A novel anti-inflammatory mechanism identified by an *in vivo* chemical genetic screen

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Abstract

Neutrophil clearance is an essential prerequisite for the successful resolution of inflammation. Dysregulation of the mechanisms that regulate inflammation resolution may lead to persistent neutrophilic inflammation and extensive host tissue damage, which contributes to the pathogenesis of chronic inflammatory diseases. Many of these respond poorly to current treatments and uncovering new mechanisms by which inflammation resolution can be accelerated is key for the development of novel, more effective therapies, which specifically target neutrophil clearance.

Using a zebrafish model of neutrophilic inflammation, I have designed and optimised a partially automated compound screening assay, with the hypothesis that this would lead to the discovery of novel or previously unsuspected anti-inflammatory compounds that accelerate the resolution of inflammation. This approach enabled the identification of multiple active compounds, acting via distinct anti-inflammatory and pro-resolution mechanisms. By hierarchical cluster analysis, I was able to accurately predict the mechanism of action of one of these compounds, isopimpinellin, which led to the development of a model for analysing PI-3K activity during neutrophil recruitment *in vivo*. Chemical structure comparison also revealed a potential structure-activity relationship within a subset of the assay positive compounds.

The most interesting compound identified, tanshinone IIA, was found to induce apoptosis of human neutrophils *in vitro*, even in the presence of survival signals, but also drive inflammation resolution *in vivo* via an additional, novel mechanism. Tanshinone IIA promotes neutrophil clearance from the wound by reverse migration, without increasing neutrophil speed, and dynamic modelling reveals that this occurs as a passive redistribution process rather than active fugetaxis. Importantly, this effect is maintained in the presence of survival signalling. This is the first evidence to demonstrate that it is possible to pharmacologically drive inflammation resolution by promoting neutrophil reverse migration.

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List of Abbreviations

AA	Arachidonic Acid
ALX	Lipoxin receptor
AKT	Protein Kinase B
ANOVA	Analysis of Variance
Anx-A1	Annexin-A1
ARDS	Acute Respiratory Distress Syndrome
ATL	Aspirin-Triggered Lipoxin
ATP	Adenosine Triphosphate
Bcl-2	B-cell Lymphoma 2
CDK	Cyclin Dependent Kinase
CGD	Chronic Granulomatous Disease
CHT	Caudal Haematopoietic Tissue
COPD	Chronic Obstructive Pulmonary Disease
COX	Cyclooxygenase
CXCR	C-X-C Chemokine Receptor
DAMP	Damage-Associated Molecular Pattern
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
dpf	Days Post Fertilisation
ECM	Extracellular Matrix
EGFP	Enhanced GFP
ERK	Extracellular Signal-Regulated Kinase
FACS	Fluorescence-Activated Cell Sorting
FCS	Foetal Calf Serum
FITC	Fluorescein Isothiocyanate
fMLP	Formyl-Methionyl-Leucyl-Phenylalanine
FPU	Fluorescence Per Unit Area
FRET	Fluorescence Resonance Energy Transfer
G-CSF	Granulocyte-Colony Stimulating Factor
GFP	Green Fluorescent Protein
GM-CSF	Granulocyte Macrophage-Colony Stimulating Factor
GPCR	G-Protein Coupled Receptor

GTP	Guanosine Triphosphate
HIF	Hypoxia Inducible Factor
HBSS	Hank's Balanced Salt Solution
hpf	Hours Post Fertilisation
hpi	Hours Post Injury
IBD	Inflammatory Bowel Disease
ICAM	Intracellular Adhesion Molecule
IFN	Interferon
IgG	Immunoglobulin G
IL-	Interleukin
PIP_3	Phosphatidylinositol-3,4,5-Triphosphate
JAK	Janus Kinase
JAM	Junctional Adhesion Molecule
JNK	c-Jun N-Terminal Kinase
LFA-1	Lymphocyte Function-Associated Antigen-1
LMP	Low Melting Point
LO	Lipooxygenase
LPS	Lipopolysaccharide
LT	Leukotriene
MAPK	Mitogen Activated Protein Kinase
Mcl-1	Myeloid Cell Leukemia Sequence-1
MMP	Matrix Metalloproteinase
MPO	Myeloperoxidase
MPX	Myeloid Specific Peroxidise
mRNA	Messenger RNA
mTOR	Mammalian Target of Rapamycin
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NET	Neutrophil Extracellular Trap
NF-κB	Nuclear Factor-Kappa Light Chain Enhancer of Activated B Cells
NLR	NACHT (NAIP, C2TA, HET-E, TP1)-Leucine-Rich Repeat
NSAID	Non-Steroidal Anti-Inflammatory Drug
PAF	Platelet Activating Factor
PE	Phycoerythrin
PAMP	Pathogen-Associated Molecular Pattern

PBMC	Peripheral Blood Mononuclear Cell
PBS	Phosphate Buffered Saline
PBT	Phosphate Buffered Saline + Tween
PDE	Phosphodiesterase
PFA	Paraformaldehyde
PG	Prostaglandin
PH	Pleckstrin Homology
PI-3K	Phosphoinositide 3-Kinase
PIP_3	Phosphatidylinositol (3,4,5)-Triphosphate
PKC	Protein Kinase C
PMN	Polymorphonuclear Neutrophil
PP2A	Protein Phosphatase 2A
PRR	Pattern Recognition Receptor
PTEN	Phosphatase and Tensin Homologue
RA	Rheumatoid Arthritis
RNA	Ribonucleic acid
ROI	Region Of Interest
ROS	Reactive Oxygen Species
RPMI	Roswell Park Memorial Institute Medium
SDF	Stromal Cell Derived Factor
SEM	Standard Error of the Mean
SGK	Serum and Glucocorticoid Regulated Kinase
STAT	Signal Transducer and Activator of Transcription
TGF	Transforming Growth Factor
TLR	Toll-Like Receptor
TNF	Tumor Necrosis Factor
TSA	Tyramide Signal Amplification
TUNEL	Terminal Deoxynucleotidyl Transferase dUTP Nick End Labelling
UAS	Upstream Activation Sequence
WHIM	Warts, Hypogammaglobulinaemia, Infection and Myelokathesis
WISH	Whole Mount *in situ* Hybridisation

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Chapter 1: Introduction

1.1 Overview of inflammation

The inflammatory response is a multistep process initiated by the innate immune system in the event of tissue injury or infection. It is a rapid and highly controlled host-defence mechanism, which functions to detect and destroy invading pathogens (Medzhitov, 2007). The response is triggered by recognition of an inflammatory stimulus by immune cell receptors, which leads to activation of multiple inflammatory signalling cascades and the production of pro-inflammatory mediators. These promote the rapid recruitment and activation of innate immune cells such as neutrophil granulocytes, which function to eliminate infectious agents.

It is critical that the inflammatory response resolves effectively to allow successful tissue repair and the return to normal homeostasis. Host tissue damage may be required in order to contain an infection, but this must be selective and controlled to avoid extensive tissue destruction. Without adequate control, detrimental damage to healthy tissue may ensue and failure of the acute inflammatory response to resolve may result in chronic inflammation (Medzhitov, 2010). Persistent inflammation and dysregulation of the mechanisms governing resolution of inflammation are associated with the progression of chronic inflammatory diseases, such as rheumatoid arthritis (RA) and chronic obstructive pulmonary disease (COPD) (Serhan et al., 2007).

1.1.1 Evolution of inflammation

The innate immune response is the first line of host defence, designed to identify and destroy foreign invaders. It is an evolutionarily ancient system, many components of which are highly conserved and exist in all metazoan organisms (Barton, 2008). The concept of cellular immunity was first documented by Elie Metchnikoff in 1892, when he observed the accumulation of phagocytes at a site of injury induced by a rose thorn in starfish larvae. He recognised the beneficial function of phagocytosis for host defence and maintenance of homeostasis (Metchnikoff, 1905).

Inflammation was initially defined in the first century AD by the Roman encyclopaedist Cornelius Celsus, who described the four principal signs as *rubor* (redness), *tumor* (swelling), *calore* (heat) and *dolore* (pain) (Medzhitov, 2010). These simple observations are an accurate portrayal of inflammation as we understand it today. Vasodilation and extravasation of red blood cells results in heat and redness, whilst swelling is caused by fluid accumulation and pain may ensue via the stimulation of sensory nerve endings by chemical mediators such as bradykinin. A fifth cardinal sign of inflammation, *functio laesa* (disturbance of function), was defined centuries later in 1858, by the German physician Rudolph Virchow (Medzhitov, 2010).

1.1.2 Initiation of the inflammatory response

Inflammation can be initiated by either endogenous or exogenous stimuli. The most well characterised inflammatory response is that induced by microbial infection (Medzhitov, 2008). Tissue resident macrophages, dendritic cells and mast cells detect the presence of foreign microorganisms via the recognition of pathogen-associated molecular patterns (PAMPs). These are conserved microbial components such as lipopolysaccharides (LPS), flagellin and double- or single-stranded RNA, which bind to Pattern Recognition Receptors (PRRs), such as the family of transmembrane Toll-like receptors (TLRs) (Medzhitov, 2001). Binding causes phagocytosis of the foreign microbe and initiates an intracellular, pro-inflammatory signalling cascade, resulting in the activation of transcription factors such as nuclear factor-κB (NF-κB) and interferon-regulatory factors (IRFs) (Medzhitov, 2007; Barton, 2008). These important regulators drive the expression of pro-inflammatory genes including Tumor Necrosis Factor-α (TNF-α) and interleukin-1 (IL-1), resulting in immune cell activation and the subsequent release of multiple pro-inflammatory mediators (Nathan, 2002).

Alternatively, the inflammatory response can be initiated via the activation of cytosolic receptors, amongst which are the family of NACHT-leucine-rich repeat containing receptors (NLRs) (Pétrilli et al., 2007). The Nod1 and Nod2 proteins form a subfamily of NLRs, which function in a comparable manner to TLRs, by binding bacterial products such as peptidoglycan to activate NF-κB and enhance pro-inflammatory gene expression (Strober et al., 2006). Nod2 mutations have been associated with Crohn's

disease, emphasising the significance of these proteins in coordination of the inflammatory response (Strober et al., 2006).

Other intracellular NLRs function in a different manner, including the NLR family apoptosis inhibitory proteins (NAIPs) and NACHT-, LRR-, and pyrin domain-containing proteins (NALPs), which target internalised exogenous ligands such as bacterial flagellin and RNA, and the endogenous uric acid crystals characteristic of gout (Mariathasan et al., 2007). Binding triggers activation of a multi-protein complex known as the inflammasome, which sequentially activates caspase-1 to allow cleavage and processing of the IL-1 cytokine family, members of which include IL-1β, IL-18 and IL-33 (Mariathasan & Monack, 2007; Medzhitov, 2007). It is likely that crosstalk between the TLR and NLR pathways exists, as both lead to inflammasome activation and subsequent IL-1 secretion (Barton, 2008).

In terms of sterile tissue injury in which there is an absence of microbial infection, it is likely that TLRs are responsible for induction of the inflammatory response via the recognition of endogenous, intracellular ligands that are released by necrotic cells, such as heat shock proteins and β-defensin (Ohashi et al., 2000; Biragyn et al., 2002). For example, knockout mice studies have revealed that TLR2 and TLR4, along with MyD88, are necessary for the detection of hyaluronan, an extracellular matrix (ECM) glycosaminoglycan that is present in the serum of acute lung injury patients and is responsible for stimulating inflammation via activation of NF-κB (Jiang et al., 2005). These endogenous stimuli are collectively referred to as damage-associated molecular patterns (DAMPs) and also include adenosine triphosphate (ATP), high-mobility group box 1 protein (HMGB1) and various members of the S100 calcium-binding protein family (Bianchi, 2007). ATP binding to purinoceptors such as P2X7 on macrophages decreases intracellular K^+ levels and contributes to activation of the inflammasome (Mariathasan et al., 2006). In addition to the inflammatory response induced by innate receptor signalling, activation of afferent neurons in response to pain from tissue injury results in the release of bioactive peptides such as calcitonin gene-related peptide and substance P (Steinhoff et al., 2000).

1.1.3 Inflammatory mediators

Following initiation of the inflammatory response, pro-inflammatory mediators released by macrophages and other tissue-resident inflammatory cells amplify the response and have various effects on the local environment (Table 1.1). This cascade of inflammatory 'go' signals (Nathan, 2002) results in a combination of systemic effects and local morphological and biochemical changes. The release of cytokines such as TNF-α, IL-1 and IL-6 causes priming and activation of the endothelium and other leukocytes, to promote their transmigration and recruitment (Medzhitov, 2008). Vasodilation is triggered by the release of histamine, eicosanoids and tryptases, particularly from mast cells, and increased vascular permeability results in extravasation of fluid into the surrounding tissue, forming the inflammatory exudate (Nathan, 2002).

Platelet-activating factors (PAFs), produced via the acetylation of lysophosphatidic acid, are released from leukocytes and also function to promote vascular changes and increase the adhesive properties of the endothelium (Nathan, 2002; Medzhitov, 2008). Other lipid mediators, the eicosanoids, are generated from either the cyclooxygenase (COX)- or lipoxygenase (LO)-mediated metabolism of arachidonic acid (AA), to produce prostaglandins (PGs) and thromboxanes, or leukotrienes and lipoxins, respectively (Medzhitov, 2008). Prostaglandins can induce fever and vasodilation and leukotrienes promote leukocyte recruitment. Lipoxins play a role in the resolution phase of inflammation and will be discussed later. Another important class of inflammatory mediators is the chemokine family, a group of approximately 50 secreted proteins that mediate extravasation and directed migration (Moser et al., 2004). Other inflammatory mediators are indicated in Table 1.1.

The combined and co-ordinated effect of these inflammatory mediators is the recruitment of circulating leukocytes, the first of which to arrive at the site of inflammation is the neutrophil (Barton, 2008).

Inflammatory mediator	Examples	Effects
Vasoactive amines	Histamine Serotonin	Vasodilation, ↑ vascular permeability (may cause vasoconstriction in some circumstances)
Vasoactive peptides	Substance P Fibrinopeptides Kinins	Mast cell degranulation, vasodilation, ↑ vascular permeability, pro-algesic
Complement fragments	C3a, C3b C4a C5a	Mast cell degranulation, ↑ leukocyte recruitment, opsonisation of pathogens, formation of membrane attack complex
Lipid mediators	Eicosanoids: - Prostaglandins - Thromboxanes - Leukotrienes - Lipoxins - Resolvins - Protectins Platelet-activating factors	Vasodilation, hyperalgesia, ↑ fever ↓ Inflammation, ↑ resolution, ↑ tissue repair ↑ Recruitment of leukocytes, vasodilation/constriction, platelet activation
Cytokines	TNF-α IL-1 IL-6	Activate endothelium and leukocytes, induce acute-phase systemic response (↑ fever, ↑ metabolism)
Chemokines	IL-8 MCP-1 MIP-1α	Affect leukocyte extravasation and chemotaxis
Proteolytic enzymes	Elastin Cathepsin G Matrix metallo-proteases	Degrade extracellular matrix and basement membrane proteins, affect leukocyte migration

Table 1.1: Inflammatory mediators and their effects.

Following initiation, the inflammatory response is amplified by pro-inflammatory mediators, which trigger a combination of systemic effects and local morphological and biochemical changes. There are seven groups of inflammatory mediators, classified by their biochemical attributes. Adapted from (Medzhitov, 2008).

1.2 The neutrophil in inflammation

1.2.1 The neutrophil lifespan

Neutrophils have evolved as key components of the inflammatory response and are critical for the first line of host defence. They are polymorphonuclear leukocytes (PMNs), characterised by a multi-lobed nucleus connected by thin bridges of nuclear material and a plethora of cytoplasmic granules. Neutrophils are the most abundant human leukocyte and, along with other granulocytes and macrophages, are derived from common myeloid progenitors, which arise from haematopoietic stem cells (Akashi et al., 2000).

In the adult vertebrate, haematopoiesis takes place in the bone marrow, where key cytokines such as Granulocyte Colony Stimulating Factor (G-CSF), Granulocyte-Macrophage Colony Stimulating Factor (GM-CSF) and IL-3, drive the maturation and functional activation of neutrophils (Berliner, 2008; Dale et al., 2008). It is estimated that under basal conditions, between 5×10^{10} and 10×10^{10} neutrophils are produced in the bone marrow every day (Summers et al., 2010). The majority of differentiated neutrophils reside here to form a reserve population up to ten-fold higher than the circulating population (Berliner, 2008). In addition to these populations, there also exist a number of marginated neutrophil pools within the capillary beds of specific organs such as the spleen, liver and lung (Summers et al., 2010).

For homeostasis to be maintained, a tightly regulated balance must exist between granulopoiesis, the release of neutrophils from the bone marrow and their elimination from the circulation. G-CSF is a particularly important regulator of granulopoiesis and the loss of functional G-CSF receptors has been shown to cause neutropenia in both mice and humans (Lieschke et al., 1994; Druhan et al., 2005). Mature neutrophils express low levels of the chemokine receptor CXCR4, which binds to stromal-derived factor-1 (SDF-1), a chemokine that is constitutively produced in the bone marrow (Sugiyama et al., 2006). The CXCR4-SDF-1 axis mediates neutrophil retention and disruption of this interaction induces neutrophil release (Ma et al., 1999; Devine et al.,

2008). Failure of neutrophil mobilisation can result in neutropenia, as demonstrated in WHIM syndrome (warts, hypogammaglobulinaemia, infection and myelokathesis), a rare disorder in which neutrophils have increased sensitivity to SDF-1 due to a mutation in *CXCR4* (Hernandez et al., 2003). There is evidence to suggest that signalling via another chemokine receptor, CXCR2, also plays a role in neutrophil trafficking and acts in an antagonist manner to CXCR4, as neutrophils in *Cxcr2*$^{-/-}$ mice are retained in the bone marrow (Eash et al., 2010).

Despite their abundance, neutrophils possess a relatively short half-life due to their predisposition to undergo spontaneous apoptosis. Circulating neutrophils are generally accepted to have a half-life of approximately 8 hours (Dancey et al., 1976). More recent evidence obtained by *in vivo* $^{2}H_2O$ labelling of human neutrophils proposed that circulating neutrophils have a mean lifespan of 5.4 days (Pillay et al., 2010), although this has been met with some criticism (Pillay et al., 2011; Tofts et al., 2011). Studies performed in mice suggest that under physiological conditions, senescent neutrophils return to the bone marrow where they undergo apoptosis, followed by phagocytosis by stromal macrophages, which then stimulates G-CSF production (Furze et al., 2008). This homing behaviour is believed to be CXCR4-dependent, as senescent neutrophils have up-regulated expression of the chemokine receptor and increased sensitivity to SDF-1α (Martin et al., 2003). This process has not yet been fully defined, particularly in humans, and it is likely that other mechanisms may also be important in the regulation of neutrophil clearance from the circulation.

