **5** Sepharose probe.

(B) Summary of the conditions used in the pilot chemical proteomics to enrich the intracellular protein targets of ABT-869. ABT-869 Sepharose beads (2 $\mu\text{mol/mL}$) and Compound **5** Sepharose beads (2 $\mu\text{mol/mL}$) were competed with DMSO, 1 μM ABT-869 or 100 μM ABT-869.

(C) Gel analysis of the pilot pulldown assays. MDF cell lysate supernatants were incubated with increasing concentrations of ABT-869 compound as specified in **(B)**. ABT-869 Sepharose beads (2 $\mu\text{mol/mL}$) and Compound **5** Sepharose beads (2 $\mu\text{mol/mL}$) were incubated with the pre-treated lysates

and washed to remove proteins binding non-specifically. Pellet (cell lysate pellet), input (cell lysate supernatant), unbound (supernatant following incubation with the beads), wash 1 (supernatant following first wash with RIPA buffer), wash 5 (supernatant following last wash with ModRIPA buffer) and bead (proteins captured by beads) samples were resolved by SDS-PAGE and stained with SYPRO Ruby. One representative result shown from n = 3 independent experiments.

To identify the proteins enriched in the pilot pulldown assays, proteomic analysis of the ABT-869 and Compound **5** beads samples was performed. Following on-bead trypsin digestion of the enriched proteins, mass spectrometry was performed and analysed by Dr Jarrod Sandow, a Postdoctoral Researcher in the Proteomics Lab at WEHI. However, the quality control box plot demonstrated that there was variability between experimental repeats within a given condition (**Figure 3.4A**). In addition, poor competition profiles were generally observed and the enriched proteins (**Figure 3.4B**) did not appear to be competed by ABT-869, as the intensity did not decrease with increasing concentrations of ABT-869 competitor (**Figure 3.4C**). This could be attributed to the high on-bead concentration of ABT-869 and, as a result, the concentrations of ABT-869 competitor were likely insufficient to compete with this high concentration on the beads. Therefore, these factors made it difficult to draw any reliable conclusions from these initial pulldown assays.

A**B**

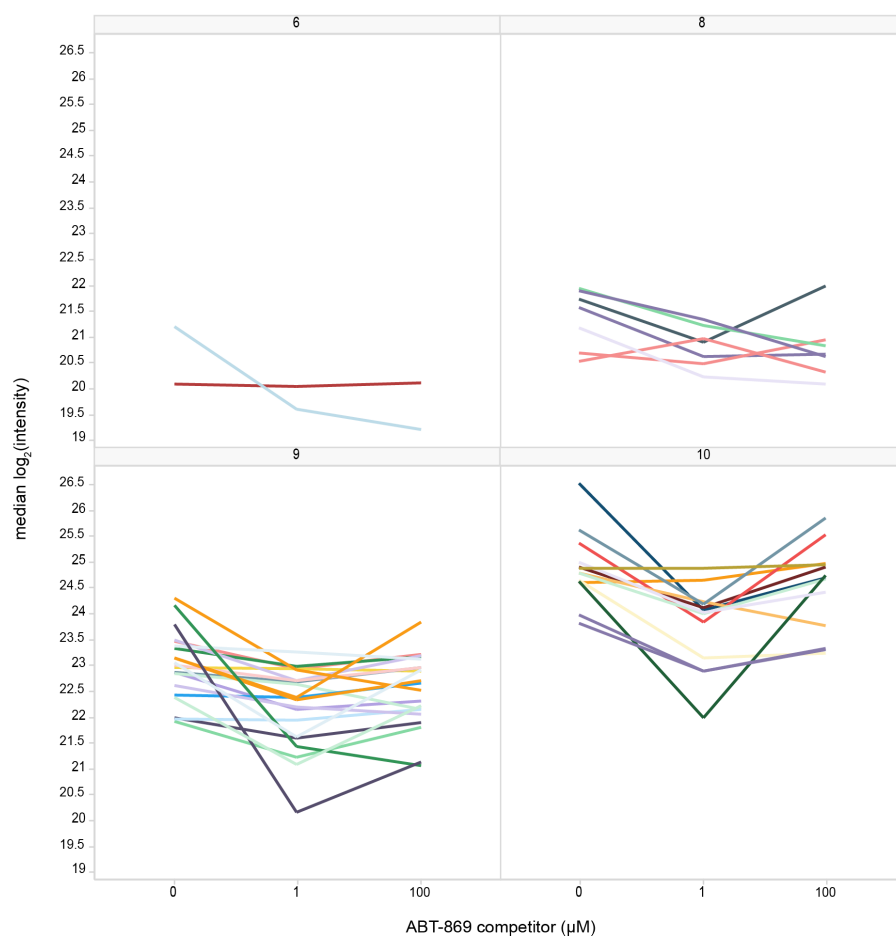
C

Figure 3.4 Mass spectrometry analysis of pilot chemical proteomics.

(A) Quality control box plot from the proteomic analysis depicting the number of peptides identified from each condition across all 3 repeats, as a plot of intensity against experimental conditions. Each condition (as specified in **Figure 3.3B**) is shown in a different colour, each individual box depicts a single repeat of that condition and the line across the middle of each box is the median value. The letters C and D refer to the Compound **5** beads and ABT-869 beads, respectively, and the number following the letter refers to the concentration of ABT-869 competitor. Data analysed from $n = 3$ independent experiments.

(B) Volcano plots from the proteomic analysis depicting proteins enriched by the Sepharose probes (2 $\mu\text{mol/mL}$), as a plot of p-value against fold change where each circle represents one protein. Proteins towards the top right indicate proteins enriched by the ABT-869 beads, but not the Compound **5** beads. Proteins towards the top left indicate proteins enriched by the Compound **5** beads, but not the ABT-869 beads. Red circles represent 4-fold enrichment, blue circles represent 2-fold enrichment and grey circles represent less than 2-fold enrichment. Data analysed from $n = 3$ independent experiments.

(C) Competition profiles from the proteomic analysis depicting ABT-869 competition, as plots of median intensity against increasing concentrations of ABT-869 competitor (1 μM and 100 μM).

Competition profiles of proteins with 4-fold enrichment, depicted by the red circles in **(B)**, are shown. Proteins with similar competition profiles are clustered together. Data analysed from $n = 3$ independent experiments.

Accordingly, the pulldown conditions were modified to address these issues and to improve the reliability and robustness of the experiments. I adopted several key changes to optimise the pulldown protocol. Firstly, the compounds were conjugated to Sepharose at two concentrations, 2 $\mu\text{mol/mL}$ and 0.2 $\mu\text{mol/mL}$, and the concentration of compounds on the Sepharose was taken into account when determining the concentrations of ABT-869 competitor. Furthermore, it was decided to use additional concentrations of ABT-869 competitor to increase the number of points in the competition profile. Secondly, a different wild-type MDF cell line, WT #1 MDF, was used and the cells were first stimulated with TSI for 3 hours to activate necroptosis signalling prior to performing the pulldown, to ensure necroptotic targets were enriched by the probes. Thirdly, additional repeats were performed relative to the pilot experiment to minimise the effect of variability between experimental repeats.

Using these optimised conditions, a larger scale chemical proteomics experiment was performed. The TSI-stimulated WT #1 MDF cell lysates were pre-treated with increasing concentrations of ABT-869 compound or DMSO alone and then incubated with the ABT-869 beads at both 2 $\mu\text{mol/mL}$ and 0.2 $\mu\text{mol/mL}$, while cell lysates pre-treated with DMSO alone were incubated with the Compound **5** control beads at both 2 $\mu\text{mol/mL}$ and 0.2 $\mu\text{mol/mL}$ (**Figure 3.5A**). SDS-PAGE analysis of samples from various steps of this modified protocol demonstrated that both concentrations of compounds on the beads were able to capture proteins from TSI-stimulated MDF cell lysates (**Figure 3.5B**). The 2 $\mu\text{mol/mL}$ beads showed

greater enrichment of proteins than the 0.2 $\mu\text{mol/mL}$ beads, as expected due to the higher concentration of compound on the Sepharose. In addition, the washes successfully removed proteins binding non-specifically, with the complete absence of bands in the last wash compared to the first wash.

A

Compound on Beads	Concentration of Compound on Beads	Concentration of ABT-869 Competitor
Compound 5	2 $\mu\text{mol/mL}$	0 μM
ABT-869	2 $\mu\text{mol/mL}$	0 μM
ABT-869	2 $\mu\text{mol/mL}$	40 μM
ABT-869	2 $\mu\text{mol/mL}$	80 μM
ABT-869	2 $\mu\text{mol/mL}$	160 μM
Compound 5	0.2 $\mu\text{mol/mL}$	0 μM
ABT-869	0.2 $\mu\text{mol/mL}$	0 μM
ABT-869	0.2 $\mu\text{mol/mL}$	4 μM
ABT-869	0.2 $\mu\text{mol/mL}$	20 μM
ABT-869	0.2 $\mu\text{mol/mL}$	100 μM

B

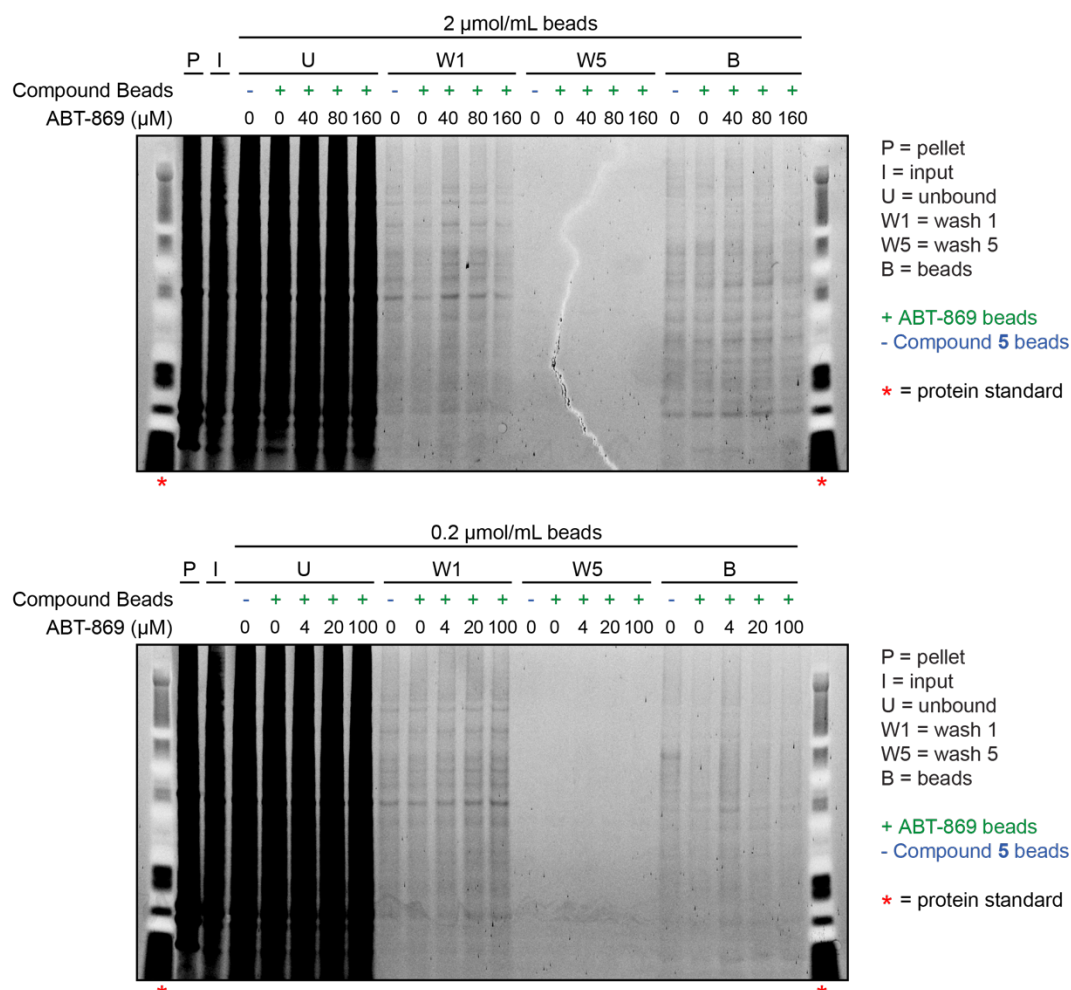


Figure 3.5 Larger scale chemical proteomics with ABT-869 and Compound 5 Sepharose probes.

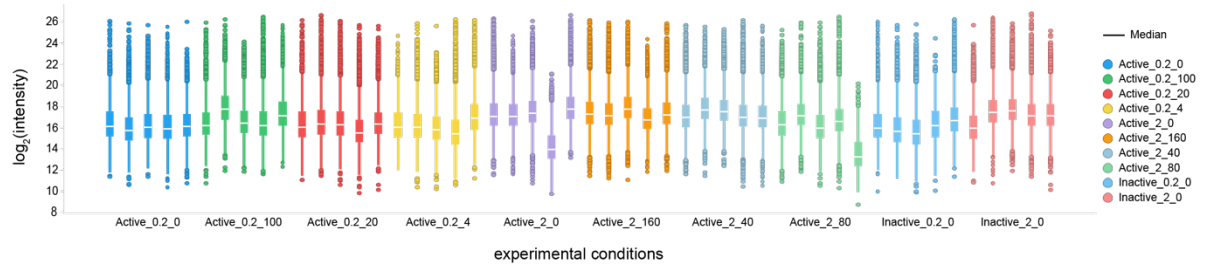
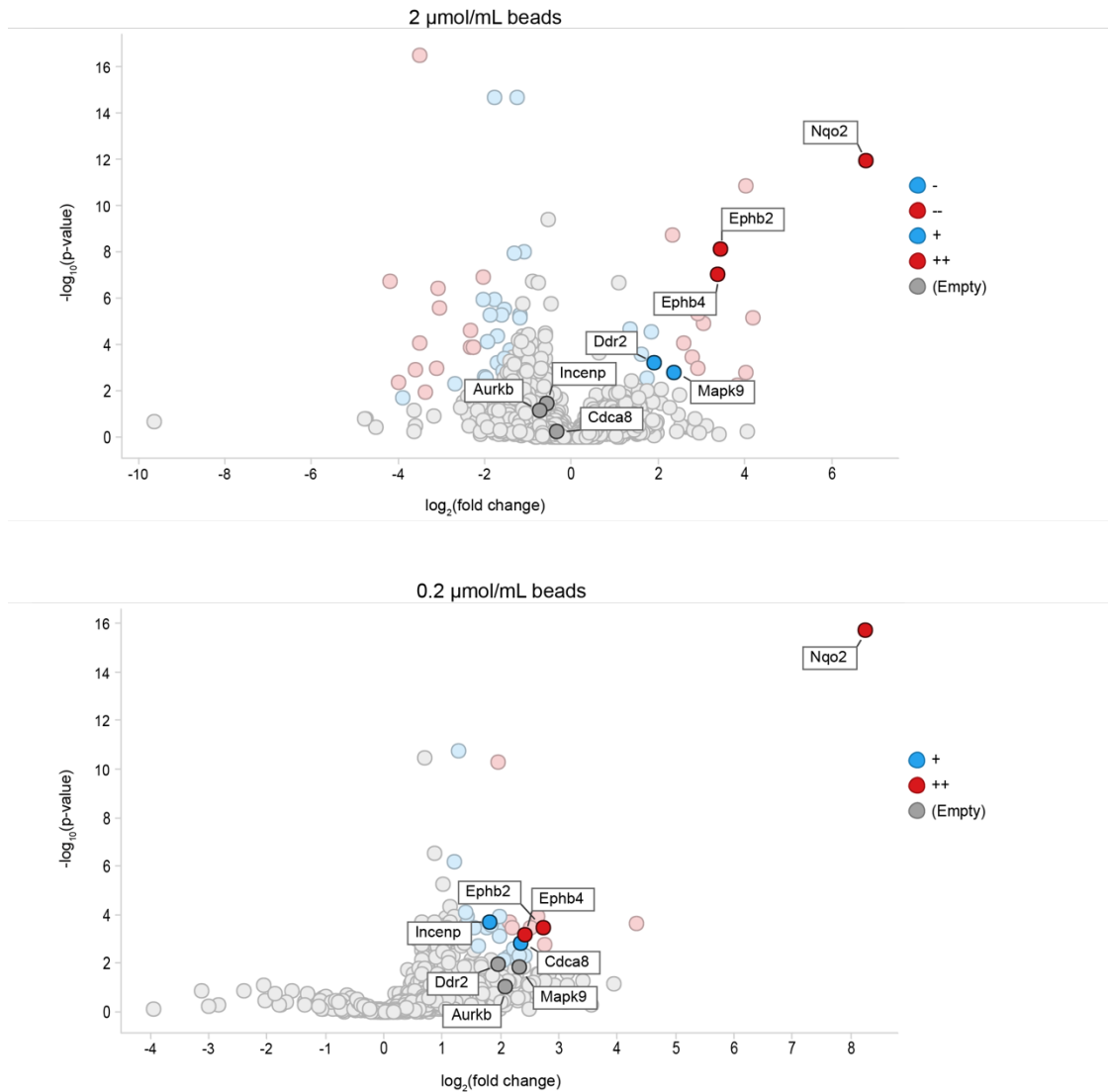
(A) Summary of conditions used in the larger scale chemical proteomics to enrich the intracellular protein targets of ABT-869. ABT-869 Sepharose beads at two concentrations (2 $\mu\text{mol/mL}$ and 0.2

$\mu\text{mol/mL}$) were competed with DMSO or increasing concentrations of ABT-869 compound (40, 80, 160 μM and 4, 20, 100 μM). Compound **5** Sepharose beads were competed with DMSO alone.

(B) Gel analysis of the larger scale pulldown assays. MDF cells were stimulated with TSI (TNF, Smac-mimetic, IDN-6556) for 3 hours, then the cell lysate supernatants were incubated with increasing concentrations of ABT-869 compound as specified in **(A)**. ABT-869 Sepharose beads (2 $\mu\text{mol/mL}$ and 0.2 $\mu\text{mol/mL}$) and Compound **5** Sepharose beads (2 $\mu\text{mol/mL}$ and 0.2 $\mu\text{mol/mL}$) were incubated with the pre-treated lysates and washed to remove proteins binding non-specifically. Pellet (cell lysate pellet), input (cell lysate supernatant), unbound (supernatant following incubation with the beads), wash 1 (supernatant following first wash with RIPA buffer), wash 5 (supernatant following last wash with ModRIPA buffer) and bead (proteins captured by beads) samples were resolved by SDS-PAGE and stained with SYPRO Ruby. Data shown are from $n = 1$ experiment.

Following on-bead trypsin digestion of the proteins captured by the ABT-869 and Compound **5** beads, mass spectrometry was once again performed and analysed by Dr Jarrod Sandow. The quality control box plot demonstrated that there was generally good consistency across the five repeats in each condition (**Figure 3.6A**). This relatively good consistency between the experimental repeats illustrated that this larger scale pulldown experiment was robust and, therefore, that the proteomic data were reliable. The volcano plots depicting the identified proteins enriched by the ABT-869 beads compared to the Compound **5** beads, demonstrated that more proteins overall were enriched by the 2 $\mu\text{mol/mL}$ beads than the 0.2 $\mu\text{mol/mL}$ beads (**Figure 3.6B**). This could be due to avidity effects, whereby increased avidity resulting from the higher concentration of compound on the Sepharose resin enables weaker interactors to be captured. Furthermore, some of the enriched proteins displayed the desired competition profile, whereby the intensity decreased as the concentration of ABT-869 competitor increased (**Figure 3.6C**).

From the proteomic data of the larger scale pulldowns, the top protein hits were selected based on two key criteria. The first criterion was that proteins were enriched by the ABT-869 beads, but not the Compound **5** beads, indicating that they were potential targets of ABT-869, but not Compound **5**. The second criterion was that proteins were competed off the beads by increasing concentrations of ABT-869, confirming that they were potential targets of ABT-869. The top eight protein hits that fulfilled these criteria were: NQO2, EphB2, EphB4, DDR2, MAPK9, AurKB, Borealin and INCENP.

A**B**

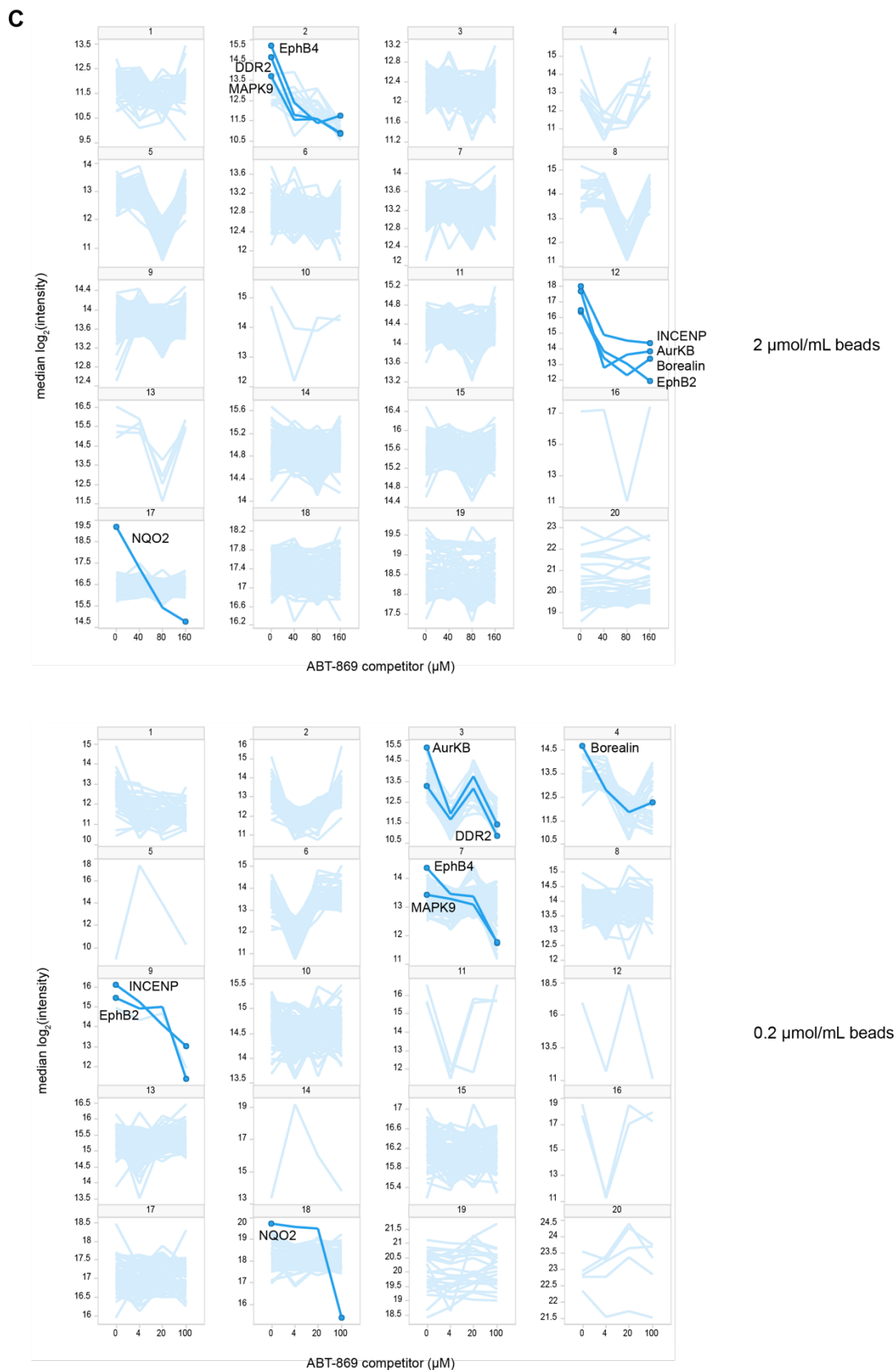


Figure 3.6 Mass spectrometry analysis of larger scale chemical proteomics.

(A) Quality control box plot from the proteomic analysis depicting the number of peptides identified from each condition across all five repeats, as a plot of intensity against experimental conditions. Each

condition (as specified in **Figure 3.5A**) is shown in a different colour, each individual box depicts a single repeat of that condition and the line across the middle of each box is the median value. Active refers to the ABT-869 beads and inactive refers to the Compound **5** beads. The number in between the underscore symbols refers to the concentration of compound on the beads, while the last number refers to the concentration of ABT-869 competitor. Data analysed from $n = 5$ independent experiments.

(B) Volcano plots from the proteomic analysis depicting proteins enriched by the Sepharose probes (2 $\mu\text{mol/mL}$ and 0.2 $\mu\text{mol/mL}$), as a plot of p-value against fold change where each circle represents one protein. Proteins towards the top right indicate proteins enriched by the ABT-869 beads, but not the Compound **5** beads. Proteins towards the top left indicate proteins enriched by the Compound **5** beads, but not the ABT-869 beads. Red circles represent 4-fold enrichment, blue circles represent 2-fold enrichment and grey circles represent less than 2-fold enrichment. The top eight protein hits are highlighted and labelled. Data analysed from $n = 5$ independent experiments.

(C) Competition profiles from the proteomic analysis depicting ABT-869 competition, as plots of median intensity against increasing concentrations of ABT-869 competitor, for the 2 $\mu\text{mol/mL}$ beads and 0.2 $\mu\text{mol/mL}$ beads. Proteins with similar competition profiles are clustered together. The top eight protein hits are highlighted and labelled. Data analysed from $n = 5$ independent experiments.

While none of these proteins are known to participate in necroptosis signalling, they have been attributed various functions in other cell signalling pathways. N-ribosyldihydronicotinamide:quinone oxidoreductase 2 (NQO2) is a cytosolic quinone reductase whose precise physiological function is not known, but is believed to be broadly involved in detoxification, metabolic and biosynthetic pathways (Janda et al., 2020). Ephrin type-B receptors 2 and 4 (EphB2 and EphB4) are receptor tyrosine kinases, with roles in neurogenesis and angiogenesis, respectively (Pasquale, 2008). The discoidin domain-containing receptor 2 (DDR2) is also a receptor tyrosine kinase that is activated by fibrillar collagen and is involved in the remodelling of the extracellular matrix for cell adhesion and migration (Leitinger, 2014). Mitogen-activated protein kinase 9 (MAPK9), also known as c-Jun N-terminal kinase 2 (JNK2), is a serine-threonine kinase involved in a wide range of cellular

processes including proliferation, differentiation and migration (Zeke et al., 2016). Aurora kinase B (AurKB) is also a serine-threonine kinase, while Borealin (also known as cell division cycle-associated protein 8 (CDCA8)) and inner centromere protein (INCENP) are both non-kinase proteins (Carmena et al., 2012). Together AurKB, Borealin and INCENP are components of the chromosomal passenger complex, a key regulator of mitosis that ensures correct chromosome alignment and segregation (Carmena et al., 2012).