During inflammation, neutrophil release from the bone marrow is increased by pro-inflammatory mediators such as IL-8, TNF-α, complement component C5a and leukotriene B_4 (LTB_4) (Terashima et al., 1998; Summers et al., 2010). G-CSF and CXC chemokines can also mediate neutrophil release under inflammatory conditions (Semerad et al., 2002; Burdon et al., 2008), and it is likely that this involves disruption of the CXCR4-SDF-1 axis (Wengner et al., 2008). Activated neutrophils that have migrated into inflamed tissue may have a lifespan of up to 4 days (Edwards et al., 2004). This longevity results from exposure to pro-inflammatory mediators such as G-

CSF, IL-1β, TNF-α and interferon-γ (IFN-γ), which interfere with apoptotic pathways (Colotta et al., 1992), as will be discussed later.

1.2.2 Neutrophil priming and recruitment

Neutrophils must respond rapidly in the event of tissue injury or infection. Upon exposure to pro-inflammatory mediators including cytokines, chemokines and bacterial products, neutrophils become partially activated, or 'primed', and are recruited towards the inflamed area (Hallett & Lloyds, 1995). This initial activation step is required to prevent inappropriate degranulation before the neutrophils have reached their destination. It is also essential for maximising the neutrophil effector functions, such as the activation of NADPH oxidase, once they arrive (Summers et al., 2010). Priming by agents such as LPS and GM-CSF also extends neutrophil lifespan, to maximise the duration of the response and facilitate efficient elimination of pathogens (Condliffe et al., 1998).

Recruitment of circulating neutrophils to sites of inflammation or infection involves a complex process of rolling and adhesion on endothelial cells, directed by a chemotactic gradient. Once firmed adhered to the endothelium, neutrophils transmigrate into the tissues, a process known as extravasation. From here, neutrophils migrate to the required tissue site by chemotaxis.

1.2.3 Extravasation

Changes in hemodynamic flow as blood enters the post-capillary venules results in leukocytes passing through in close proximity to the vessel wall (Simon et al., 2002). In areas of inflammation, cytokines such as TNF-α and IL-1 promote the expression of an array of adhesion molecules on vascular endothelial cells (Spertini et al., 1991; Kansas, 1996). These bind their corresponding ligands, including E-, P-, and L-selectin, to facilitate a weak interaction between neutrophils and the endothelial cells, known as tethering. Transient and sequential selectin-binding also mediates neutrophil rolling along the vessel wall, which is a prerequisite for neutrophil arrest (Ley et al., 1995; Kansas, 1996). Immobilised chemokines are presented on the luminal surface of endothelial cells by interacting with glycosaminoglycans, and these also promote neutrophil tethering and activation via G-protein coupled receptor (GPCR) binding

(Grabovsky et al., 2000; Middleton et al., 2002). The reduction of neutrophil rolling speed and their firm adhesion to the endothelial surface is achieved via 'inside-out' signalling, which alters the conformation of β2-integrins including lymphocyte function antigen (LFA-1) and macrophage antigen-1 (Mac-1) on the neutrophil surface, enabling them to bind ligands such as intracellular adhesion molecule-1 (ICAM-1; also known as CD54) (Zimmerman et al., 1992; Dunne et al., 2002; Zarbock et al., 2008). Additional priming of neutrophils by TNF-α, C5a and other inflammatory mediators causes release of PAF and elastase, resulting in cleavage of CD43, an anti-adhesive sialoprotein, from the cell surface (Nathan et al., 1993). This enables neutrophil spreading and firm adhesion to the ECM through β2-integrin interactions.

Following neutrophil arrest, 'outside-in' signalling triggers neutrophil diapedesis and transmigration through the endothelium (Zarbock et al., 2008). It is believed that this emigration may occur either paracellularly, between the junction of two tightly apposed endothelial cells, or transcellularly, across one epithelial cell (Petri et al., 2013). The latter phenomenon has been investigated by electron microscopy of sections of inflamed venular epithelium from guinea pigs, in which in most cases neutrophils were observed to emigrate through trans-endothelial cell pores, rather than inter-endothelial cell gaps (Feng et al., 1998). This form of transmigration is believed to be highly dependent on the interaction between ICAM-1 and LFA-1, which may trigger the extension of neutrophil pseudopodia into the endothelial cells, at sites away from cell-cell junctions (Yang et al., 2005). Emigration is also facilitated by the redistribution and recycling of many other adhesion molecules on the surface of endothelial cells, including members of the junctional adhesion molecule (JAM) family, platelet endothelial cell adhesion molecule-1 (PECAM-1) and the vascular endothelial cadherin complex (Williams et al., 2011).

1.2.4 Neutrophil chemotaxis

Following extravasation, neutrophils continue their migration through the tissue microenvironment towards the site of inflammation by a process known as chemotaxis. They are directed by gradients of soluble chemoattractants, including complement fragments, LTB_4 and the CXC family of chemokines (Sadik et al., 2012).

More than 40 chemokines and 19 different chemokine receptors have been identified, which enables specificity between leukocyte subtypes (Kim, 2004). Selective trafficking is achieved via the differential expression of chemokine receptors and adhesion molecules on immune cells and within different tissues.

The predominant receptors that regulate neutrophil trafficking are the CXC chemokine receptors, CXCR1 and CXCR2 (Holmes et al., 1991; Murphy & Tiffany, 1991). These are GPCRs, which are activated by glutamic acid-leucine-arginine containing (ELR^+) chemokines. The receptors are highly homologous, sharing 77% amino acid identity, but have different binding affinities for IL-8 (Holmes et al., 1991; Murphy & Tiffany, 1991). CXCR1 binds IL-8 with high affinity and also binds CXCL6 (granulocyte chemotactic protein-2) (Wolf et al., 1998). CXCR2 binds both of these ligands, along with other ELR^+ chemokines including CXCL7 (neutrophil-activating protein 2) and CXCL5 (epithelial cell-derived neutrophil-activating peptide-78) (Ahuja et al., 1996). CXCR1 and CXCR2 activate a number of intracellular signalling pathways to regulate neutrophil polarisation and motility in response to chemotactic stimuli, which are described below (Stillie et al., 2009).

A complex spatial and temporal array of different chemoattractant signals may exist within the tissue environment and neutrophils must be able to respond appropriately. In some circumstances, neutrophils can migrate against a chemokine gradient in response to a different, opposing chemotactic signal (Foxman et al., 1997). It has been proposed that during inflammation, neutrophils first respond to 'intermediary' chemoattractants such as IL-8 and LTB_4, which activate phosphatidylinositol-3 kinase (PI-3K) signalling (Hirsch, 2000; Heit et al., 2002). A signalling hierarchy exists and once neutrophils encounter 'end-target' chemoattractants such as C5a and bacterial N-formyl peptides (fMLPs), these signals take precedence, leading to inactivation of PI-3K signalling and activation of p38 mitogen-activated protein kinase (MAPK) signalling (Nick et al., 1997; Heit et al., 2002). Chemoattractant receptor cross-desensitisation is also believed to regulate the responsiveness of neutrophils to competing chemotactic signals (Ali et al., 1999).

1.2.5 Intracellular pathways involved in neutrophil recruitment

The activation of chemoattractant GPCRs triggers an increase in cAMP levels and the mobilisation of intracellular calcium, which can regulate chemotaxis (Partida-Sánchez et al., 2001; Partida-Sánchez et al., 2004). This, along with the activation of multiple intracellular signalling cascades, is responsible for the cytoskeletal rearrangements and F-actin polymerisation required for directed neutrophil migration. Much of our current understanding of chemotaxis has been established from study of the simple eukaryotic organism *Dictyostelium discoideum* (Bagorda et al., 2006). It was originally believed that cells possess an internal 'compass' and once they encounter a chemokine gradient, actin polymerisation and pseudopod formation ensue to direct the cell towards the appropriate location (Bourne et al., 2002). However, it has since been proposed that during chemotaxis, pseudopods are formed independently of chemoattractant signalling, are usually generated by the bifurcation of existing pseudopods, and only those which detect the chemokine gradient are maintained in order to direct migration (Andrew et al., 2007; Insall, 2010). Neutrophil chemotaxis is highly complex, context-dependent and involves cross-talk between a number of intracellular signalling pathways in order to co-ordinate effective gradient sensing and create a functional response.

The PI-3K pathway

The PI-3K pathway plays an integral role in the amplification of chemoattractant gradients and neutrophil polarisation. Following PI-3K activation, phosphatidylinositol (3,4,5)-triphosphate (PIP_3) accumulates at the leading edge of the neutrophil and recruits various Pleckstrin Homology (PH) domain proteins, such as AKT, to the membrane, where they activate the downstream components required for migration (Stephens et al., 2002) (Figure 1.1). This process is believed to be mediated by the small GTPase Ras, as its loss or mutation is associated with defects in directional cell movement (Sasaki et al., 2004).

Studies indicate that PI-3Kγ is the principal class I PI-3K isoform involved in leukocyte migration. In PI-3K$\gamma^{-/-}$ mice, neutrophils are unable to produce PIP_3 or phosphorylate AKT upon stimulation with chemotactic agents such as IL-8, fMLP and C5a, resulting in

an impaired motility and also reduced respiratory burst (Hirsch, 2000). Additionally, in PI-3Kγ$^{-/-}$ neutrophils, the correct spatial and temporal accumulation of PIP_3, AKT and polymerised F-actin at the leading edge are impaired, leading to defects in cell movement (Hannigan et al., 2002; Ferguson et al., 2007). A class Ia PI-3K isoform, PI-3Kδ, may also facilitate directional neutrophil migration, as its specific blockade inhibits PIP_3 production and polarisation and reduces recruitment of neutrophils to the lung in a mouse model of acute pulmonary inflammation (Sadhu et al., 2003; Puri et al., 2004).

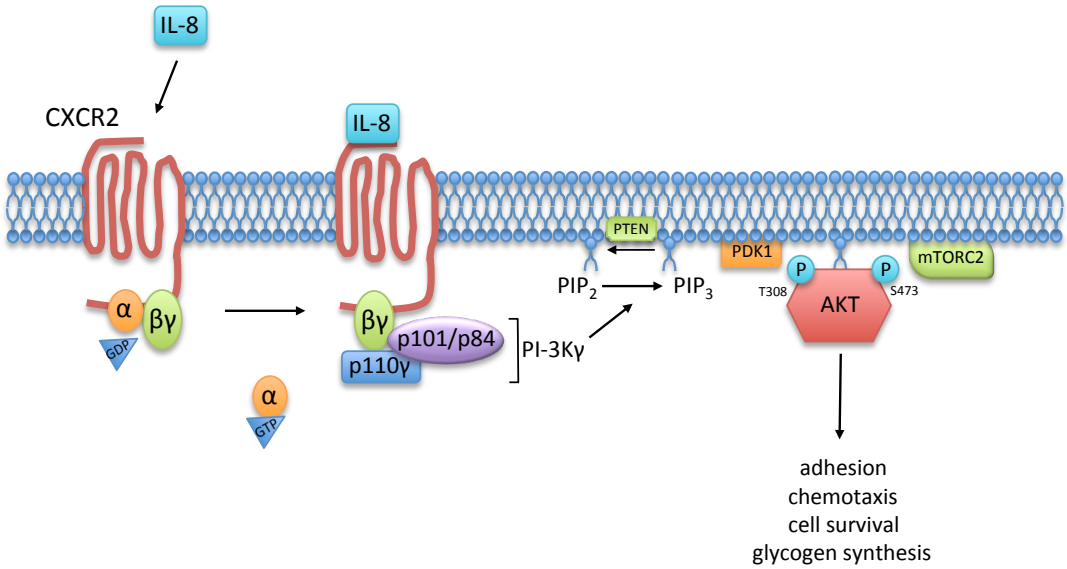

Figure 1.1: Schematic representation of PI-3Kγ signalling.

Activation of GPCRs such as CXCR2 by IL-8 binding causes activation of the heterotrimeric G-protein complex, via dissociation of GDP and binding of GTP. The Gβγ subunit recruits PI-3Kγ, the activation of which results in accumulation of PIP_3 at the plasma membrane. This causes subsequent recruitment of PH domain containing proteins such as AKT and also activates GTPases such as Ras (not shown) to promote neutrophil migration. AKT is activated by phosphorylation by mTORC2 at serine 473 and PDK1 at threonine 308, which leads to activation of its downstream targets such as NF-κB, BCL-2 family members and FOXO transcription factors. Endogenous regulators of this pathway include the phosphatase PTEN, which dephosphorylates PIP_3 back to PIP_2. Adapted from (Rommel et al., 2007; Stillie et al., 2009).

The MAPK pathway

In response to certain stimuli, neutrophil chemotaxis can occur independently of PI-3K signalling. For example, PI-3K is not required for signal transduction of the end-target chemoattractant fMLP, although it does appear to accelerate the response (Heit et al., 2008). Instead, fMLP and other end-target chemotactic signals can activate the p38 MAPK pathway to induce neutrophil polarisation and migration (Heit et al., 2002). F-actin polymerisation at the leading edge is disrupted in the presence of p38 MAPK inhibitors and in neutrophils that are deficient in MK2, a downstream signalling component of the p38 MAPK pathway (Wu et al., 2004; Heit et al., 2008). MAPK inhibition has also been shown to reduce neutrophil expression of the β2-integrin Mac-1, suggesting that this pathway may regulate multiple stages of neutrophil recruitment (Xu et al., 2013).

Other MAP kinases, c-Jun amino-terminal kinase (JNK) and extracellular signal-regulated kinase (ERK) may also be involved in neutrophil migration (Johnson et al., 2002). Interestingly, it has recently been reported that the p38 and ERK MAPKs differentially regulate neutrophil chemotaxis, with p38 mediating neutrophil 'go' signals whilst ERK may control the 'stop' signals that take precedence once the neutrophil has reached its destination (Liu et al., 2012). The role of JNK is less well defined, although pharmacological inhibition of this kinase reduces neutrophil recruitment to the lung in a murine model of acute lung inflammation and is likely to alleviate the symptoms of RA (Han et al., 2001; Arndt, Young, Lieber, et al., 2005).

1.2.6 The role of the neutrophil at the site of inflammation

Under physiological conditions, neutrophils are recruited until approximately 6 hours after initiation of the inflammatory response (Serhan and Savill, 2005). Neutrophils are highly specialised for host defence and once they arrive at the site of inflammation, they perform a myriad of functions in order to eliminate invading pathogens. These include phagocytosis, degranulation and the formation of reactive oxygen species and neutrophil extracellular traps (Dahlgren et al., 1999; Brinkmann et al., 2004; Liu & Pope, 2004).

Neutrophil phagocytosis and degranulation

The binding of microbial products to specific cell surface receptors activates neutrophils, triggering reorganisation of the actin cytoskeleton to allow endocytosis of foreign material into phagosomes (Dale et al., 2008). These include TLRs, which detect conserved PAMPs, and Fc-γ receptors, which interact with pathogens that have been targeted for destruction via opsonisation. This process involves the coating of phagocytic targets with immunoglobulin G (IgG). Pathogens can also be opsonised by C3 complement fragments and these bind to complement receptors on neutrophils, triggering a different form of phagocytosis that does not promote neutrophil effector functions, in contrast to Fc-γ-dependent phagocytosis (Caron et al., 1998). During phagocytosis, there is a rapid rise in intracellular calcium levels and this mediates fusion of the phagosome with neutrophil granules, exposing microbes to a lethal microenvironment containing proteases, defensins and other toxic products (Jaconi et al., 1990).

Various types of cytoplasmic granules exist, dependent on their contents and time of formation (Borregaard et al., 1997). Primary, or azurophil, granules stain positive for myeloperoxidase (MPO) and are formed during the early stages of granulopoiesis. These granules are highly antimicrobial and, along with MPO, contain α-defensins, elastase, cathepsin G and proteinase-3. Granules formed later in neutrophil development, after promyelocytes differentiate into myelocytes, do not contain MPO (Borregaard et al., 1997). These include secondary (specific) granules, which contain an abundance of the antibacterial glycoprotein lactoferrin, and tertiary (gelatinase) granules. These contain high levels of elastase, gelatinase and metalloproteinase enzymes, which are required for degradation of the ECM during neutrophil extravasation (Delclaux et al., 1996). Therefore, at times it is necessary for neutrophils to degranulate, releasing their toxic granule contents into the extracellular environment. Degranulation can also help to destroy pathogens at sites of inflammation, however this process must be tightly controlled in order to limit host tissue damage.

The respiratory burst

Neutrophil phagocytosis also triggers a prolonged 'respiratory burst', in which the NADPH oxidase complex catalyses the formation of hydrogen peroxide and reactive oxygen species (ROS) (Babior et al., 1976). Within the phagosome, MPO from primary granules catalyses the reaction between hydrogen peroxide and chloride to form hypochlorous acid, a potent oxidant that is responsible for microbe destruction (Klebanoff, 2005). Activation of TLRs, for example by bacterial LPS, also induces generation of ROS, along with shedding of L-selectin and down-regulation of the chemokine receptor, CXCR2 (Sabroe et al., 2005). The NADPH oxidase complex can also be recruited to the neutrophil plasma membrane, resulting in the release of ROS into the extracellular environment and rapid elimination of invading pathogens, but at the expense of host tissue damage (El-Benna et al., 2008). Mutations in the NADPH oxidase complex can cause chronic granulomatous disease (CGD), in which the respiratory burst is impaired and patients suffer from recurrent infections, due to their inability to destroy pathogens (Gallin et al., 1992).

Neutrophil extracellular traps (NETs)

Another putative neutrophil defence strategy is the formation of neutrophil extracellular traps (NETs), which can be triggered by a range of stimuli including IL-8 and LPS (Brinkmann et al., 2004). These are web-like structures composed of DNA, histones, antimicrobial peptides and proteases, which capture invading pathogens within a highly concentrated anti-microbial milieu, destroying them extracellularly. NETs can be visualised *in vitro* by confocal microscopy techniques, using fluorescent probes to label nucleic acids in stimulated neutrophils (Yousefi et al., 2009). Early studies indicate that NET production is an alternative mechanism of cell death, termed NETosis, as it involves nuclear and membrane disintegration (Fuchs et al., 2007; Steinberg et al., 2007). In neutrophils, this was evidenced by the loss of a viable cell marker, calcein blue, and the simultaneous detection of Annexin V, a marker of apoptosis, along with fluorescent antibodies against various histone components (Fuchs et al., 2007). The precise mechanisms that control NET formation are not fully understood, although they are believed to be NADPH oxidase-dependent, as NET

production is impaired in neutrophils isolated from CGD patients (Fuchs et al., 2007). More recent evidence, obtained using a fluorescent probe specific for mitochondrial DNA and that can enter living cells, suggests that NETs consisting of mitochondrial rather than nuclear DNA may also be released from viable neutrophils in a ROS-dependent manner (Yousefi et al., 2009).