A 2011 paper by Davis *et al.* reported a comprehensive study of kinase inhibitor selectivity where 72 known kinase inhibitors were profiled against 442 kinases, covering >80% of the human catalytic protein kinome, using DiscoverX competition binding assays (Davis et al., 2011). Interestingly, ABT-869 was one of the 72 kinase inhibitors evaluated in this study and all of the kinases identified as hits from the chemical proteomics – EphB2, EphB4, DDR2, MAPK9 and AurKB – were included in the kinase panel. Indeed, ABT-869 was reported to bind to all of these kinases with the following binding affinities (K_D): 1.4 μ M for EphB2, 1.4 μ M for EphB4, 3.8 μ M for DDR2, 240 nM for MAPK9 and 390 nM for AurKB (Davis et al., 2011). However, as the other three protein hits – NQO2, Borealin and INCENP – are not kinases, they were not evaluated in this study. Nevertheless, these data support the possibility that the proteins identified from the chemical proteomics could be the targets of ABT-869 mediating its anti-necroptotic activity in cells.

Investigating NQO2 as a Potential Necroptotic Target Protein of ABT-869

The first approach I employed to validate the protein hits identified from the chemical proteomics was to genetically delete the proteins from MDF cells using the well-established CRISPR gene editing system. Following this, the knockout cell lines would then be evaluated in necroptosis assays to determine whether deletion of the protein ablated the anti-necroptotic activity of ABT-869 and/or prevented cell death upon necroptosis induction. As NQO2 was the top hit identified and was much more significantly enriched than the other seven protein hits in the proteomic analysis (**Figure 3.6B**), this was chosen as the first candidate to investigate as a potential novel target of ABT-869 and a potential novel component of the necroptosis pathway downstream of MLKL.

Using CRISPR-Cas9 gene editing (Ran et al., 2013), two single guide RNA (sgRNA) targeting *Nqo2* were each cloned into the pSpCas9(BB)-2A-GFP (PX458) plasmid encoding Cas9 endonuclease and green fluorescent protein (GFP). In the initial attempt, WT #164 MDF cells were transfected with plasmids containing either the *Nqo2* sgRNA, denoted sg1A and sg2B, or the empty PX458 vector, as a control, using the lipid transfection reagent FuGENE 6, which had been previously used by others at WEHI for murine embryonic fibroblast (MEF) cells. However, this yielded no GFP-positive cells by fluorescence-activated cell sorting (FACS) for either the empty PX458 vector or the sg1A or sg2B plasmids, indicating that the MDF cells had not taken up the plasmid DNA (data not shown). As performing CRISPR experiments in MDF cells was a new approach, some optimisation was required.

Subsequently, I performed an optimisation of the lipid transfection reagent by determining the ability of different reagents to enable the transfection of the empty PX458 vector into

wild-type MDF cells, as measured by GFP fluorescence. Compared to untransfected cells, GFP-positive cells were obtained with all four transfection reagents tested, albeit to varying extents (**Figure 3.7A**). Lipofectamine 3000 was the most efficient transfection reagent, with a transfection rate of 16%, while Effectene was the least efficient, with a transfection rate of 1.6%. Lipofectamine 2000 and FuGENE HD displayed intermediate transfection efficiencies, with transfection rates of 5.5% and 3%, respectively. For all future experiments, Lipofectamine 2000 was used as the transfection reagent as it was more readily available than Lipofectamine 3000.

I then transfected WT #164 MDF cells with the sg1A plasmid, sg2B plasmid or empty vector control using Lipofectamine 2000 and GFP-positive cells were single cell sorted by FACS. Following expansion of the single cell clones in culture, NQO2 protein levels were analysed by Western blot to determine whether *Nqo2* had been successfully deleted from MDFs (**Figure 3.7B**). For the 15 clones obtained from the sg1A plasmid transfection and 10 clones obtained from the sg2B plasmid transfection, all but one clone for each cell line (clone #9 for sg1A and clone #3 for sg2B) contained no detectable NQO2 protein. As a control, NQO2 protein was present in all 11 empty vector clones. This demonstrates that NQO2 was able to be deleted from MDF cells, with very high knockout efficiency, to generate *Nqo2*^{-/-} MDF cell lines.

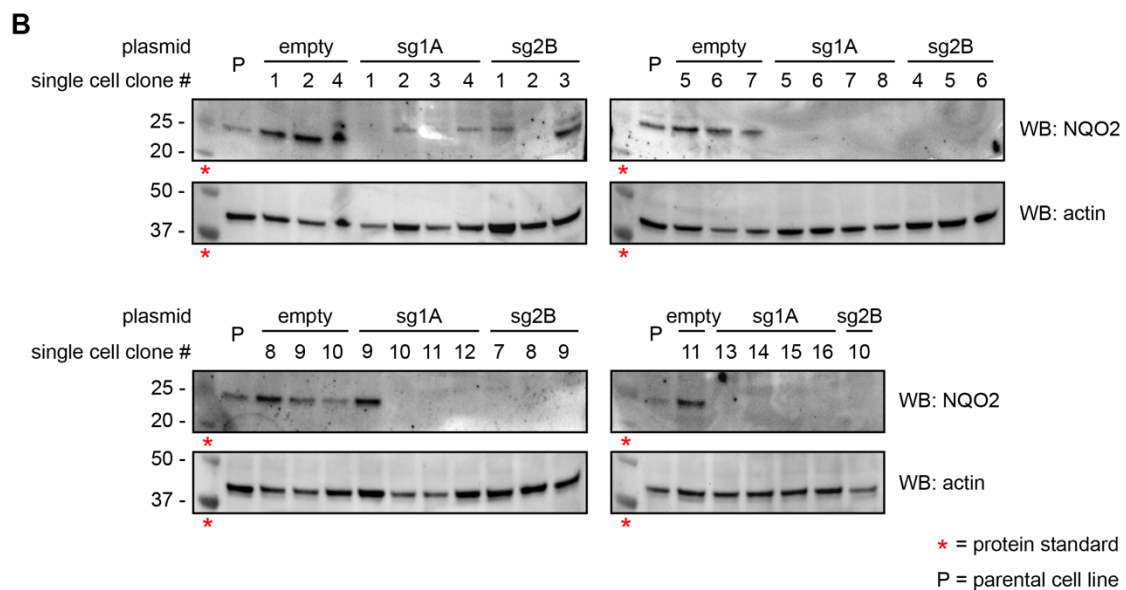
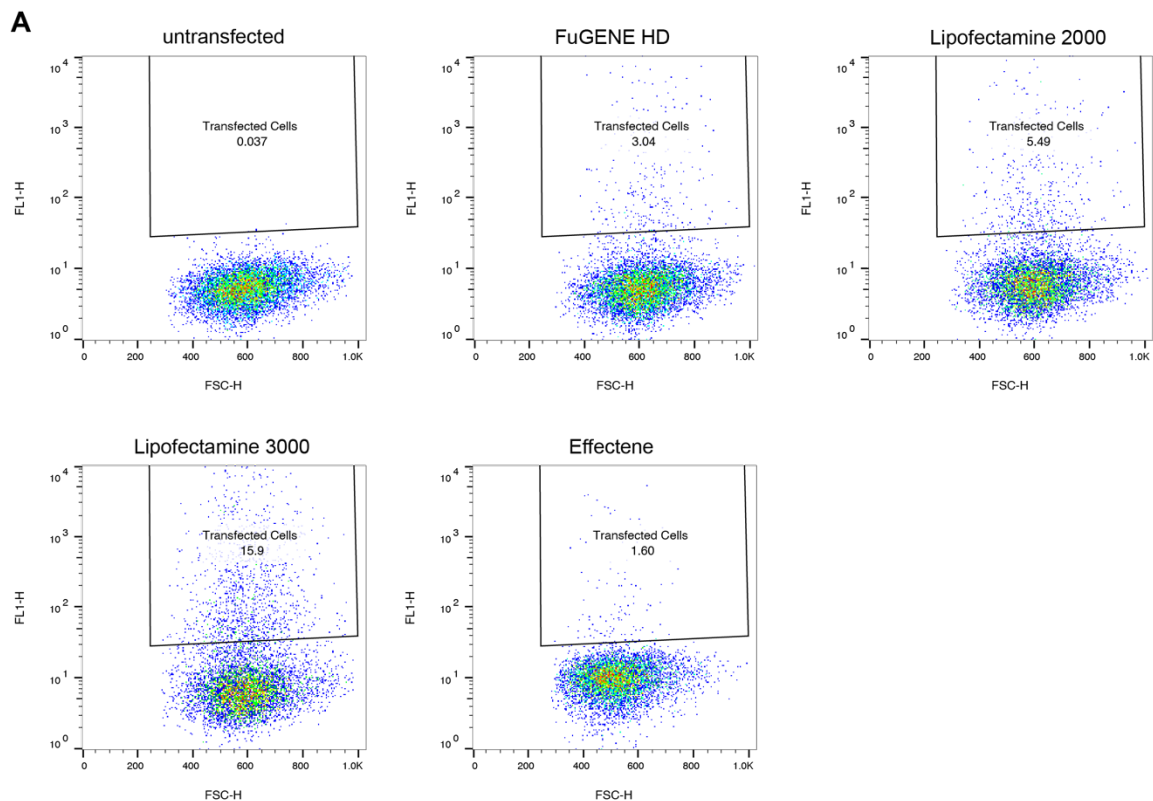


Figure 3.7 Generation of *Nqo2*^{-/-} CRISPR MDF single cell clones.

(A) WT #164 MDF cells were transfected with the empty pSpCas9(BB)-2A-GFP (PX458) vector using four different lipid transfection reagents (FuGENE HD, Lipofectamine 2000, Lipofectamine 3000, Effectene) or left untransfected. The transfection efficiency of each lipid transfection reagent was measured by GFP fluorescence using flow cytometry, as depicted by the dot plots of fluorescence (FL1) against forward scatter (FSC). The transfection rate was quantified as the percentage of GFP-positive cells. Data shown are from n = 1 experiment.

(B) WT #164 MDF cells were transfected with two *Nqo2* sgRNA cloned into the PX458 plasmid, sg1A and sg2B, or the empty PX458 vector, as a control. GFP-positive cells were single cell sorted by fluorescence-activated cell sorting (FACS) and expanded in culture. Cell lysates were prepared from the empty, sg1A and sg2B single cell clones and NQO2 protein levels were detected by Western blot. Red asterisks denote protein standards and the letter P refers to the parental cell line. Data shown are from n = 1 experiment.

To determine whether the *Nqo2*^{-/-} MDF cells behaved differently to wild-type MDF cells in response to necroptosis induction or ABT-869 treatment, one representative clone from each of the empty vector, sg1A and sg2B cell lines were assessed in cell death assays (**Figure 3.8A**). When empty vector clone #8, sg1A clone #10 and sg2B clone #8 were treated with ABT-869 and stimulated with TSI to induce necroptosis for 24 hours, the same time point previously used to evaluate cell death (**Figure 3.2**), no difference was observed between the wild-type and *Nqo2*^{-/-} cell lines in their response to ABT-869 treatment or TSI stimulation. However, surprisingly, TSI did not trigger cell death in the empty vector cell line, with PI-positive levels resembling those of the non-TSI treated cells.

To investigate why the empty vector control cells did not respond to necroptotic stimuli, a panel of independently-derived wild-type MDF cell lines available in-house were collated, including the parental (untransfected) cell line used to generate the *Nqo2*^{-/-} cells. These various wild-type MDFs were tested, alongside the *Nqo2*^{-/-} MDFs, in cell death assays to check their response to TSI stimulation (**Figure 3.8B**). Even more surprisingly, most of these wild-type MDF cell lines did not undergo cell death upon treatment with TSI, including the parental cell line used to generate the *Nqo2*^{-/-} cells. Only WT #1 and WT #2 showed substantial cell death upon TSI stimulation, compared to the non-TSI treated cells. This provided an

explanation as to why the empty vector control cells were unable to undergo necroptosis, as it was discovered that the parental cell line used for CRISPR was resistant to necroptosis.

To investigate why different wild-type MDF cell lines displayed different sensitivity to necroptosis induction, protein levels of the necroptotic effectors were evaluated by Western blot (**Figure 3.8C**). While MLKL protein levels displayed some variability between the cell lines, there was no obvious correlation to their cell death response. RIPK1 protein levels were consistent across all cell lines. Strikingly, marked differences in RIPK3 protein levels were found between the different cell lines, to the extent that no detectable RIPK3 was observed in some cell lines. Furthermore, the RIPK3 levels detected by Western blot generally correlated with the level of response to TSI stimulation in the cell death assays. WT #1 and WT #2, the two cell lines that underwent necroptosis in response to TSI stimulation, were the two cell lines with the most RIPK3. All the other MDF cell lines, which did not respond to necroptosis, contained less or no RIPK3 protein. Lastly, it was also confirmed that NQO2 was present in all the wild-type cell lines, except the *Nqo2*^{-/-} sg1A and sg2B clones. Together this demonstrated that the loss of, or decrease in, RIPK3 accounted for the necroptosis resistance observed in some MDF cell lines. This finding was unexpected, but consistent with observations that cell lines derived from MEF cells, another source of murine fibroblasts, also exhibit variable expression of critical cell death proteins (Cook et al., 2014).

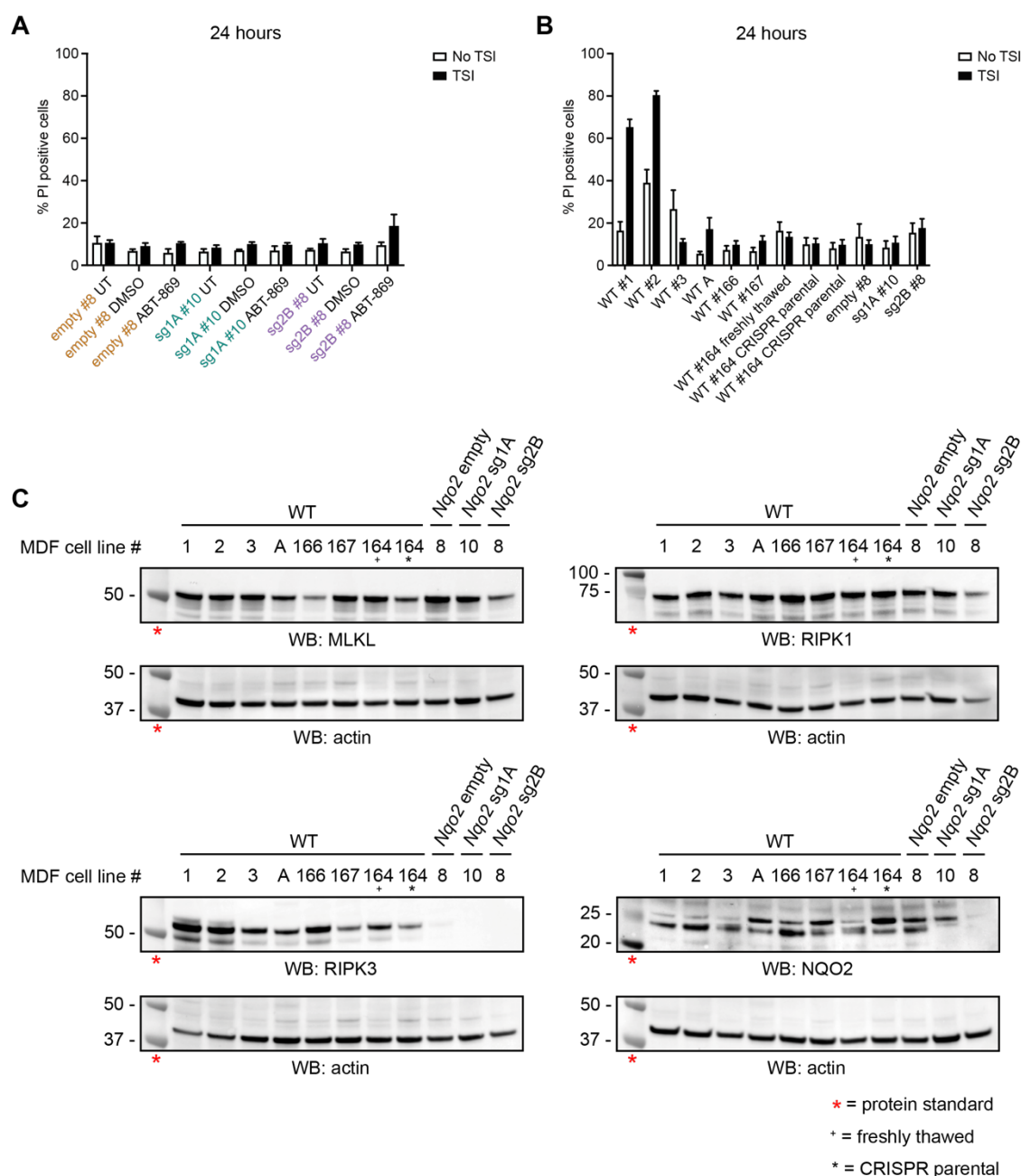


Figure 3.8 Evaluation of *Nqo2*^{-/-} CRISPR MDF single cell clones.

(A) *Nqo2* CRISPR MDF empty clone #8, sg1A clone #10 and sg2B clone #8 from **Figure 3.7B** were treated with ABT-869 (1 μ M), DMSO alone or left untreated (UT), and immediately stimulated with TSI (TNF, Smac-mimetic, IDN-6556) for 24 hours to induce necroptosis. Cell death was quantified by propidium iodide (PI) staining using flow cytometry. Data represent the mean of $n = 2$ independent experiments and error bars represent SEM.

(B) A panel of independently-derived wild-type MDF cell lines available in-house and a selection of the *Nqo2* CRISPR MDF clones (empty clone #8, sg1A clone #10, sg2B clone #8) were stimulated with TSI (TNF, Smac-mimetic, IDN-6556) for 24 hours to induce necroptosis. Cell death was quantified by

propidium iodide (PI) staining using flow cytometry. WT #164 CRISPR parental cells were performed in duplicate. Data represent the mean of n = 3 independent experiments and error bars represent SEM.

(C) Cell lysates were generated from the panel of independently-derived wild-type MDF cell lines available in-house and a selection of the *Nqo2* CRISPR MDF clones (empty clone #8, sg1A clone #10, sg2B clone #8). Protein levels of MLKL, RIPK1, RIPK3 and NQO2 were detected by Western blot. Red asterisks denote protein standards, black crosses denote WT #164 freshly thawed cells and black asterisks denote WT #164 CRISPR parental cells. One representative result shown from n = 3 independent experiments.

Based on the identification that WT #1 and WT #2 were the wild-type MDF cell lines most responsive to necroptosis, I remade the CRISPR *Nqo2*^{-/-} cells using these two cell lines as the parental cells. WT #1 and WT #2 MDFs were transfected with the sg1A plasmid, sg2B plasmid or empty PX458 vector control using Lipofectamine 2000 and GFP-positive cells were sorted by FACS. However, this time, the GFP-positive cell pools were collected rather than single cell sorting, given the high knockout efficiency previously observed in the single cell clones. When the empty, sg1A and sg2B cell pools were analysed by Western blot, no detectable NQO2 protein was observed in the cells transfected with either sgRNA, compared to the empty vector control, on either the WT #1 or WT #2 background (**Figure 3.9A**). Concurrent analysis of the necroptotic effectors showed no substantial difference in the protein levels of MLKL, RIPK1 or RIPK3 between the empty, sg1A and sg2B cell pools on either the WT #1 or WT #2 background.

To determine the impact of *Nqo2* deletion on necroptosis and ABT-869 activity, the *Nqo2*^{-/-} MDF cell pools were subjected to a series of cell death assays to evaluate their response to necroptosis stimulation and ABT-869 treatment. While similar results were obtained with the *Nqo2*^{-/-} cell lines derived from either the WT #1 or WT #2 parental cells, the *Nqo2*^{-/-} cell lines

on the WT #2 background were found to be more robust and thus were preferentially used in subsequent experiments. In initial preliminary assays ($n = 1$), the empty vector, sg1A *Nqo2*^{-/-} and sg2B *Nqo2*^{-/-} cell lines derived from the WT #2 parental cells were treated with various necroptosis-inducing stimuli for either 24 hours or 5 hours. When stimulated with TSQ, TSZ or TSI for 24 hours, no striking difference was observed between the empty, sg1A and sg2B cells in their cell death response following necroptosis stimulation, compared to the untreated cells (**Figure 3.9B**). Similarly, no striking difference between the control and *Nqo2*^{-/-} cell lines was observed after TSI stimulation for 5 hours, while little or no cell death was induced by TSQ or TSZ at this time point (**Figure 3.9C**). Nevertheless, this earlier time point was investigated to mitigate potentially overlooking any subtle protection afforded by *Nqo2* loss that might not be evident following 24 hour death stimulation. However, these data are preliminary ($n = 1$) and therefore more repeats need to be performed to confirm that these results are reproducible. Although preliminary, these data suggested that *Nqo2* loss does not impact necroptosis in murine cells.

Subsequently, the impact of *Nqo2* deletion on the ability of ABT-869 to block necroptosis was investigated. When the *Nqo2*^{-/-} MDF cell lines on the WT #2 background were treated with ABT-869 for 1 hour prior to stimulation with TSQ or TSZ for 24 hours, ABT-869 inhibited cell death in the sg1A or sg2B cells to comparable levels as the empty vector control cells (**Figure 3.9D**). Preliminary data ($n = 1$) for *Nqo2*^{-/-} cells treated with ABT-869 for 1 hour then stimulated with TSI for 5 hours, indicated that there was no striking difference between the ability of ABT-869 to block death in the empty vector and sg1A cells (**Figure 3.9E**). There was somewhat more of an apparent difference between the sg2B and control cells in response to ABT-869 treatment, however, as these data are preliminary ($n = 1$), more experimental

repeats are required prior to drawing any conclusions from this data. Nevertheless, given that the deletion of *Nqo2* did not attenuate the ability of ABT-869 to block TSQ or TSZ-stimulated necroptosis (**Figure 3.9D**), this suggests that NQO2 does not mediate the anti-necroptotic activity of ABT-869 in MDFs. Furthermore, as *Nqo2* deletion had no marked effect on the level of cell death induced by TSQ or TSZ (**Figure 3.9B and 3.9D**), or in preliminary studies, by TSI (**Figure 3.9C and 3.9E**), this suggests that NQO2 is most likely not involved in TNF-driven necroptosis. However, there is still the possibility that one or more of the other seven protein hits identified from the chemical proteomics could be the cellular targets of ABT-869 that mediate its ability to inhibit necroptosis.

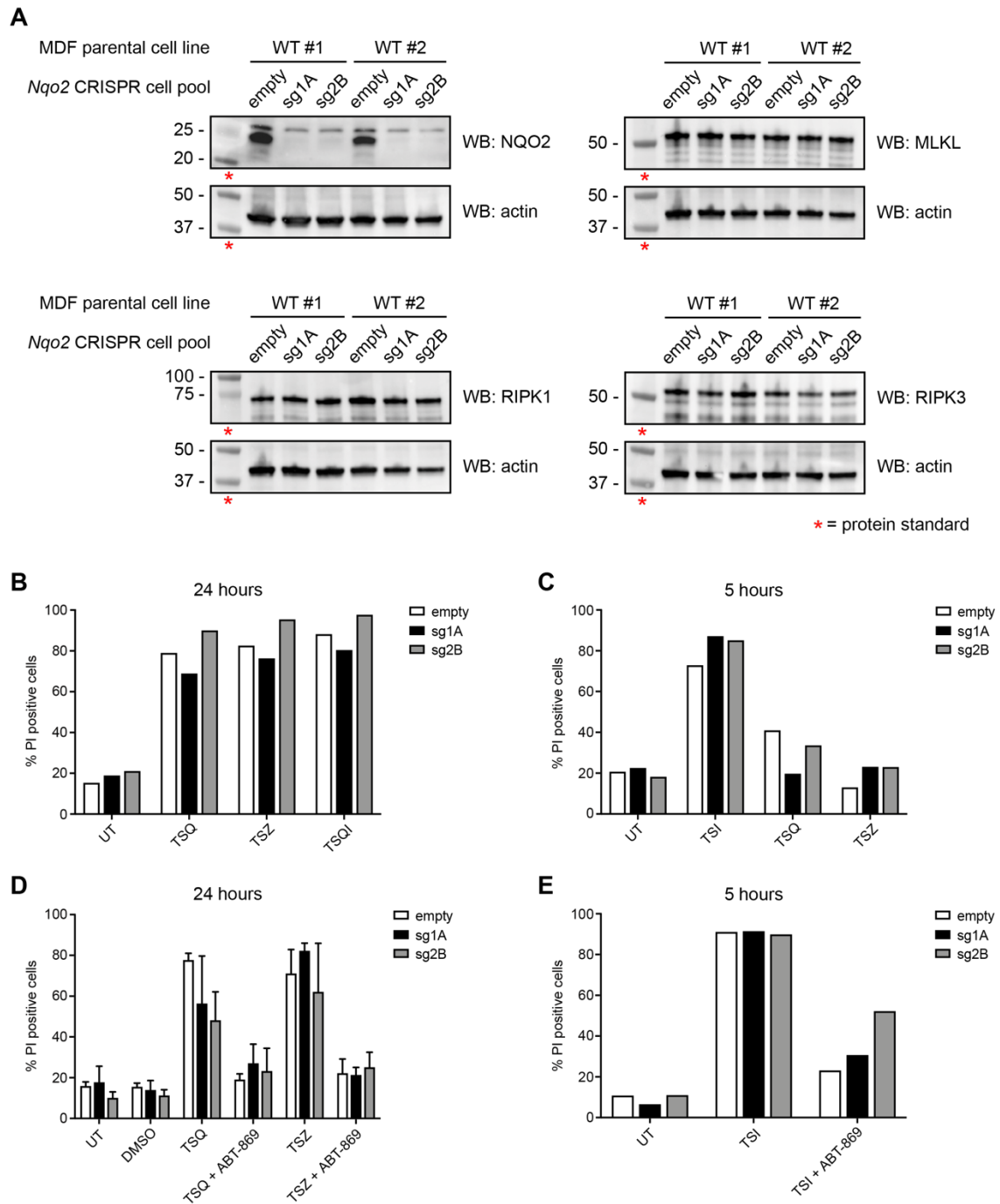


Figure 3.9 Generation of *Nqo2*^{-/-} CRISPR MDF cell pools.

(A) WT #1 or WT #2 MDF cells were transfected with two *Nqo2* sgRNA cloned into the PX458 plasmid, sg1A and sg2B, or the empty PX458 vector, as a control. GFP-positive cells were sorted by FACS and GFP-positive cell pools were collected and expanded in culture. Whole cell lysates were prepared from the empty, sg1A and sg2B cell pools and protein levels of NQO2, MLKL, RIPK1 and RIPK3 were detected by Western blot. Red asterisks denote protein standards. One representative result shown from $n = 3$ independent experiments.

(B) *Nqo2* CRISPR empty, sg1A and sg2B cell pools generated from the WT #2 MDF parental cell line were left untreated (UT) or stimulated with TSQ (TNF, Smac-mimetic, Q-VD-OPh), TSZ (TNF, Smac-mimetic, z-VAD-fmk) or TSQI (TNF, Smac-mimetic, Q-VD-OPh, IDN-6556) for 24 hours to induce necroptosis. Cell death was quantified by propidium iodide (PI) staining using flow cytometry. Data shown are from n = 1 experiment.

(C) *Nqo2* CRISPR empty, sg1A and sg2B cell pools generated from the WT #2 MDF parental cell line were left untreated (UT) or stimulated with TSI (TNF, Smac-mimetic, IDN-6556), TSQ (TNF, Smac-mimetic, Q-VD-OPh) or TSZ (TNF, Smac-mimetic, z-VAD-fmk) for 5 hours to induce necroptosis. Cell death was quantified by propidium iodide (PI) staining using flow cytometry. Data shown are from n = 1 experiment.

(D) *Nqo2* CRISPR empty, sg1A and sg2B cell pools generated from the WT #2 MDF parental cell line were left untreated (UT) or stimulated with TSQ (TNF, Smac-mimetic, Q-VD-OPh) or TSZ (TNF, Smac-mimetic, z-VAD-fmk) for 24 hours to induce necroptosis, with or without pre-treatment with ABT-869 (5 μ M) or DMSO alone for 1 hour. Cell death was quantified by propidium iodide (PI) staining using flow cytometry. Data represent the mean of n = 3 independent experiments and error bars represent SEM.

(E) *Nqo2* CRISPR empty, sg1A and sg2B cell pools generated from the WT #2 MDF parental cell line were left untreated (UT) or stimulated with TSI (TNF, Smac-mimetic, IDN-6556) for 5 hours to induce necroptosis, with or without pre-treatment with ABT-869 (5 μ M) for 1 hour. Cell death was quantified by propidium iodide (PI) staining using flow cytometry. Data shown are from n = 1 experiment.

In Vitro Binding Studies of ABT-869 with the Necroptotic Effectors RIPK1, RIPK3 and MLKL

Recent studies have implicated various auxiliary proteins involved in necroptosis execution, as discussed in Chapter 1. Some of these appear to vary depending on the cell type studied, but the core machinery comprising RIPK1, RIPK3 and MLKL are essential components. As RIPK1, RIPK3 and MLKL are kinases or pseudokinases, I sought to establish whether ABT-869, a kinase inhibitor, could target these proteins to inhibit necroptosis. Further rationale to interrogate these proteins as potential targets stemmed from findings with two other small molecule necroptosis inhibitors identified in the same MLKL(Q343A) pilot screen as ABT-869 (Jacobsen et al., 2016, Jacobsen et al.). One of these small molecules, the kinase inhibitor AMG-47a, was found to directly target RIPK1 and RIPK3 (Jacobsen et al.). The other small molecule, the HSP90 inhibitor 17-AAG, was found to indirectly affect RIPK1, RIPK3 and MLKL as all three are HSP90 client proteins (Jacobsen et al., 2016).

Firstly, using the DiscoverX KINOMEScan platform (Fabian et al., 2005), the binding affinity (K_D) of ABT-869 for the human necroptotic effector proteins was determined (**Figure 3.10A**). Interestingly, ABT-869 bound to the RIPK1 kinase domain with a K_D of 1.6 μ M, but did not bind to the RIPK3 kinase domain at the highest concentration tested (30 μ M). ABT-869 showed weaker binding for full-length MLKL with a K_D of 12 μ M. However, the DiscoverX platform only offers binding assays with human proteins and thus the binding affinity of ABT-869 for murine MLKL, RIPK1 and RIPK3 could not be determined from these assays. To address this limitation, I used Surface Plasmon Resonance (SPR) to examine the binding of ABT-869 to the murine and human necroptotic effector proteins in parallel.

SPR is a biophysical binding assay that measures the interaction between two binding partners (Schasfoort, 2017). One binding partner, called the ligand, is attached to the surface of a sensor chip, while the other partner, called the analyte, is flowed over the surface in solution. The binding interaction is recorded by the SPR instrument in the form of a sensorgram; a graph of response (resonance units, RU) against time (seconds). In a typical sensorgram, as the analyte is injected into the SPR instrument, the analyte binds to the ligand, which results in an increase in response that is termed the association phase. At the end of the injection, the analyte dissociates from the ligand, which results in a decrease in response that is termed the dissociation phase. This is often, but not always, followed by the injection of a regeneration solution to regenerate the surface of the sensor chip and ensure that all the analyte is removed from the ligand to prevent carry over into the next run. From the association rate constant (k_a) and dissociation rate constant (k_d), the binding affinity (K_D) can be calculated, which is a quantitative measure of the strength of the binding interaction between the analyte and ligand. From the k_a , k_d and RU level, additional information on the kinetics and stoichiometry of binding can also be obtained.

To perform SPR binding studies, recombinant murine and human full-length MLKL, RIPK1 kinase domain and RIPK3 kinase domain were cloned and expressed with an N-terminal Streptavidin-Binding Peptide (SBP)-tag to enable conjugation to a streptavidin coated sensor chip (**Figure 3.10B**). All proteins were expressed using the baculovirus expression system and purified by affinity chromatography via a TEV protease-cleavable GST or His-tag, followed by size exclusion chromatography. The protein cloning, expression, purification and SPR studies were performed in conjunction with Annette Jacobsen, fellow PhD student in the Murphy Lab at WEHI. In the SPR studies presented herein, the Biacore S200 instrument was used for all

SPR experiments. The purified murine and human MLKL, RIPK1 and RIPK3 proteins were immobilised to a streptavidin coated sensor chip via their SBP-tag and increasing concentrations of the small molecules of interest were flowed over the sensor chip in solution. The aim of these experiments was to determine whether ABT-869 binds to the necroptotic effector proteins (murine and human) by comparison with a series of control compounds that are known binders of these proteins.

Small molecule SPR with SBP-mMLKL and SBP-hMLKL had been previously performed and optimised by Wilco Kersten, a Research Assistant in the Lessene Lab at WEHI, and thus, his SPR conditions and protocols were adopted for these experiments. Compared to Compound **1** and Compound **2** binding to murine and human full-length MLKL (Chapter 2, **Figure 1B**), which were used as positive control compounds for MLKL in these experiments, both ABT-869 and Compound **5** did not bind appreciably to mMLKL or hMLKL (**Figure 3.10C**). The sensorgrams obtained for ABT-869 and Compound **5** reflected only background or non-specific binding, indicated by the atypical sensorgrams and very low response.

The MLKL SPR conditions were then employed for binding studies with the murine and human RIPK1 and RIPK3 proteins. While mRIPK3 detectably bound the control Compound **2** at the start of the experiment, the binding was almost completely lost by the end of the experiment, indicating that the stability of mRIPK3 on the sensor chip deteriorated over the course of the experiment (**Figure 3.10D**). However, mRIPK3 did not bind its other control compounds, the RIPK3 inhibitors GSK'872 or GSK'843, nor did mRIPK3 bind ABT-869 or Compound **5** (**Figure 3.10E**). Surprisingly, no binding to mRIPK1, hRIPK3(2-356) or hRIPK1 was observed with any of the control compounds tested, nor with ABT-869 or Compound **5** (**Figure 3.10E**).

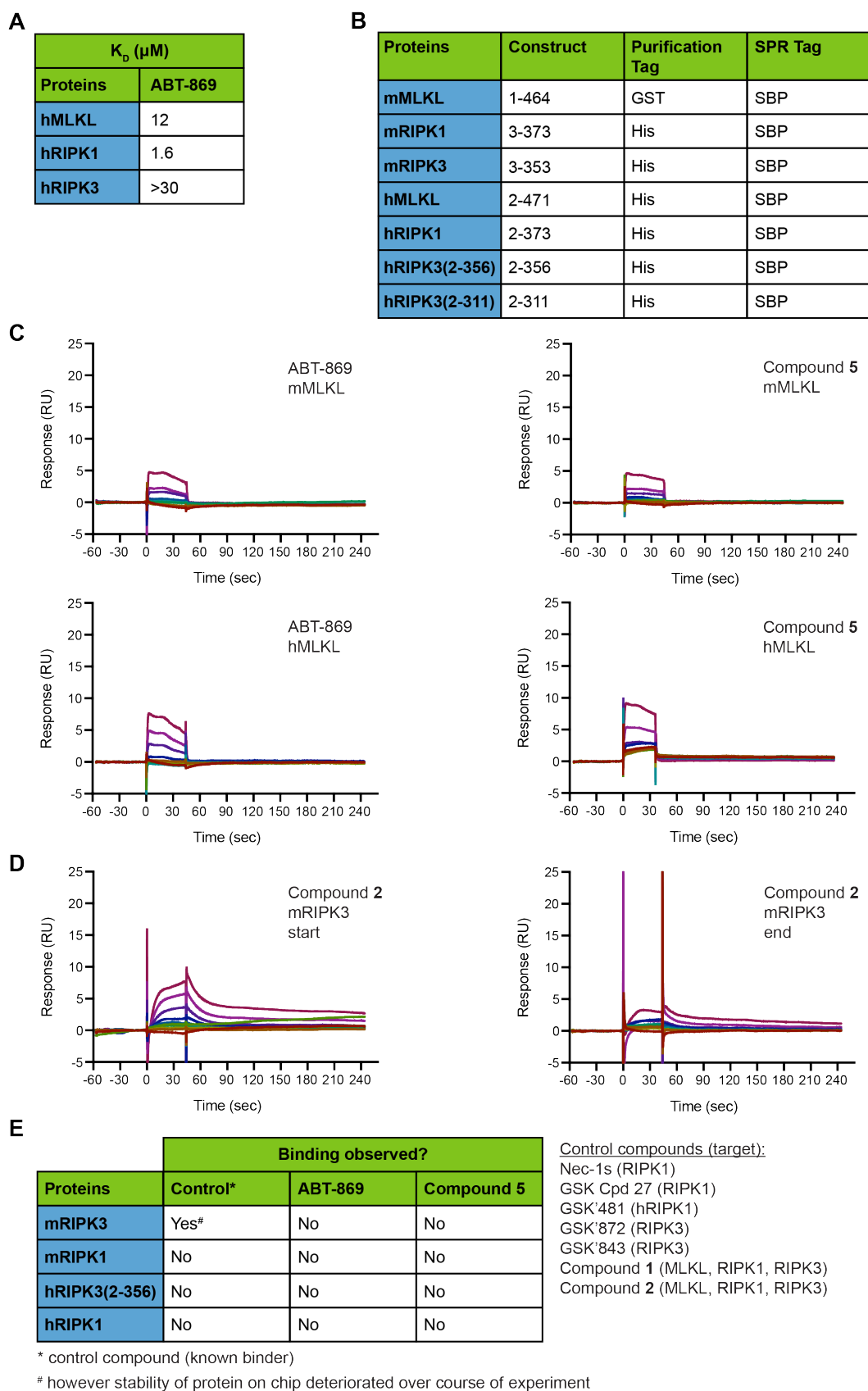


Figure 3.10 *In vitro* binding studies of ABT-869 and Compound 5 with the murine and human necroptotic effectors RIPK1, RIPK3 and MLKL.

(A) Binding affinities (K_D) of ABT-869 for human full-length MLKL, RIPK1 kinase domain and RIPK3 kinase domain obtained from the DiscoverX KINOMEScan platform using the *Kd*ELECT service, where each value is the mean of two replicates.

(B) Summary of murine and human necroptotic effector proteins expressed and purified for Surface Plasmon Resonance (SPR) studies. Murine full-length MLKL (1-464), RIPK1 kinase domain (3-373), RIPK3 kinase domain (3-353) along with human full-length MLKL (2-471), RIPK1 kinase domain (2-373) and two RIPK3 kinase domain constructs (2-356 and 2-311) were generated. All proteins were expressed with an N-terminal Streptavidin-Binding Peptide (SBP)-tag to enable conjugation to a streptavidin coated SPR chip, along with a TEV protease-cleavable GST-tag or His-tag for purification.

(C) SPR sensorgrams for ABT-869 and Compound **5** against murine and human MLKL. Increasing concentrations of each compound (0.0024 μ M to 2.5 μ M) were flowed over MLKL (50 μ g/mL for mMLKL and 110 μ g/mL for hMLKL) immobilised on the biosensor surface. SPR binding experiments were performed at 25°C using HEPES buffer (20 mM HEPES pH 8.0, 200 mM NaCl, 0.05% P20, 2% DMSO) and 1 M NaCl for regeneration. One representative result shown from $n = 3$ independent experiments.

(D) SPR sensorgrams for Compound **2** binding to murine RIPK3, run at the start and end of a single experiment. Increasing concentrations of Compound **2** (0.0024 μ M to 2.5 μ M) were flowed over mRIPK3 (187 μ g/mL) immobilised on the biosensor surface. SPR binding experiments were performed at 25°C using HEPES buffer (20 mM HEPES pH 8.0, 200 mM NaCl, 0.05% P20, 2% DMSO) and 1 M NaCl for regeneration. Data shown are from $n = 1$ experiment.

(E) Summary of SPR results testing the binding of control compounds (known binders), ABT-869 and Compound **5** to murine and human RIPK1 and RIPK3. Increasing concentrations of each compound (0.16 μ M to 2.5 μ M or 0.0024 μ M to 2.5 μ M for control compounds, 0.0024 μ M to 2.5 μ M for ABT-869 and Compound **5**) were flowed over the kinases (187 μ g/mL for mRIPK3, 227 μ g/mL for mRIPK1, 173 μ g/mL for hRIPK3(2-356), 313 μ g/mL for hRIPK1) immobilised on the biosensor surface. SPR binding experiments were performed at 25°C using HEPES buffer (20 mM HEPES pH 8.0, 200 mM NaCl, 0.05% P20, 2% DMSO) and 1 M NaCl for regeneration. Data shown are from $n = 1$ experiment.

Given that RIPK1 and RIPK3 did not detectably interact with their known binders and that Compound **2** binding to mRIPK3 markedly decreased over the course of a single SPR experiment, it was hypothesised that the proteins were not folded properly or not stable under the SPR conditions. Accordingly, a technique called Differential Scanning Fluorimetry

(DSF) was employed to assess the stability of the SBP-tagged RIPK1 and RIPK3 proteins. DSF is based on the same principle as Thermal Shift Assay (TSA), where a fluorescent dye is used to monitor the folded state of a protein as it is heated (Niesen et al., 2007). Upon increasing temperature, proteins will begin to unfold and become denatured, allowing the fluorescent dye to bind hydrophobic residues that become exposed as the proteins unfold, which results in a measurable increase in fluorescence. This is then followed by a decrease in fluorescence due to aggregation of the unfolded proteins and expulsion of the dye. From the resulting thermal melt curve of fluorescence (relative fluorescence units, RFU) against temperature (°C), the melting temperature (T_m) of the protein can be calculated.

Firstly, the DSF thermal melt curves were measured for SBP-tagged RIPK1 and RIPK3 in their purification buffer (performed at the C3 Facility, CSIRO, Parkville) (Seabrook and Newman, 2013, Rosa et al., 2015, Ristic et al., 2015). The mRIPK3 thermal melt curve demonstrated that mRIPK3 was a folded, stable protein at room temperature, as indicated by the low initial fluorescence at 20°C, with a typical thermal melt profile (**Figure 3.11**). However, all the other RIPK1 and RIPK3 proteins were either partially or completely misfolded or unfolded at room temperature, indicated by the higher initial fluorescence at 20°C (**Figure 3.11**). In particular, both hRIPK3(2-356) and hRIPK3(2-311) were very unstable and almost completely misfolded or unfolded at room temperature. The stabilities of mRIPK1 and hRIPK1 were in between those of mRIPK3 and the hRIPK3 proteins, with hRIPK1 exhibiting slightly higher stability at 20°C than mRIPK1.

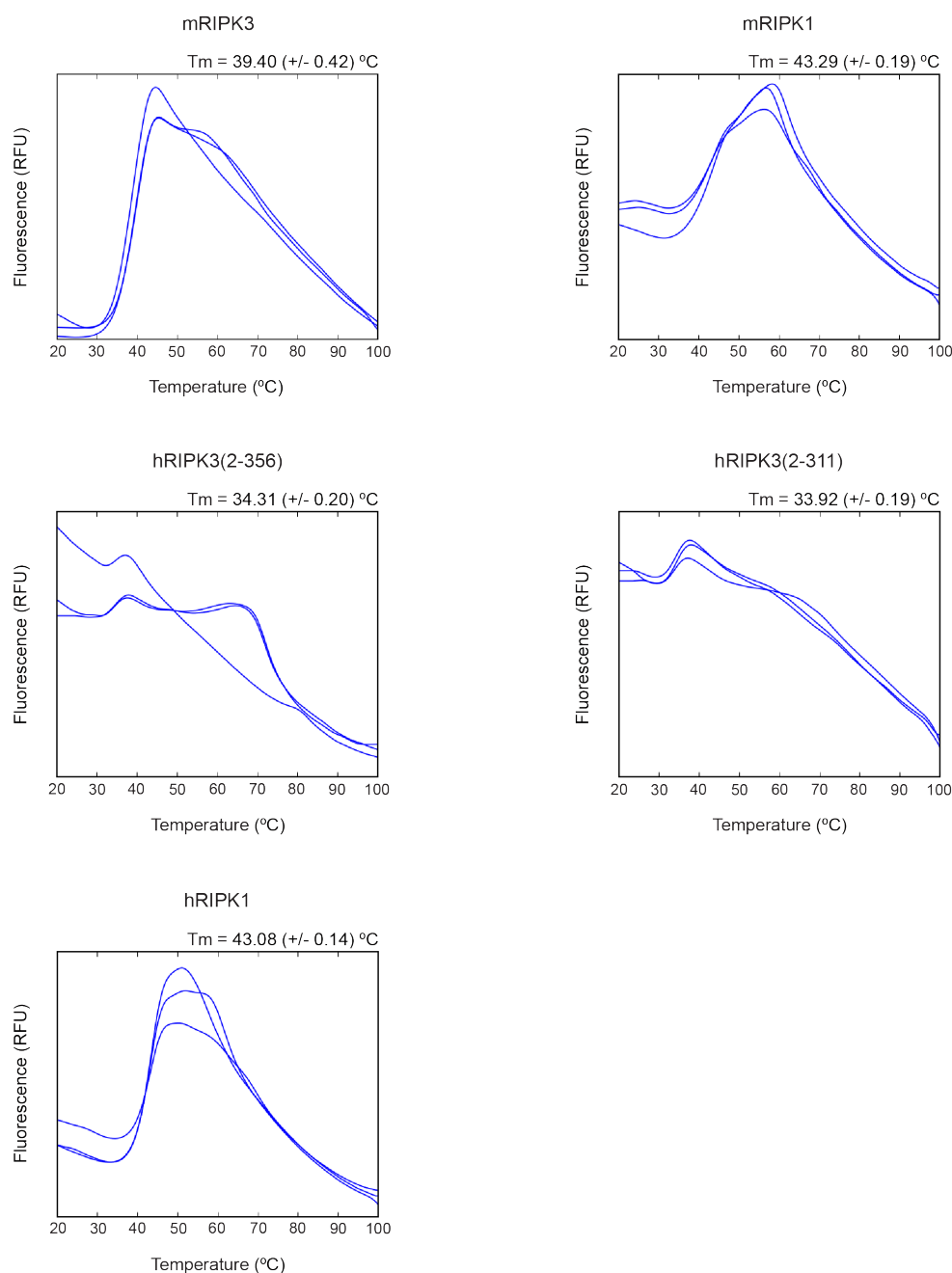


Figure 3.11 DSF protein thermal stability of RIPK1 and RIPK3.

Differential Scanning Fluorimetry (DSF) thermal melt curves of the SBP-tagged proteins (mRIPK3, mRIPK1, hRIPK3(2-356), hRIPK3(2-311), hRIPK1) in protein purification buffer (SEC buffer), obtained from the CSIRO C3 facility. Proteins were heated from 20-100°C in the presence of a fluorescent dye, which binds to hydrophobic residues that become exposed upon protein unfolding, and fluorescence was detected at 570 nm as a function of temperature. Each protein was performed in triplicate. The melting temperature (T_m) of each protein was determined as the inflection point of the sigmoidal curve, averaged for the three replicate thermal melt curves.

The stability of these proteins was then examined across various buffer, pH and salt conditions via a panel of 14 different buffers with a pH range of 5.0 to 9.0 and either 50 mM or 200 mM NaCl (performed at the C3 Facility, CSIRO, Parkville) (Seabrook and Newman, 2013, Rosa et al., 2015, Ristic et al., 2015). It was observed that mRIPK3 was generally more stable in buffers with lower salt concentration (**Figure 3.12**). In addition, it was found that the stability of hRIPK1 displayed a pH dependence, whereby the protein stability increased as the pH increased from 5.0 to 8.5 (**Figure 3.12**). No such obvious trends were observed for mRIPK1, hRIPK3(2-356) or hRIPK3(2-311) (**Figure 3.12**). Overall, none of the buffer conditions improved the stability of mRIPK1 or hRIPK1, compared to the original buffer. Two buffer conditions appeared to slightly increase the stability of mRIPK3, however this increase was less than 1°C and therefore not a substantial difference (Seabrook and Newman, 2013). The hRIPK3(2-356) and hRIPK3(2-311) buffer screen results were difficult to interpret, as both hRIPK3 proteins were very unstable and already completely misfolded or unfolded at 20°C. As a result, they did not display typical thermal melt profiles and these proteins simply aggregated upon heating, making it difficult to reliably determine their T_m and difficult to draw reliable conclusions from this data.

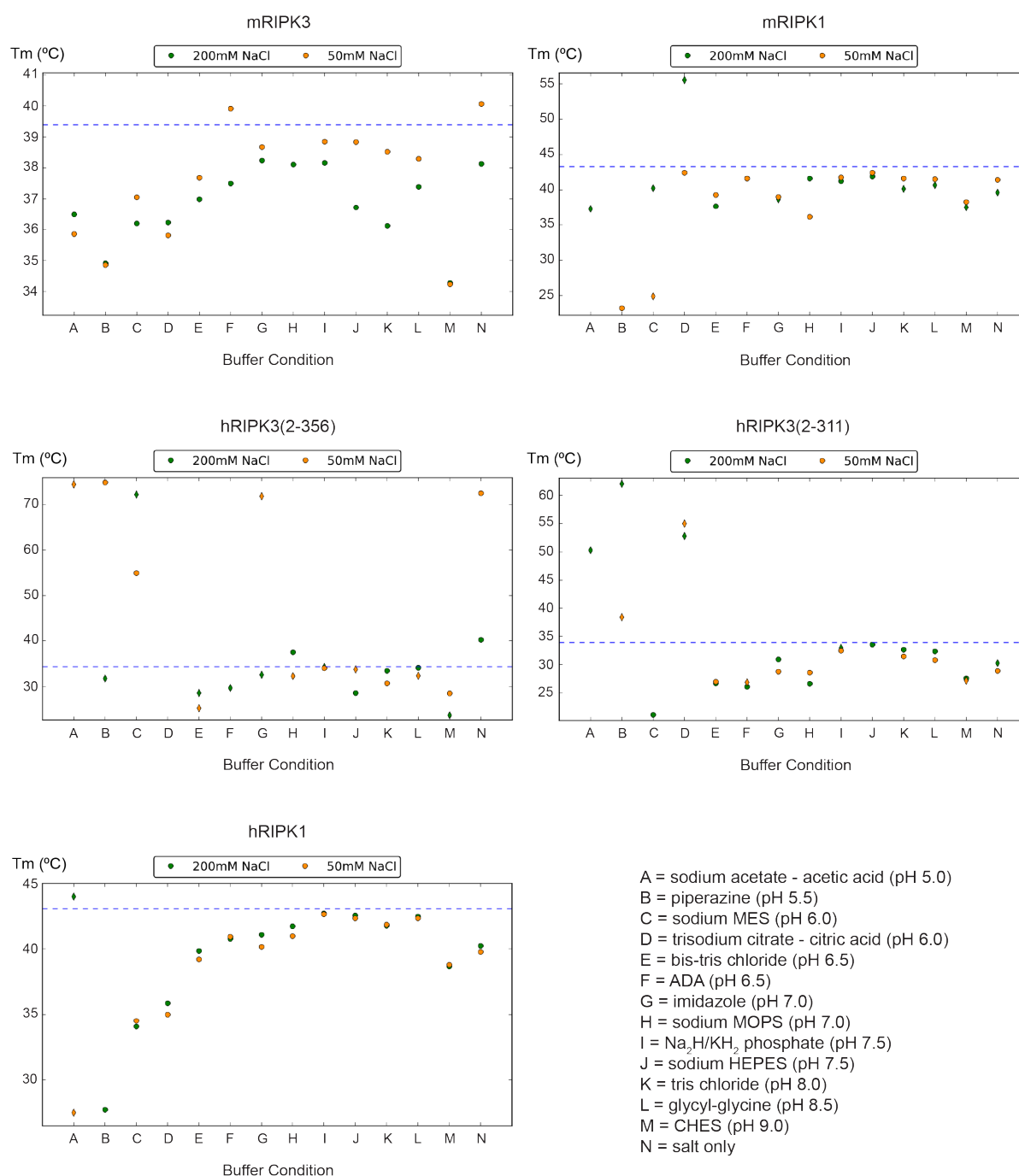


Figure 3.12 DSF protein stability buffer screen of RIPK1 and RIPK3.