1.2.7 Neutrophil survival factors

At the site of inflammation, neutrophil half-life is significantly extended to allow successful elimination of pathogens and control of the infection. There are a number of survival factors that can prolong neutrophil longevity and these include both host- and pathogen-derived signals, as well as physical conditions of the inflammatory environment. Multiple pro-inflammatory cytokines have been shown to act as potent neutrophil survival factors *in vitro*, including GM-CSF, G-CSF, IL-2 and IFN-γ (Klebanoff et al., 1992; Lee et al., 1993; Pericle et al., 1994). Macrophages are a source of neutrophil survival cytokines at the site of inflammation where, along with G-CSF and GM-CSF, they also release IL-1β and TNF-α (Kumar & Sharma, 2010). The latter plays a complex role in the regulation of neutrophil lifespan; high levels of TNF-α induce apoptosis, but at low doses, transcription of pro-survival proteins such as BFL-1 is increased (van den Berg et al., 2001; Cross et al., 2008).

On binding to their receptors, cytokines activate intracellular signalling, predominantly via the PI-3K, MAPK, NF-κB and JAK/STAT pathways, which regulate members of the BCL-2 family of proteins and the production of cytokines (Epling-Burnette et al., 2001; Luo et al., 2008). For example, GM-CSF induces phosphorylation and inactivation of the pro-apoptotic protein BAD and a reduction in *Bad* mRNA (Cowburn et al., 2002). Bacterial products such as LPS and lipoteichoic acid (LTA) can promote neutrophil survival via TLR binding and these, along with GM-CSF and IL-1β, have been shown to increase levels of the anti-apoptotic proteins Mcl-1 and Bcl-x(L) (Moulding et al., 1998; François et al., 2005; Ocaña et al., 2008). The release of MPO has also been reported to prolong neutrophil lifespan in a paracrine manner, via binding to the β2-integrin Mac-1 (El Kebir et al., 2008).

Typically, sites of inflammation are oxygen-depleted and neutrophils are required to function within hypoxic conditions (Hunt et al., 1967). Neutrophil survival is prolonged in hypoxia and this effect is mediated via the hypoxia-inducible factor (HIF) signalling pathway, which activates NF-κB (Hannah et al., 1995; Walmsley et al., 2005). Studies also indicate that hypoxic neutrophil survival is associated with up-regulation of Mcl-1, which occurs in a MAPK dependent manner (Leuenroth et al., 2000). It has been suggested that hypoxic conditions promote the activity of anti-apoptotic signals at the site of inflammation, in preference to pro-apoptotic signalling (Cross et al., 2006). Additionally, activation of hypoxia signalling has been shown to delay neutrophil apoptosis in a zebrafish model of inflammation (Elks et al., 2011).

1.3 The resolution of inflammation

Once any infection or injury has been controlled, it is critical that inflammation resolves effectively in order to restore homeostasis and enable tissue repair. There is a switch from the local production of pro-inflammatory mediators to anti-inflammatory ones, including lipoxins, resolvins and protectins, which trigger pro-resolution events and promote healing (Serhan & Savill, 2005). This is an active process that is inherent to inflammatory cells, such that as soon as inflammation is initiated, resolution is programmed to occur in a timely and controlled manner (Serhan & Savill, 2005).

For successful resolution, there are a number of necessary pre-requisites, including removal of the inciting stimulus, catabolism of pro-inflammatory mediators and the inhibition of further neutrophil recruitment. Another essential step is the clearance of extravasated neutrophils, a process that was originally believed to be primarily dependent on the apoptosis of neutrophils and their clearance by phagocytic macrophages (Savill et al., 1989). More recently, evidence from various model systems has indicated that an alternative mechanism may also be involved. Potentially viable neutrophils have been observed to leave sites of inflammation by reverse migration (Mathias et al., 2006; Elks et al., 2011; Woodfin et al., 2011).

1.3.1 Neutrophil apoptosis and macrophage clearance

Neutrophils are unique among cell types in that they can undergo spontaneous apoptosis in the absence of external signals, a feature that may be attributed to their constitutive expression of pro-apoptotic BCL-2 family proteins (Akgul et al., 2001). These proteins have a relatively long half-life in comparison to anti-apoptotic members such as Mcl-1, which is estimated to be between 2 and 3 hours (Akgul et al., 2001). As described above, neutrophil survival can be extended, for example by the modulation of apoptotic pathways by cytokines. Likewise, apoptosis can also be accelerated, by both intracellular and extracellular signals, and is regulated by survival signalling pathways such as PI-3K and MAPK (Akgul et al., 2001). The apoptosis of inflammatory neutrophils represents a key step towards successful inflammation resolution, along with their subsequent recognition and clearance by phagocytic macrophages (Savill et al., 1989). It is critical that neutrophil cell membrane integrity is preserved during apoptosis, to avoid leakage of cytotoxic granule products that have the potential to damage host tissues.

Neutrophil apoptosis is a highly regulated and complex process that can proceed via numerous signalling pathways and is mostly dependent on the activation of cysteine proteases known as caspases, which cleave a host of proteins that are important for maintaining cellular function (Cohen, 1997). Essentially, the extrinsic apoptotic pathway involves death receptor activation by extracellular ligands, which include TNF binding to TNF-related apoptosis-inducing ligand receptor (TNFR) and Fas ligand (FasL) binding to the Fas receptor (Renshaw et al., 2000; Renshaw et al., 2003). These lead to assembly of the death-inducing signalling complex (DISC) and clustering of inactive caspase-8 peptides, which leads to their processing and activation (Cohen, 1997). Alternatively, neutrophil death may be induced via the intrinsic apoptotic pathway, which involves permeabilisation of the mitochondrial outer membrane, either by pore formation or the insertion of pro-apoptotic proteins from the BCL-2 family such as BAX (Crompton, 1999). This intrinsic pathway can be induced by oxidative stress, DNA damage or the presence of cytotoxic factors, and mitochondrial disruption leads to the release of pro-apoptotic agents into the cytosol (Hallett et al., 2008). These include cytochrome *c*, apoptotic-protease activating factor-1 (APAF-1) and second

mitochondria-derived activator of caspases (SMAC), which inhibit IAP (inhibitor of apoptosis) proteins to enable formation of the apoptosome and caspase-9 activation (Hallett et al., 2008). Both the extrinsic and intrinsic apoptotic pathways lead to cleavage and activation of the executioner caspases, such as caspase-3, which cleave key functional proteins (Cohen, 1997). There is evidence that cross-talk exists between the intrinsic and apoptotic pathways, as the activation of caspase-8 via the extrinsic pathway can cause cleavage of BID, resulting in its translocation to the mitochondria and disruption of mitochondrial membrane potential (Li et al., 1998).

Apoptotic neutrophils express 'find me' and 'eat me' signals such as sphingosine-1-phosphate and phosphatidylserine residues, in order to facilitate their detection and engulfment by phagocytic macrophages, a process known as efferocytosis (Bratton et al., 2011). If correctly regulated, efferocytosis should occur in a non-phlogistic manner, inhibiting macrophage production of pro-inflammatory mediators such as thromboxanes, LTs, IL-1β and GM-CSF, and triggering the production of anti-inflammatory ones, such as transforming growth factor-β1 (TGF-β1), IL-10 and prostaglandin E_2 (PGE_2) (Fadok et al., 1998). Macrophages can also function to promote further neutrophil apoptosis at sites of inflammation. Upon ingestion of apoptotic neutrophils, macrophages release FasL, initiating a positive feedback loop that triggers Fas-mediated neutrophil apoptosis (Brown et al., 1999). They can also induce apoptosis of neighbouring neutrophils via a cell-cell contact-dependent mechanism that involves integrin-ligand binding (Meszaros et al., 2000). Efferocytosis triggers the emigration of macrophages away from the site of inflammation via the lymphatic system in order to clear the site of inflammation and promote resolution (Bellingan et al., 1996). Apoptotic neutrophils may also be removed in exudates, for example by egression into the lumen of the lung airways (Uller et al. , 2006).

1.3.2 Neutrophil reverse migration

Neutrophil clearance is not limited to macrophage uptake, but may also be accomplished by the movement of neutrophils away from sites of inflammation; a process defined as reverse migration. This has been observed *in vivo* in a mouse model of ischemia-reperfusion injury, in which fluorescently-labelled neutrophils were

reported to undergo reverse transendothelial migration and this process was augmented following genetic or pharmacological inhibition of JAM-C (Woodfin et al., 2011). In this investigation, the reverse migrated neutrophils displayed elevated cell surface expression of ICAM-1. The up-regulation of this adhesion molecule was consistent with *in vitro* studies, in which cultured human neutrophils were observed to reverse migrate back across an endothelial monolayer, and these neutrophils displayed a unique cell-surface receptor phenotype (Buckley et al., 2006). This consisted of elevated expression of ICAM-1 and the cluster of differentiation molecule 11b (CD11b) and reduced expression of the key neutrophil chemokine receptors CXCR1 and CXCR2, in comparison to neutrophils that did not undergo reverse migration. It may be that this altered expression of cell-surface markers is important for mediating neutrophil reverse migration, but currently the precise mechanisms involved in the regulation of this process remain undefined.

The lack of knowledge in this area may be partly due to the technical difficulties associated with the investigation of this process in mammalian models. Over the last few years, the zebrafish model has provided some much needed insight into neutrophil reverse migration and this activity has been observed by multiple research groups (Mathias et al., 2006; Hall et al., 2007; Elks et al., 2011; Yoo and Huttenlocher, 2011). Interestingly, it has been shown that hypoxia can regulate reverse migration in zebrafish, as genetic or pharmacological activation of Hypoxia-inducible factor-1α (Hif-1α) can delay the resolution of inflammation, predominantly by causing increased neutrophil retention at a wound (Elks et al., 2011).

The significance of neutrophil reverse migration *in vivo* is not yet fully understood. A small percentage of peripheral blood neutrophils have been reported to express the reverse migrated receptor phenotype and this proportion is increased to 1-2% of total circulating neutrophils in patients suffering with RA or atherosclerotic disease (Buckley et al., 2006). It is likely that reverse migration functions as an alternative mechanism of neutrophil removal from sites of inflammation, promoting their clearance by organs such as the liver and spleen. However, some reports suggest that this primed and activated subset of neutrophils might contribute to the dissemination of inflammation,

which may have pathophysiological consequences (Buckley et al., 2006; Woodfin et al., 2011). The function of reverse migrated neutrophils is currently under debate, with evidence also indicating that neutrophils might influence the adaptive immune response (Yamashiro et al., 2001). Further work is required in order to establish the functional relevance of neutrophil reverse migration.

1.3.3 Pro-resolution signalling and tissue repair

A number of mediators have been identified that promote inflammation resolution, shifting the balance from pro- to anti-inflammatory signalling. Some mediators, such as TNF-α, TGF-β1 and PGE_2, have differential and temporal-dependent effects throughout the initiation and resolution phases of inflammation, illustrating the importance of controlled regulation of the inflammatory response and mediator production (Nathan, 2002). The mechanisms governing inflammation resolution and tissue repair must be tightly controlled in order to avoid failure of resolution or inadequate repair.

Over the course of the inflammatory response, there is a co-ordinated switch in lipid mediator production, which promotes inflammation resolution. Production of the pro-inflammatory eicosanoids, leukotrienes (LTs) and prostaglandins (PGs), is reduced in a self-limiting manner, in favour of the generation of pro-resolution lipids including lipoxins, resolvins and protectins (Serhan & Savill, 2005). LTs are formed by the oxygenation of AA by 5-lipoxygenase (5-LO), but this switches to 15-LO-dependent oxygenation and the biosynthesis of lipoxins, a process that is mediated by PGE_2 (Levy et al., 2001). One of the main ways in which lipoxins promote inflammation resolution is by reducing neutrophil recruitment to sites of inflammation by inhibiting their chemotaxis and adhesion (Serhan et al., 1995). They also attenuate cytokine production, superoxide anion generation and elastase secretion, and have both vasodilatory and anti-fibrotic properties (Hachicha et al., 1999; Levy et al., 2002; Maderna & Godson, 2009). Moreover, lipoxins A_4 and B_4 stimulate the recruitment of monocytes to inflammatory sites, where they mature into macrophages and promote wound healing (Maddox et al., 1996). These lipoxins have also been shown to enhance the non-phlogistic phagocytosis of apoptotic neutrophils by macrophages *in vivo* (Godson et al., 2000; Mitchell et al., 2002). It has been proposed that lipoxins may be

released from neutrophils in NETs (Papayannopoulos et al., 2009). Resolvins and protectins are synthesised from eicosapentaenoic and docosahexaenoic acid precursors, respectively, are released from macrophages following uptake of apoptotic material, and function similarly to lipoxins (Schwab et al., 2007; Serhan, 2007).

As mentioned previously, the ingestion of apoptotic neutrophils by macrophages promotes a switch in signalling and the release of a number of pro-resolution mediators, one of the most important of which is TGF-β1, a cytokine that aids inflammation resolution and tissue repair (Fadok et al., 1998; Huynh et al., 2002). TGF-β1 expression is enhanced by lipoxin A_4 (Serhan & Savill, 2005). Macrophage phagocytosis of apoptotic cells also causes release of vascular endothelial growth factor (VEGF), which is required for the repair and proliferation of endothelial cells (Golpon et al., 2004). The clearance of cells and debris by macrophages must of course be tightly regulated to avoid excessive removal that might incorporate cells that are required for the healing process. It is thought that the phagocytosis of apoptotic neutrophils might act as a negative feedback mechanism to limit this function (Duffield et al., 2001). Another factor secreted by macrophages to promote healing is secretory leukocyte protease inhibitor (SLPI), which limits the activity of elastase and other proteases and maintains TGF-β1 activity (Ashcroft et al., 2000). Overall, the macrophage response is fundamental to efficient wound healing and the formation of scar tissue. In macrophage-ablated mouse models, the depletion of TGF-β1 and other mediators contributes to reduced collagen deposition, limited neovascularisation and poor wound healing (Mirza et al., 2009; Lucas et al., 2010).

1.4 Inflammatory disease

Neutrophil clearance is a critical prerequisite for successful resolution of inflammation and evasion of host tissue damage. If the inflammatory response fails to resolve, persistent neutrophilic inflammation may cause significant host tissue damage and chronic inflammation, which contributes to the pathogenesis of many inflammatory

diseases. Many of these are associated with dysregulation of the mechanisms governing inflammation resolution and respond poorly to conventional treatments. Uncovering new mechanisms by which inflammation can be resolved is key for the development of novel, more effective therapies. Ideally these must have the potential to override aberrant neutrophil survival signalling and counteract the damage caused by persistent neutrophilic inflammation.

1.4.1 Neutrophil mediated host tissue damage

The neutrophil has a powerful destructive capacity that is essential for the innate immune response to infection. However, the combination of potent oxidative and enzymatic killing mechanisms are not specific to invading pathogens and can cause extensive damage to host cells if not precisely regulated. In the early stages of the acute inflammatory response, the array of granule proteases, oxidants and other toxic metabolites produced by neutrophils are necessary to ensure infection is contained. Mice lacking essential proteases such as elastase and cathepsin G are unable to prevent the spread of bacteria, rendering them susceptible to prolonged infections (Belaaouaj et al., 1998; Tkalcevic et al., 2000).

The phagocytosis of foreign microbes attempts to restrict cytotoxic enzymes to the phagosome. However, sometimes it is necessary for neutrophils to degranulate, releasing potent ROS and enzymes that can potentially damage the surrounding ECM and other tissue resident cells. Host protease inhibitors function to keep small amounts of leakage in check, but the release of ROS can inactivate these inhibitors, allowing elastase and other proteases to degrade the ECM (Babior, 1984; Weiss, 1989). If injurious degranulation continues unchecked it can wreak havoc on host tissues, preventing successful inflammation resolution and contributing to the pathogenesis of chronic inflammatory disease.

1.4.2 Neutrophil dominated inflammatory diseases

Unresolved neutrophilic inflammation and the associated tissue damage is a hallmark of many inflammatory diseases, affecting multiple organ systems. These are often triggered by dysregulation of inflammation resolution and tissue repair, which contributes to disease progression. Abnormally high neutrophil numbers are

characteristic of neutrophilic inflammatory diseases such as COPD, inflammatory bowel disease (IBD) and RA, and the prolonged presence of activated neutrophils can amplify the immune response (Hallett et al., 2008). Enhanced levels of neutrophils and cytokines such as IL-1β, IL-6 and TNF-α have been recorded in sputum from COPD patients and up-regulated expression of chemokines including IL-8 and LTB_4 is thought to account for increased neutrophil recruitment (Chung, 2005). Similarly, this has been reported in acute respiratory distress syndrome (ARDS), in which enhanced levels of IL-8 augments neutrophil accumulation and activation (Donnelly et al., 1992). Neutrophilic inflammation is also directly implicated in the progression of other inflammatory lung diseases, including idiopathic pulmonary fibrosis (IPF), in which neutrophil numbers in the lung correlate with mortality (Kinder et al., 2008). High neutrophil numbers are also observed at the cartilage-pannus junction of affected joints in RA sufferers (Bromley et al., 1984). Alternatively, chronic inflammatory disease may arise when neutrophil function is impaired, such as in CGD, as mentioned previously. In CGD patients, neutrophils are unable to destroy phagocytosed pathogens due to mutations in the NADPH oxidase complex, which results in a failure to generate ROS (Gallin et al., 1992).

Neutrophil proteases are considered one of the main culprits of neutrophil mediated tissue damage and enhanced protease activity is associated with chronic inflammatory lung diseases including COPD and ARDS (Selby et al., 1991; Donnelly et al., 1992). Neutrophil proteases are responsible for the destruction of elastic lung tissue that can contribute to the development of emphysema (Stone et al., 1990) and can accelerate the progression of RA by cartilage degradation (Edwards et al., 1997).