Summary graphs of the DSF protein stability screen, Buffer Screen 9, obtained from the CSIRO C3 facility. The stabilities of the SBP-tagged proteins (mRIPK3, mRIPK1, hRIPK3(2-356), hRIPK3(2-311), hRIPK1) were evaluated across 14 different buffers (A - N) with a pH range of 5.0 to 9.0 at low salt concentration (50 mM NaCl; yellow circles) and high salt concentration (200 mM NaCl; green circles), performed as described in **Figure 3.11**. Robust T_m estimations are plotted as circles, while less reliable or unreliable T_m estimations are plotted as diamonds. Blue dotted line indicates the T_m of the protein in its original buffer from **Figure 3.11**.

Based on the DSF results, which demonstrated that not all the RIPK1 and RIPK3 proteins were folded or stable at room temperature, the SPR conditions were modified with the aim of improving the stability of these proteins. As such, several key changes were implemented. Firstly, all subsequent SPR experiments were performed at 4°C instead of room temperature and the regeneration step was omitted. Secondly, a 2005 study by Nordin *et al.* reported an optimised kinase buffer for small molecule SPR (50 mM Tris pH 7.5, 150 mM NaCl, 10 mM MgCl₂, 1 mM MnCl₂, 0.05% P20, 2% DMSO) (Nordin *et al.*, 2005) and this buffer was used in subsequent SPR experiments to determine if this also increased the stability of the proteins.

Prior to testing any compounds, the ability of RIPK1 and RIPK3 to bind ATP was evaluated to determine whether the proteins were more stable under these new SPR conditions. Encouragingly, robust binding of mRIPK3 to ATP was detected and this ATP-binding was maintained over the course of the experiment (**Figure 3.13A**). However, mRIPK1, hRIPK3(2-356), hRIPK3(2-311) and hRIPK1 did not show any ATP-binding under these new conditions (**Figure 3.13B**). A series of control compounds were then tested, and while mRIPK3 displayed good binding for Compound **1** in the first experiment, this was not reproducible (**Figure 3.13A**). Unsurprisingly given the lack of ATP-binding observed, mRIPK1, hRIPK3(2-356), hRIPK3(2-311) and hRIPK1 did not bind any of the control compounds (**Figure 3.13B**). In addition, ABT-869 or Compound **5** did not bind to any of the proteins (**Figure 3.13B**).

Given that the modified SPR conditions did not improve the detection of mRIPK1, hRIPK3(2-356), hRIPK3(2-311) or hRIPK1 ATP-binding, the SPR buffer conditions were optimised as an alternative approach to improve the stability of these proteins. The ability of RIPK1 and RIPK3 to bind ATP was assessed using a range of different buffer conditions (**Figure 3.13C**). However,

none of these buffer conditions resulted in any detectable ATP-binding by mRIPK1, hRIPK3(2-356), hRIPK3(2-311) or hRIPK1. As previously observed, mRIPK3 continued to exhibit ATP-binding in most of the buffer conditions, however none of the buffers appeared to enhance ATP-binding. Consistent with mRIPK3 being a conventional kinase, removing MgCl_2 from the SPR buffer markedly reduced the ability of mRIPK3 to bind ATP. In summary, while the SPR assays demonstrated that ABT-869 does not bind to mMLKL or hMLKL, it was not possible to establish reliable SPR assays for murine and human RIPK1 and RIPK3, and thus I was not able to determine whether ABT-869 binds to RIPK1 and RIPK3 using SPR.

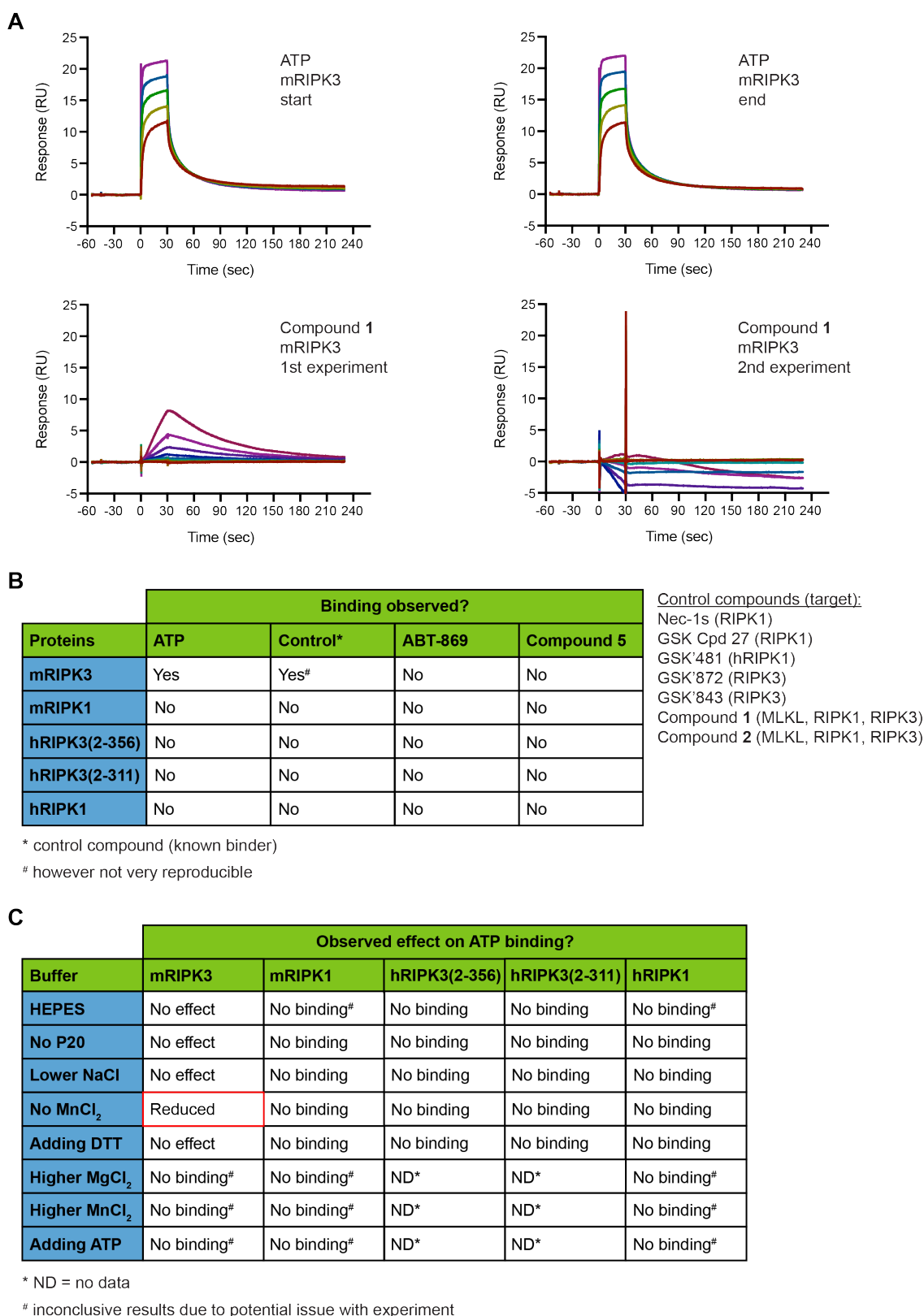


Figure 3.13 SPR binding studies with murine and human RIPK1 and RIPK3.

(A) SPR sensorgrams for ATP binding to murine RIPK3, run at the start and end of a single experiment, and Compound 1 binding to murine RIPK3, in two independent experiments. Increasing

concentrations of ATP (6.25 μ M to 100 μ M) or Compound **1** (0.0024 μ M to 2.5 μ M) were flowed over mRIPK3 (373 μ g/mL) immobilised on the biosensor surface. SPR binding experiments were performed at 4°C using Tris buffer (50 mM Tris pH 7.5, 150 mM NaCl, 10 mM MgCl₂, 1 mM MnCl₂, 0.05% P20, 2% DMSO) and no regeneration. One representative result shown from n = 2 independent experiments.

(B) Summary of SPR results testing the binding of ATP, control compounds (known binders), ABT-869 and Compound **5** to murine and human RIPK1 and RIPK3. Increasing concentrations of ATP (6.25 μ M to 100 μ M) or each compound (0.16 μ M to 2.5 μ M for control compounds, 0.0024 μ M to 2.5 μ M for ABT-869 and Compound **5**) were flowed over the kinases (373 μ g/mL for mRIPK3, 680 μ g/mL for mRIPK1, 347 μ g/mL for hRIPK3(2-356), 333 μ g/mL for hRIPK3(2-311), 627 μ g/mL for hRIPK1) immobilised on the biosensor surface. SPR binding experiments were performed at 4°C using Tris buffer (50 mM Tris pH 7.5, 150 mM NaCl, 10 mM MgCl₂, 1 mM MnCl₂, 0.05% P20, 2% DMSO) and no regeneration. Data are representative of n = 1 - 2 independent experiments.

(C) Summary of SPR results optimising the buffer conditions by testing the binding of ATP to murine and human RIPK1 and RIPK3. Increasing concentrations of ATP (6.25 μ M to 100 μ M) were flowed over the kinases (373 μ g/mL for mRIPK3, 680 μ g/mL for mRIPK1, 347 μ g/mL for hRIPK3(2-356), 333 μ g/mL for hRIPK3(2-311), 627 μ g/mL for hRIPK1) immobilised on the biosensor surface. SPR binding experiments were performed at 4°C using the buffer conditions stated and no regeneration. Data shown are from n = 1 experiment.

Investigating the Binding of ABT-869 to RIPK1, RIPK3 and MLKL in Cells using CETSA

Given that it was not possible to determine whether ABT-869 binds to RIPK1 and RIPK3 from the SPR studies, an alternative approach was required. Furthermore, as these recombinant proteins, especially the hRIPK3 constructs, were unstable *in vitro*, I decided to use Cellular Thermal Shift Assay (CETSA) as an alternative technique to determine if ABT-869 interacts with RIPK1 and RIPK3. CETSA is based on the principle of conventional TSA, but is performed in live cells rather than with purified protein (Martinez Molina et al., 2013, Jafari et al., 2014). When a heat treatment is applied to intact cells, proteins inside the cells will denature, aggregate and precipitate. The binding of a small molecule to its target protein will alter the thermal stability of that protein and thus will impact the ratio of soluble protein to precipitated protein compared to an untreated or DMSO-treated sample. CETSA enables compound-protein binding to be measured in live cells, enabling direct target engagement in a cellular context (Martinez Molina et al., 2013, Jafari et al., 2014).

Firstly, the melting temperature of each protein of interest had to be determined to select an appropriate temperature range to perform the CETSA. The melting temperature and the CETSA temperature range for murine MLKL in MDF cells and human MLKL in U937 cells had been previously optimised by Sarah Garnish and Dr Anne Hempel, fellow scientists in the Inflammation Division at WEHI, and were used in all MLKL CETSA herein. As the melting temperatures of murine RIPK1 and RIPK3 in MDF cells and human RIPK1 and RIPK3 in U937 cells had not yet been established, I performed a series of CETSA optimisation experiments.

For the mRIPK1 temperature optimisation, WT #2 MDF cells were subjected to an 8-point temperature gradient from 42.0 to 52.0°C (**Figure 3.14A**). The cells were then lysed and

centrifuged to separate the soluble and insoluble protein fractions. Proteins in the soluble fraction were separated by SDS-PAGE and mRIPK1 was detected by Western blot. The complete disappearance of the mRIPK1 band was observed at higher temperatures and the melting temperature of mRIPK1 in MDF cells was found to be ~48°C. In the mRIPK3 temperature optimisation, the initial temperature range of 45.0 to 55.0°C was found to be too low, as the melting temperature was towards the upper end of the range, rather than in the centre as desired (**Figure 3.14A**). Subsequently, a higher temperature gradient of 47.0 to 57.0°C resulted in the complete disappearance of the mRIPK3 band at the highest temperatures and a melting temperature of ~55°C in MDF cells (**Figure 3.14A**).

For the hRIPK1 temperature optimisation, the same approach was employed with U937 cells, the human cell line of choice. An 8-point temperature gradient from 44.0 to 54.0°C was found to be suitable for hRIPK1, with a melting temperature of ~48°C (**Figure 3.14B**). For hRIPK3, in addition to determining an adequate temperature range, I also wanted to optimise the positive control I would go on to use in my CETSA experiments testing ABT-869. While my cell death assay data indicated that GSK'872 is generally a more potent inhibitor of necroptosis in murine cells than GSK'843 (**Figure 3.2**), their relative potency was observed to vary between different human cell lines (Annette Jacobsen and Dr Ying Zhang, personal communication). I therefore wanted to establish which RIPK3 inhibitor would be more suitable to use as a positive control for hRIPK3 CETSA. In the hRIPK3 CETSA optimisation, U937 cells were treated with GSK'872, GSK'843 or DMSO for 1 hour prior to being subjected to a heat treatment ranging from 44.0 to 54.0°C. While a robust CETSA melt profile was observed over this temperature range for hRIPK3, neither GSK'872 nor GSK'843 impacted the thermal stability of hRIPK3 as both were comparable to the DMSO control (**Figure 3.14B**). This finding was

unexpected as both GSK'872 and GSK'843 are well-established RIPK3 inhibitors, however, there are several possible explanations for this observation. While false positives are rare in CETSA, false negatives can occur due to several reasons, including that compound binding does not induce substantial stabilisation (or destabilisation) of the target protein and that multidomain proteins or protein complexes are more likely to give no or weaker compound-induced stabilisation (or destabilisation) (Jafari et al., 2014). Furthermore, in some cellular contexts, other protein interactors that affect the stability of the protein of interest could confound interpretation of CETSA results.

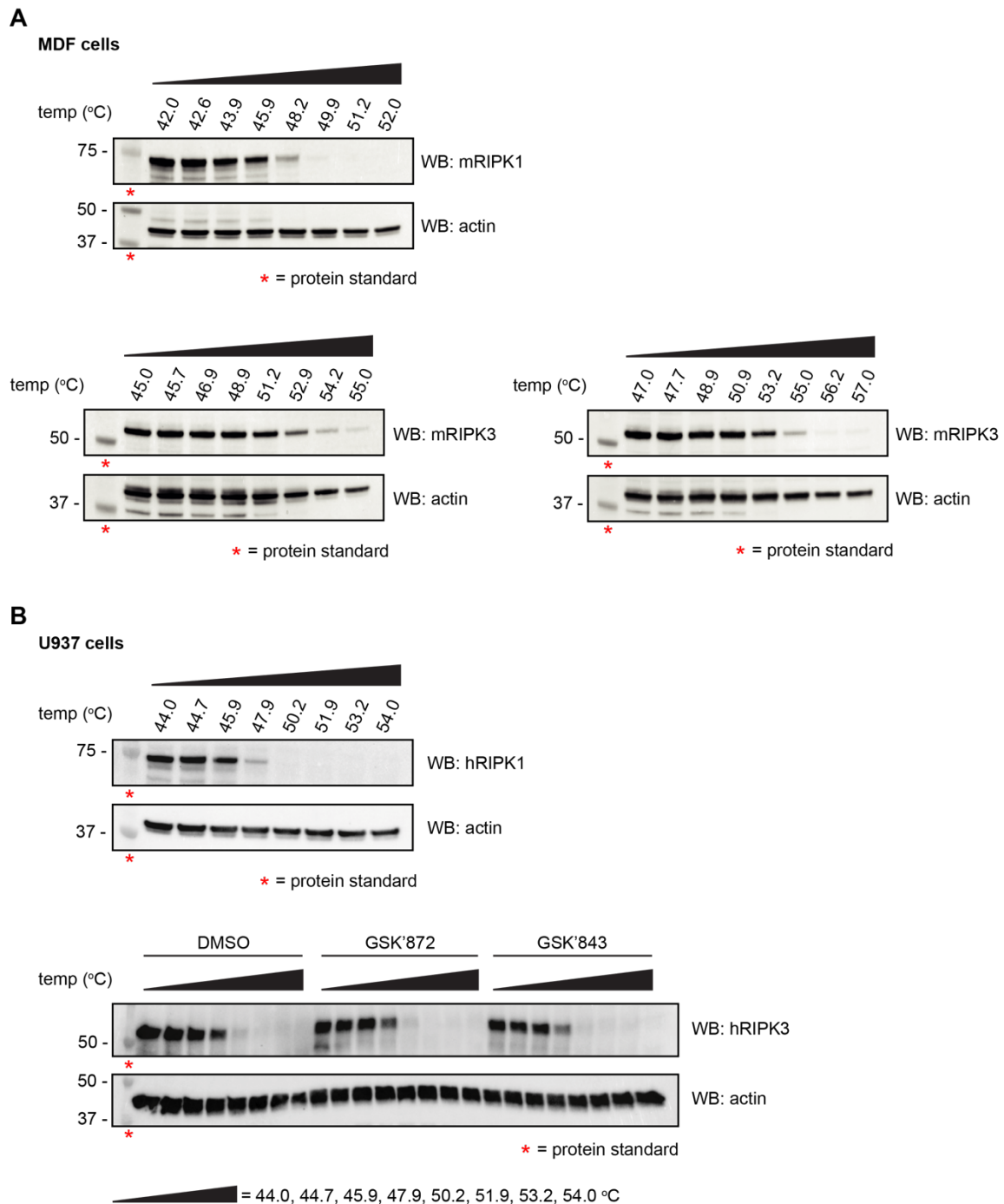


Figure 3.14 CETSA optimisation of RIPK1 and RIPK3 in murine and human cells.

(A) WT #2 MDF cells were subjected to an increasing temperature gradient to determine the melting temperature of mRIPK1 and mRIPK3. Following the separation of soluble and insoluble proteins, the remaining soluble proteins were detected by Western blot. Red asterisks denote protein standards. Data shown are from $n = 1$ experiment.

(B) Human U937 cells were subjected to an increasing temperature gradient to determine the melting temperature of hRIPK1 and hRIPK3. For the hRIPK3 experiment, cells were pre-treated with DMSO, GSK'872 (20 μ M) or GSK'843 (20 μ M) to determine if these known RIPK3 inhibitors resulted in the

thermal stabilisation of RIPK3 in cells. Following the separation of soluble and insoluble proteins, the remaining soluble proteins were detected by Western blot. Red asterisks denote protein standards. Data shown are from n = 1 experiment.

To determine whether ABT-869 binds to MLKL, RIPK1 and RIPK3 in cells, I used CETSA to perform cellular target validation in murine and human cells. WT #2 MDF cells or U937 cells were treated with ABT-869, Compound **5** and a positive control compound (a known binder of each protein, except for MLKL) at 20 μ M. Based on the temperature range for each protein obtained from the temperature optimisation experiments (**Figure 3.14A and 3.14B**), or those previously optimised for MLKL, cells were subjected to a 6-point temperature gradient focused around the melting temperature of each protein. Following lysis and centrifugation, proteins in the soluble fraction were analysed by Western blot.

Consistent with the SPR binding data, neither ABT-869 nor Compound **5** had any effect on the thermal stability of mMLKL in MDFs or hMLKL in U937s (**Figure 3.15A and 3.15B**), which suggests that ABT-869 does not bind to MLKL in cells. In contrast, ABT-869 as well as Compound **5** increased the thermal stability of mRIPK1 in MDF cells, to a similar extent as Nec-1s (**Figure 3.15A**). Both ABT-869 and Compound **5** also increased the thermal stability of hRIPK1 in U937 cells, albeit not as profoundly as GSK'481 (**Figure 3.15B**). Together, this suggests that both ABT-869 and Compound **5** bind to RIPK1 in cells. Neither ABT-869 nor Compound **5** impacted the thermal stability of mRIPK3 in MDFs or hRIPK3 in U937s (**Figure 3.15A and 3.15B**), however due to the lack of effect observed with GSK'872, it is difficult to draw conclusions about whether or not ABT-869 and Compound **5** bind to RIPK3. Together, these data indicate that ABT-869 and Compound **5** bind to RIPK1, but not to MLKL, in both murine and human cells.

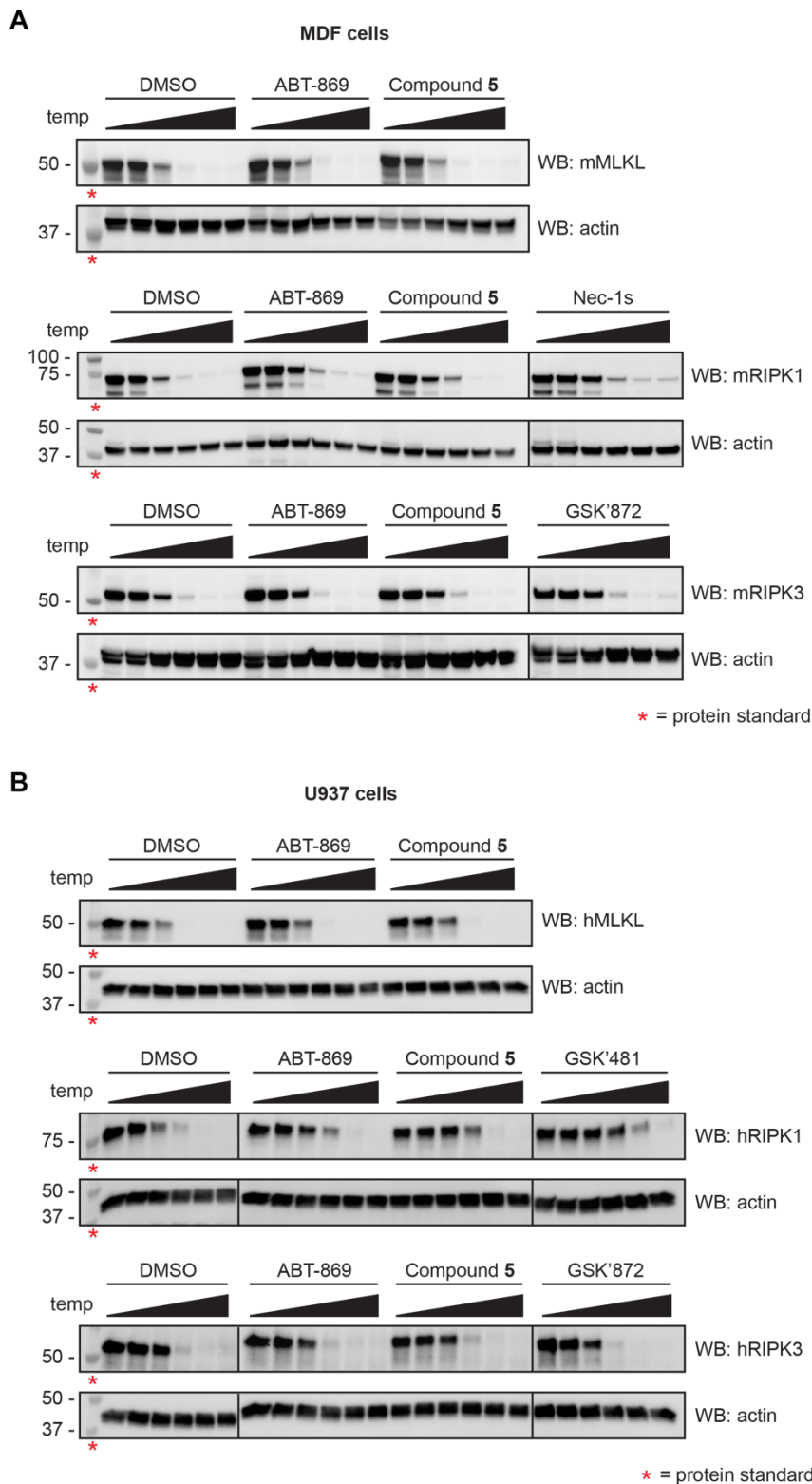


Figure 3.15 CETSA binding studies of ABT-869 with RIPK1, RIPK3 and MLKL in murine and human cells.

WT #2 MDF cells (**A**) or human U937 cells (**B**) were treated with DMSO, ABT-869 or Compound **5** then subjected to an increasing temperature gradient focused around the melting temperature of each

protein of interest. Following the separation of soluble and insoluble proteins, the remaining soluble proteins were detected by Western blot. The RIPK1 inhibitor Nec-1s, RIPK3 inhibitor GSK'872 and hRIPK1 inhibitor GSK'481 were included as controls. All compounds were tested at 20 μ M. Red asterisks denote protein standards. One representative result shown from n = 2 - 3 independent experiments.

ABT-869 Inhibits RIPK1 and RIPK3 Phosphorylation in Murine Cells

The combined data obtained from the CETSA, SPR and DiscoverX binding assays suggested that ABT-869 binds to RIPK1, and potentially RIPK3, but not MLKL *in vitro* and in cells. In TNF-driven necroptosis, the autophosphorylation of RIPK1 and RIPK3 are key events in this cell death signalling pathway (He et al., 2009, Cho et al., 2009, Chen et al., 2013, Cook et al., 2014, Wu et al., 2014, Laurien et al., 2020). To determine whether ABT-869 binding to RIPK1 and/or RIPK3 impacts their activity in cells, I investigated the effect of ABT-869 on RIPK1 and RIPK3 autophosphorylation.

Firstly, to determine the timing of these key phosphorylation events in TNF-driven necroptosis, I performed time course experiments to monitor the phosphorylation of the necroptotic effectors following necroptosis induction over a defined period. WT #2 MDF cells were stimulated with TSI and the phosphorylation of murine MLKL, RIPK1 and RIPK3 were analysed by Western blot over a 4 hour time period (**Figure 3.16A**). Robust phosphorylation of the MLKL activation loop residue, S345, as detected by the pS345 antibody (Abcam, cat #ab196436), was observed 2 hours after TSI stimulation, with levels maintained at 3 hours and 4 hours. Phosphorylation of S166 in the RIPK1 activation loop, as detected by the pS166 antibody (Cell Signaling Technology, cat #31122), was observed at the earlier time point of 1 hour and maintained at 2 hours, however phospho-RIPK1 levels decreased at 3 hours and 4 hours. Phosphorylation of the RIPK3 C-lobe residues, T231/S232, as detected by the pT231/pS232 antibody (kindly supplied by Genentech, GEN135-35-9 (Newton et al., 2016b)), was found to occur at a similar time point as MLKL phosphorylation, with phospho-RIPK3 observed at 2 hours and still present at 4 hours. Total MLKL, RIPK1 and RIPK3 levels remained relatively constant over this 4 hour time period following TSI stimulation. As maximal

phosphorylation of all three proteins occurred 2 hours after TSI stimulation, this was chosen as the time point at which to investigate the effect of ABT-869 on the phosphorylation of RIPK1 and RIPK3.

To determine the impact of ABT-869 on RIPK1 and RIPK3 autophosphorylation, WT #2 MDF cells were treated with 1 μ M or 10 μ M ABT-869, Compound **5** and control inhibitors for 2 hours, then stimulated with TSI for 2 hours to induce the autophosphorylation of RIPK1 and RIPK3 (**Figure 3.16B**). Following TSI stimulation for 2 hours, a robust phospho-RIPK1 signal was observed by Western blot, which was completely ablated by Nec-1s at 10 μ M. ABT-869 blocked RIPK1 phosphorylation to a similar extent as Nec-1s, whereas Compound **5** only partially reduced phospho-RIPK1 levels at 10 μ M. Similarly, a robust phospho-RIPK3 signal was observed by Western blot following TSI stimulation for 2 hours, which was almost completely inhibited by GSK'872 at 10 μ M. ABT-869 also blocked RIPK3 phosphorylation to a similar extent as GSK'872, while Compound **5** only partially reduced phospho-RIPK3 levels at 10 μ M. Together, this suggests that ABT-869 targets both RIPK1 and RIPK3 in murine cells, although the possibility that the effect on RIPK3 could simply be attributable to RIPK1 inhibition, cannot be ruled out.

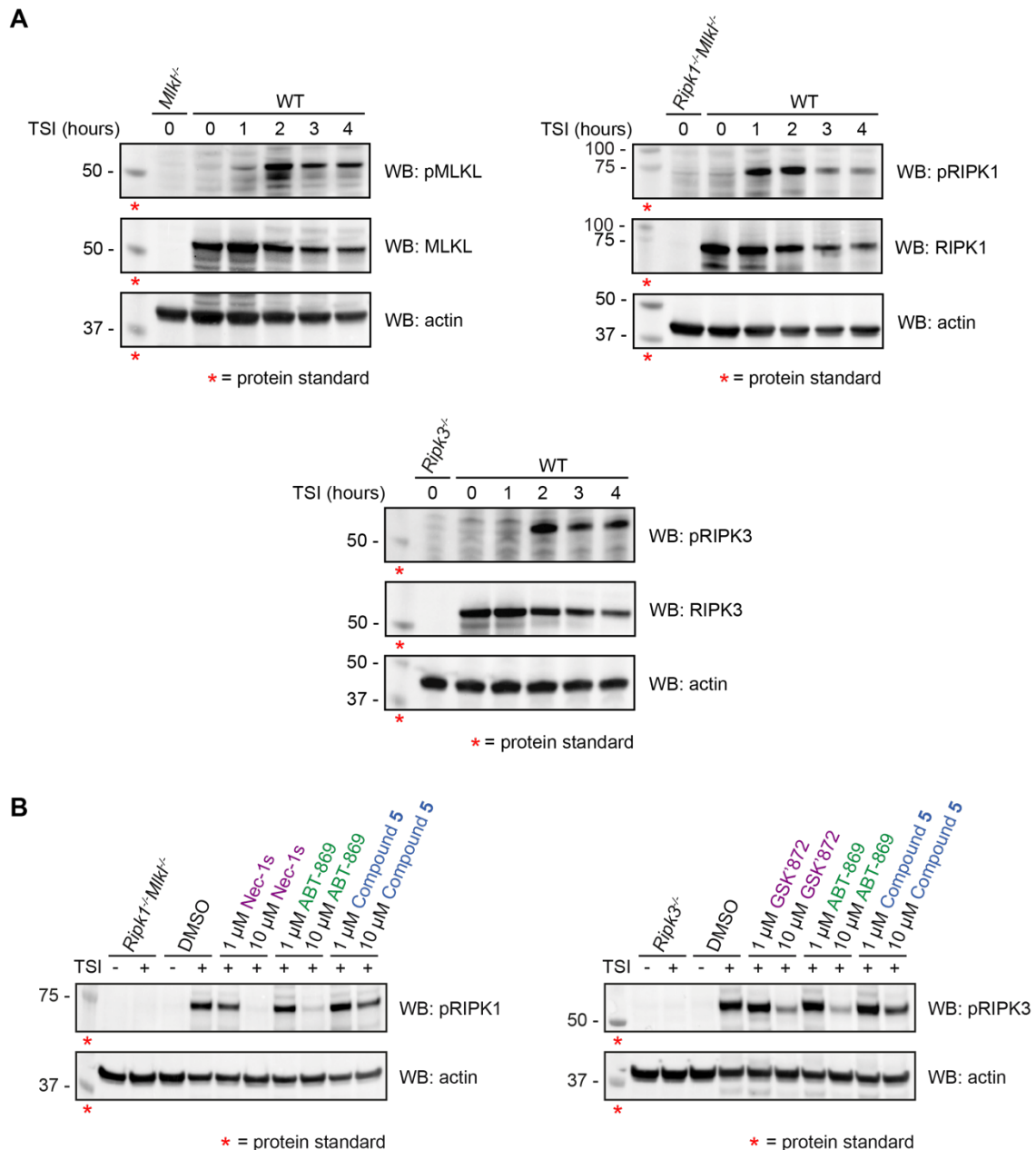


Figure 3.16 ABT-869 inhibits phosphorylation of RIPK1 and RIPK3 in murine cells.

(A) WT #2 MDF cells were stimulated with TSI (TNF, Smac-mimetic, IDN-6556) for 1, 2, 3 or 4 hours to monitor the phosphorylation of MLKL, RIPK1 and RIPK3 across this time period. Whole cell lysates were taken at each time point and the protein levels of phospho-MLKL, phospho-RIPK1 and phospho-RIPK3 were detected by Western blot. *MLK1^{-/-}* #393, *Ripk1^{-/-}MLK1^{-/-}* #569 and *Ripk3^{-/-}* #19 MDF cells were included as control cell lines. Red asterisks denote protein standards. One representative result shown from n = 3 - 4 independent experiments.

(B) WT #2 MDF cells were treated with DMSO, ABT-869 or Compound 5 for 2 hours then stimulated with TSI (TNF, Smac-mimetic, IDN-6556) for 2 hours to induce phosphorylation of RIPK1 and RIPK3. Phospho-RIPK1 and phospho-RIPK3 protein levels were detected from whole cell lysates by Western

blot. *Ripk1*^{-/-}*Mlkl*^{-/-} #569 and *Ripk3*^{-/-} #19 MDF cells were included as control cell lines, while the RIPK1 inhibitor Nec-1s and RIPK3 inhibitor GSK'872 were included as control inhibitors. Red asterisks denote protein standards. One representative result shown from n = 3 independent experiments.

Conclusions

The small molecule ABT-869 was identified from a phenotypic screen as a novel inhibitor of necroptosis. In this study, I showed that ABT-869 inhibits the death of murine cells stimulated with various necroptosis-inducing stimuli that activate the TNF-dependent necroptosis pathway. To deduce the cellular targets of ABT-869, chemical proteomics was performed using a chemical probe derivative of ABT-869 to enrich ABT-869 target proteins from cells, yielding eight candidate protein hits. The top hit identified by mass spectrometry, NQO2, was investigated using CRISPR-Cas9 to genetically delete *Nqo2* in MDF cells. However, no difference was observed between the *Nqo2*^{-/-} cells and wild-type cells in their response to necroptosis induction or ABT-869 treatment, indicating that NQO2 is not the target of ABT-869 that mediates its ability to inhibit necroptosis. Nevertheless, the possibility remains that one or more of the other seven protein hits identified from the chemical proteomics – EphB2, EphB4, DDR2, MAPK9, AurKB, Borealin and INCENP – could be the targets of ABT-869 in the context of its necroptosis inhibition.

Given that the three essential necroptotic effectors – RIPK1, RIPK3 and MLKL – are kinases or pseudokinases, and that other small molecules identified from the same screen as ABT-869 were found to directly or indirectly target these proteins, the ability of ABT-869 to engage RIPK1, RIPK3 and MLKL was investigated. From the DiscoverX KINOMEScan *in vitro* competition binding assays, I found that ABT-869 binds to human RIPK1 with a K_D of 1.6 μ M, does not bind to human RIPK3 and binds more weakly to human MLKL with a K_D of 12 μ M. Subsequent SPR binding studies demonstrated that ABT-869 does not bind to murine or human MLKL *in vitro*, however, reliable SPR binding data was not able to be obtained for RIPK1 and RIPK3 due to the instability of these recombinant proteins.

By employing a CETSA approach to determine cellular target engagement, I showed that ABT-869 had no effect on the thermal stability of MLKL in murine or human cells, consistent with the lack of binding observed by SPR. Moreover, I found that ABT-869 stabilises RIPK1 in murine and human cells by CETSA and that ABT-869 impedes the autophosphorylation of RIPK1 in cells. While ABT-869 did not affect the thermal stability of RIPK3 in murine or human cells, ABT-869 did block RIPK3 autophosphorylation in cells. Together, the results from the DiscoverX binding assays, CETSA and phosphorylation assays demonstrated that ABT-869 directly targets RIPK1 and possibly RIPK3, as it is difficult to ascertain whether ABT-869 directly targets RIPK3 or whether its effect on RIPK3 phosphorylation is due to RIPK1 inhibition.

Materials and Methods

Synthesis of Compounds and Probes

ABT-869 (Compound **4**), Compound **5**, Compound **6**, ABT-869 Sepharose probes and Compound **5** Sepharose probes were prepared by Dr Christoph Grohmann, a Postdoctoral Researcher in the Lessene Lab.

Necroptosis Control Inhibitors

GSK'872 (kindly supplied by Catalyst Therapeutics) (Kaiser et al., 2013)

GSK'843 (kindly supplied by Catalyst Therapeutics) (Kaiser et al., 2013)

Nec-1s (Sigma-Aldrich) (Teng et al., 2005, Degterev et al., 2005, Takahashi et al., 2012)

GSK'481 (kindly supplied by Catalyst Therapeutics) (Harris et al., 2016)

Necroptosis-Inducing Stimuli

TSQ: 100 ng/ml TNF (produced in-house (Bossen et al., 2006)), 500 nM Smac-mimetic (Compound A; Tetralogic), 10 μ M Q-VD-OPh (MP Biologicals)

TSZ: 100 ng/ml TNF (produced in-house (Bossen et al., 2006)), 500 nM Smac-mimetic (Compound A; Tetralogic), 10 μ M z-VAD-fmk (ApexBio)

TSI: 100 ng/ml TNF (produced in-house (Bossen et al., 2006)), 500 nM Smac-mimetic (Compound A; Tetralogic), 5 μ M IDN-6556 (emricasan; Tetralogic)

Western Blot Antibodies

General

actin: chicken anti- β -actin-HRP, Santa Cruz Biotechnology, cat #sc-47778 HRP, 1:5000

secondary antibodies: goat anti-rat-HRP (cat #3010-05), goat anti-mouse-HRP (cat #1010-05), goat anti-rabbit-HRP (cat #4010-05), all from Southern Biotech and used at 1:5000 or 1:10000

Ngo2 CRISPR

mNQO2: rabbit anti-NQO2, Novus Biologicals, cat #NBP1-31563, 1:1000

mMLKL: rat anti-MLKL clone 3H1, produced in-house as previously described (Murphy et al., 2013), available from Sigma-Aldrich cat #MABC604, 1:1000

mRIPK1: rabbit anti-RIPK1, Cell Signaling Technology, cat #3493, 1:1000

mRIPK3: rat anti-mouse RIPK3 clone 8G7, produced in-house as previously described (Petrie et al., 2019), 1:1000

CETSA

mMLKL and hMLKL: rat anti-MLKL clone 3H1, produced in-house as previously described (Murphy et al., 2013), available from Sigma-Aldrich cat #MABC604, 1:1000

mRIPK1: rabbit anti-RIPK1, Cell Signaling Technology, cat #3493, 1:1000

mRIPK3: rabbit anti-RIPK3, ProSci, cat #2283, 1:1000

hRIPK1: mouse anti-RIPK1, BD Transduction Laboratories, cat #610458, 1:1000

hRIPK3: rat anti-human RIPK3 clone 1H2, produced in-house as previously described (Petrie et al., 2019), 1:1000

Phosphorylation Time Course and Phosphorylation Assays

phospho-mMLKL: rabbit anti-mouse MLKL pS345, Abcam, cat #ab196436, 1:1000

mMLKL: rat anti-MLKL clone 3H1, produced in-house as previously described (Murphy et al., 2013), available from Sigma-Aldrich cat #MABC604, 1:1000

phospho-mRIPK1: rabbit anti-mouse RIPK1 pS166, Cell Signaling Technology, cat #31122, 1:1000

mRIPK1: rabbit anti-RIPK1, Cell Signaling Technology, cat #3493, 1:1000

phospho-mRIPK3: rabbit anti-mouse RIPK3 pT231/pS232, kindly supplied by Genentech, GEN135-35-9 (Newton et al., 2016b), 1:5000

mRIPK3: rat anti-mouse RIPK3 clone 8G7, produced in-house as previously described (Petrie et al., 2019), 1:1000

Western Blotting General Procedure

Proteins were transferred to nitrocellulose membranes using the iBlot Dry Blotting System (Invitrogen). Membranes were blocked with 5% w/v skim milk in PBS-T (PBS + 0.1% v/v Tween-20) for 1 hour at room temperature, washed 3 x 20 minutes with PBS-T at room temperature, probed overnight at 4°C with the primary antibody for the protein of interest in PBS + 2% w/v BSA + 0.02% w/v NaN₃, washed as above, probed for 1 hour at room temperature with the relevant secondary antibody in PBS-T + 5% w/v BSA and washed as above. Immobilon Forte Western HRP Substrate (Merck Millipore) or Pierce ECL Western Blotting Substrate (Thermo Scientific) was added to the membranes and Western blots were developed using chemiluminescence on the ChemiDoc Imaging System (Bio-Rad). Membranes were then washed overnight with PBS-T, reprobed with the loading control (actin-HRP) for 1 hour at room temperature, washed 3 x 20 minutes with PBS-T at room temperature and developed as described above.

Cell Culture and Cell Lines

Murine dermal fibroblast (MDF) cells were cultured in DMEM (in-house) and U937 cells were cultured in human tonicity RPMI (in-house). All media contained penicillin and streptomycin (100X solution, 10000-12000 units/mL penicillin and 10-12 mg/mL streptomycin in 0.9% w/v NaCl; Sigma-Aldrich) and was supplemented with 8-10% v/v Fetal Calf Serum (FCS; GE

Healthcare, cat #SH30084.03). *Nqo2*^{-/-} CRISPR MDF cell lines were cultured in DMEM analogous to wild-type MDF cells. Wild-type MDF cell lines (WT #1, WT #2, WT #3, WT A, WT #164, WT #166, WT #167) were generated from different mice to generate biologically independent cell lines. Knockout MDF cell lines (*Mkl1*^{-/-} #393, *Ripk1*^{-/-}*Mkl1*^{-/-} #569 and *Ripk3*^{-/-} #19) were also generated from individual mice. All MDF cell lines (aside from the *Nqo2*^{-/-}) were made previously by other scientists in the Inflammation Division at WEHI, as previously described (Murphy et al., 2013), where the number refers to the mouse from which they were derived. U937 cells were kindly provided by the Silke Lab at WEHI from the American Type Culture Collection (ATCC).

Cell Death Assays

WT #2 MDF cells were seeded at 5.0×10^4 cells in 500 μ L per well into 24-well plates (Falcon, cat #353047) and allowed to settle for 24 hours at 37°C 10% CO₂. Cells were treated with DMSO alone or compounds at the specified concentrations, or left untreated. Cells were then stimulated with TSQ, TSZ, TSI or TSQI to induce necroptosis, for the specified duration of time, or left unstimulated. For all compounds/reagents, 0.5 μ L of 1000X stocks were used. Cells were then harvested, stained with 2 μ g/mL propidium iodide (PI; Sigma-Aldrich) and PI-positive cells were quantified by flow cytometry on a BD FACSCalibur instrument. Cell death assays with *Nqo2*^{-/-} CRISPR MDF single cell clones were performed analogously, except that cells were treated with DMSO, ABT-869 (1 μ M) or left untreated and immediately stimulated with TSI for 24 hours to induce necroptosis. Cell death assays testing the panel of various MDF cell lines were performed analogously, except that cells were stimulated with TSI for 24 hours to induce necroptosis. Cell death assays with *Nqo2*^{-/-} CRISPR MDF cell pools were performed analogously, except that cells were either stimulated with TSQ, TSZ, TSI or TSQI to induce

necroptosis, or treated for 1 hour with DMSO, ABT-869 (5 μ M) or left untreated prior to necroptosis induction.

Pilot Pulldown Assays

WT #2 MDF cells were seeded to obtain a total of 50-100 x 10⁶ cells grown in 10 cm dishes (Falcon, cat #353003). Cells were washed with 2 x 5 mL DPBS (Gibco) and then 1 mL ice-cold RIPA lysis buffer (25 mM Tris-Cl pH 7.5, 150 mM NaCl, 1% v/v Nonidet P-40, 1% v/v Na Deoxycholate, 0.1% v/v SDS), containing cOmplete EDTA-free Protease Inhibitor Cocktail (Roche) and PhosSTOP Phosphatase Inhibitor Cocktail (Roche), was added. Cells were collected using a cell scraper and lysed for 20 minutes on ice. To prepare the Sepharose probes, 60 μ L of 50% slurries of ABT-869 beads (2 μ mol/mL) and Compound **5** beads (2 μ mol/mL), per condition, were washed with 2 x 1 mL ice-cold DPBS (Gibco) and 1 mL ice-cold RIPA lysis buffer containing protease/phosphatase inhibitor cocktails, and the supernatant discarded. Cell lysates were centrifuged at 13000 x g for 15 minutes at 4°C. Cell lysate supernatants were incubated with DMSO, 1 μ M ABT-869 or 100 μ M ABT-869 for 30 minutes on ice. 1 mL of each pre-treated cell lysate was incubated with ABT-869 beads and Compound **5** beads for 3 hours at 4°C on a rotating wheel. Beads were pelleted at 2000 x g for 5 minutes at 4°C and the supernatant discarded. Beads were washed with 3 x 1 mL ice-cold RIPA buffer and 1 mL ice-cold ModRIPA buffer (50 mM Tris-Cl pH 7.5, 150 mM NaCl, 1% v/v Nonidet P-40, 0.1% v/v Na Deoxycholate, 1 mM EDTA). Beads were resuspended in 900 μ L ice-cold ModRIPA buffer and divided into 2 aliquots (600 μ L for SDS-PAGE and 300 μ L for proteomics) in clean microcentrifuge tubes. Beads were pelleted at 2000 x g for 1 minute at 4°C, the supernatant discarded and the beads stored at -20°C.

Larger Scale Pulldown Assays

WT #1 MDF cells were seeded to obtain $4.0\text{--}7.0 \times 10^6$ cells per condition in 10 mL in 10 cm dishes (Falcon, cat #353003). Cells were stimulated with TSI (10 μL of 1000X stocks of each component) for 3 hours to induce necroptosis. Cells were washed with 2 x 5 mL DPBS (Gibco) and then 600 μL ice-cold RIPA lysis buffer (25 mM Tris-Cl pH 7.5, 150 mM NaCl, 1% v/v Nonidet P-40, 1% v/v Na Deoxycholate, 0.1% v/v SDS), containing cOmplete EDTA-free Protease Inhibitor Cocktail (Roche) and PhosSTOP Phosphatase Inhibitor Cocktail (Roche), was added. Cells were collected using a cell scraper and lysed for 30 minutes on ice. To prepare the Sepharose probes, 20 μL of 50% slurries of ABT-869 beads (2 $\mu\text{mol/mL}$ and 0.2 $\mu\text{mol/mL}$) and Compound **5** beads (2 $\mu\text{mol/mL}$ and 0.2 $\mu\text{mol/mL}$), per condition, were washed with 2 x 500 μL ice-cold DPBS (Gibco) and 500 μL ice-cold RIPA lysis buffer containing protease/phosphatase inhibitor cocktails, and the supernatant discarded. Cell lysates were centrifuged at $5000 \times g$ for 15 minutes at 4°C and then 500 μL of cell lysate supernatants were incubated with DMSO or increasing concentrations of ABT-869, as specified, for 1 hour on ice. Pre-treated cell lysates were incubated with ABT-869 beads and Compound **5** beads, as specified, for 3 hours at 4°C on a rotating wheel. Beads were pelleted at $2000 \times g$ for 5 minutes at 4°C and the supernatant discarded. Beads were washed with 2 x 500 μL ice-cold RIPA buffer and 2 x 500 μL ice-cold ModRIPA buffer (50 mM Tris-Cl pH 7.5, 150 mM NaCl, 1% v/v Nonidet P-40, 0.1% v/v Na Deoxycholate, 1mM EDTA). Beads were resuspended in 500 μL ice-cold ModRIPA buffer and transferred into clean microcentrifuge tubes for either SDS-PAGE or proteomics. Beads were pelleted at $2000 \times g$ for 1 minute at 4°C , the supernatant discarded and the beads stored at -20°C .

Gel Analysis of Pulldown Assays

For SDS-PAGE, 50 μL samples of the pellet (cell lysate pellet), input (cell lysate supernatant), unbound (supernatant following incubation with the beads), wash 1 (supernatant following first wash with RIPA buffer) and wash 5 (supernatant following last wash with ModRIPA buffer) were collected during the pulldown assays. To each sample, 25 μL of NuPAGE LDS Sample Buffer (Invitrogen) containing TCEP (Sigma-Aldrich) was added. Samples were heated at 95°C for 10 minutes and 10 μL of each sample (5 μL for pellet and input samples) was run on either NuPAGE 4-12% Bis-Tris 1.0 mm x 15-well Mini Gels (Invitrogen) or NuPAGE 4-12% Bis-Tris 1.0 mm x 26-well Midi Gels (Invitrogen), using NuPAGE MES SDS Running Buffer (Invitrogen) along with the SeeBlue Plus2 Pre-stained Protein Standard (Invitrogen). Gels were fixed 2 x 30 minutes in fix solution (50% v/v MeOH, 7% v/v $\text{CH}_3\text{CO}_2\text{H}$), stained with SYPRO Ruby (Invitrogen) overnight at 4°C protected from light, washed 2 x 30 minutes in wash solution (10% v/v MeOH, 7% v/v $\text{CH}_3\text{CO}_2\text{H}$) and rinsed 2 x 30 minutes in Milli-Q water. Gels were imaged on the Typhoon FLA 7000 Imaging System (GE Healthcare).

Trypsin Digestion of Pulldown Assays

On-bead trypsin digest of enriched proteins was performed using the Filter-Aided Sample Preparation (FASP) protocol (Wisniewski, 2018). All centrifugation steps were performed at 14000 x g for 8-10 minutes at room temperature unless otherwise specified. Beads samples were reduced with 200 μL of 10 mM TCEP in urea/Tris-Cl buffer (75% w/v urea in 100 mM Tris-Cl) in FASP spin columns for 30 minutes and spun down. Samples were washed with 200 μL of urea/Tris-Cl buffer, spun down and the flow-through discarded. Samples were alkylated with 100 μL of 50 mM iodoacetamide in urea/Tris-Cl buffer in the dark for 20 minutes and spun down. Samples were washed with 100 μL of urea/Tris-Cl buffer, spun down, repeated a

total of three times and the flow-through discarded. Samples were exchanged with 100 μ L of 50 mM ammonium bicarbonate, spun down and repeated a total of three times. Samples were digested with 75 μ L of trypsin gold (containing 0.25 μ g trypsin; Promega) overnight at 37°C with gentle agitation. FASP spin column were transferred to new FASP collection tubes, then 40 μ L of 50 mM ammonium bicarbonate was added and samples were spun down, and repeated with a further 70 μ L of 50 mM ammonium bicarbonate. Samples were acidified with 4 μ L neat formic acid and the flow-through transferred to mass spectrometry vials, which were frozen at -80°C prior to lyophilisation.

Mass Spectrometry of Pulldown Assays

Mass spectrometry was performed by Dr Jarrod Sandow, a Postdoctoral Researcher in the Proteomics Lab at WEHI, as previously described (Rautela et al., 2019). Briefly, peptides were resuspended (2% v/v acetonitrile, 1% v/v formic acid) and resolved by reverse-phase chromatography on a C18 fused silica column (75 μ m ID, 360 μ m OD $\times$ 25 cm length, 1.6 μ m C18 beads) packed into an emitter tip (IonOpticks) using an M-class nanoflow HPLC (Waters) coupled to a Bruker Daltonics Impact II UHR-QqTOF mass spectrometer with a CaptiveSpray source and nanoBooster. Peptides were loaded onto the column at 400 nL/min in buffer A (99.9% v/v Milli-Q water, 0.1% v/v formic acid) and eluted with a gradient of 2–34% buffer B (99.9% v/v acetonitrile, 0.1% v/v formic acid) over 90 minutes. Mass spectra were acquired over a mass range of 200–2000 m/z and peptide fragmentation was achieved using collision-induced dissociation (CID).

Data Analysis of Pulldown Assays

Analysis of the mass spectrometry data was also performed by Dr Jarrod Sandow, as previously described (Rautela et al., 2019, Delconte et al., 2016). Briefly, high-resolution MS/MS spectra raw files were processed using the MaxQuant software package and Andromeda search engine. Extracted peak lists were searched against the *Mus Musculus* protein database (Uniprot 2016), along with a separate reverse decoy database to empirically determine the false discovery rate (FDR). A strict trypsin specificity was applied, where up to 2 missed cleavages were allowed and the minimum required peptide length was set as 7 amino acids. The carbamidomethylation of Cys was set as a fixed modification. The N-terminal acetylation of peptides, oxidation of Met and addition of pyroglutamate to the N-terminus of Glu and Gln were set as variable modifications. The mass tolerance for precursor ions was 20 ppm and for fragment ions was 0.5 Da. The “match between runs” option in MaxQuant was used to transfer identifications between runs based on matched precursors with high mass accuracy. Protein identification was based on a minimum of two unique peptides, and peptide-spectrum match (PSM) and protein identifications were filtered using a target-decoy approach at an FDR of 1%. Subsequent analysis was performed using a custom pipeline developed in R, which employed the allPeptides.txt, peptides.txt and evidence.txt MaxQuant output files. Features (defined as the combination of peptide sequence, charge and modification) were removed if they were not found in at least half the number of replicates in each group. Proteins were removed if they were identified from hits to the reverse database. Corrections were performed to account for injection volume variability and for features assigned to the same protein differing in intensity due to chemico-physical properties and charge state. The mean set was at the mean of the real distribution of values minus 1.8 standard deviation (SD), and a SD of 0.5 times the SD of the distribution of the

measured intensities, to impute missing values using a random normal distribution of values. The probability of differential expression between groups was calculated using the Mann-Whitney U test, probability values were corrected for multiple testing using the Benjamini-Hochberg method and the output of the R function `wilcox.test` included P value, confidence interval and ratio estimate. Cut-off lines with the function $y = -\log_{10}(0.05) + c/(x - x_0)$ were used to identify significantly enriched proteins, where c was set to 0.2 and x_0 was set to 1, representing proteins with a twofold ($\log_2$ protein ratio of 1) or fourfold ($\log_2$ protein ratio of 2) change in protein expression, respectively.