Persistent neutrophilia is often associated with chronic inflammatory disease and the failure of successful inflammation resolution and neutrophil clearance may be a result of aberrant neutrophil survival signalling at inflammatory sites and the disruption of constitutive apoptotic pathways. Extended neutrophil survival has been reported in disease states, for example, little neutrophil apoptosis is observed in bronchoalveolar lavage fluid extracted from ARDS patients compared to healthy volunteers (Matute-Bello et al., 1997). Additionally, in acute pancreatitis, there appears to be a delay in

neutrophil apoptosis that is mediated by altered caspase expression (O'Neill et al., 2000). It is not only the apoptosis of neutrophils that is important for inflammation resolution but also the removal of cell corpses. If apoptotic neutrophils are not effectively cleared from sites of inflammation, they may ultimately undergo secondary necrosis, losing their membrane integrity and releasing their destructive contents (Majno et al., 1995). Thus, the impaired clearance of apoptotic neutrophils by macrophages may also contribute to tissue damage and chronic inflammation and the dysregulation of this process has been associated with multiple auto-immune inflammatory diseases, including COPD, CGD and cystic fibrosis (Bratton et al., 2011).

Chronic inflammatory diseases remain a significant, worldwide problem and there is a major unmet need to develop novel, more effective therapies. Although the acute inflammatory response is required in order to promote tissue repair, this must be controlled in order to limit host tissue destruction. A lack of neutrophil apoptosis and clearance prevents inflammation resolution, even in circumstances when the original inflammatory stimulus or infection has been eliminated. Targeting neutrophil apoptosis and removal pathways to pharmacologically drive inflammation resolution is required and is currently a key research aim.

1.4.3 Existing inflammatory disease therapies

Most currently available therapeutics antagonise pro-inflammatory signalling pathways and limit the recruitment and accumulation of neutrophils at sites of inflammation. Although these may alleviate symptoms, they do not specifically target infiltrating neutrophils, often causing widespread adverse side effects that can severely impair the immune system. Nor do they promote long-lasting inflammation resolution to cure the underlying condition. There is considerable evidence that pharmacological inhibition of the components of key neutrophil survival pathways, such as PI-3K, MAPK and NF-κB, might promote inflammation resolution in animal models of inflammation by inducing neutrophil apoptosis (Hallett et al., 2008). However, for these approaches to be successful therapeutically, the clearance of apoptotic neutrophils by macrophages must be able to proceed unhindered. Other research focuses on targeting mediators of the resolution phase of inflammation, to

agonise rather than antagonise endogenous signalling pathways, which might prove more effective and limit potential side effects. A selection of current and potential inflammatory disease therapies are described here.

Glucocorticoids

Most patients suffering from inflammatory diseases such as RA, asthma, IBD and COPD are treated with glucocorticoids, however some patients are unresponsive and these drugs can cause numerous side effects, likely due to the extensive effects of glucocorticoids on gene expression (Stewart, 2009). Glucocorticoids bind specifically to intracellular type II glucocorticoid receptors, expressed in neutrophils and other cell types, in which they can regulate a plethora of biochemical signalling events (Goulding et al., 1998). They suppress neutrophilic inflammation predominantly by limiting neutrophil recruitment, for example by inhibiting the release of pro-inflammatory mediators such as IL-6 and IL-8, and down-regulating the expression of adhesion molecules including L-selectin and β2-integrins (Wertheim et al., 1993; Mianji et al., 1996; Filep et al., 1997).

Glucocorticoids may also potentiate inflammation resolution, by enhancing the phagocytosis of apoptotic neutrophils by macrophages (Liu et al., 1999). It has been proposed that this effect may be mediated by the extracellular protein Annexin-A1 (Anx-A1), which is released from both neutrophils and macrophages upon stimulation with glucocorticoids (Goulding et al., 1990; Maderna et al., 2005). Anx-A1 is thought to enhance efferocytosis by binding to the lipoxin receptor, ALX, thus there is a potential link between this peptide and the lipid mediators of inflammation resolution (Scannell et al., 2007). Glucocorticoids enhance protein expression and binding capacity of Anx-A1, which alongside its effects on phagocytosis, also inhibits proteolytic enzymes such as elastase and cathepsin G and suppresses neutrophil transmigration (Mancuso et al., 1995; Perretti et al., 1996).

Anx-A1 binding capacity of neutrophils is reduced by 90% in RA patients compared to healthy controls, indicating that it may play a role in inflammatory disease progression

and might be a potential therapeutic target (Goulding et al., 1992). The active N-terminal peptide of Anx-A1, Ac2-26, has been shown to promote inflammation resolution *in vivo*, in a model of LPS-induced pleurisy (Vago et al., 2012). This effect appeared to be a consequence of decreased neutrophil survival and indeed, Anx-A1 has been shown to induce neutrophil apoptosis (Solito et al., 2003; Scannell et al., 2007).

The effect of Anx-A1 on neutrophil apoptosis is perhaps surprising given that glucocorticoids are believed to directly promote neutrophil survival, but accelerate apoptosis in other cell types, such as eosinophils (Cox, 1995; Kato et al., 1995; Liles et al., 1995). It has been suggested that glucocorticoids such as dexamethasone can increase the stability of Mcl-1 in neutrophils but not in eosinophils (Sivertson et al., 2007). These opposing effects may partially explain why glucocorticoids have proven more beneficial in treating asthma, a predominantly eosinophilic disease, compared to COPD, a largely neutrophil mediated disease, the progression of which is not reduced with inhaled glucocorticoids (Barnes, 2000). Glucocorticoids can act via a histone deacetylase 2 (HDAC2) dependent mechanism to 'switch off' pro-inflammatory genes, but evidence suggests that HDAC2 expression and activity is down-regulated in COPD lungs (Ito et al., 2005). This is due to some extent by the increased level of nitrative stress in the disease state, which causes increased ubiquitination and degradation of HDAC2, reducing the ability of the glucocorticoid receptor to down-regulate the pro-inflammatory transcription factor, NF-κB (Ito et al., 2006). This may account for the glucocorticoid resistance of COPD patients and novel therapies designed to augment HDAC2 activity could potentially improve glucocorticoid sensitivity.

PI-3K inhibitors

As PI-3K signalling can regulate both neutrophil recruitment during the initiation of inflammation and neutrophil survival, which can influence inflammation resolution, the therapeutic blockade of PI-3Ks has been explored. In mouse models of RA, both the PI-3Kγ inhibitor AS605240 and genetic knockdown of PI-3Kγ attenuate neutrophil chemotaxis and reduce joint inflammation (M Camps et al., 2005; Hayer et al., 2009).

Similar effects are observed by inhibition of PI-3Kδ (Randis et al., 2008). PI-3K blockade may also be a promising therapeutic candidate in COPD, as a dual-specific inhibitor of PI-3Kδ and PI-3Kγ, TG100-115, has been shown to suppress neutrophil accumulation and pulmonary inflammation in both cigarette smoke and LPS-induced murine models of COPD (Doukas et al., 2009).

Chemokine receptor inhibitors

Chemokines and their receptors represent another potential target for inflammatory disease therapy. High levels of IL-8, for example, have been reported in patients suffering from COPD, RA and ARDS, and this is thought to contribute to increased neutrophil recruitment and disease progression (Donnelly et al., 1992; Beaulieu et al., 1994; Chung, 2005). Some non-steroidal anti-inflammatory drugs (NSAIDs) and corticosteroids have been shown to reduce the production of IL-8 and other chemokines such as CCL2 in both animal models of arthritis and human patients (Loetscher et al., 1994; López-Armada et al., 2002), however alternative strategies have been developed in order to specifically target chemokines and their receptors. In animal models of arthritis, antibodies against IL-8, CXCL1, CXCL5 and other chemokines seem to limit disease severity, however they are only effective when administered before the onset of disease, or in its early stages of progression (Akahoshi et al., 1994; Kasama et al., 1995; Halloran et al., 1999). Inhibition of chemokine receptors such as CXCR1 and CXCR2 has also provided some therapeutic benefit in arthritis models (Podolin et al., 2002; Barsante et al., 2008).

Despite some encouraging results from *in vivo* testing, neither chemokine receptor inhibitors nor chemokine blockade using neutralising antibodies have been a huge success in human clinical trials (Haringman et al., 2006; Lebre et al., 2011; Fleishaker et al., 2012). Perhaps to design more effective chemokine-based therapies, a more complete knowledge of chemokine receptor signalling throughout the entire course of the inflammatory response and in disease states is required.

Cyclin-dependent kinase inhibitors

Cyclin-dependent kinases (CDKs) are members of the serine/threonine protein kinase family. They were originally discovered as essential regulators of the cell cycle; hence it is somewhat surprising that CDK inhibitors have any effect in the terminally differentiated neutrophil. It is now widely accepted that, via their phosphorylating activity, CDKs play critical roles in a number of physiological processes, including cell differentiation and apoptosis (Leitch et al., 2009).

Caspase-dependent neutrophil apoptosis is enhanced *in vitro* by incubation with roscovitine, a CDK inhibitor, which importantly can override the effects of powerful survival factors such as GM-CSF, TNF-α and LPS (Rossi et al., 2006; Leitch et al., 2010). Roscovitine significantly accelerates the resolution of inflammation in murine models of neutrophil dominant inflammation, including bleomycin-induced lung injury and passively induced arthritis, by increasing granulocyte apoptosis and decreasing the release of pro-inflammatory cytokines such as IL-6 and IFN-γ (Rossi et al., 2006). It also effectively accelerates resolution in a zebrafish model of neutrophilic inflammation (Loynes et al., 2009). The pro-apoptotic effect of roscovitine is mediated by down-regulation of the neutrophil survival protein Mcl-1 at the mRNA level and this occurs independently of an effect on NF-κB or ERK survival signalling (Leitch et al., 2010). It seems that this may be explained by the inhibition of CDK7 and CDK9, which results in dephosphorylation of the RNA polymerase II enzyme and disruption of the pre-initiation complex that is required for transcription (Leitch et al., 2012). This causes down-regulation of Mcl-1 and other genes that may be important regulators of neutrophil survival. Additionally, it has been proposed that CDK regulation and the effects on transcription may be responsible for the transition from a pro-inflammatory to anti-inflammatory phenotype in neutrophils during the inflammatory response, but further investigation is required (Leitch et al., 2009).

Interestingly, an alternative mechanism of action for CDK inhibitors has also been identified. Roscovitine and the CDK9 inhibitor flavopiridol have been shown to inhibit leukocyte extravasation by the down-regulation of endothelial cell adhesion

molecules, suggesting that they may also act in an anti-inflammatory manner to suppress neutrophil accumulation (Berberich et al., 2011; Schmerwitz et al., 2011).

CDK inhibitors, including roscovitine, induce apoptosis in actively proliferating cancer cell lines by disruption of the cell cycle (McClue et al., 2002), and some have reached clinical trial stage as anti-cancer therapies with reasonably manageable side-effects (Cicenas et al., 2011). Roscovitine induces apoptosis in various cancer cell lines and this effect is also mediated by disruption of transcription and down-regulation of Mcl-1 (MacCallum et al., 2005; Bettayeb et al., 2010; Mitchell et al., 2010). For the successful development of CDK inhibitors as anti-inflammatory agents, their specificity and activity must be well characterised in order to avoid off-target effects.

Lipid mediators

Much current research focuses on targeting endogenous pro-resolution signalling pathways, particularly those regulated by lipid mediators such as lipoxins, resolvins and protectins. The temporal switch in lipid mediator production over the course of the inflammatory response is integral to driving active inflammation resolution, thus promoting the function of these mediators pharmacologically may provide therapeutic benefit in persistent inflammation. Indeed, a failure to synthesise lipoxins has been associated with chronic inflammation and low levels of lipoxins have been reported in the airways of cystic fibrosis and asthma patients, and in PMNs from RA patients (Thomas et al., 1995; Levy et al., 2002; Karp et al., 2004). Interestingly, it is believed that the NSAID aspirin exerts its therapeutic effects not only by inhibition of prostaglandin and thromboxane synthesis, but also by stimulating the production of a unique class of lipoxins, termed the aspirin-triggered lipoxins (ATLs) (Clària et al., 1995). These also bind ALX receptors, along with the peptide Anx-A1, illustrating the potential of currently available anti-inflammatory drugs to activate pro-resolution signalling pathways (Perretti et al., 2002).

Under normal conditions, lipoxins are rapidly metabolised and inactivated, predominantly through dehydrogenation at carbon-15, which prompted the design of

more stable, synthetic lipoxin analogues for therapeutic use (Serhan et al., 1995). Analogues of LXs and ATLs have been shown to reduce airway inflammation in murine models of asthma (Levy et al., 2002; Levy et al., 2007) and inhibit neutrophil accumulation in murine models of dermal inflammation (Bannenberg et al., 2004; Guilford et al., 2004) and Crohn's disease (Fiorucci et al., 2004). Other pro-resolving lipid mediators, the resolvins and protectins, may also have the potential for therapeutic use. For example, resolvins are reported to improve symptoms in mouse models of colitis, peritonitis and dermal inflammation (Arita, Bianchini, et al., 2005; Arita, Yoshida, et al., 2005).

There is a need to develop novel and more effective therapies for inflammatory diseases. For this to be achieved, it is evident that understanding the molecular mechanisms regulating inflammation resolution and a thorough knowledge of the mediators involved is key. The identification of compounds that can stimulate endogenous pro-resolution signalling pathways and override neutrophil survival signals may be key for driving the resolution of persistent inflammation.

1.5 The zebrafish

The zebrafish, *Danio rerio*, belongs to the teleostei class of ray-finned fishes and over the last few decades has emerged as a powerful and versatile vertebrate model, bridging the gap between *Drosophila melanogaster* (*D. melanogaster*) and murine systems. There are a growing number of publications describing the application of zebrafish for biomedical and genetic research, reflecting the value of this emerging whole-animal model system. It is a useful organism not only for modelling human disease, but also for the application of high-throughput compound screening.

1.5.1 The zebrafish as a model organism

From a practical perspective, the zebrafish is relatively cheap and easy to maintain. This, combined with its high fecundity and generation time of only 3 months, makes it a preferable model to other vertebrate organisms such as the mouse. Due to a

genomic duplication event, zebrafish have a particularly high number of genes, however many of these have been shown to function similarly to their human counterparts. In addition, the zebrafish genome has higher homology to the human genome than non-vertebrate organisms such as *Caenorhabditis elegans* (*C. elegans*) and *D. melanogaster*. The ability to study complex molecular and cellular events and importantly the onset and progression of pathological processes *in vivo* also favours the use of zebrafish over *in vitro* cell culture systems.

Owing to the external fertilisation and rapid development of small, optically transparent embryos that permit the *in vivo* visualisation of cellular processes, the zebrafish has provided a useful and informative tool, particularly for developmental studies. It is relatively straightforward to label specific cell populations or proteins in transparent zebrafish larvae by the generation of fluorescent reporter lines, which can be established simply by injecting DNA constructs into zebrafish embryos immediately after fertilisation. Transgenesis, mutagenesis and cloning techniques have revealed the genetic tractability of the zebrafish, leading to its successful use in large-scale mutagenic screens. These forward genetics approaches have led to the identification of multiple orthologous human disease genes within the zebrafish genome and it is now widely accepted that the zebrafish is an ideal candidate for the development of translational models of human disease (Lieschke & Currie, 2007; Renshaw & Trede, 2012; Santoriello & Zon, 2012). It is now used extensively to study neurodegenerative disorders (Xi et al., 2011), muscular dystrophies (Xi et al., 2011) cancers (Teittinen et al., 2012) and infectious diseases (Novoa et al., 2012).

The *Danio rerio* Sequencing Project has assisted the identification of mutations causing disease and advances in reverse genetics techniques such as transient gene silencing and overexpression, TILLING (targeting local lesions in genomes) and targeted gene expression using the yeast GAL4-UAS marker system, have enabled the development of zebrafish disease models by a candidate gene-driven approach. Genetic techniques are becoming increasingly more advanced and zebrafish mutants are now being generated by targeted mutagenesis using zinc finger nucleases (ZFNs) and

transcription activator-like effector nucleases (TALENs), which enable knockdown of specific genes (Ekker, 2008; Huang et al., 2011).

1.5.2 Using zebrafish for high-throughput compound screening

The discovery of new cures for human disease is an on-going endeavour for scientific research. Following the initial identification of a lead compound, it may take up to 15 years of testing and development until it is safe and suitable to be marketed as a drug for human patients (Bowman et al., 2010). It is estimated that under 1% of new drugs successfully pass through all the stages of research and development. New methods to screen large numbers of compounds rapidly and robustly are required in order to improve the development process and reduce long-term costs.

Over the last few years, the zebrafish has risen to the forefront of small molecule drug discovery and numerous large scale, high-throughput compound screens are currently underway. Many of these incorporate the use of zebrafish models of human disease and focus on discovering novel pharmacological agents with therapeutic properties. The zebrafish has unique advantages for drug discovery, particularly over *in vitro* screening assays, as the use of a whole-organism system allows recognition of toxicity and embryonic lethality at an early stage of drug development. As many drugs fail due to problems associated with their absorption, distribution, metabolism, excretion and toxicity (ADMET) properties *in vivo*, identifying these issues in the preliminary stages would greatly improve the development process (Bowman et al., 2010). These properties and any potential problems can be directly addressed in zebrafish, prior to more costly and time-consuming testing in mammalian systems. Other advantages of the zebrafish that make them particularly valuable for high-throughput drug screening include their small size, which enables arraying of numerous larvae into multi-well plates, and their optical transparency, which facilitates phenotype-based screening approaches and allows changes induced by compound treatment to be readily visualised. By using this system there is the potential to identify previously undiscovered signalling pathways that are associated with human disease processes, which can be targeted for therapeutic benefit.

Small-molecule screening, also known as 'chemical genetics', in zebrafish has already effectively identified novel compounds that can suppress human disease phenotypes. For example, the zebrafish *gridlock* mutant displays a comparable phenotype to that seen in coarctation of the aorta, a human congenital disorder, and a screen of 5000 small molecules identified 2 lead compounds that overcame this aortic defect (Peterson et al., 2004). More recently, zebrafish have been used to screen for compounds that can protect against tuberculosis (Takaki et al., 2012), leukaemia (Ridges et al., 2012) and hearing loss (Owens et al., 2008). A particular success story that reflects the value of the zebrafish model and its translation to human disease is a zebrafish screen that identified dimethyl-PGE_2 ($dmPGE_2$) as a promoter of haematopoietic stem cell (HSC) expansion (North et al., 2007). Therapeutic use of $dmPGE_2$ to encourage HSC expansion following transplantation or bone marrow depletion has now reached the clinical trial stage (Goessling et al., 2011). Another example is the identification of a novel mechanism of action for the RA drug, leflunomide, which has been shown to inhibit melanoma growth in zebrafish (White et al., 2011). The application of high-throughput zebrafish screening is not limited to the direct search for potential therapeutics, but has also proved effective for the identification of novel compounds that affect behaviour (Rihel et al., 2011), and development (Hao et al., 2010).