Nqo2^{-/-} CRISPR Cloning

Two sgRNA targeting *Nqo2*, denoted sgRNA1 and sgRNA2, were designed with 20 nucleotide target sequences (shown in bold below) and the DNA oligonucleotides (below) were purchased from Integrated DNA Technologies (IDT).

sgRNA1 FWD: CACCG**TTCCAGCAATCCTAAAAGGT**

sgRNA1 REV: AAACACCTTTTAGGATTGCTGGAAC

sgRNA2 FWD: CACCG**TGGATAGGGTGCTGTGCCGA**

sgRNA2 REV: AAACCTCGGCACAGCACCTATCCAC

Each *Nqo2* sgRNA was cloned into the pSpCas9(BB)-2A-GFP (PX458) vector (Addgene) as previously described (Ran et al., 2013). Briefly, each pair of DNA oligonucleotides were phosphorylated and annealed using T4 Polynucleotide Kinase (PNK; New England BioLabs) then ligated into the *BbsI/BpiI* digested PX458 vector using Quick Ligase (New England BioLabs). Plasmids were transformed into NEB Stable Competent *E. coli* (New England BioLabs) and cells were plated on carbenicillin agar plates and incubated overnight at 37°C. Two individual colonies, denoted A and B, for each sgRNA plasmid (sg1A, sg1B, sg2A, sg2B)

were picked and used to inoculate overnight cultures containing carbenicillin (Sigma-Aldrich). Plasmid DNA was isolated and purified using the QIAprep Spin Miniprep Kit (Qiagen) according to the manufacturer's instructions. Miniprep DNA concentrations were quantified using the NanoDrop (Thermo Scientific) and samples were prepared for Sanger sequencing using a sequencing primer annealing to the U6 promoter upstream of the sgRNA cloning site (IDT). Sanger sequencing was performed by the Australian Genome Research Facility (AGRF). Sequencing results indicated that colonies sg1A and sg2B contained the correct insertion of sgRNA1 and sgRNA2, respectively.

Optimisation of Lipid Transfection Reagent for *Nqo2*^{-/-} CRISPR

WT #164 MDF cells were seeded at 1.0×10^5 cells in 2.5 mL per well into 5 wells of a 6-well plate (Corning, cat #3516) and allowed to settle overnight at 37°C 10% CO₂. Cells were checked under the microscope and were observed to be ~25-30% confluent. Solutions containing 50 µL of Opti-MEM Reduced Serum Media (Gibco), 1 µg of PX458 plasmid DNA and 3 µL of transfection reagent (FuGENE HD (Promega), Lipofectamine 2000 (Invitrogen) or Lipofectamine 3000 (Invitrogen)) were prepared. For the transfection with Lipofectamine 3000, 4 µL of P3000 Reagent (Invitrogen) was also added. A separate solution with Effectene Transfection Reagent (Qiagen) was prepared according to the manufacturer's instructions. DNA/lipid samples were mixed well, allowed to stand for 10 minutes and then added dropwise evenly across 5 wells of cells, while one well of cells was left untransfected. Cells were incubated for 48 hours at 37°C 10% CO₂, with the media changed in the Effectene well after 24 hours. Cells were harvested, spun down, resuspended in 200 µL FACS buffer (KDS BSS (in-house) + 2% v/v FCS) and GFP-positive cells were quantified by flow cytometry on a BD FACSCalibur instrument.

Nqo2^{-/-} CRISPR Transfections

WT #164 MDF cells were seeded at 1.0×10^5 cells per well for the CRISPR single cell clones, while WT #2 and WT #1 MDF cells were seeded at 1.0×10^5 and 1.5×10^5 cells per well, respectively, for the CRISPR cell pools. All cells were seeded in 2.5 mL per well into 4 wells of a 6-well plate (Corning, cat #3516) and allowed to settle overnight at 37°C 10% CO₂. Cells were checked under the microscope and were observed to be ~25%-30% confluent. Solutions containing 50 µL of Opti-MEM Reduced Serum Media (Gibco), 1 µg of DNA (PX458 plasmid DNA, sg1A miniprep DNA or sg2B miniprep DNA) and 3 µL of Lipofectamine 2000 Transfection Reagent (Invitrogen) (or 3 µL of FuGENE 6 Transfection Reagent (Promega) in the case of the initial unsuccessful transfection) were prepared. DNA/lipid samples were mixed well, allowed to stand for 10 minutes and then added dropwise evenly across 3 wells of cells, while one well of cells was left untransfected. Cells were incubated for 48 hours (WT #164 cells), 41 hours (WT #2 cells) or 22 hours (WT #1 cells) at 37°C 10% CO₂. Cells were harvested, spun down, resuspended in 200-300 µL cell culture media (DMEM + 8-10% v/v FCS containing pen/strep) and sorted for GFP-positive cells by flow cytometry on a BD FACS Aria Fusion instrument. For the CRISPR single cell clones, GFP-positive cells were single cell sorted into 96-well plates (Falcon, cat #353072) for the empty vector, sg1A and sg2B transfections then expanded in culture. For the CRISPR cell pools, GFP-positive cell pools were sorted into FACS tubes (Falcon, cat #352063) for the empty vector, sg1A and sg2B transfections. Cell pools were spun down, resuspended in fresh cell culture media, plated into 6-well plates (Corning, cat #3516) and expanded in culture.

Western Blot Analysis of *Nqo2*^{-/-} CRISPR Cell Lines

Nqo2^{-/-} CRISPR MDF single cell clones were grown in 12-well plates (Corning, cat #3512), while the panel of various MDF cell lines and *Nqo2*^{-/-} CRISPR MDF cell pools were grown in 6-well plates (Corning, cat #3516). For each cell line, 5.0×10^5 cells were collected, spun down and the supernatant discarded. Cells were resuspended in 30 μ L ice-cold RIPA lysis buffer (25 mM Tris-Cl pH 7.5, 150 mM NaCl, 1% v/v Nonidet P-40, 1% v/v Na Deoxycholate, 0.1% v/v SDS), containing cOmplete EDTA-free Protease Inhibitor Cocktail (Roche) and PhosSTOP Phosphatase Inhibitor Cocktail (Roche), and lysed for 30 minutes on ice. Cell lysates were centrifuged at 13000 x g for 30 minutes at 4°C, snap frozen in liquid N₂ and stored at -80°C until SDS-PAGE and Western blots were performed. For the *Nqo2*^{-/-} CRISPR MDF single cell clones, cell lysates were thawed on ice and 10 μ L of each lysate supernatant was added to 10 μ L of NuPAGE LDS Sample Buffer (Invitrogen) containing TCEP (Sigma-Aldrich). Samples were heated at 95°C for 10 minutes and 20 μ L of each sample was run on NuPAGE 4-12% Bis-Tris 1.0 mm x 12-well Mini Gels (Invitrogen). For the panel of MDF cell lines, cell lysates were thawed on ice and 20 μ L of each lysate supernatant was added to 20 μ L of NuPAGE LDS Sample Buffer (Invitrogen) containing TCEP (Sigma-Aldrich). Samples were heated at 95°C for 10 minutes and 10 μ L of each sample was run on NuPAGE 4-12% Bis-Tris 1.0 mm x 12-well Mini Gels (Invitrogen). For the *Nqo2*^{-/-} CRISPR MDF cell pools, cell lysates were thawed on ice and 30 μ L of NuPAGE LDS Sample Buffer (Invitrogen) containing TCEP (Sigma-Aldrich) was added directly to each lysate. Samples were heated at 95°C for 10 minutes and 20 μ L (NQO2 blots) or 10 μ L (MLKL, RIPK1, RIPK3 blots) of each sample was run on NuPAGE 4-12% Bis-Tris 1.5 mm x 10-well Mini Gels (Invitrogen). All gels were run using NuPAGE MES SDS Running Buffer (Invitrogen) with the Precision Plus Kaleidoscope Prestained Protein Standard (Bio-Rad). Western blots were performed according to the Western Blotting General Procedure.

DiscoverX KINOMEscan Binding Assays

Binding affinities (K_D) of ABT-869 for human MLKL, RIPK1 and RIPK3 were obtained from the DiscoverX KINOMEscan, a commercial kinase inhibitor binding platform, using the *KdELECT* service (Fabian et al., 2005). Briefly, active/pseudoactive site competition binding assays were performed by measuring compound binding to a given kinase/pseudokinase through competition with a promiscuous probe. The ability of ABT-869 to compete with this immobilised, active-site directed ligand was quantitatively measured via qPCR of the DNA tagged-protein in competition binding assays. For the binding affinities (K_D) from *KdELECT*, ABT-869 was tested in an 11-point 3-fold dilution series against human MLKL (full-length), RIPK1 (kinase domain) and RIPK3 (kinase domain) where binding affinities (K_D) represent the mean of two replicates.

Cloning Recombinant Protein Expression Constructs

Cloning of SBP-mMLKL, SBP-mRIPK1, SBP-mRIPK3, SBP-hMLKL, SBP-hRIPK1, SBP-hRIPK3(2-356) and SBP-hRIPK3(2-311) protein constructs was performed in conjunction with Annette Jacobsen, fellow PhD student in the Murphy Lab at WEHI. Modifications of the pFastBac vector to include an N-terminal SBP-tag, preparation of the plasmid encoding SBP-tagged mRIPK3, and preparation of the bacmids encoding SBP-tagged hMLKL, hRIPK3(2-356) and hRIPK3(2-311) were performed by Associate Professor James Murphy and Sam Young in the Murphy Lab at WEHI. DNA encoding each protein was ligated into the pFastBac HTB SBP vector (modified from the commercial pFastBac HTB vector (Invitrogen)) as in-frame fusions with a TEV-protease cleavable N-terminal His₆-tag followed by a non-cleavable N-terminal SBP-tag, as previously described (Petrie et al., 2018, Petrie et al., 2019). For hMLKL, an in-house vector, pFastBac GST SBP, was used instead, encoding a TEV-protease cleavable N-

terminal GST-tag followed by a non-cleavable N-terminal SBP-tag, as previously described (Pierotti et al., 2020).

Plasmid Generation

Insert DNA was amplified from template DNA by PCR using manually-designed forward and reverse oligonucleotide primers (IDT), dNTPs (Bioline) and Phusion Polymerase (Thermo Scientific) in supplied Phusion HF Buffer (Thermo Scientific) (**Table 1**). PCR products were purified using the MinElute PCR Purification Kit (Qiagen) according to the manufacturer's instructions, eluted in 30 μ L of EB buffer (Qiagen; 10 mM Tris-Cl pH 8.5). Purified PCR products were digested using *Bam*HI-HF (New England BioLabs) and *Eco*RI-HF (New England BioLabs) restriction enzymes in supplied CutSmart Buffer (New England BioLabs) for 1.5 hours at 37°C (**Table 2**). Digested PCR products were resolved by DNA agarose gel electrophoresis, by adding 7 μ L of 6X DNA loading dye (Qiagen) and loading the entire volume onto 1% w/v agarose (Bioline) in TAE buffer (in-house; 50 mM Tris pH 8.2-8.3, 20 mM NaOAc, 3.1 mM EDTA) containing 0.5 μ g/mL EtBr (Sigma) along with the 1 kb Plus DNA Ladder (Invitrogen). DNA inserts were excised and extracted from the agarose gel using the QIAquick Gel Extraction Kit (Qiagen) according to the manufacturer's instructions, eluted in 50 μ L of EB buffer (Qiagen; 10 mM Tris-Cl pH 8.5). Purified inserts were ligated into either the pFastBac HTB SBP vector or pFastBac GST SBP vector, as specified above, using T4 DNA Ligase (Promega) in supplied T4 DNA Ligase Buffer (Promega) for at least 1 hour at room temperature (**Table 3**). The ligation reaction mix was transformed into electrocompetent DH10B T1-resistant *E. coli* cells (prepared in-house by Sam Young; available from Invitrogen) via electroporation, by adding 2 μ L of ligation to 50 μ L of DH10B cells in ice-cold 0.2 cm electroporation cuvettes (Bio-Rad) using the MicroPulser Electroporator (Bio-Rad). The cells

were allowed to recover by adding 200 μ L of S-Broth (in-house; 3.5% w/v tryptone, 2% w/v yeast extract, 0.5% w/v NaCl, 5 mM NaOH pH 7.1-7.3) and incubating for 10 minutes at room temperature. 200 μ L of transformed cells were plated onto LG-agar plates (in-house; 1% w/v tryptone, 0.5% w/v yeast extract, 0.5% w/v NaCl, 0.2% w/v D-glucose, 10 mM Tris pH 7.4, 1 mM $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 1.5% w/v agar agar) containing 100 $\mu\text{g}/\text{mL}$ ampicillin (Roche) and incubated overnight at 37°C. Individual colonies were picked and used to inoculate overnight cultures, consisting of 3 mL of S-Broth containing 100 $\mu\text{g}/\text{mL}$ ampicillin grown shaking at 180 rpm overnight at 37°C. Plasmid DNA was isolated and purified using the QIAprep Spin Miniprep Kit (Qiagen) according to the manufacturer's instructions, eluted in 50 μ L of EB buffer (Qiagen; 10 mM Tris-Cl pH 8.5), and miniprep DNA concentrations were quantified using a NanoDrop spectrophotometer (Thermo Scientific). To verify the presence of the insert, a diagnostic digest was performed using *Bam*HI-HF and *Eco*RI-HF restriction enzymes in supplied CutSmart Buffer for 1.5 hours at 37°C (**Table 4**). Digested miniprep DNA was resolved by DNA agarose gel electrophoresis, by adding 4 μ L of 6X DNA loading dye (Qiagen) and loading 15 μ L onto 1% w/v agarose in TAE buffer containing 0.5 $\mu\text{g}/\text{mL}$ EtBr along with the 1 kb Plus DNA Ladder. Plasmid DNA was prepared for Sanger sequencing by PCR using the BacUp forward primer (GeneWorks) and BacDown reverse primer (GeneWorks) with BigDye Terminator v3.1 (Applied Biosystems) in supplied BigDye Terminator v3.1 Sequencing Buffer (Applied Biosystems) (**Table 5**), followed by a sequencing PCR clean-up using ethanol precipitation. Briefly, 80 μ L of ice-cold sequencing clean-up buffer (375 mM NaOAc, 67.5% v/v EtOH) was added to the 20 μ L sequencing PCR reaction, vortexed briefly and incubated for 15 minutes on ice. Precipitated sequencing reactions were centrifuged at 15000 x g for 20 minutes and the supernatant discarded. DNA pellets were washed with 250 μ L of ice-cold 70% v/v EtOH, the supernatant discarded and dried with lids open for 45-60 minutes at 37°C. Sanger

sequencing was performed by Micromon Genomics at Monash University. Plasmids were stored at -20°C.

Table 1 PCR recipe and PCR cycling conditions.

template DNA (50 ng/μL)	1 μL
forward primer (50 ng/μL)	2 μL
reverse primer (50 ng/μL)	2 μL
dNTPs (10 mM)	1.5 μL
Phusion Polymerase (2 U/μL)	0.5 μL
Phusion HF Buffer (5x)	10 μL
Milli-Q water	32 μL
Total	50 μL
PCR: 98°C for 3 min {98°C for 15 sec; 55°C for 15 sec; 72°C for 15 sec/kb} x 30 cycles 72°C for 1 min hold at 12°C	

Table 2 Restriction digest recipe.

PCR product	28.3 μL
<i>Bam</i> HI-HF (20000 U/mL)	0.7 μL
<i>Eco</i> RI-HF (20000 U/mL)	0.7 μL
CutSmart Buffer (10x)	3.3 μL
Total	33 μL

Table 3 Ligation recipe.

DNA insert	2.5 μL
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pFastBac HTB SBP or pFastBac GST SBP vector	0.8 μ L
T4 DNA Ligase (3 U/ μ L)	0.8 μ L
T4 DNA Ligase Buffer (10x)	1 μ L
Milli-Q water	4.9 μ L
Total	10 μ L

Table 4 Diagnostic digest recipe.

miniprep DNA (250-500 ng/ μ L)	3 μ L
<i>Bam</i> HI-HF (20000 U/mL)	0.3 μ L
<i>Eco</i> RI-HF (20000 U/mL)	0.3 μ L
CutSmart Buffer (10x)	2 μ L
Milli-Q water	14.4 μ L
Total	20 μ L

Table 5 Sequencing PCR recipe and PCR cycling conditions.

miniprep DNA (250-500 ng/ μ L)	1 μ L
forward or reverse primer (1 μ M)	4 μ L
BigDye Terminator v3.1	1 μ L
BigDye Terminator v3.1 Sequencing Buffer (5x)	4 μ L
Milli-Q water	10 μ L
Total	20 μ L

PCR: 96°C for 2 min | {96°C for 10 sec; 50°C for 5 sec; 60°C for 4 min} x 25 cycles | hold at 12°C

Bacmid Generation

Bacmids were generated using the Bac-to-Bac system (Invitrogen), as previously described (Babon and Murphy, 2013). Miniprep plasmid DNA was transformed into chemically competent DH10MultiBac *E. coli* cells (prepared in-house by Sam Young; available from ATG Biosynthetics) via heat shock, by adding 1.3 µL of 1 ng/µL miniprep plasmid DNA to 50 µL of DH10MultiBac cells, which were incubated for 30 minutes on ice before cells were heat shocked in 42°C water bath for 45 seconds followed by 2 minutes on ice. The cells were allowed to recover by adding 800 µL of S-Broth (in-house; 3.5% w/v tryptone, 2% w/v yeast extract, 0.5% w/v NaCl, 5 mM NaOH pH 7.1-7.3) and incubated shaking at 220 rpm for 5 hours at 37°C. 300 µL of transformed cells were plated onto LG-agar plates (in-house; 1% w/v tryptone, 0.5% w/v yeast extract, 0.5% w/v NaCl, 0.2% w/v D-glucose, 10 mM Tris pH 7.4, 1 mM MgCl₂·6H₂O, 1.5% w/v agar agar) containing 7 µg/mL gentamicin (Sigma), 10 µg/mL tetracycline (Sigma), 50 µg/mL kanamycin (Roche), 40 µg/mL IPTG (UBP Bio) and 0.1 mg/mL BluoGal (Invitrogen), and incubated for 48 hours at 37°C to enable blue-white screening. White colonies were picked, streaked onto fresh plates (as above) and incubated for 48 hours at 37°C. Individual white colonies were picked and used to inoculate overnight cultures, consisting of 2.5 mL of S-Broth containing 7 µg/mL gentamicin, 10 µg/mL tetracycline and 50 µg/mL kanamycin grown shaking at 180 rpm overnight at 37°C. Bacmid DNA was isolated and purified as previously described (Babon and Murphy, 2013). Briefly, 1.5 mL of bacmid overnight culture was centrifuged at 13000 x g for 1 minute to pellet the cells and the supernatant discarded. The pellet was resuspended in 300 µL of ice-cold Solution I (15 mM

Tris pH 8.0, 10 mM EDTA, 0.1 mg/mL RNaseA) by gently pipetting (not vortexing). The cells were lysed in 300 µL of room-temperature Solution II (200 mM NaOH, 1% v/v SDS), mixed by gentle inversion and incubated for 4.5 minutes at room temperature. 300 µL of ice-cold Solution III (3 M KOAc pH 5.4) was added, mixed by gentle inversion and incubated for 10 minutes on ice. Cell lysates were centrifuged at 15000 x g for 10 minutes to eliminate cellular debris and then 750 µL of lysate supernatant was added to 750 µL of ice-cold isopropanol, mixed by gentle inversion and incubated for 10 minutes on ice. Samples were centrifuged 15000 x g for 15 minutes at 4°C to pellet the bacmid DNA and the supernatant discarded. Bacmid DNA was washed with 2 x 500 µL of ice-cold 70% v/v EtOH, mixing by gentle inversion and centrifuging at 15000 x g for 3 minutes at 4°C, and the supernatant discarded. DNA pellets were dried with lids open for 15 minutes at room temperature, then 50 µL of TE buffer (Invitrogen; 10 mM Tris-Cl pH 8.0, 0.1 mM EDTA) was added and left for several hours at room temperature to allow bacmid to dissolve. Bacmid DNA concentrations were quantified using a NanoDrop spectrophotometer (Thermo Scientific). To check the presence of the insert within each bacmid, a quality control PCR was performed using an internal forward primer within the gene of interest (IDT), M13 Reverse primer (GeneWorks), dNTPs (Bioline) and Phire Polymerase (Thermo Scientific) in supplied Phire Buffer (Thermo Scientific) (**Table 6**). PCR products were resolved by DNA agarose gel electrophoresis, by loading 15 µL onto 1% w/v agarose (Bioline) in TAE buffer (in-house; 50 mM Tris pH 8.2-8.3, 20 mM NaOAc, 3.1 mM EDTA) containing 0.5 µg/mL EtBr (Sigma) along with the 1 kb Plus DNA Ladder (Invitrogen). Bacmids were stored at 4°C.

Table 6 Bacmid quality control PCR recipe and PCR cycling conditions.

bacmid DNA (400 ng/µL)	0.25 µL
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internal forward primer (10 μ M)	1.25 μ L
M13 Reverse primer (10 μ M)	1.25 μ L
dNTPs (10 mM)	1 μ L
Phire Polymerase	0.05 μ L
Phire Buffer (5x)	4 μ L
Milli-Q water	12.2 μ L
Total	20 μ L
PCR: 98°C for 3 min {98°C for 10 sec; 53°C for 10 sec; 72°C for 15 sec/kb} x 30 cycles 72°C for 30 sec hold at 12°C	

Recombinant Protein Expression

Protein expression was performed in conjunction with Annette Jacobsen, aside from the expression of mMLKL and mRIPK1, which were performed solely by Annette Jacobsen, and the expression of hMLKL and hRIPK3(2-356), which were performed by other members of the Murphy Lab at WEHI. All proteins were expressed using the baculoviral expression system with Sf21 insect cells cultured in Insect-XPRESS (Lonza). To generate DNA/lipid complexes for transfection, 6 μ L Cellfectin II Reagent (Invitrogen) in 100 μ L media was added to 1 μ g bacmid DNA in 100 μ L media and incubated for 30-45 minutes at room temperature. Sf21 insect cells were seeded at 0.45×10^6 cell/mL in 2 mL per well into 6-well plates (Corning, cat #3516) and allowed to adhere for at least 20 minutes at 27°C. Once cells had adhered, cell media was removed and 800 μ L fresh media was added to the DNA/lipid complexes, which were then added slowly dropwise to the cell monolayer and incubated for 5 hours at 27°C. The transfection mix was removed from the cells, then 2 mL fresh media was added slowly dropwise to the cell monolayer and incubated for 4 days at 27°C. P1 virus was harvested via

centrifugation of the cell supernatant at 500 x g to remove dead cells and following centrifugation, the supernatant was collected and stored at 4°C protected from light, if not used immediately. P1 virus, added at 1% v/v, was used to infect 100 mL Sf21 insect cells at 1.5×10^6 cell/mL in 1 L Schott bottles shaking at 130 rpm for 4 days at 27°C. P2 virus was harvested via centrifugation to remove the Sf21 insect cells and the supernatant was collected and stored at 4°C protected from light, if not used immediately. P2 virus, added at 10% v/v, was used to infect 2 x 0.5 L Sf21 insect cell cultures at $3-4 \times 10^6$ cells/mL in 2.8 L Fernbach flasks shaking at 90 rpm for 48 hours at 27°C. Cells were harvested via centrifugation and the supernatant discarded. Cell pellets were snap frozen in liquid N₂ and stored at -80°C until protein purification was performed.

Recombinant Protein Purification

Protein purification was performed in conjunction with Annette Jacobsen, aside from the purification of mMLKL, which was performed solely by Annette Jacobsen. All protein purification steps were performed on ice or at 4°C unless otherwise specified.

GST-tag Purification

SBP-hMLKL: Cell pellets were thawed in a cold-water bath and, per 1 L expression, were resuspended in ~40 mL lysis buffer (20 mM Tris pH 8.0, 200 mM NaCl, 10% v/v glycerol, 0.5 mM TCEP (Bond-Breaker), protease inhibitor (Roche cOmplete EDTA-free Cocktail)). Cells were lysed via sonication and then centrifuged at 45000 x g for 45 minutes at 4°C to separate the soluble protein from the cell debris. Per 1 L expression, 1 mL of 50% slurry of Glutathione Agarose Resin (UBP Bio) was equilibrated with 3 x 10 mL lysis buffer. Lysate supernatant was applied to the pre-equilibrated Glutathione Agarose Resin for 1 hour at 4°C with gentle

agitation on rollers. Beads were collected via centrifugation, washed with 3 x 25 mL wash buffer (20 mM Tris pH 8.0, 200 mM NaCl, 10% v/v glycerol, 0.5 mM TCEP (Bond-Breaker), resuspended in 1 mL wash buffer and incubated with 150 µg His-tagged TEV protease (in-house) standing at room temperature for 1 hour. The purification was monitored by reducing SDS-PAGE using standard methods and protein bands were observed using Stain-Free imaging. To collect the cleaved protein from the beads, 5 mL of wash buffer was added and the supernatant collected. Beads were then further washed with 3 x 5 mL wash buffer and the supernatant collected. His-tagged TEV protease was removed by passing protein/TEV solution over pre-equilibrated (as above) His-Tag Purification Resin for 15 minutes at room temperature with gentle agitation on rollers. Beads were collected via centrifugation and the supernatant was filtered through a 0.45 µm filter (Sartorius). Proteins were concentrated via centrifugal ultrafiltration using a 30 kDa MWCO Amicon Ultra Centrifugal Filter (Merck Millipore) until a volume of <1 mL was obtained. Proteins were purified by size exclusion chromatography (SEC) by loading the concentrated protein sample onto a Superdex 200 120 mL column (GE Healthcare) equilibrated in SEC buffer (20 mM HEPES pH 7.5, 200 mM NaCl, 5% v/v glycerol). SEC fractions were analysed by reducing SDS-PAGE using standard methods and protein bands were observed using Stain-Free imaging. The fractions in which the target protein was eluted, with the minimal contaminants, were pooled and concentrated via centrifugal ultrafiltration using a 10 kDa MWCO Amicon Ultra Centrifugal Filter (Merck Millipore) to approximately 1-5 mg/mL, then snap frozen in liquid N₂ for storage at -80°C.

His-tag Purification

SBP-mMLKL, SBP-mRIPK1, SBP-mRIPK3, SBP-hRIPK1, SBP- hRIPK3(2-356), SBP- hRIPK3(2-311):
Cell pellets were thawed in a cold-water bath and, per 1 L expression, were resuspended in

~40 mL lysis buffer (20 mM Tris pH 8.0, 500 mM NaCl, 20% v/v glycerol, 15 mM imidazole, 0.5 mM TCEP (Bond-Breaker), protease inhibitor (Roche cOmplete EDTA-free Cocktail)). Cells were lysed via sonication and then centrifuged at 45000 x g for 45 minutes at 4°C to separate the soluble protein from the cell debris. Per 1 L expression, 2 mL of 50% slurry of cOmplete His-Tag Purification Resin (Roche) was equilibrated firstly with 10 mL Milli-Q water and then with 10 mL lysis buffer. Lysate supernatant was applied to the pre-equilibrated His-Tag Purification Resin for 1 hour at 4°C with gentle agitation on rollers. Beads were collected via centrifugation then washed with 2 x 10 mL lysis buffer and 2 x 10 mL wash buffer (20 mM Tris pH 8.0, 500 mM NaCl, 20% v/v glycerol, 40 mM imidazole, 0.5 mM TCEP (Bond-Breaker)). Proteins were eluted in 15 mL elution buffer (20 mM Tris pH 8.0, 500 mM NaCl, 20% v/v glycerol, 250 mM imidazole, 0.5 mM TCEP (Bond-Breaker)) and the supernatant was filtered through a 0.45 µm filter (Sartorius). The eluted protein was incubated with 300 µg His-tagged TEV protease (in-house) standing at room temperature for 1 hour. The purification was monitored by reducing SDS-PAGE using standard methods and protein bands were observed using Stain-Free imaging. Proteins were dialysed into SEC buffer (20 mM HEPES pH 7.5, 200 mM NaCl, 5% v/v glycerol) using 6-8 kDa MWCO Dialysis Membranes (CelluSep) to remove the imidazole. His-tagged TEV protease was removed by passing dialysed protein/TEV solution over pre-equilibrated (as above) His-Tag Purification Resin for 15 minutes at room temperature with gentle agitation on rollers. For hRIPK3(2-311), His-tagged TEV protease was added to the eluted protein and the protein/TEV solution was dialysed, followed by removal of TEV protease for 30 minutes at 4°C, to ensure the protein was always kept at 4°C. Beads were collected via centrifugation and the supernatant was filtered through a 0.45 µm filter (Sartorius). Beads were washed with 3 x 5 mL wash buffer and the supernatant was collected and filtered as above. Proteins were concentrated via centrifugal ultrafiltration

using a 30 kDa or 10 kDa MWCO Amicon Ultra Centrifugal Filter (Merck Millipore) until a volume of <1 mL was obtained. Proteins were purified by size exclusion chromatography (SEC) by loading the concentrated protein sample onto a Superdex 200 120 mL column (GE Healthcare) equilibrated in SEC buffer. SEC fractions were analysed by reducing SDS-PAGE using standard methods and protein bands were observed using Stain-Free imaging. The fractions in which the target protein was eluted, with the minimal contaminants, were pooled and concentrated via centrifugal ultrafiltration using a 10 kDa MWCO Amicon Ultra Centrifugal Filter (Merck Millipore) to approximately 1-5 mg/mL, then snap frozen in liquid N₂ for storage at -80°C.

Surface Plasmon Resonance

SPR was performed as previously described (Pierotti et al., 2020) in conjunction with Annette Jacobsen. Briefly, all SPR experiments were performed on a Biacore S200 instrument (GE Healthcare) and SBP-tagged proteins were immobilised on a Biacore Series S Sensor Chip SA (GE Healthcare). The chip surface was first activated with a solution of 1 M NaCl in 50 mM NaOH. SBP-tagged proteins, at the specified concentrations, were injected in immobilisation buffer to obtain immobilisation levels of 3500-5000 Response Units (RU). The immobilisation buffer was either HEPES buffer (20 mM HEPES pH 8.0, 200 mM NaCl) or Tris buffer (50 mM Tris pH 7.5, 150 mM NaCl, 10 mM MgCl₂, 1 mM MnCl₂), according to the running buffer used. As a control, the first flow cell was left blank as the reference surface. Increasing concentrations of each compound (5-point 2-fold dilution from 2.5 µM or 11-point 2-fold dilution from 2.5 µM) or ATP (5-point 2-fold dilution from 100 µM) were flowed over the sensor chip in running buffer with an association time of 30-45 seconds and dissociation time of 200 seconds at a flow rate of 30 µL/min. The running buffer was either HEPES buffer (20

mM HEPES pH 8.0, 200 mM NaCl, 0.05% v/v P20, 2% v/v DMSO) or Tris buffer (50 mM Tris pH 7.5, 150 mM NaCl, 10 mM MgCl₂, 1 mM MnCl₂, 0.05% v/v P20, 2% v/v DMSO). The protein-immobilised surface was either regenerated with an injection of 1 M NaCl for 30 seconds at 30 μ L/min or the regeneration was omitted. All immobilisation and binding experiments were performed at either 25°C or 4°C. The amount of compound or ATP binding to the immobilised proteins as a function of time was recorded as sensorgrams and data were reference subtracted by the Biacore Evaluation Software.

Differential Scanning Fluorimetry

DSF protein stability screen was performed by the CSIRO C3 facility (Seabrook and Newman, 2013, Rosa et al., 2015, Ristic et al., 2015). 50 μ L of each SBP-tagged protein mRIPK1 (3.4 mg/mL), mRIPK3 (5.6 mg/mL), hRIPK1 (4.7 mg/mL), hRIPK3(2-356) (1.3 mg/mL) and hRIPK3(2-311) (5.0 mg/mL) were submitted for the DSF Buffer Screen 9, which was performed as previously described (Seabrook and Newman, 2013). Briefly, proteins were provided to the CSIRO C3 facility in SEC buffer, and were diluted directly into test formulations consisting of 14 different buffer conditions with a pH range of 5.0 to 9.0 at low and high salt concentrations (50 nM and 200 nM NaCl). The assay was performed in a high-throughput approach in 96-well PCR microplates with a total volume of 20 μ L per well. Each sample contained the protein of interest (0.3 μ L of 1-10 mg/mL protein sample), SYPRO Orange dye (0.3 μ L of a 1:10 dilution in water) and the test formulation (19.4 μ L). Four controls were also included: a benchmark control (protein sample in its original buffer), two negative controls (protein only, SYPRO Orange dye only) and a positive control (lysozyme; a readily available protein with a known T_m). All samples and controls were performed in triplicate. DSF was performed in a RT-PCR machine with a fluorescence excitation of 490 nm and emission of 570 nm. Plates were

subjected to the following temperature protocol: 20°C was maintained for 30 seconds and then a measurement taken, followed by a temperature increase of 0.5°C every 30 seconds with a measurement taken at each increment until 100°C. Data were analysed using the Bio-Rad CFX Manager Software and the Meltdown Program (Rosa et al., 2015).

Cellular Thermal Shift Assays

CETSA was performed as previously described (Pierotti et al., 2020). Briefly, WT #2 MDF or U937 cells were seeded to obtain 1.5×10^6 cells per temperature point. For the CETSA experiments with compounds, cells were treated with DMSO or compounds (20 μ M), as specified, for 1 hour at 37°C 10% CO₂. For the CETSA temperature optimisation experiments, this step was omitted. Cells were spun down, washed with 5 mL DPBS (Gibco), resuspended in DPBS containing cOmplete EDTA-free Protease Inhibitor Cocktail (Roche) and aliquoted into 100 μ L samples per temperature point into PCR tubes. Live cell suspensions were heated across a temperature gradient (below or as specified) for 3 minutes in a T100 Thermal Cycler (Bio-Rad), cooled at room temperature for 3 minutes, snap frozen in liquid N₂ and stored at -80°C overnight.

CETSA compound temperature gradients (°C)

hMLKL (U937 cells): 46.6, 47.7, 49.5, 51.6, 53.2, 54.3

hRIPK1 (U937 cells): 44.0, 44.7, 45.9, 47.9, 50.2, 51.9

hRIPK3 (U937 cells): 44.7, 45.9, 47.9, 50.2, 51.9, 53.2

mMLKL (MDF cells): 45.3, 47.2, 49.4, 51.0, 52.2, 53.0

mRIPK1 (MDF cells): 43.9, 45.9, 48.2, 49.9, 51.2, 52.0

mRIPK3 (MDF cells): 48.9, 50.9, 53.2, 55.0, 56.2, 57.0

Samples were freeze-thawed once to lyse the cells. Soluble protein was separated from precipitated protein via centrifugation at 17000 x g for 30 minutes at 4°C. Soluble protein fractions were resolved by SDS-PAGE and analysed by Western blot. Briefly, 10 µL of each soluble protein sample was added to 10 µL of NuPAGE LDS Sample Buffer (Invitrogen) containing TCEP (Sigma-Aldrich). Samples were heated at 95°C for 10 minutes and then 15 µL of each sample was run on a gel along with the Precision Plus Kaleidoscope Prestained Protein Standard (Bio-Rad). For the CETSA temperature optimisation experiments, NuPAGE 4-12% Bis-Tris 1.5 mm x 10-well Mini Gels (Invitrogen) with NuPAGE MES SDS Running Buffer (Invitrogen) were used. For the CETSA compound experiments, either NuPAGE 4-12% Bis-Tris 1.0 mm x 20-well Midi Gels (Invitrogen) with NuPAGE MES SDS Running Buffer (Invitrogen), or 4-15% Criterion TGX Stain-Free 26 well 15 µL Protein Gels (Bio-Rad) with Tris/Glycine Buffer (Bio-Rad) were used. Western blots were performed according to the Western Blotting General Procedure.

Phosphorylation Time Course

WT #2, *Mlkl*^{-/-} #393, *Ripk1*^{-/-}*Mlkl*^{-/-} #569 and *Ripk3*^{-/-} #19 MDF cell lines were seeded at 5.0 x 10⁵ cells in 3 mL per well into 6-well plates (Corning, cat #3516) and allowed to settle for 24 hours at 37°C 10% CO₂. WT #2 cells were stimulated with TSI for 1, 2, 3, or 4 hours, or left unstimulated, to induce phosphorylation of MLKL, RIPK1 and RIPK3. *Mlkl*^{-/-} #393, *Ripk1*^{-/-}*Mlkl*^{-/-} #569 and *Ripk3*^{-/-} #19 were left unstimulated. Cells were harvested, washed with 1 mL DPBS (Gibco) and lysed in 30 µL of ice-cold RIPA buffer (25 mM Tris-Cl pH 7.5, 150 mM NaCl, 1% v/v Nonidet P-40, 1% v/v Na Deoxycholate, 0.1% v/v SDS) containing cOmplete EDTA-free Protease Inhibitor Cocktail (Roche), PhosSTOP Phosphatase Inhibitor Cocktail (Roche) and benzonase nuclease (Sigma-Aldrich) for 30 minutes on ice. 30 µL of NuPAGE LDS Sample

Buffer (Invitrogen) containing TCEP (Sigma-Aldrich) was added to the whole cell lysates, which were then stored at -20°C until SDS-PAGE and Western blots were performed. Briefly, samples were thawed at room temperature, heated at 95°C for 10 minutes and 10 µL of each sample was run on NuPAGE 4-12% Bis-Tris 1.5 mm x 10-well Mini Gels (Invitrogen) using NuPAGE MES SDS Running Buffer (Invitrogen) along with the Precision Plus Kaleidoscope Prestained Protein Standard (Bio-Rad). Western blots were performed according to the Western Blotting General Procedure.

Phosphorylation Assays

WT #2, *Ripk1*^{-/-}*Mlkl*^{-/-} #569 and *Ripk3*^{-/-} #19 MDF cell lines were seeded at 3.0 x 10⁵ cells in 1 mL per well into 12-well plates (Corning, cat #3512) and allowed to settle for 24 hours at 37°C 10% CO₂. WT #2 cells were treated with DMSO alone or compounds (1 µM or 20 µM) at the specified concentrations for 2 hours. *Ripk1*^{-/-} #569 and *Ripk3*^{-/-} #19 cells were left untreated. Cell lines were stimulated with TSI to induce phosphorylation of RIPK1 and RIPK3, or left unstimulated, for 2 hours. For all compounds/reagents, 1 µL of 1000X stocks were used. Cells were harvested, washed with 500 µL DPBS (Gibco) and lysed in 25 µL of ice-cold RIPA buffer (25 mM Tris-Cl pH 7.5, 150 mM NaCl, 1% v/v Nonidet P-40, 1% v/v Na Deoxycholate, 0.1% v/v SDS) containing cOmplete EDTA-free Protease Inhibitor Cocktail (Roche), PhosSTOP Phosphatase Inhibitor Cocktail (Roche) and benzonase nuclease (Sigma-Aldrich) for 30 minutes on ice. Whole cell lysates were stored at -80°C until protein quantification, SDS-PAGE and Western blots were performed. Briefly, whole cell lysates were thawed on ice then quantified using the BCA Protein Assay with bicinchoninic acid solution (Sigma-Aldrich) and copper sulfate solution (Sigma-Aldrich). NuPAGE LDS Sample Buffer (Invitrogen) containing TCEP (Sigma-Aldrich) was added to the whole cell lysates to obtain 20 µg protein in 20 µL total

volume per sample. Samples were heated at 95°C for 10 minutes and 20 µL of each sample was run on NuPAGE 4-12% Bis-Tris 1.0 mm x 12-well Mini Gels (Invitrogen) using NuPAGE MES SDS Running Buffer (Invitrogen) along with the Precision Plus Kaleidoscope Prestained Protein Standard (Bio-Rad). Western blots were performed according to the Western Blotting General Procedure.

Chapter 4: Discussion, Conclusions and Future Directions

Necroptosis is an inflammatory form of programmed cell death that has important roles in human physiology and diseases. TNF-driven necroptosis, the best characterised form of necroptotic cell death, is mediated by three key protein effectors: RIPK1, RIPK3 and MLKL. The pathophysiological role of necroptosis in various diseases has sparked recent interest in the discovery and development of small molecules that target this pathway, both as potential novel therapeutics and as tools to better understand how necroptosis is regulated at the molecular level. This PhD thesis detailed the investigation into two novel inhibitors of necroptosis, Compound **2** and ABT-869, identified from small molecule screens performed at WEHI. The overarching aim of this thesis was to deduce the targets and mechanism of action of these two novel necroptosis inhibitors. As such, a range of chemical biology, biochemistry and cell biology techniques were employed for target identification and target validation studies.

Chapter 2 of this thesis is based on previous work performed at WEHI, where we reported the identification of a novel inhibitor of necroptosis, Compound **1**, from a small molecule screen against the murine MLKL pseudokinase domain (Hildebrand et al., 2014). We demonstrated that Compound **1** binds to the pseudoactive site of MLKL and inhibits necroptosis in murine cells (Hildebrand et al., 2014). Chapter 2 of this thesis outlined the identification of Compound **2**, an analogue of Compound **1** with more potent anti-necroptotic activity. Using a combination of *in vitro* binding assays, photoaffinity labelling experiments and CETSA, I identified that Compound **2** interacts with all three key necroptotic effectors – MLKL, RIPK1 and RIPK3 – *in vitro* and in cells. As a result, Compound **2** inhibits necroptosis at

nanomolar concentrations in a range of human cell lines, in addition to murine cells. Furthermore, Compound **2** shows efficacy *in vivo* and provides protection in murine model of TNF-induced SIRS.

Compound **1** and its analogues were first described by GSK as VEGFR2 inhibitors (Sammond et al., 2005). The X-ray crystal structure of Compound **2** bound to the human MLKL pseudokinase domain demonstrates that kinase inhibitors are able to bind to the pseudoactive site of pseudokinases in a similar fashion to the active site of kinases, despite key differences between kinases and pseudokinases in their canonical residues or motifs typically required to catalyse phosphoryl transfer. However, as pseudokinases lack catalytic activity, the impact of a small molecule binding to the MLKL pseudoactive site is largely unknown. Given that MLKL acts as a molecular switch in the necroptosis pathway, it is plausible that the binding of a small molecule jams the molecular switch and prevents the conformational change within MLKL that releases the 4-helix bundle domain from the constraint of the pseudokinase domain, thus preventing the activation of MLKL (Hildebrand et al., 2014).

It was reported in a recent study that Compound **1** is only able to weakly inhibit necroptosis in FADD-deficient Jurkat cells and that small molecules binding to the MLKL pseudoactive site are unable to inhibit necroptosis (Ma et al., 2016). However, the cellular activity was only evaluated in one cell line, while our previous study in murine cells (Hildebrand et al., 2014) and this current study in multiple human cell lines demonstrate that the potent anti-necroptotic activity of Compounds **1** and **2** is consistent across different cell lines and species. Furthermore, their conclusion regarding the impact of small molecules targeting MLKL was

based on a very small number of compounds, and therefore a larger number and greater diversity of compounds with more potent binding affinity need to be evaluated before such a conclusion can be made. Moreover, selective small molecule inhibitors of MLKL are needed to better understand the effect of compounds engaging MLKL on necroptotic signalling and to remove any confounding effects due to simultaneous RIPK1 or RIPK3 inhibition.

As RIPK1, RIPK3 and MLKL operate in the same pathway, it is difficult to determine the exact contribution of Compound **2** targeting each protein on the overall anti-necroptotic activity. In the DiscoverX competition binding assays, Compound **2** exhibited higher affinity for RIPK1 than for RIPK3 or MLKL, and in the CETSA, Compound **2** displayed a more profound impact on the thermal stability of RIPK1 than of MLKL or RIPK3. However, in the photoaffinity labelling experiments using photoaffinity probe Compound **3**, the strongest labelling was observed for MLKL, although this could be due to differences in the residues located within the ATP pocket of the three proteins that lend themselves to being photo-crosslinked more or less readily. Together, these data illustrate that the individual contributions of MLKL, RIPK1 and RIPK3 inhibition on the ability of Compound **2** to block necroptosis is difficult to determine. This is the main outstanding question arising from this project, which will be the subject of future research.

One possible approach to address this outstanding question could be to design mutations in MLKL, RIPK1 and RIPK3 that impair compound binding, yet do not affect the ability of these proteins to mediate necroptosis. This type of approach was performed by Li *et al.* in 2010, where dasatinib-resistant mutant kinases, containing gatekeeper residue mutations, were expressed in cells to identify which dasatinib target proteins contributed to the cellular effects

of dasatinib in lung cancer (Li et al., 2010). A similar approach could be employed to generate cell lines that individually express the gatekeeper residue mutant of either MLKL, RIPK1 or RIPK3 to disrupt compound binding to each protein. The ability of Compound **2** to block necroptosis in wild-type cells compared to the gatekeeper mutant cell lines would provide insight into the relative contribution of each protein on the overall anti-necroptotic activity of Compound **2**.

Interestingly, it was observed that higher concentrations of Compounds **1**, **2** and **3** induced cell death both in the presence and absence of necroptotic stimuli. Investigations into the cause of this toxicity and the type of cell death induced, using engineered cell lines lacking key necroptotic or apoptotic effector proteins, revealed that the toxicity was independent of all three necroptotic effectors. This toxicity was mostly blocked by the caspase inhibitor Q-VD-OPh, suggesting an apoptotic cell death. However, at the highest compound concentration of 10 μ M some cell death was still observed even in the presence of Q-VD-OPh, suggesting a non-apoptotic cell death. Overall, the observed toxicity was most likely due to the off-target activity of the compounds inhibiting other kinases, as illustrated by the kinome selectivity profile.

Overall, Chapter 2 provides a detailed study into the mechanism of action of Compound **2**. I identified that Compound **2** targets MLKL, RIPK1 and RIPK3 to potently inhibit necroptosis in cells and *in vivo* (**Figure 4.1**). It is plausible that there is a common ancestor in this pathway, as pseudoenzymes typically originate from gene duplication and adaptation to function in the same pathway as the cognate parent enzyme (Adrain and Freeman, 2012, Murphy et al., 2017). While at present, it is difficult to determine the exact contribution of each target to

the overall anti-necroptotic activity, the combined inhibition of all three proteins may contribute to the nanomolar necroptosis inhibition. Indeed, this research suggests that using small molecules to target multiple key players in the necroptosis pathway may be an optimal approach for attaining potent necroptosis inhibition for therapeutic benefit. In addition, this work suggests that targeting MLKL may be a viable strategy to modulate necroptosis and more broadly, that targeting pseudokinases may be a plausible yet mostly unexplored strategy for developing novel therapeutics.

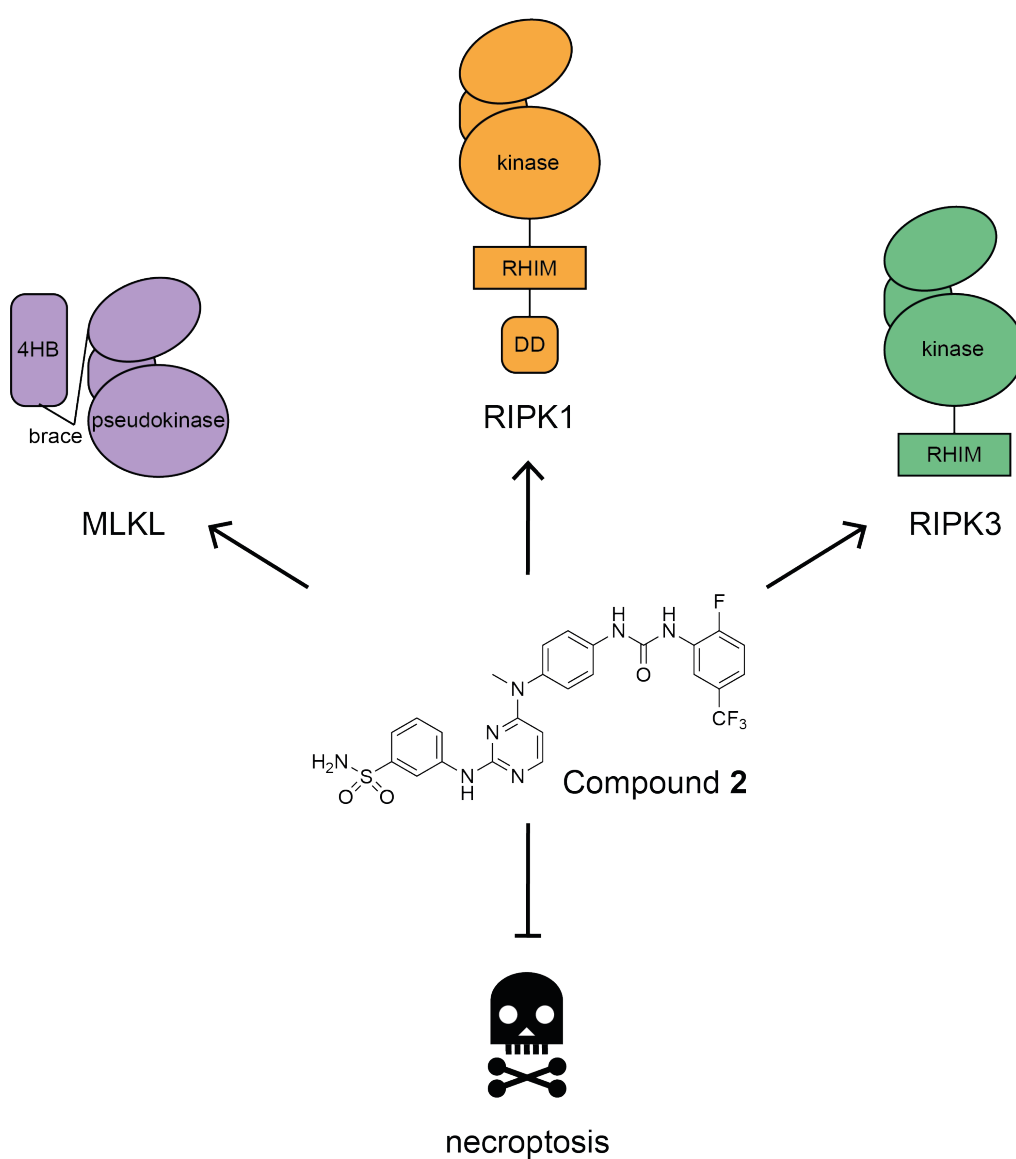


Figure 4.1 Compound 2 targets MLKL, RIPK1 and RIPK3 to potentially block necroptosis.

Chapter 3 of this thesis is based on previous work by Associate Professor James Murphy and colleagues at WEHI, where they identified certain mutations in the murine MLKL pseudoactive site that resulted in a constitutively active form of MLKL (Murphy et al., 2013). These constitutively active MLKL mutants, such as MLKL(Q343A), trigger necroptotic cell death upon expression alone, even in the absence of external necroptotic stimuli (Murphy et al., 2013). While the structure, localisation pattern and interactors of MLKL(Q343A) have not yet been investigated, these were predicted to be similar to wild-type MLKL, as the Q343A mutation causes a conformational change within the pseudokinase domain that mimics the RIPK3-mediated activation of MLKL (Murphy et al., 2013). A cell-based phenotypic screen was established at WEHI with the aim of identifying small molecules that can block MLKL(Q343A)-induced necroptosis and therefore modulate necroptosis downstream of MLKL activation (Jacobsen et al., 2016). Chapter 3 of this thesis outlined the identification of ABT-869, a hit from the MLKL(Q343A) phenotypic screen. I explored the cellular activity of ABT-869 and validated that ABT-869 was a true novel inhibitor of necroptosis in murine cells, which attenuated cell death in a dose-dependent manner. Using a combination of *in vitro* binding assays, CETSA and phosphorylation assays, I identified that ABT-869 directly targets RIPK1, and potentially RIPK3, to inhibit necroptotic cell death.

From the data obtained thus far in this project, it is difficult to ascertain whether ABT-869 targets RIPK3 directly or indirectly, as a result of ABT-869 also inhibiting RIPK1. However, given that ABT-869 did not impact the thermal stability of RIPK3 in CETSA, this suggests that ABT-869 does not directly target RIPK3 and that the reduced RIPK3 autophosphorylation observed in cells following ABT-869 treatment is due to RIPK1 inhibition. Nevertheless,

further investigations are needed to conclusively demonstrate whether ABT-869 has a direct or indirect effect on RIPK3 activity in necroptosis signalling.