1.5.3 The zebrafish immune system

The unique advantages of the zebrafish make it a particularly useful model organism for the study of inflammation, infection and immunity. The function of innate immune cells including neutrophils and macrophages can be readily studied in zebrafish and it has also been possible to investigate the link between inflammation and cancer initiation (Feng et al., 2012). Models of inflammatory diseases such as colitis and WHIM syndrome are emerging (Walters et al., 2010; Oehlers et al., 2011). There has also been successful development of techniques to initiate both bacterial and viral infections in zebrafish in order to study the effects of pathogens on the immune response (LaPatra et al., 2000; Davis et al., 2002; Neely et al., 2002; Prajsnar et al., 2008). This has enabled the development of models of infectious disease, for example with *Staphylococcus aureus* (Li & Hu, 2012; Prajsnar et al., 2012) and *Mycobacterium*

marinum, which causes the formation of granulomas characteristic of tuberculosis (Davis et al., 2002). Models such as these are currently under development for the identification of novel therapeutics by drug screening approaches (Takaki et al., 2012; Lore Lambein and Stephen Renshaw, personal communication).

Despite 450 million years of divergence between zebrafish and humans, the immune system remains remarkably highly conserved (Kumar & Hedges, 1998). Zebrafish possess both adaptive immunity and innate components, including phagocytic macrophages and neutrophils, TLRs, complement factors and homologues to mammalian cytokines such as *tnfa* and *il1b* (Lieschke & Currie, 2007). Although *C. elegans* and *D. melanogaster* have been used effectively to model vertebrate disease and key physiological events such as apoptosis, they are less useful for studying immunity. For instance, *C.elegans* lacks professional phagocytes such as macrophages, *D. melanogaster* do not possess granulocytes, and adaptive immune components are absent in both models (Eisenhut et al., 2005; Stramer et al., 2005; Lieschke & Currie, 2007).

Zebrafish possess two types of granulocyte, one being analogous in structure and function to the human neutrophil and another comparable to the human eosinophil, but which also exhibits basophil-like characteristics. Granulopoiesis occurs primarily in the kidney, a site considered equivalent to haematopoietic human bone marrow (Lieschke, 2001; Crowhurst et al., 2002). Neutrophil granulocytes, sometimes referred to as 'heterophils' in other teleosts, have been observed by 48 hours post fertilisation (hpf) in both the circulation and in tissue, whereas eosinophil granulocytes are not evident until after 5 days post fertilisation (dpf) (Bennett et al., 2001; Lieschke, 2001).

Zebrafish neutrophils are morphologically similar to their human counterparts, containing segmented nuclei and characteristic cytoplasmic granules. A myeloperoxidase homologue, myeloid-specific peroxidase (*mpx*), is specifically expressed in zebrafish neutrophils and its expression is apparent in the posterior inner cell mass (ICM) at 18 hpf. From 24 hpf, cells expressing *mpx* are detected within the vasculature and shortly afterwards, they are visible throughout the whole embryo

(Bennett et al., 2001; Lieschke, 2001). These *mpx*-expressing cells have functional capacity analogous to human neutrophils as early as 2 dpf, as indicated by migration of neutrophil granulocytes towards a site of inflammation induced by injury to the zebrafish caudal fin (Lieschke, 2001).

Functional and motile macrophages are observed in zebrafish from the 13-somite stage (15.5 hpf), and they migrate through the circulation to become scattered throughout the embryo by 20 hpf (Herbomel et al., 1999). Their role in zebrafish is highly comparable to their human function, for example they phagocytose bacteria in infected zebrafish (Herbomel et al., 1999; Davis et al., 2002; Prajsnar et al., 2008), and have been implicated in the uptake of apoptotic neutrophils during inflammation (Loynes et al., 2009).

Markers for the adaptive immune system are not detectable until 4 dpf, when expression of the recombination-activating genes *rag1* and *rag2* is evident in the thymus (Willett, Cherry, et al., 1997; Willett, Zapata, et al., 1997). These genes are responsible for the rearrangement of immunoglobulin and T-cell receptor genes in immature B and T lymphocytes, respectively. *In situ* hybridisation techniques have revealed the expression of other adaptive immune genes, such as T cell receptor alpha, at 4 dpf (Danilova et al., 2004), however it is accepted that the adaptive immune system is not fully differentiated and functional until 4-6 weeks post fertilisation (Lam et al., 2002). The advantage of this is that it allows the examination and manipulation of innate immune system components, without interference from adaptive immune modulators.

1.5.4 The zebrafish as a model of inflammation and inflammatory disease

The striking homology between components of the zebrafish immune system and their human counterparts permits in-depth investigation into factors influencing the immune response. Through knowledge of multiple inflammatory cell markers, it is possible to generate transgenic zebrafish lines that allow straightforward examination and manipulation of the immune response *in vivo*. For instance, the Renshaw lab have developed a transgenic zebrafish line, *Tg(mpx:GFP)i114*, which expresses green

fluorescent protein (GFP) under the *mpx* promoter to specifically label neutrophils, allowing their *in vivo* visualisation and tracking (Renshaw et al., 2006). Injury to the zebrafish tail fin initiates an acute inflammatory response, which spontaneously resolves with kinetics comparable to mammalian systems. This model is susceptible to pharmacological manipulation and exposure of zebrafish larvae to chemical agents that are known to target the pathways regulating neutrophil apoptosis can influence inflammation resolution (Loynes et al., 2009). Other transgenic lines labelling neutrophils and macrophages have also been established (Ward et al., 2003; Mathias et al., 2006; Redd et al., 2006; Hall et al., 2007; Ellett et al., 2011). In addition, it has been possible to perform forward mutagenesis screens to identify mutants with persistent inflammation, such as *hai1*, *fad24* and *clint1* (Mathias et al., 2007; Walters et al., 2009; Martin et al., 2009). These have enabled the generation of zebrafish models of chronic inflammation, which may provide insight into the mechanisms regulating inflammation in disease states and facilitate high-throughput compound screening for novel inflammatory disease therapeutics.

One of the most important findings from zebrafish research relating to inflammation was the identification of hydrogen peroxide as the initial signal for neutrophil recruitment. Following wounding, an extracellular gradient of hydrogen peroxide is established at the site of injury via the activation of the NADPH oxidase enzyme, Dual oxidase (Duox) (Niethammer et al., 2009). It is thought that neutrophils detect and respond to this signal by activation of Lyn, a Src family kinase (Yoo et al., 2011), and that this also provides a functional link between initiation of the inflammatory response and the regeneration required for wound healing (Yoo et al., 2012).

The zebrafish model has been particularly useful for investigating the dynamics of neutrophil chemotaxis, due to the ease of visualising individual migrating cells *in vivo* in real time. The signalling pathways that regulate neutrophil chemotaxis are analogous to those in mammals, for example it has been shown that inhibition of PI-3Kγ in zebrafish disrupts neutrophil polarisation and directionality (Yoo et al., 2010). The function of chemokine signalling pathway components such as the small GTPase Rac2 and signalling molecules such as IL-8 homologous chemokines can also be readily

investigated in this system (Hartl et al., 2008; Deng et al., 2011; Sarris et al., 2012). As described previously, the zebrafish has proved instrumental for the study of neutrophil reverse migration as a mechanism of neutrophil clearance and factors that promote neutrophil survival (Mathias et al., 2006; Hall et al., 2007; Elks et al., 2011). Findings such as these illustrate the value of the zebrafish model for inflammation research.

1.6 Thesis aims

Persistent neutrophilic inflammation and the associated tissue damage is a significant cause of many chronic inflammatory diseases, which respond poorly to current treatments. There is a major requirement to develop novel, more effective therapies that specifically target the neutrophil, to promote the removal of these cells and drive inflammation resolution. This may be accomplished by targeting neutrophil apoptosis pathways or other, currently unknown signalling pathways that regulate alternative neutrophil clearance mechanisms such as reverse migration. Ideally, novel compounds must have the ability to override persistent neutrophil survival signalling and drive inflammation resolution without compromising the host immune system. As it is possible to initiate a robust inflammatory response in zebrafish larvae using the simple tail fin injury assay, this model provides an ideal system for the screening of diverse compound libraries. I hypothesised that novel accelerators of inflammation resolution could be identified in this manner.

The aims of this thesis are:

- To design and optimise a screening assay in the *Tg(mpx:GFP)i114* zebrafish model with the hypothesis that this approach will reveal novel accelerators of inflammation resolution

- To perform a robust screen of a diverse compound library and identify compounds that can reproducibly drive inflammation resolution in zebrafish

- To further investigate the activity of assay positive compounds in zebrafish, explore any potential structure-activity relationships and generate and test new hypotheses to attempt to define their precise mechanisms of action

- To investigate whether the effects of assay positive compounds can be replicated in human neutrophils *in vitro* and in the presence of neutrophil survival signals

Chapter 2: Materials and Methods

2.1 Reagents

All general reagents and chemicals were obtained from Sigma-Aldrich (Poole, UK), unless stated otherwise. All positive hit compounds that were used in secondary assays were acquired from Sigma-Aldrich, with the exception of isopimpinellin and 2-benzoyl-5-methoxybenzoquinone, which were obtained directly from MicroSource Discovery Systems Inc. (Gaylordsville, CT, USA). Nedocromil was obtained as a kind gift from Professor Rod Flower (William Harvey Research Institute, UK). The CXCR2 antagonist, SB225002 was obtained from Tocris Bioscience (Bristol, UK).

2.1.1 Compound libraries

The Spectrum compound collections were acquired from MicroSource Discovery Systems, Inc. The natural product collection of caspase-3 activators was obtained from Merlion Pharmaceuticals (Singapore). The library of known inflammatory signalling pathway inhibitors was acquired from GlaxoSmithKline (GSK, Stevenage, UK).

2.1.2 Antibodies for flow cytometry

Human CXCR1 Fluorescein isothiocyanate (FITC) MAb, Human CXCR2 Phycoerythrin (PE) MAb, Mouse IgG2A FITC Isotype Control and Mouse IgG2A PE Control were obtained as kind gifts from Professor Ian Sabroe and Dr Lisa Parker (University of Sheffield). Additional Human CXCR2 PE MAb and Human CXCR4 FITC were acquired from R & D Systems (Abingdon, UK).

2.2 General zebrafish techniques

2.2.1 Zebrafish husbandry

Zebrafish were raised and maintained according to standard protocols (Nüsslein-Volhard and Dahm, 2002), in a continuous re-circulating closed aquarium system at 28^{o}C and with a daily light cycle of 14 light hours and 10 dark hours, at UK Home Office approved aquaria in the MRC Centre for Developmental and Biomedical Genetics at

the University of Sheffield. At 5 days post fertilisation (dpf), zebrafish larvae were fed Tetra A-Z powdered fish feed, and from 13 dpf onwards, were fed live artemia twice daily. Transgenic zebrafish lines used throughout this project are listed in Appendix 1.

Embryos were acquired through either individual pair mating or collective marble tank mating. For pair mating, one female and one male zebrafish were placed into a small mating tank with a plastic grid at the base to allow embryos to fall into a separate space below. For collective mating, a 'marble tank' was placed into a tank of up to 40 adult zebrafish, consisting of a plastic container in which a wire-mesh sieve was inserted and filled with marbles. In all cases, mating set-up was carried out in the evening and embryos were collected the following morning by pouring the mating or marble tank water through a plastic tea strainer. Fertilised, healthy embryos were sorted using a Pasteur pipette into Petri dishes (Scientific Laboratory Supplies Ltd. (SLS), Coatbridge, UK) containing approximately 30 ml of E3 medium (Appendix 2), at a density of 50-60 viable embryos per dish. All embryos were incubated at 28^{o}C unless stated otherwise. At the endpoint of all experiments and prior to 5.2 dpf, embryos were sacrificed by immersion in bleach. All procedures were performed in accordance with UK Home Office legislation, which permits use of embryos up to 5.2 dpf outside of the Animals (Scientific Procedures) Act.

2.2.2 Microinjection of zebrafish embryos

For injection of ribonucleic acid (RNA), embryos were collected at the one-cell stage and viewed under a dissecting microscope (Leica Microsystems Ltd., Milton Keynes, UK). Embryos were positioned against a glass microscope slide (Menzel-Gläser, Braunschweig, Germany) in a Petri dish lid and excess liquid was removed. RNA was prepared in sterile water at the appropriate concentration required, with 10 % phenol red to allow visualisation. Injections were performed into the yolk using non-filament glass capillary needles (Kwik-Fil^{TM} Borosilicate Glass Capillaries, World Precision Instruments (WPI), Herts, UK) pulled using a Flaming Brown micropipette puller (Sutter Instrument Co., Novato, USA), and attached to a microinjection rig (WPI). Following injection, embryos were incubated in E3 at 28^{o}C and those that remained viable were transferred to fresh E3 every few hours.

2.2.3 Tail fin transection

Zebrafish larvae were anaesthetised by immersion in 0.017% 3-amino benzoic acid ethyl ester, referred to as tricaine, and were transferred onto a strip of masking tape adhered to a Petri dish lid. Excess liquid was removed to ensure minimal movement of the larvae, which were viewed under a dissecting microscope. A micro-scalpel (15°, 5.0 mm depth; WPI) was used to fully transect the caudal fin in order to initiate an inflammatory response. For consistency, injury was always performed within the region where the pigment pattern is disrupted, as posterior as possible to the circulatory loop, as illustrated by the red line (Figure 2.1). Following tail transection, larvae were transferred to fresh E3 medium and incubated at 28^{o}C to allow recovery. Tail fin transection was carried out at either 2 or 3 dpf, as stated.

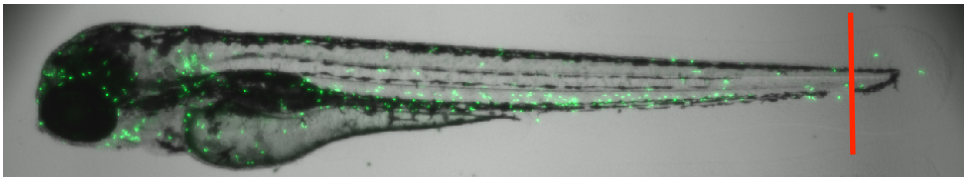

Figure 2.1: Site of zebrafish tail fin transection.
Image shows a 3 dpf *Tg(mpx:GFP)i114* zebrafish larva. Red line indicates point at which tail fin transection is performed using a micro-scalpel to initiate an inflammatory response.

2.2.4 Compound treatment

Chemical compounds were dissolved in dimethyl sulfoxide (DMSO), E3 or distilled water, as indicated. Larvae were treated by immersion in either 24- or 96-well plate format (SLS), as stated. The appropriate volume of stock solution was added to E3 to make a working solution at double the final concentration and half the final volume required, then larvae in an equal volume of E3 were added to result in the correct final concentration. Compounds were always tested alongside the appropriate vehicle control and a positive control as indicated. Unless otherwise stated, well plates were wrapped in aluminium foil and incubated at 28^{o}C.

2.2.5 Mounting of larvae for microscopy

For imaging, larvae were anaesthetised in tricaine then transferred to a mounting dish, prepared by adhering a coverslip (number 0; SLS) underneath a 10 mm puncture in a 25 mm Petri dish using high vacuum grease (Dow Corning®, Seneffe, Belgium). Excess E3 was removed then larvae were mounted in a 1% solution of low melting point (LMP) agarose dissolved in sterile water and containing 0.017% tricaine. Larvae were manipulated into a flat position using a human hair. For time-lapse microscopy, E3 containing 0.017% tricaine and if required, the appropriate concentration of drug treatment, was applied to the Petri dish after the agarose had solidified, to avoid dehydration of the larvae.

2.3 Zebrafish drug screening

The transgenic zebrafish line *Tg(mpx:GFP)i114* (Renshaw et al., 2006), which specifically labels neutrophils with green-fluorescent protein (GFP), was used for all drug screening experiments in this project and is subsequently referred to as *mpx*:GFP. The Merlion Collection was screened at a final concentration of 250 μg/ml for crude extracts and 62.5 μg/ml for pre-fractions. The Spectrum Collection was screened at 25 μM and each plate was screened on 2 independent occasions.

2.3.1 Compound library screening protocol

Tail fin transection was performed as described and at 4 hpi, larvae were viewed under a fluorescent dissecting microscope (Leica MZ10F, GFP plus filter). Larvae with 25-30 neutrophils at the site of injury were selected and transferred to fresh E3. Screening plates were set up blinded to experimental conditions, to contain 4 wells each of E3 control, DMSO vehicle control and either 10 μM GSK650394 (Tocris Bioscience) or 30 μM SP600125 as a positive control. Selected larvae were added to the compounds using a pipette, at a density of 3 larvae per well. Screening plates were wrapped in aluminium foil and incubated at 28^{o}C.

At 12 hpi, larvae were anaesthetised and the well plate was imaged using the Phenosight High-Content plate reader (Ash Biotech, Cambridge, UK), which captured both bright field and GFP images, at an exposure of 40 ms. Prior to imaging, larvae were removed from any wells containing auto-fluorescent compounds, which included the SP600125 positive control, and the wells were washed out using fresh E3 to remove remaining traces of auto-fluorescence. Larvae were then returned to their wells in fresh E3 containing tricaine.

2.3.2 Manual scoring procedure

Individual fluorescent and bright field *.tif* images were generated for each well by the Phenosight plate scanner at 12 hpi, were observed in Preview (Macintosh) and given a score between 0 and 3 (Table 2.1). The scoring system was dependent on the number of larvae within the well that were considered to have a significantly reduced neutrophil population at the site of injury in comparison to the DMSO control larvae. In all experiments, there was more than one well of 3 fish per compound, so a mean score was calculated.

Table 2.1: Guidelines for the manual scoring method.