A chemical proteomics approach was employed for target identification studies of ABT-869. A chemical probe derivative of ABT-869, consisting of ABT-869 conjugated to Sepharose resin, was employed to enrich putative targets and mass spectrometry analyses identified eight candidate protein hits. However, CRISPR deletion of the top hit, *Nqo2*, did not affect the ability of MDF cells to undergo cell death in response to necroptotic stimuli and did not affect the ability of ABT-869 to block necroptotic cell death. While the remaining seven hits were not evaluated during the course of this project, this will be the subject of future work, to determine if any of these other proteins are relevant targets of ABT-869 in the context of necroptosis.

Interestingly, two other small molecules identified as novel necroptosis inhibitors from the same MLKL(Q343A) phenotypic screen, AMG-47a and 17-AAG, were also found to directly or indirectly target RIPK1 and RIPK3 by others at WEHI. AMG-47a, a multi-kinase inhibitor, was found to directly inhibit RIPK1 and RIPK3 (Jacobsen et al.), while 17-AAG, a HSP90 inhibitor, was found to indirectly affect RIPK1, RIPK3 and MLKL as all three proteins are HSP90 clients (Jacobsen et al., 2016).

The combined discoveries that ABT-869, AMG-47a and 17-AAG all target necroptotic effectors upstream of MLKL raises several interesting questions. Firstly, how did the MLKL(Q343A) screen, which was designed to be independent of upstream signalling, identify small molecules that target RIPK1 and RIPK3? Some potential clues to this conundrum come from

the parallel study that identified the targets of AMG-47a, where two constitutively active forms of necroptosis in human cells were identified that are both dependent on the presence of RIPK1 (Jacobsen et al.). In one of these systems, fusion of a gyrase domain to the N-terminal domain of MLKL (NTD-gyrase) results in constitutive necroptosis upon dimerisation of the gyrase domain (Tanzer et al., 2016, Jacobsen et al.), while in the other system, RIPK3 overexpression triggers constitutive necroptosis (Jacobsen et al.). Intriguingly, while both of these systems could drive necroptosis in wild-type U937 cells, neither could do so in *RIPK1*^{-/-} U937 cells, implicating an additional role for RIPK1 downstream of MLKL activation in human cells (Jacobsen et al.). This is further supported by recent data in HT29 cells demonstrating that RIPK1 co-localises with MLKL following MLKL phosphorylation (Samson et al., 2020).

This raises a second important question: is MLKL(Q343A)-induced necroptosis indeed independent of upstream signalling, as was thought when the phenotypic screen was established? It has been shown that this system is independent of RIPK3, as expression of MLKL(Q343A) in *Ripk3*^{-/-} MDF cells still results in constitutive necroptosis (Murphy et al., 2013). However, this system is yet to be evaluated in *Ripk1*^{-/-} MDF cells and thus future work will involve determining whether necroptosis driven by MLKL(Q343A) and other constitutively active MLKL mutants is independent of RIPK1 in the context of murine cells.

It is plausible that, in addition to its upstream role, RIPK1 plays a scaffold function downstream of MLKL activation. This additional role of RIPK1 in mediating necroptosis after MLKL activation appears to be independent of its kinase activity, as RIPK1 kinase inhibitors are unable to block necroptotic cell death triggered by either MLKL NTD-gyrase or RIPK3 overexpression in human cells (Jacobsen et al.). It is possible that RIPK1 could be required to

support the conformational change of MLKL following phosphorylation by RIPK3, perhaps by stabilising the MLKL N-terminal domain when released from the constraint of the pseudokinase domain. Alternatively, RIPK1 could be required to directly facilitate or recruit the necessary chaperones for either the assembly of MLKL into productive oligomers or for the trafficking of activated MLKL to the plasma membrane.

Overall, Chapter 3 details the investigation into the cellular targets of ABT-869. I identified that ABT-869 inhibits necroptosis by targeting RIPK1, and possibly RIPK3, although it is yet to be determined whether ABT-869 impacts RIPK3 directly or indirectly (**Figure 4.2**). This study, in combination with a parallel study (Jacobsen et al.), raises questions regarding a potential role for RIPK1 downstream of MLKL activation in murine cells. As such, this research contributes to an improved understanding of necroptosis and how it is regulated at the molecular level.

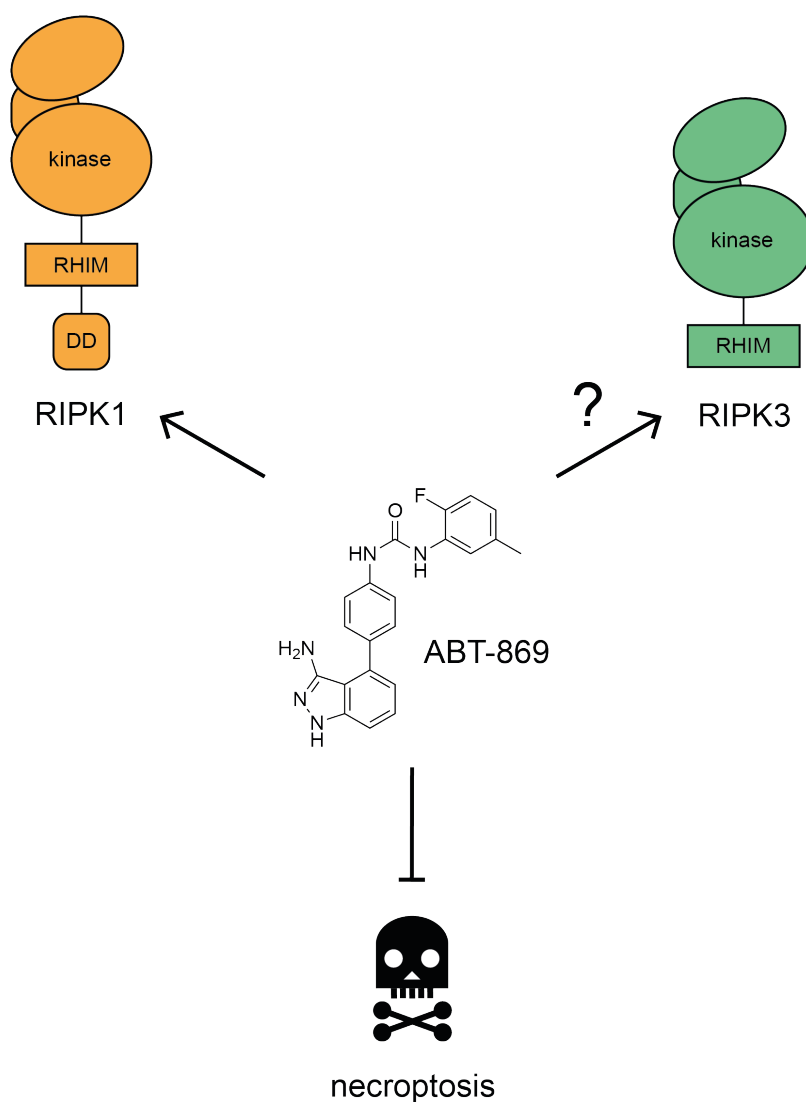


Figure 4.2 ABT-869 inhibits necroptosis by targeting RIPK1 and potentially RIPK3.

It is interesting to note that Compounds **1/2** and ABT-869 were originally described as inhibitors of VEGFR (Sammond et al., 2005, Dai et al., 2007). However, we and others have shown that other VEGFR inhibitors, such as sorafenib, do not inhibit necroptosis (Li et al., 2014, Hildebrand et al., 2014), suggesting that VEGFR is not involved in the necroptotic signalling pathway. Furthermore, Compounds **1/2** and ABT-869 were originally developed as anti-cancer compounds. Indeed, several other anti-cancer compounds have also been recently reported to inhibit necroptosis including ponatinib, pazopanib, tozasertib and

dabrafenib (Fauster et al., 2015, Najjar et al., 2015, Martens et al., 2018, Li et al., 2014). This suggests that repurposing other anti-cancer compounds could lead to the identification of novel necroptosis inhibitors and may serve as a useful starting point in medicinal chemistry campaigns to develop selective inhibitors of necroptosis.

Together, the investigation into the targets and mechanism of action of Compound **2** and ABT-869 described in this PhD thesis, demonstrates that these two novel necroptosis inhibitors block cell death by targeting known components of the TNF-driven necroptosis pathway. This provides insight into how small molecules can modulate necroptosis by targeting key necroptotic effectors to inhibit cell death. This work demonstrates that small molecules are valuable tools to dissect necroptotic signalling and investigate the molecular details of necroptosis. Furthermore, this research highlights that a variety of target validation approaches are required to deduce the targets of small molecules.

Appendices

Appendix 1

The results table below contains the raw data represented schematically in the TREEspot interaction maps presented in Chapter 2, **Figure 3A**.

Table 7 Kinome selectivity profile of Compound 2 tested at 1 μ M against 468 kinases from the DiscoverX KINOMEScan scanMAX kinase assay panel.

DiscoverX Gene Symbol	Entrez Gene Symbol	Percent Control
AAK1	AAK1	100
ABL1(E255K)-phosphorylated	ABL1	13
ABL1(F317I)-nonphosphorylated	ABL1	17
ABL1(F317I)-phosphorylated	ABL1	54
ABL1(F317L)-nonphosphorylated	ABL1	7.1
ABL1(F317L)-phosphorylated	ABL1	30
ABL1(H396P)-nonphosphorylated	ABL1	0.1
ABL1(H396P)-phosphorylated	ABL1	9.6
ABL1(M351T)-phosphorylated	ABL1	9
ABL1(Q252H)-nonphosphorylated	ABL1	0
ABL1(Q252H)-phosphorylated	ABL1	13
ABL1(T315I)-nonphosphorylated	ABL1	2.9
ABL1(T315I)-phosphorylated	ABL1	11
ABL1(Y253F)-phosphorylated	ABL1	5.5
ABL1-nonphosphorylated	ABL1	2.3

ABL1-phosphorylated	ABL1	6.9
ABL2	ABL2	1.2
ACVR1	ACVR1	100
ACVR1B	ACVR1B	100
ACVR2A	ACVR2A	72
ACVR2B	ACVR2B	68
ACVRL1	ACVRL1	69
ADCK3	CABC1	100
ADCK4	ADCK4	100
AKT1	AKT1	100
AKT2	AKT2	100
AKT3	AKT3	100
ALK	ALK	37
ALK(C1156Y)	ALK	58
ALK(L1196M)	ALK	80
AMPK-alpha1	PRKAA1	100
AMPK-alpha2	PRKAA2	94
ANKK1	ANKK1	69
ARK5	NUAK1	92
ASK1	MAP3K5	100
ASK2	MAP3K6	100
AURKA	AURKA	5.2
AURKB	AURKB	13

AURKC	AURKC	23
AXL	AXL	0.6
BIKE	BMP2K	74
BLK	BLK	0.2
BMPR1A	BMPR1A	65
BMPR1B	BMPR1B	99
BMPR2	BMPR2	100
BMX	BMX	50
BRAF	BRAF	43
BRAF(V600E)	BRAF	20
BRK	PTK6	2.2
BRSK1	BRSK1	100
BRSK2	BRSK2	100
BTK	BTK	98
BUB1	BUB1	100
CAMK1	CAMK1	100
CAMK1B	PNCK	100
CAMK1D	CAMK1D	100
CAMK1G	CAMK1G	100
CAMK2A	CAMK2A	96
CAMK2B	CAMK2B	100
CAMK2D	CAMK2D	100
CAMK2G	CAMK2G	100

CAMK4	CAMK4	100
CAMKK1	CAMKK1	62
CAMKK2	CAMKK2	100
CASK	CASK	100
CDC2L1	CDK11B	8.2
CDC2L2	CDC2L2	4.9
CDC2L5	CDK13	22
CDK11	CDK19	0
CDK2	CDK2	5.3
CDK3	CDK3	4.1
CDK4	CDK4	100
CDK4-cyclinD1	CDK4	90
CDK4-cyclinD3	CDK4	92
CDK5	CDK5	22
CDK7	CDK7	5
CDK8	CDK8	0.05
CDK9	CDK9	4.8
CDKL1	CDKL1	42
CDKL2	CDKL2	0
CDKL3	CDKL3	0
CDKL5	CDKL5	98
CHEK1	CHEK1	100
CHEK2	CHEK2	91

CIT	CIT	26
CLK1	CLK1	31
CLK2	CLK2	100
CLK3	CLK3	81
CLK4	CLK4	62
CSF1R	CSF1R	0.25
CSF1R-autoinhibited	CSF1R	100
CSK	CSK	100
CSNK1A1	CSNK1A1	84
CSNK1A1L	CSNK1A1L	100
CSNK1D	CSNK1D	100
CSNK1E	CSNK1E	94
CSNK1G1	CSNK1G1	100
CSNK1G2	CSNK1G2	97
CSNK1G3	CSNK1G3	100
CSNK2A1	CSNK2A1	99
CSNK2A2	CSNK2A2	93
CTK	MATK	100
DAPK1	DAPK1	100
DAPK2	DAPK2	100
DAPK3	DAPK3	100
DCAMKL1	DCLK1	95
DCAMKL2	DCLK2	100

DCAMKL3	DCLK3	100
DDR1	DDR1	0.25
DDR2	DDR2	17
DLK	MAP3K12	100
DMPK	DMPK	93
DMPK2	CDC42BPG	97
DRAK1	STK17A	98
DRAK2	STK17B	87
DYRK1A	DYRK1A	100
DYRK1B	DYRK1B	85
DYRK2	DYRK2	100
EGFR	EGFR	63
EGFR(E746-A750del)	EGFR	72
EGFR(G719C)	EGFR	46
EGFR(G719S)	EGFR	60
EGFR(L747-E749del, A750P)	EGFR	60
EGFR(L747-S752del, P753S)	EGFR	63
EGFR(L747-T751del,Sins)	EGFR	97
EGFR(L858R)	EGFR	59
EGFR(L858R,T790M)	EGFR	95
EGFR(L861Q)	EGFR	37
EGFR(S752-I759del)	EGFR	85
EGFR(T790M)	EGFR	53

EIF2AK1	EIF2AK1	94
EPHA1	EPHA1	3.5
EPHA2	EPHA2	3.6
EPHA3	EPHA3	0.9
EPHA4	EPHA4	3.7
EPHA5	EPHA5	5.1
EPHA6	EPHA6	2.5
EPHA7	EPHA7	2.2
EPHA8	EPHA8	0.1
EPHB1	EPHB1	1.2
EPHB2	EPHB2	5.3
EPHB3	EPHB3	94
EPHB4	EPHB4	5.2
EPHB6	EPHB6	0.1
ERBB2	ERBB2	100
ERBB3	ERBB3	77
ERBB4	ERBB4	71
ERK1	MAPK3	92
ERK2	MAPK1	100
ERK3	MAPK6	100
ERK4	MAPK4	100
ERK5	MAPK7	33
ERK8	MAPK15	0.65

ERN1	ERN1	100
FAK	PTK2	0.75
FER	FER	3
FES	FES	12
FGFR1	FGFR1	5.7
FGFR2	FGFR2	5.2
FGFR3	FGFR3	17
FGFR3(G697C)	FGFR3	56
FGFR4	FGFR4	11
FGR	FGR	2.2
FLT1	FLT1	3.7
FLT3	FLT3	0.5
FLT3(D835H)	FLT3	1.2
FLT3(D835V)	FLT3	3.2
FLT3(D835Y)	FLT3	3
FLT3(ITD)	FLT3	2
FLT3(ITD,D835V)	FLT3	12
FLT3(ITD,F691L)	FLT3	19
FLT3(K663Q)	FLT3	0
FLT3(N841I)	FLT3	0.2
FLT3(R834Q)	FLT3	13
FLT3-autoinhibited	FLT3	94
FLT4	FLT4	0.1

FRK	FRK	0.35
FYN	FYN	0.85
GAK	GAK	100
GCN2(Kin.Dom.2,S808G)	EIF2AK4	96
GRK1	GRK1	100
GRK2	ADRBK1	100
GRK3	ADRBK2	90
GRK4	GRK4	75
GRK7	GRK7	94
GSK3A	GSK3A	19
GSK3B	GSK3B	100
HASPIN	GSG2	100
HCK	HCK	1.4
HIPK1	HIPK1	17
HIPK2	HIPK2	35
HIPK3	HIPK3	7.3
HIPK4	HIPK4	2
HPK1	MAP4K1	14
HUNK	HUNK	85
ICK	ICK	98
IGF1R	IGF1R	58
IKK-alpha	CHUK	32
IKK-beta	IKBKB	80

IKK-epsilon	IKBKE	100
INSR	INSR	81
INSRR	INSRR	6.3
IRAK1	IRAK1	11
IRAK3	IRAK3	56
IRAK4	IRAK4	100
ITK	ITK	4.8
JAK1(JH1domain-catalytic)	JAK1	3.1
JAK1(JH2domain-pseudokinase)	JAK1	76
JAK2(JH1domain-catalytic)	JAK2	8.5
JAK3(JH1domain-catalytic)	JAK3	42
JNK1	MAPK8	100
JNK2	MAPK9	31
JNK3	MAPK10	100
KIT	KIT	0.45
KIT(A829P)	KIT	9.9
KIT(D816H)	KIT	39
KIT(D816V)	KIT	1.2
KIT(L576P)	KIT	0.7
KIT(V559D)	KIT	0.25
KIT(V559D,T670I)	KIT	1.1
KIT(V559D,V654A)	KIT	6
KIT-autoinhibited	KIT	100

LATS1	LATS1	80
LATS2	LATS2	91
LCK	LCK	0.1
LIMK1	LIMK1	18
LIMK2	LIMK2	18
LKB1	STK11	90
LOK	STK10	0.05
LRRK2	LRRK2	100
LRRK2(G2019S)	LRRK2	100
LTK	LTK	1.7
LYN	LYN	2.3
LZK	MAP3K13	86
MAK	MAK	6.5
MAP3K1	MAP3K1	100
MAP3K15	MAP3K15	100
MAP3K2	MAP3K2	97
MAP3K3	MAP3K3	71
MAP3K4	MAP3K4	100
MAP4K2	MAP4K2	40
MAP4K3	MAP4K3	9.6
MAP4K4	MAP4K4	0
MAP4K5	MAP4K5	2.1
MAPKAPK2	MAPKAPK2	73

MAPKAPK5	MAPKAPK5	100
MARK1	MARK1	100
MARK2	MARK2	98
MARK3	MARK3	100
MARK4	MARK4	100
MAST1	MAST1	100
MEK1	MAP2K1	99
MEK2	MAP2K2	90
MEK3	MAP2K3	84
MEK4	MAP2K4	83
MEK5	MAP2K5	0.65
MEK6	MAP2K6	95
MELK	MELK	100
MERTK	MERTK	0.35
MET	MET	7.3
MET(M1250T)	MET	1.2
MET(Y1235D)	MET	2.1
MINK	MINK1	9.9
MKK7	MAP2K7	100
MKNK1	MKNK1	62
MKNK2	MKNK2	2.6
MLCK	MYLK3	42
MLK1	MAP3K9	23

MLK2	MAP3K10	59
MLK3	MAP3K11	38
MRCKA	CDC42BPA	100
MRCKB	CDC42BPB	100
MST1	STK4	28
MST1R	MST1R	2.6
MST2	STK3	36
MST3	STK24	100
MST4	MST4	100
MTOR	MTOR	82
MUSK	MUSK	7.5
MYLK	MYLK	84
MYLK2	MYLK2	25
MYLK4	MYLK4	100
MYO3A	MYO3A	52
MYO3B	MYO3B	17
NDR1	STK38	85
NDR2	STK38L	100
NEK1	NEK1	100
NEK10	NEK10	100
NEK11	NEK11	100
NEK2	NEK2	100
NEK3	NEK3	94

NEK4	NEK4	100
NEK5	NEK5	48
NEK6	NEK6	100
NEK7	NEK7	100
NEK9	NEK9	38
NIK	MAP3K14	100
NIM1	MGC42105	100
NLK	NLK	49
OSR1	OXS1	100
p38-alpha	MAPK14	86
p38-beta	MAPK11	8.4
p38-delta	MAPK13	0.6
p38-gamma	MAPK12	0
PAK1	PAK1	100
PAK2	PAK2	100
PAK3	PAK3	3.9
PAK4	PAK4	100
PAK6	PAK6	100
PAK7	PAK7	100
PCTK1	CDK16	79
PCTK2	CDK17	1.3
PCTK3	CDK18	6.7
PDGFRA	PDGFRA	19

PDGFRB	PDGFRB	0
PDPK1	PDPK1	54
PFCDPK1(P.falciparum)	CDPK1	100
PFPK5(P.falciparum)	MAL13P1.279	100
PFTAIRE2	CDK15	8.8
PFTK1	CDK14	3.3
PHKG1	PHKG1	100
PHKG2	PHKG2	100
PIK3C2B	PIK3C2B	73
PIK3C2G	PIK3C2G	100
PIK3CA	PIK3CA	75
PIK3CA(C420R)	PIK3CA	64
PIK3CA(E542K)	PIK3CA	100
PIK3CA(E545A)	PIK3CA	91
PIK3CA(E545K)	PIK3CA	93
PIK3CA(H1047L)	PIK3CA	100
PIK3CA(H1047Y)	PIK3CA	74
PIK3CA(I800L)	PIK3CA	96
PIK3CA(M1043I)	PIK3CA	97
PIK3CA(Q546K)	PIK3CA	100
PIK3CB	PIK3CB	100
PIK3CD	PIK3CD	98
PIK3CG	PIK3CG	74

PIK4CB	PI4KB	100
PIKFYVE	PIKFYVE	99
PIM1	PIM1	100
PIM2	PIM2	100
PIM3	PIM3	100
PIP5K1A	PIP5K1A	82
PIP5K1C	PIP5K1C	100
PIP5K2B	PIP4K2B	97
PIP5K2C	PIP4K2C	8.7
PKAC-alpha	PRKACA	100
PKAC-beta	PRKACB	92
PKMYT1	PKMYT1	100
PKN1	PKN1	100
PKN2	PKN2	100
PKNB(M.tuberculosis)	pknB	96
PLK1	PLK1	100
PLK2	PLK2	74
PLK3	PLK3	38
PLK4	PLK4	2.6
PRKCD	PRKCD	100
PRKCE	PRKCE	97
PRKCH	PRKCH	100
PRKCI	PRKCI	68

PRKCQ	PRKCQ	100
PRKD1	PRKD1	94
PRKD2	PRKD2	100
PRKD3	PRKD3	100
PRKG1	PRKG1	100
PRKG2	PRKG2	94
PRKR	EIF2AK2	100
PRKX	PRKX	100
PRP4	PRPF4B	82
PYK2	PTK2B	2.9
QSK	KIAA0999	100
RAF1	RAF1	7.4
RET	RET	0
RET(M918T)	RET	0
RET(V804L)	RET	0.1
RET(V804M)	RET	0.3
RIOK1	RIOK1	1.2
RIOK2	RIOK2	100
RIOK3	RIOK3	12
RIPK1	RIPK1	0.35
RIPK2	RIPK2	10
RIPK4	RIPK4	97
RIPK5	DSTYK	91

ROCK1	ROCK1	100
ROCK2	ROCK2	97
ROS1	ROS1	1
RPS6KA4(Kin.Dom.1-N-terminal)	RPS6KA4	55
RPS6KA4(Kin.Dom.2-C-terminal)	RPS6KA4	100
RPS6KA5(Kin.Dom.1-N-terminal)	RPS6KA5	100
RPS6KA5(Kin.Dom.2-C-terminal)	RPS6KA5	100
RSK1(Kin.Dom.1-N-terminal)	RPS6KA1	98
RSK1(Kin.Dom.2-C-terminal)	RPS6KA1	100
RSK2(Kin.Dom.1-N-terminal)	RPS6KA3	100
RSK2(Kin.Dom.2-C-terminal)	RPS6KA3	92
RSK3(Kin.Dom.1-N-terminal)	RPS6KA2	46
RSK3(Kin.Dom.2-C-terminal)	RPS6KA2	100
RSK4(Kin.Dom.1-N-terminal)	RPS6KA6	100
RSK4(Kin.Dom.2-C-terminal)	RPS6KA6	100
S6K1	RPS6KB1	71
SBK1	SBK1	100
SGK	SGK1	79
SgK110	SgK110	89
SGK2	SGK2	85
SGK3	SGK3	100
SIK	SIK1	63
SIK2	SIK2	100

SLK	SLK	0.75
SNARK	NUAK2	100
SNRK	SNRK	74
SRC	SRC	0.15
SRMS	SRMS	37
SRPK1	SRPK1	98
SRPK2	SRPK2	95
SRPK3	SRPK3	76
STK16	STK16	100
STK33	STK33	12
STK35	STK35	100
STK36	STK36	100
STK39	STK39	100
SYK	SYK	3.4
TAK1	MAP3K7	16
TAOK1	TAOK1	26
TAOK2	TAOK2	7.6
TAOK3	TAOK3	11
TBK1	TBK1	64
TEC	TEC	100
TESK1	TESK1	55
TGFBR1	TGFBR1	95
TGFBR2	TGFBR2	41

TIE1	TIE1	0.25
TIE2	TEK	0.4
TLK1	TLK1	100
TLK2	TLK2	100
TNIK	TNIK	12
TNK1	TNK1	1.8
TNK2	TNK2	98
TNNI3K	TNNI3K	4.3
TRKA	NTRK1	2.3
TRKB	NTRK2	6.8
TRKC	NTRK3	7.3
TRPM6	TRPM6	100
TSSK1B	TSSK1B	98
TSSK3	TSSK3	86
TTK	TTK	97
TXK	TXK	40
TYK2(JH1domain-catalytic)	TYK2	94
TYK2(JH2domain-pseudokinase)	TYK2	98
TYRO3	TYRO3	8.1
ULK1	ULK1	93
ULK2	ULK2	100
ULK3	ULK3	97
VEGFR2	KDR	23

VPS34	PIK3C3	100
VRK2	VRK2	100
WEE1	WEE1	67
WEE2	WEE2	100
WNK1	WNK1	100
WNK2	WNK2	51
WNK3	WNK3	100
WNK4	WNK4	100
YANK1	STK32A	100
YANK2	STK32B	100
YANK3	STK32C	100
YES	YES1	2.3
YSK1	STK25	100
YSK4	MAP3K19	4
ZAK	ZAK	11
ZAP70	ZAP70	100

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