Score	Phenotype
0	0 out of 3 larvae with reduced neutrophil population at site of injury
1	1 out of 3 larvae with reduced neutrophil population at site of injury
2	2 out of 3 larvae with reduced neutrophil population at site of injury
3	3 out of 3 larvae with reduced neutrophil population at site of injury

2.3.3 Automated analysis using a pilot version of the Phenosight software

Phenosight analysis software (Ash Biotech), designed to complement the automated plate reader by generating a quantitative readout of fluorescence, is currently under development. To aid progression, a trial version of the software was tested using results from the Merlion Collection screen. Fluorescent images from each 96-well plate were imported into the program and a 'constellation', indicated by a large yellow circle, containing 2 'features', indicated by 2 small red circles, was placed onto each

larva (Figure 2.2). One feature was placed in the tail region, covering as much of the site of injury as possible, and the other feature was placed anterior to the first, at a site where an unbiased background reading of fluorescence could be measured. Once constellations were placed on all 3 larvae in all 96 wells, the software measured the level of fluorescence within each feature, producing a Microsoft® Excel worksheet containing these values expressed as 'fluorescence per unit area' (FPU). The worksheet was manipulated using a series of formulae to generate a final readout of FPU at the site of injury as a percentage of the background fluorescence.

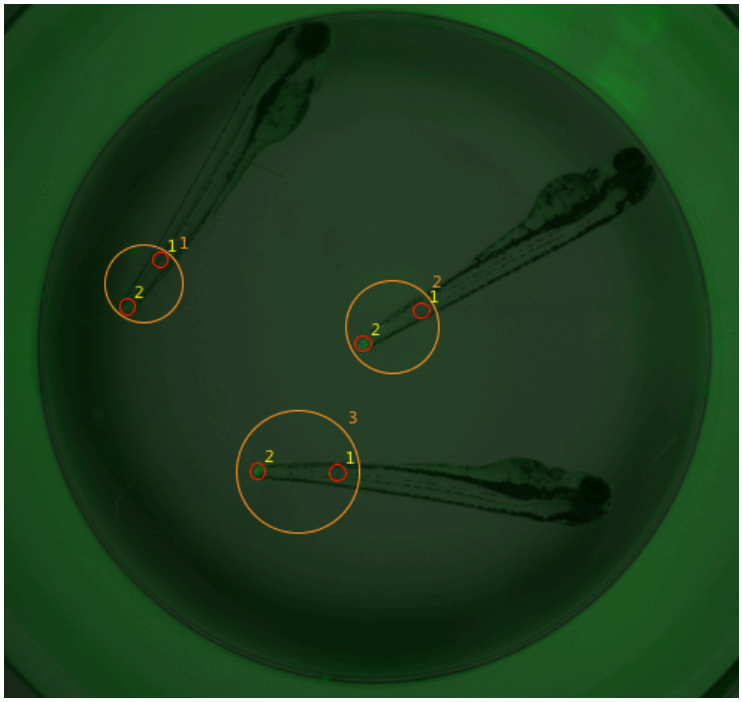

Figure 2.2: Illustrative example of constellation location in the Phenosight Analysis software.

Constellations (1 – 3) containing 2 features were placed onto each larva. Feature 1 was placed in a region where a representative level of background fluorescence could be measured. Feature 2 was placed in the tail region to encompass the site of injury.

2.4 Assays to analyse neutrophil response and behaviour in zebrafish

2.4.1 Neutrophil recruitment assay

To assess the ability of compounds to inhibit neutrophil recruitment, 3 dpf *mpx*:GFP larvae were injured as described above and treated immediately by immersion with compounds diluted in E3 at the concentrations indicated. At 6 hpi, larvae were anaesthetised and the numbers of neutrophils at the injury site were counted by eye under a fluorescent dissecting microscope (Leica MZ10F, GFP plus filter). Where possible, counting was performed blind to experimental conditions.

2.4.2 Inflammation resolution assay

To investigate the effect of compounds on the acceleration of inflammation resolution, 3 dpf *mpx*:GFP larvae were injured and good responders were selected at 6 hpi for treatment by immersion. At 12 hpi, larvae were anaesthetised and neutrophil numbers were counted as above. On all possible occasions, counting was performed blind to experimental conditions.

2.4.3 Analysis of total neutrophil number

To examine the effect of compounds on the total neutrophil number of intact larvae, 3 dpf *mpx*:GFP larvae were treated with compounds by immersion for 24 hours, anaesthetised and then mounted in 1% LMP agarose. Imaging was carried out using an Eclipse TE2000-U inverted compound fluorescence microscope (Nikon UK Ltd., Kingston upon Thames, UK) with a 1394 ORCA-ERA (Hamamatsu Photonics Inc., NJ, USA) camera and Volocity® software (PerkinElmer Life and Analytical Sciences, Cambridge, UK). Images were acquired using a 2x NA 0.06 Plan UW objective, with a GFP-specific filter and excitation at 488 nm, with 10 Z slices. Images were compressed into a single focal plane and exported as *.tif* files. Automated analysis of total neutrophil number was performed using ImageJ software. As each image acquired contained 3 larvae, images were cropped to contain a single larva of interest, and converted to 8-bit resolution and binary form after appropriate adjustment of brightness and contrast to exclude background fluorescence. Neutrophil number was measured in an unbiased manner using the 'analyse particles' function.

2.4.4 Neutrophil tracking during recruitment to the site of injury

For investigation of changes in neutrophil behaviour during the initial recruitment phase of the inflammatory response, 3 dpf *mpx*:GFP larvae were incubated with compounds for 2 hours before tail fin injury. After approximately 10 minutes, larvae were mounted in 1% LMP agarose. A one hour time-lapse series was captured using a 1394 ORCA-ERA camera on an Eclipse TE2000-U inverted compound fluorescence microscope. Acquisition occurred at 2 minute intervals, using a 10x NA 0.3 Plan Fluor objective, with 10 Z planes over a total focal depth of 100 µM, and using a GFP-specific filter with excitation at 488 nm. For analysis, a region of interest (ROI) was drawn around each injured tail fin, to exclude stationary developing neutrophils in the posterior blood island. Tracking of GFP-labelled cells was performed using Volocity® software, which generated various parameters for each neutrophil track, including speed, displacement, meandering index and bearing. These data were also used to calculate displacement towards the wound.

2.4.5 Analysis of PI-3K activity

To investigate PI-3K activation during neutrophil recruitment *in vivo*, *Tg(lyz:PHAkt-EGFP)* larvae, subsequently referred to as *lyz*/PHAkt, were incubated with test compounds for 2 hours prior to tail fin transection as described above. In all experiments, compounds were tested alongside 50 µM LY294002 and a DMSO vehicle control. To avoid bias, experiments were performed blind to treatment conditions. Approximately 10 minutes after injury, larvae were mounted in 1% LMP agarose and observed on an Ultra*VIEW*VoX spinning disk confocal imaging system (PerkinElmer Life and Analytical Sciences) with an inverted Olympus IX81 microscope, using an Olympus UPlanSApo 60x oil immersion objective (Hamburg, Germany). GFP-positive cells in the region between the injury site and the posterior blood island were imaged individually, using the 488 nm laser line with 20 Z slices. Single slices in which a leading and trailing edge of each cell could be defined were exported as *.tif* files and analysed in ImageJ. Using the straight-line tool, a transection was drawn through each neutrophil from the trailing edge towards the leading edge. In cases where there were no clear leading or trailing edges, the straight line was drawn through the most longitudinal section of the cell. A plot profile was generated to measure the fluorescence intensity per pixel along

the length of the line (Figure 2.3) and these values were inserted into a Microsoft® Excel worksheet. A threshold was applied to account for background fluorescence, and the trailing and leading edges were defined by selecting the appropriate pixels. Mean intensity values for each parameter, as illustrated in Figure 4.3, were used to calculate the polarity index with the equation:

$$polarity\ index = \left(log_{10}\, {}^{a}/_{b}\right) \times \frac{a+b}{c}$$

$$where\ a = trailing\ edge\ of\ cell;\ b = leading\ edge\ of\ cell; c = whole\ cell$$

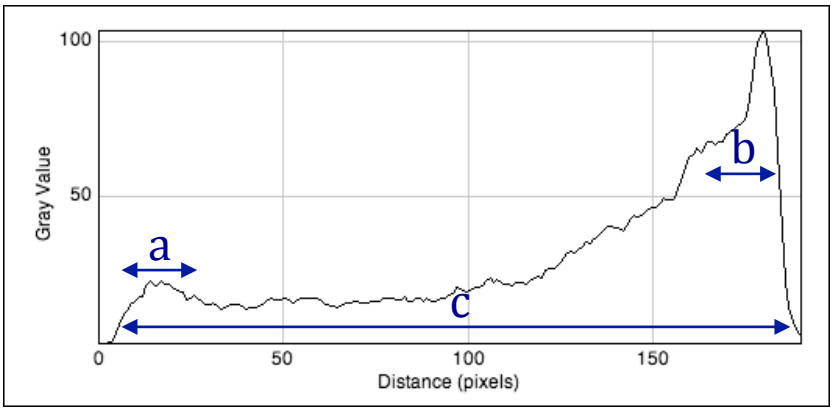

Figure 2.3: Fluorescence intensity plot profile of a migrating neutrophil.

Figure shows representative plot profile generated in ImageJ by drawing a straight line through a migrating neutrophil, from the trailing edge towards the leading edge. The fluorescence intensities per pixel were imported into Excel, a threshold was applied and the appropriate pixels were selected to define the trailing edge (a), leading edge (b) and the whole cell (c).

2.4.6 Reverse migration assay

Reverse migration assays were performed using embryos expressing the photoactivatable fluorescent protein Kaede in neutrophils, raised from the double transgenic line *Tg(mpx:Gal4;UAS:Kaede)i222*, subsequently termed *mpx*/Kaede. At either 2 or 3 dpf as specified, Kaede-positive larvae were selected and tail transection was performed**.** At 4 hpi, larvae with high numbers of neutrophils recruited to the site of injury were immersed with test compounds as indicated or DMSO vehicle control. At 5 hpi for 2 dpf, and 6 hpi for 3 dpf, larvae were mounted in a chamber slide (SLS) in 1% LMP agarose containing the required concentration of test compound or DMSO. Kaede-labelled cells at the site of injury were photoconverted at 10x magnification using an Ultra*VIEW*PhotoKinesis™ device (PerkinElmer Life and Analytical Sciences) on the Ultra*VIEW*VoX laser imaging system described above. The PhotoKinesis™ device was calibrated using fluorescent ink (Stabilo Boss™ Berks, UK) as a photo-bleachable substrate, drawn onto a glass microscope slide. The 405 nm laser line was used for photoconversion, with 120 cycles at 40% laser energy (settings optimised by previous student, Giles Dixon and published in (Dixon et al., 2012)). A ROI was drawn to selectively photoconvert neutrophils at the site of injury and successful photoconversion of Kaede was ensured by observation of a loss of green fluorescence in the 488 nm laser line and gain of red fluorescence in the 561 nm laser line. Larvae were then transferred to the Eclipse TE2000-U inverted compound fluorescence microscope and a time-lapse series was captured as described for neutrophil tracking experiments (2.4.4), with 2.5 minute intervals, for the duration indicated. Both GFP- and RFP-specific filters were used, with excitation at 488 nm and 561 nm respectively. For analysis in Volocity®, a ROI was drawn in the first time frame, to exclude the site of injury and majority of red photoconverted cells. The number of red cells that migrated into the ROI was recorded at every subsequent time frame.

The behaviour of reverse migrating neutrophils was also analysed using Volocity® software as described in section 2.4.4, to analyse the speed and meandering index of red-labelled cell tracks, without prior selection of a ROI.

2.5 Zebrafish staining assays

2.5.1 Analysis of neutrophil apoptosis

To assess the rate of neutrophil apoptosis *in vivo*, resolution assays were carried out as described above, using 3 dpf *mpx*:GFP larvae. At 12 hpi, rather than counting numbers of neutrophils at the site of injury, larvae were fixed overnight at 4^{o}C in 4% paraformaldehyde (PFA; Fisher Scientific, Loughborough, UK). The following day, Tyramide Signal Amplification (TSA) staining was performed using the TSA^{TM}-Plus Fluorescein System (PerkinElmer Life and Analytical Sciences), as per the manufacturer's instructions. This labels myeloperoxidase in neutrophils to enable their visualisation under a GFP filter. Prior to staining, larvae were washed in PBT (phosphate buffered saline (PBS) + 0.1% tween 20), followed by amp diluent. Larvae were then incubated in 50 µl of 1:50 FITC TSA:amp diluent in the dark at 28^{o}C for 10 minutes. To remove background staining, larvae were washed in PBT, then fixed for 20 minutes in 4% PFA at room temperature before storage in PBT overnight at 4^{o}C.

The ApopTag® Red *In Situ* Apoptosis Detection Kit (Millipore Corporation, Herts, UK) was used for terminal deoxynucleotidyl transferase (TdT) dUTP nick end labelling (TUNEL) as per the manufacturer's instructions. This stains the double strand breaks in DNA to allow detection of apoptotic cell death. Initially, larvae were digested with proteinase K at 10 µg/ml for 90 minutes, washed in PBT, then fixed for 20 minutes in 4% PFA at room temperature. After further washing in PBT, larvae were immersed in 1:2 acetone:ethanol for 7 minutes at -20^{o}C, then rewashed in PBT. Larvae were then incubated in 50 µl equilibration buffer for 1 hour at room temperature, which was then replaced with 30 µl reaction buffer and 16 µl TdT enzyme, for 90 minutes at 37^{o}C. This was replaced with 200 µl stop buffer for 3 hours at 37^{o}C. Following PBT washing, larvae were incubated with 62 µl anti-dig-rhodamine and 68 µl blocking solution overnight at 4^{o}C with gentle rocking. The next day, larvae were washed in PBT, fixed for 4% PFA for 30 minutes at room temperature, then stored at 4^{o}C in 80% glycerol until imaging.

For imaging, the tail fin was removed from each larva using a surgical blade (Swann Morton Ltd., Sheffield, UK), mounted on a glass microscope slide and held in place with an adhered coverslip. The wound region was imaged at 20x magnification using the Ultra*VIEW*VoX spinning disk confocal imaging system (PerkinElmer Life and Analytical Sciences) with the 488 nm and 561 nm laser lines and 20 Z slices. The total neutrophil number at the site of injury (cells labelled with TSA) and number of apoptotic neutrophils (cells labelled with both TSA and TUNEL) were analysed using Volocity® software (PerkinElmer Life and Analytical Sciences).

2.5.2 Whole mount *in situ* hybridisation (WISH)

WISH studies were performed to examine the spatial expression of *sdf-1a* during the inflammatory response in injured 3 dpf zebrafish larvae. At the appropriate time points, larvae were fixed in 4% PFA overnight at 4^{o}C, then transferred through a methanol series and stored in 100% methanol at -20^{o}C until required. No more than 20 larvae were stored per tube. For rehydration, the larvae were transferred through a reverse methanol series and then washed in PBT. Following this, larvae were digested with proteinase K at 10 µg/ml for 40 minutes, washed in PBT, then fixed for 20 minutes in 4% PFA at room temperature. After re-washing in PBT, pre-hybridisation was performed by incubating larvae with PreHyb buffer for 3 hours at 70^{o}C. Buffers and wash solutions were prepared as described (Appendix 2). The PreHyb was replaced with ProbeHyb and this was incubated overnight at 70^{o}C. The probe was removed the following day by a series of washes consisting of 3:1, 1:1 then 1:3 HybWash:2x SSC. Following another wash in 2x SSC at 70^{o}C, larvae were transferred through an SSC series at room temperature until a final wash in PBT alone. Larvae were then incubated with blocking solution (Appendix 2) for 2 hours at room temperature, with gentle rocking. This was followed by overnight incubation with an anti-DIG-AP antibody (Roche, Herts, UK) at a 1:5000 dilution in blocking solution, at 4^{o}C with gentle rocking. The antibody was removed and larvae were washed extensively in PBT at room temperature, followed by further washes in staining wash (Appendix 2). Staining solution (Appendix 2) was applied until staining developed and then the reaction was terminated using 1 mM EDTA. Larvae were washed in PBT, fixed in 4% PFA for 20 minutes at room temperature, then stored in methanol at -20^{o}C. For imaging, larvae

were rehydrated through a methanol series then transferred into 80% glycerol. Imaging was performed using a fluorescent dissecting microscope with a Leica DFC310 camera and Leica Application Suite software.

2.6 Molecular biology

2.6.1 Transformation

To transform DNA, 1 µl of the plasmid indicated was added to 25 µl of Top10 competent cells (Invitrogen, Paisley, UK), followed by gentle mixing and incubation on ice for 30 minutes to allow membrane permeabilisation. The cells were then heat shocked for 45 seconds at 42^{o}C and returned to ice for a further 10 minutes before addition of 150 µl SOC medium (Invitrogen) and incubation for 1 hour at 37^{o}C with vigorous shaking at 200 rpm. Following this, the cells were spread onto a dry selection plate and cultured at 37^{o}C overnight. In all cases, selection plates were made using autoclaved LB agar and 50 µg/ml carbenicillin.

2.6.2 DNA purification

Following transformation, single isolated colonies were selected and grown overnight in 50 ml of autoclaved LB broth containing 50 µg/ml carbenicillin, at 37^{o}C with vigorous shaking at 200 rpm. The DNA was purified using a HiSpeed Plasmid Midi Kit (QIAGEN, Manchester, UK) and addition purification was performed using a MinElute PCR Purification Kit (QIAGEN), as per the manufacturers' instructions. The DNA concentration was quantified using a Nanodrop^{TM} 1000 spectrophotometer (Thermo Scientific, Hemel Hempstead, UK).

2.6.3 Whole mount *in situ* Hybridisation (WISH) probe synthesis

An RNA probe was synthesised for the WISH studies described above. A plasmid vector containing the zebrafish *sdf-1a* coding sequence was obtained as a kind gift from Dr Stone Elworthy (University of Sheffield). Following transformation and DNA purification as described, the vector was linearised by restriction digest with EcoR1 (New England Biolabs (NEB), Herts, UK). For this, 20 µg of vector was incubated with 1

µl of EcoR1 and 20 µl of EcoR1 buffer (NEB) in a total volume of 200 µl with sterile water at 37^{o}C for 2 hours. The linearised DNA was then extracted by adding 200 µl of phenol:chloroform, vortexed for 3 minutes, then centrifuged at 14000 rpm for 7 minutes. The upper phase was removed and added to a new tube, followed by an equal volume of chloroform. This was vortexed for 1 minute and centrifuged at 14000 rpm for 5 minutes, then the upper phase was transferred to a new tube. To precipitate the DNA, 10% of this volume of 3 M sodium acetate was added, followed by an equal volume of isopropanol. The mixture was stored at -20^{o}C for 20 minutes, then centrifuged at 14000 rpm for 20 minutes at 4^{o}C. The supernatant was removed and the pellet washed carefully in 200 µl of 70% ethanol, by centrifugation at 14000 rpm for 5 minutes at 4^{o}C. The ethanol was removed and the pellet air-dried for 10 minutes before re-suspension in 20 µl of sterile water. The DNA concentration was quantified using a Nanodrop^{TM} 1000 spectrophotometer.

An SP6 RNA digoxigenin-labelling kit (Roche) was used to transcribe the RNA probe. For this, 1 µg of linearised DNA was incubated with 2 µl NTP-DIG-RNA labelling mix, 1 µl RNAse inhibitor, 2 µl transcription buffer and 2 µl SP6 polymerase in a final volume of 20 µl with sterile water for 2 hours at 37 ^{o}C. To remove the DNA template, 4 µl DNAse was added for 30 minutes at 37 ^{o}C. For precipitation, 1 µl pH8 0.5 M EDTA, 2.5 µl 4 M LiCl and 75 µl of 100% ethanol were added followed by storage at -80^{o}C for 1 hour. After centrifugation at 14000 rpm for 30 minutes at 4^{o}C, the supernatant was removed and the pellet was carefully washed in 100 µl of 70% ethanol, by centrifugation at 14000 rpm for 10 minutes at 4^{o}C. The supernatant was removed and the pellet air-dried for 5 minutes before re-suspension in 20 µl of sterile water. To confirm the presence of RNA, gel electrophoresis was performed using 1 µl of the re-suspension and a 1% agarose gel. After addition of 80 µl formamide, the RNA probe was stored at -80^{o}C.

2.6.4 RNA synthesis

A plasmid vector containing the dominant-active *hif-1αb* coding sequence (Elks et al., 2011) was obtained as a kind gift from Dr Phil Elks (Leiden University) and DNA was transformed and purified as described above. Synthesis of dominant-active *hif-1αb*

RNA was performed using the mMessage mMachine® High Yield Capped RNA Transcription Kit (Invitrogen), according to the manufacturer's instructions. For the transcription reaction, 6 µl of DNA was incubated with 10 µl 2x NTP/CAP, 10 µl 10x reaction buffer and 2 µl SP6 enzyme mix for 2 hours at 37°C. To remove the DNA template, 1 µl TURBO DNAse was added and the solution was mixed and incubated for 30 minutes at 37°C. This was followed by addition of 115 µl nuclease free water and 15 µl ammonium acetate stop solution. To extract the RNA, 150 µl phenol:chloroform was added prior to vortexing and centrifugation at 14000 rpm for 15 minutes. The upper phase was removed and transferred to a new tube with an equal volume of chloroform, which was again vortexed and centrifuged at 14000 rpm for 15 minutes. After transfer of the upper phase to a new tube, an equal volume of isopropanol was added to precipitate the RNA. The mixture was stored at -20°C for 1 hour, then centrifuged at 14000 rpm for 15 minutes at 4°C to pellet the RNA. The pellet was re-suspended in 20 µl sterile RNAse free water and stored at -80°C. The RNA concentration was quantified by gel electrophoresis and the appropriate volume was injected into zebrafish embryos at the one-cell stage, as described.

2.7 Human neutrophil techniques

For the *in vitro* experiments described in this thesis, human neutrophils were acquired following a preparation method performed by other members of the research team. Neutrophils were isolated from peripheral venous blood donated by healthy volunteers, under the approval of the South Sheffield Research Ethics Committee (reference number: STH13927). At one tenth of the total blood volume, 3.8% sodium citrate (Martindale Pharmaceuticals, Essex, UK) was added to avoid coagulation, prior to centrifugation at 350 x g for 20 minutes at 20°C. This allowed separation of the more dense blood cells from the platelet-rich plasma (PRP), which was further centrifuged at 800 x g for 20 minutes at 20°C to pellet the platelets and form the upper platelet-poor plasma (PPP). The lower blood cell phase was made up to a total volume of 50 ml by adding 6 ml of 6% dextran and 0.9% saline solution. Following gentle mixing, this was allowed to sediment for 20-30 minutes to separate the more dense

erythrocytes from the white blood cells. These formed an upper layer, which was removed and centrifuged at 320 x g for 6 minutes at 20°C. At this point, neutrophils were separated from other white blood cells using either the Percoll® or Optiprep™ method of separation. There are no reported differences in constitutive apoptosis rate or Mcl-1 regulation between neutrophils separated by either of these methods (Wardle et al., 2011).

2.7.1 Percoll® method of cell separation

A 90% Percoll® solution was prepared with 10% saline. This was used to make a plasma-Percoll® gradient, with the lower phase consisting of 0.98 ml 90% Percoll® and 1.02 ml PPP and an upper phase of 0.84 ml 90% Percoll® and 1.16 ml PPP. Following re-suspension in 2 ml of PPP, the cell pellet was layered over the gradient and centrifugation was performed at 350 x g for 11 minutes at 20°C. This resulted in three separate cell layers, with erythrocytes in the lowest phase, a layer of granulocytes above this and an upper layer of PBMCs. A Pasteur pipette was used to remove the PBMCs and neutrophils, which were counted using a haemocytometer. Neutrophils were pelleted by centrifugation at 320 x g for 6 minutes at 20°C, then re-suspended at a concentration of 5 x 10^6 neutrophils per ml in RPMI media with 100 U/ml penicillin, 100 g/ml streptomycin (Life Technologies, Paisley, UK) and 10% foetal calf serum (FCS) (Invitrogen).

2.7.2 Optiprep™ method of cell separation

The white blood cell pellet was re-suspended in a solution of 2 ml Hank's balanced salt solution (HBSS; Invitrogen) with 20% PPP and without Ca^{2+} or Mg^{2+}. Another 6 ml of HBSS with 20% PPP was added, followed by 4 ml of OptiPrep™ (Axis-Shield, Huntingdon, UK). An OptiPrep™ gradient was pipetted over this, firstly the 1080 layer consisting of 10.435 ml HBSS with 20% PPP and 3 ml OptiPrep™, followed by the 1095 layer, consisting of 8.036 ml HBSS with 20% PPP and 3 ml OptiPrep™. A further 10 ml of HBSS with 20% PPP was added prior to centrifugation of the gradient at 1978 x g for 30 minutes at 20°C. This resulted in three separate cell layers, with erythrocytes in the lowest phase below the 1095, a neutrophil layer between 1095 and 1080 and an upper layer of PBMCs above the 1080. A Pasteur pipette was used to remove the PBMCs and neutrophils, which were counted using a haemocytometer. Neutrophils were pelleted

by centrifugation at 320 x g for 6 minutes at 20°C, then re-suspended at a concentration of 5 x 10^6 neutrophils per ml in RPMI media with 100 U/ml penicillin, 100 g/ml streptomycin and 10% FCS.

2.7.3 Human neutrophil culture

For all experiments, preparation and compound treatment was performed in a Class II Biosafety cabinet. Neutrophils were cultured at 125 µl per well in 96-well 'Flexiwell' plates (BD Falcon, Oxford, UK or Costar, High Wycombe, UK), ensuring that compounds were added in minimal volume to avoid disruption of the final neutrophil concentration. Unless stated otherwise, neutrophils were treated in duplicate, to result in a total volume of 250 µl per condition. Neutrophils were cultured in a water-jacketed tissue culture incubator (Sanyo, Loughborough, UK) at 37°C and 5% CO_2. In experiments performed to investigate neutrophil survival signalling, GM-CSF (PeproTech, London, UK) was used at 1:400.

2.7.4 Measurement of neutrophil apoptosis by cytospin analysis

Neutrophil apoptosis was assessed at the time points indicated for each individual experiment and in all cases two cytospins were taken per treatment. Using a cytocentrifuge (Shandon Cytospin 3, Thermo Scientific), 100 µl of neutrophil suspension was centrifuged onto a glass microscope slide at 300 rpm for 3 minutes at room temperature. The neutrophils were fixed using a small volume of 100% MeOH. Slides were immersed in Reastain Quick-Diff Red (Gentaur, London, UK) to stain cytoplasmic material, followed by Reastain Quick-Diff Blue (Gentaur) to stain nuclear material. Cells were covered with a glass coverslip (Menzel-Gläser), attached using DPX mounting medium (Fisher Scientific).

To measure apoptosis, neutrophils were counted on a Zeiss Axioplan microscope (Carl Zeiss Ltd., Cambridge, UK) with a 100 x oil immersion lens and using a Microsoft Excel macro designed to calculate percentage apoptosis. Neutrophils were allocated as apoptotic or non-apoptotic based on morphology, taking into account nuclear condensation and cell shrinkage. A total of 600 neutrophils were counted per condition, with 300 from each cytospin, and the percentage of apoptotic cells was recorded. Counting was always performed blind to the experimental conditions. To

assess the purity of the neutrophil preparation, a total of 600 cells were counted from an untreated sample slide, including neutrophils, eosinophils and PBMCs.

2.7.5 Flow cytometry

To look at the cell surface expression levels of various chemokine receptors, human neutrophils were separated by the Percoll® method of separation, as described. For each condition, 100 µl of neutrophil suspension at a concentration of 5 x 10^6 neutrophils per ml in RPMI media was transferred to a 1.5 ml tube, followed by the addition of 100 µl FACS buffer 1 (Appendix 2). After centrifugation at 2000 rpm for 2 minutes at 4°C, the supernatant was removed and the cell pellet was re-suspended in the appropriate volume of FACS buffer 1, such that the appropriate volume of fluorescent-conjugated antibody or isotype control could be added to result in a 1:5 dilution. Unstained cells and the appropriate isotype controls were used at all times, to account for any non-specific binding. The tubes were wrapped in aluminium foil and incubated for 30 minutes at 4°C. This was followed by the addition of 300 µl FACS buffer 1, centrifugation at 2000 rpm for 2 minutes at 4°C, then re-suspension of the pellet in 300 µl of FACS buffer 1. The cell suspension was kept on ice at all times, unless the samples could not be analysed immediately, in which case the cells were re-suspended in BD CellFIX™ (BD Biosciences, Oxford, UK). Analysis was always carried out within the next 24 hours.

Samples were analysed using a FACSCalibur flow cytometer (BD Biosciences), in the Flow Cytometry Core Facility at the University of Sheffield Medical School. This measured cell size using the Forward Scatter Channel (FSC) and granularity on the Side Scatter Channel (SSC), capturing 10,000 events for each sample to generate a scatter plot. Fluorescence associated with the cells was measured by the appropriate Fluorescence channel (FL-1 for FITC-conjugated antibodies and FL-2 for PE-conjugated antibodies). Data were analysed using FlowJo (TreeStar Inc., OR, USA). A neutrophil gate was applied to the scatter plot generated by the unstained control sample to enable identification of the neutrophil population. The same gating was applied to each subsequent sample, but adjusted if necessary to ensure that all neutrophils were contained within the gate. Histograms were generated to show the fluorescence of the

gated neutrophils in each FL channel and the mean fluorescence values were used to compare cell surface receptor expression between samples.

In experiments performed to investigate the effect of tanshinone IIA on cell surface receptor expression, neutrophils were pre-treated with either tanshinone IIA or the appropriate DMSO vehicle control for 2 hours, prior to the flow cytometry protocol as described above. At the appropriate time point, cytospins were acquired for each condition, as previously described.

2.7.6 Chemokine receptor internalisation assay

To investigate chemokine receptor internalisation, human neutrophils were separated by the Percoll® method of separation, as described, and re-suspended in HBSS with 0.25% BSA and 10 mM HEPES (FACS buffer 2; Appendix 2), such that 5 x 10^5 neutrophils could be added to 1.5 ml tubes for each condition, in a volume of 75 µl. The appropriate concentration of chemokine receptor ligand was added in 25 µl to result in the correct final concentration, as indicated. Recombinant Human IL-8 (PeproTech) was used to investigate CXCR1 and CXCR2 internalisation and recombinant Human SDF-1α (PeproTech) for CXCR4 internalisation. For each antibody and isotype, a control sample was stored on ice immediately to measure basal expression levels before ligand stimulation. All other cells were incubated for 30 minutes at 37°C and then cooled rapidly by adding 1 ml of cold FACS buffer 2 and transferring tubes to an ice-water bath. Cells were centrifuged at 2000 rpm for 2 minutes at 4°C, washed with 1 ml FACS buffer 2, re-spun, then re-suspended in the appropriate volume of FACS buffer 2 for antibody staining as described in section 2.7.5. Samples were analysed using the FACSCalibur flow cytometer, as described, and chemokine receptor internalisation was measured by calculating the mean fluorescence of each sample as a percentage of the appropriate basal fluorescence.

To explore the effects of tanshinone IIA on chemokine receptor internalisation, neutrophils were exposed to tanshinone IIA or the appropriate DMSO vehicle control in combination with the receptor ligand. In some cases, as indicated, neutrophils were pre-treated with either tanshinone IIA or DMSO for 2 hours, then centrifuged at 2000

rpm for 2 minutes at 4°C, followed by re-suspension in FACS buffer 2 as described at the beginning of the protocol.

2.7.7 Chemokine receptor recycling assay

The receptor internalisation assay was performed as described above, but more cell samples were included to measure recycling of the receptors back to the cell surface after internalisation. Following the ligand stimulation step as described above, these samples were centrifuged at 2000 rpm for 2 minutes at 4°C and washed extensively with FACS buffer 2 to remove all traces of the ligand. Care was taken to ensure all samples were kept on ice during washing steps. Cells were re-suspended in 100 µl FACS buffer 2 and incubated at 37°C to permit receptor recycling. At the appropriate time points, as indicated, cells were transferred to ice, followed by centrifugation, washing, antibody staining and analysis as described previously.

Investigation of the effect of tanshinone IIA on receptor recycling was performed as described for the receptor internalisation assay. In all subsequent washing and incubation steps, the required concentration of tanshinone IIA or DMSO vehicle control, as indicated, was added to the appropriate FACS buffer 2, to ensure constant exposure of cells to the compound. At the appropriate time point, cytospins were acquired for each condition, as previously described.

2.8 Statistical and computational analyses

2.8.1 Statistical analyses

GraphPad Prism® software was used for all statistical analyses. The D'Agostino and Pearson omnibus normality test was used to test for normal distribution of samples. For comparisons between two groups, an unpaired two-tailed *t*-test was used, and the variances were compared using an F test. For simultaneous comparisons between multiple groups, a one-way or two-way ANOVA was used alongside either Dunnett's or Bonferroni's multiple comparison post-test, as indicated. Bartlett's test for equal variances was used to compare variances. Dose response curves were analysed using

non-linear regression and IC50 values were calculated. For reverse migration assays, analysis was performed using either linear regression to compare the difference between slopes or non-linear regression to compare the difference between curves, depending on which best fitted the data. On all figures, where * $p < 0.05$, where ** $p < 0.01$ and where *** $p < 0.001$.

2.8.2 Computational clustering

For recruitment and resolution assays, data were normalised by expressing as a percentage of the effect of the positive control. For total neutrophil number experiments, data were expressed as the percentage change from the vehicle control. Hierarchical cluster analysis was performed using Cluster 3.0 (developed by Michiel de Hoon), with Pearson un-centred correlation and an average linkage clustering method. Results generated were displayed using Java TreeView (developed by Alok Saldanha). Both programs were downloaded from http://www.eisenlab.org/eisen/?page_id=4.

Chapter 3: An *in vivo* Screening Assay for Accelerators of Inflammation Resolution

3.1 Introduction

Neutrophil-dominated inflammatory diseases respond poorly to current treatments. The development of more effective therapies is required, particularly of those that can specifically target the signalling pathways regulating neutrophil clearance. Mechanisms involved in the resolution of inflammation remain to be fully defined and broadening our understanding of this complex process is required, such that we can therapeutically promote resolution via endogenous pathways and avoid unwanted side effects. Considering the size of chemical space, it seems likely that compounds must exist with the correct conformational structures to allow efficient binding to key targets, to give a functional and desirable response. However, without a complete knowledge of the precise cellular components and biochemical mediators involved, it is not possible to employ target-based approaches to pro-resolution drug discovery. Phenotype-based assays might yield results that can be more readily translated into clinical practice and new methods to screen large numbers of compounds rapidly and robustly are required.

In recent years, the zebrafish has emerged as a powerful model system. With the ease of genetic and pharmacological manipulation, along with its rapid generation time, small size and embryo transparency, it is an ideal model for high-throughput screening. Already there are multiple screening success stories, reflective of its value (North et al., 2007; Cao et al., 2009; Tan et al., 2011; Cusick et al., 2012; Namdaran et al., 2012; Choi et al., 2013). The *Tg(mpx:GFP)i114* zebrafish line, subsequently referred to as *mpx*:GFP, provides a model for studying neutrophilic inflammation and enables the visualisation and tracking of green-fluorescent neutrophils *in vivo* (Renshaw et al., 2006). Injury to the zebrafish tail fin initiates an acute inflammatory response, which is quantifiable by counting the number of neutrophils present at the injury site at various experimental time points. This response spontaneously resolves with kinetics comparable to mammalian systems (Renshaw et al., 2006). Zebrafish larvae are susceptible to

pharmacological manipulation and their post-injury exposure to chemical agents known to either inhibit or induce neutrophil apoptosis causes either a delay or acceleration in inflammation resolution, respectively (Loynes et al., 2009). Based on this experimental evidence, it was hypothesised that the *mpx*:GFP zebrafish would be a suitable model for high-throughput phenotype-based drug discovery.

3.1.1 A preliminary screen for accelerators of inflammation resolution

During my time as an undergraduate summer student in Dr. Renshaw's laboratory, I performed a preliminary drug screen to identify novel accelerators of inflammation resolution using the *mpx*:GFP zebrafish model (Loynes et al., 2009). Larvae at 3 days post fertilisation (dpf) were injured by complete transection of the tail fin, to initiate an inflammatory response involving recruitment of neutrophils expressing green-fluorescent protein (GFP) to the site of injury. By 4 hours post injury (hpi), the number of neutrophils present at the site of injury approaches the expected peak value (Renshaw et al., 2006). At this time point, larvae with high numbers of neutrophils at the site of injury were selected for treatment with chemical compounds. During the resolution phase, at a time point relative to 12 hpi, numbers of neutrophils at the wound were manually counted by eye. This enabled identification of any compound treated larvae that appeared to have considerably fewer neutrophils remaining at the site of injury compared to the vehicle controls. Compounds causing a reduction in neutrophil number were subsequently defined as positive hits, or assay positive compounds.

A total of 960 compounds were screened, 79 of which were identified as positive hits on initial testing. For validation, all positive hits were rescreened and 8 compounds were found to have reproducible effects, including the known anti-inflammatory flumethasone (Loynes et al., 2009). This preliminary screen provided encouraging evidence that by using simple experimental techniques, the *mpx*:GFP zebrafish model could be used to identify compounds with anti-inflammatory properties.

3.1.2 Aims and hypotheses

The aims of this chapter are to design and optimise a high-throughput screening assay using the *mpx*:GFP zebrafish model, based on the preliminary screening work

previously published (Loynes et al., 2009). I hypothesised that this unbiased screening approach will lead to the discovery of novel compounds that can drive inflammation resolution. I also hypothesised that the compounds identified might have the potential to uncover currently unknown signalling mechanisms involved in neutrophil clearance from sites of inflammation.

3.2 Design and optimisation of a high-throughput screening assay in zebrafish to identify novel accelerators of inflammation resolution

Despite being reasonably high-throughput, with 960 compounds being screened in 8 weeks, the preliminary screening protocol described above was certainly not without its limitations. There were a number of ways in which the techniques could be improved to develop a robust and reliable assay with therapeutic potential. The percentage of compounds that were identified as positive in the initial screen (8.2%) was almost ten-fold higher than the percentage in which the effect could be reproduced (0.83%), suggesting that the rate of detection of 'false positives' was reasonably high. It was apparent that multiple variables needed to be considered when designing and optimising the screening assay to ensure its function and reliability. In order to increase the throughput of the screening assay and determine the optimum conditions, a number of experimental factors were taken into account. Amongst others, these included age of the larvae, the optimum time points for compound treatment and identification of assay positives, incubation temperature and suitable positive control compounds.

3.2.1 Characterisation of the *Tg(mpx:GFP)i114* zebrafish model

Initially, a series of experiments were carried out to determine whether the *mpx*:GFP zebrafish was a suitable model for high-throughput drug screening. Furthermore, I wished to test whether the number of neutrophils participating in the inflammatory response could be statistically analysed using standard parametric methods. For parametric analyses to be appropriate, data should be drawn from a population with normal, or Gaussian, distribution (Altman, 1991).

I first performed a simple time course experiment to characterise the inflammatory response in zebrafish larvae following tail fin transection. At 3 dpf, *mpx*:GFP larvae were injured and the number of neutrophils present at the site of injury were recorded at time intervals of 2, 4, 6, 8, 12 and 24 hpi (Figure 3.1). This revealed that neutrophils were recruited to the wound within 2 hours and this phase of recruitment continued until around 6 hpi, when the peak number of neutrophils was reached. From 6 hpi onwards, neutrophil number declined, characteristic of the resolution phase of inflammation. By 24 hpi, neutrophil numbers had almost returned to basal levels. This timely and spontaneous resolution of the inflammatory response was in keeping with published data, occurring with comparable kinetics to mammalian systems (Renshaw et al., 2006).

Next, experiments were performed in order to determine some of the optimum conditions for the screening assay. I also wished to test for normal distribution in the number of neutrophils participating in the inflammatory response at certain experimental time points. The initial variable considered was larval age. For these experiments, *mpx*:GFP embryos were obtained by marble tank mating and raised to either 2 or 3 dpf. Larvae were injured and the numbers of neutrophils at the site of injury were counted in randomly selected populations of larvae at both 6 hpi, the peak of inflammation, and at 12 hpi. These time points were chosen based on the data obtained in Figure 3.1 and from the preliminary screen (Loynes et al., 2009), in which a time point of 12 hpi was used to identify assay positive compounds. At 12 hpi, neutrophil numbers in control, untreated larvae remain relatively high, but numbers in larvae incubated with potential pro-resolving compounds are likely to be considerably lower.

Neutrophil numbers at the wound were normally distributed in both 2 dpf (Figure 3.2 A, B) and 3 dpf larvae (Figure 3.2 C, D) obtained by marble tank mating, at both time points tested. Larvae at 4 dpf were not included in these experiments, as due to development of the swim bladder they are buoyant and difficult to manipulate, making them unsuitable for use in a high-throughput screening assay.

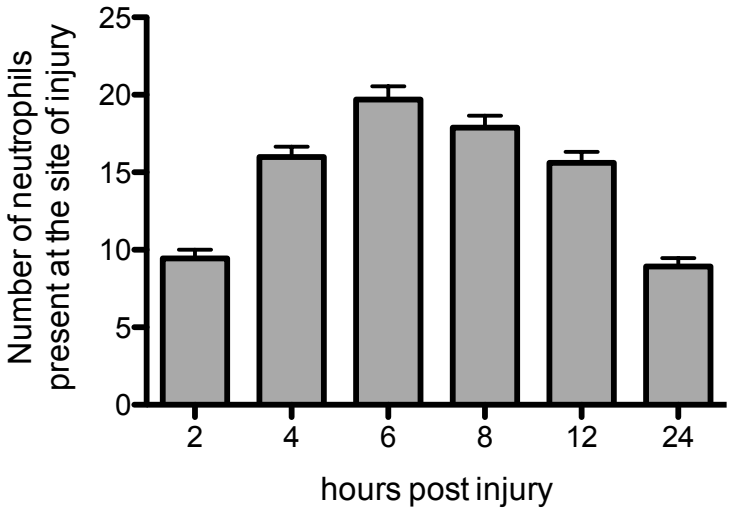

Figure 3.1: The inflammatory response in mpx:GFP larvae spontaneously resolves over time.

Tail transection was performed on *mpx*:GFP larvae at 3 dpf. The numbers of neutrophils at the site of injury were counted at 2, 4, 6, 8, 12 and 24 hpi. Neutrophil number at the wound increases up to 6 hpi then begins to decline. Neutrophilic inflammation appears to resolve from approximately 24 hpi (*n* = 72; performed on 3 independent occasions).

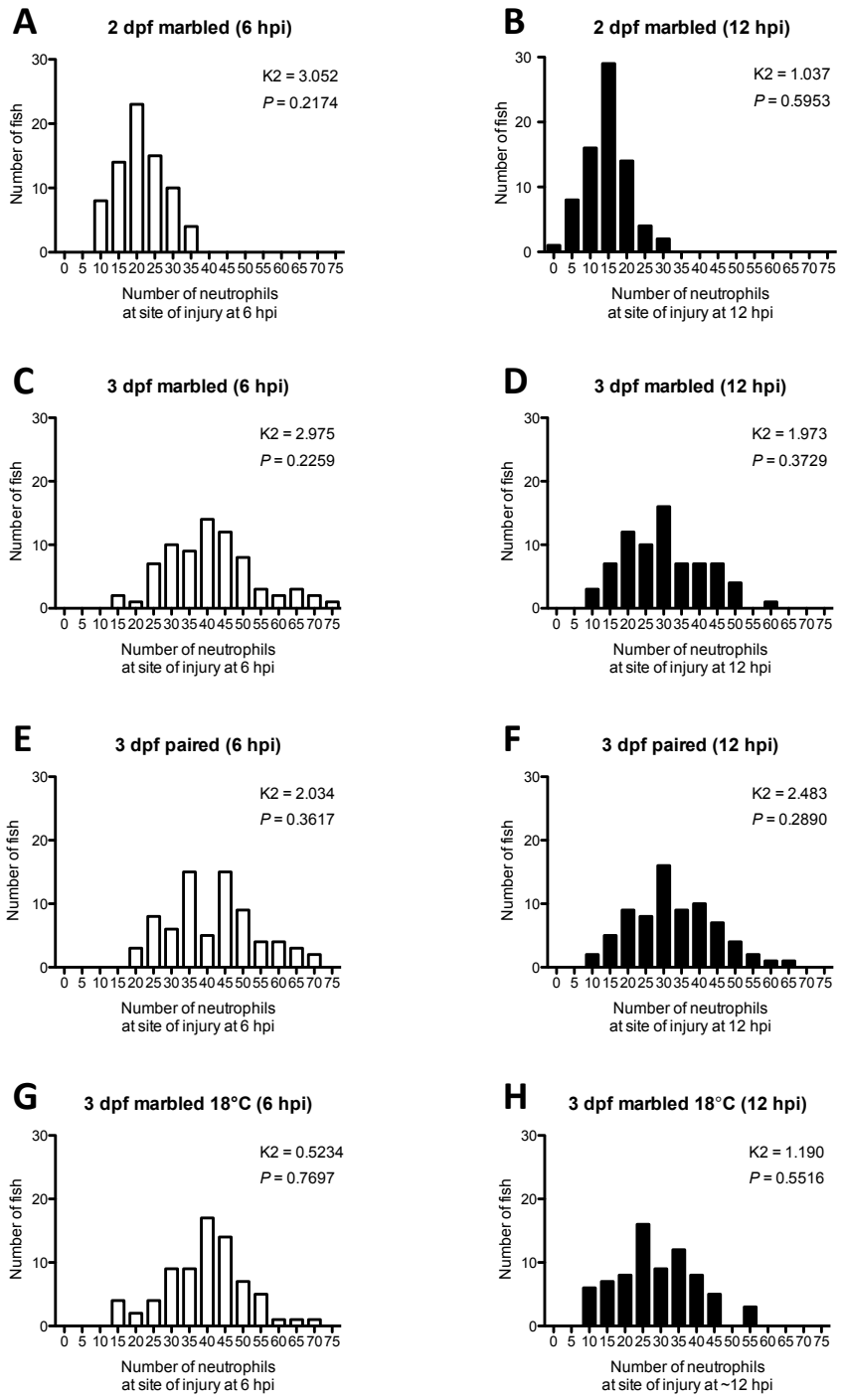

Figure 3.2: Neutrophil numbers at the site of injury are normally distributed at 6 and 12 hours post injury.

(A, B) 2 dpf; (C, D) 3 dpf marbled; (E, F) 3 dpf paired; and (G, H) 3 dpf incubated at 18°C *mpx*:GFP larvae were injured and the numbers of neutrophils at the site of injury were counted at 6 and 12 hpi. Frequency distributions in terms of the number of fish with a neutrophil number within each bin (width = 5) are represented in histograms. All data were consistent with a normal distribution (D'Agostino - Pearson omnibus K2 normality test; where $P < 0.05$ represents a non-Gaussian distribution; $n = 74$ for all groups, performed on 3 independent occasions).

It was clear that the neutrophil numbers in 2 dpf larvae were much lower than in 3 dpf larvae and there was a significant difference in variance between these groups at both time points (Bartlett's test for equal variances; $P < 0.0001$). As 12 hpi was the selected time point at which to screen and identify compounds that can reduce neutrophil numbers, it was essential to have a reasonably high number of neutrophils remaining at the site of injury in control larvae, such that positive hits could be easily and reliably detected. Neutrophil numbers were already comparatively low in 2 dpf larvae at 12 hpi and of course there was still a degree of natural variation. It is likely that this would make it potentially more difficult to detect any true effect on the reduction in neutrophil number caused by pro-resolution activity in compound treated 2 dpf larvae. For this reason, subsequent comparisons to determine assay conditions were carried out on larvae at 3 dpf only.

Another variable to consider was the method of embryo generation. Larvae obtained by pair mating were compared to those obtained from marble tank mating, following the same experimental procedures. At both 6 and 12 hpi, neutrophil numbers at the wound were normally distributed (Figure 3.2 E, F) and there was no significant difference in variance compared to larvae obtained by marble tank mating (Bartlett's test for equal variances; $P = 0.5663$).

For practical reasons, the preliminary screen involved incubation of larvae at 18°C (Loynes et al., 2009). At this temperature, the rate of larval development and cellular behaviours was slowed, to allow the identification of assay positive compounds 24 hours after injury, which was relative to 12 hpi if the larvae had been incubated at 28°C. To investigate the effect of this temperature adjustment on variability, larvae obtained by marble tank mating were incubated at 18°C after the 6 hpi count and the 12 hpi count was recorded after the appropriate number of hours incubation. Again, neutrophil numbers at the wound were normally distributed at both time points (Figure 3.2 G, H) and there was no significant difference in variance compared to larvae obtained by either marble or pair mating and incubated at 28°C (Bartlett's test for equal variances; $P = 0.6376$).

These experiments indicated that data obtained from all groups of larvae were consistent with a normal distribution, supporting the use of this zebrafish model for screening and parametric statistical analysis.

I also wished to directly compare the mean number of neutrophils present at the site of injury in all groups of larvae. This was calculated using the same data, which revealed that the mean number of neutrophils at the wound was significantly lower in 2 dpf larvae compared to 3 dpf larvae from all conditions, at both 6 hpi and 12 hpi (Figure 3.3). This further supported the use of 3 dpf larvae, given that a relatively high number of neutrophils was required in order to detect assay positive compounds, as discussed earlier.

As the number of neutrophils at the site of injury was counted in individual larvae, the percentage change in the number of neutrophils between 6 and 12 hpi could also be calculated (Figure 3.4). There was neither a statistically significant difference in the percentage change between any of the 3 dpf groups, nor a significant difference in the variance (Bartlett's test for equal variances; $P = 0.5085$).

The results obtained from this series of experiments indicated that 3 dpf larvae generated by either pair or marble tank mating would be suitable to use for screening. If it were necessary to incubate larvae at 18°C, there would be no significant effect on the normal acute inflammatory response. However, for simplicity and to avoid any potential issues with compound precipitation at lower temperatures, I chose to incubate larvae at the usual temperature of 28°C.

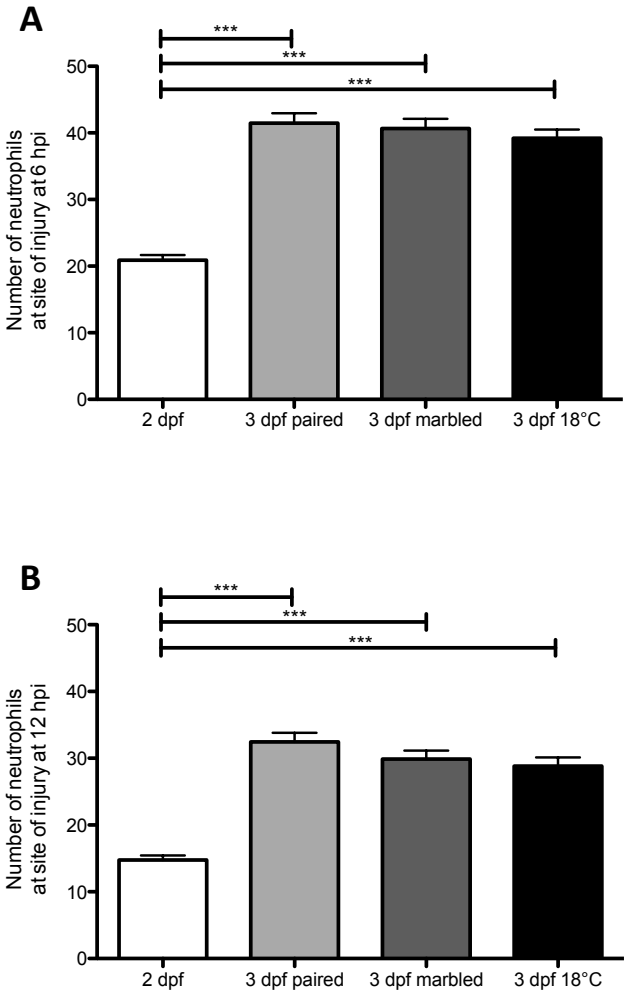

Figure 3.3: 2 dpf larvae have significantly fewer neutrophils present at the site of injury compared to 3 dpf larvae at both 6 and 12 hours post injury.

Larvae at 2 dpf and 3 dpf were injured and the numbers of neutrophils at the site of injury were counted at (A) 6 and (B) 12 hpi. At both time points, the mean number of neutrophils at the site of injury was significantly lower in 2 dpf larvae, compared to 3 dpf larvae generated by either pair mating or marble tank mating and 3 dpf larvae incubated at 18°C from 6 hpi (One-way ANOVA with Bonferroni's multiple comparison post-test; $P < 0.0001$; $n = 74$ for all groups, performed on 3 independent occasions).

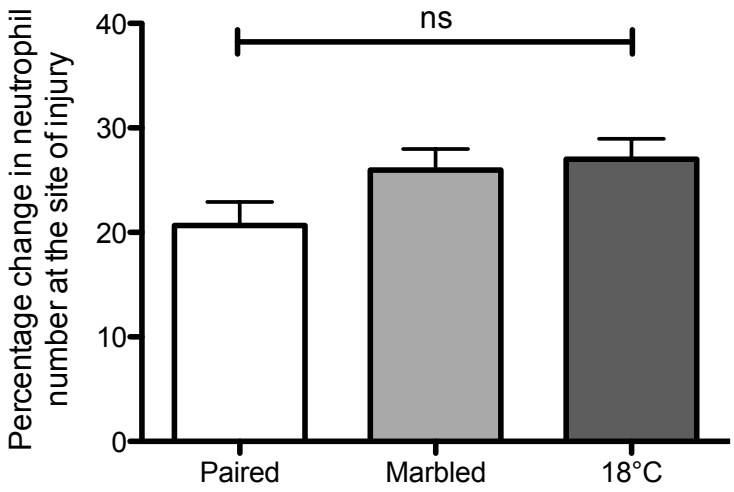

Figure 3.4: There is no difference in the percentage change in neutrophil number at the site of injury between 6 and 12 hpi in paired, marbled or 18°C incubated larvae.

At 3 dpf, larvae were injured and the numbers of neutrophils at the site of injury were counted at 6 and 12 hpi. The percentage change in neutrophil number between the two time points was calculated. No significant differences were found in the percentage change between paired, marbled or 18°C incubated larvae (One-way ANOVA with Bonferroni's multiple comparison post-test; $P = 0.0711$; $n = 74$, performed on 3 independent occasions).

3.2.2 Selection of larvae that mount a substantial inflammatory response to injury

The previous data illustrate that there is a high level of natural variability in the number of neutrophils participating in the inflammatory response in untreated 3 dpf larvae. This was of particular concern at the 12 hour time point, when variability could affect the reliable identification of positive hits and lead to an increased detection of false positives. In an attempt to overcome this, I only selected larvae with approximately 25 to 30 neutrophils recruited to the site of injury at 4 hpi (Figure 3.5). I also sought to ensure that all larvae were injured consistently, with the transection being as posterior as possible to the circulatory loop whilst remaining within the break in the pigment line. Experiments indicated that following this strict selection process, approximately 20% of injured larvae fell into the category of 'good responders'.

3.2.3 Use of the Phenosight High-Content screening system

To date, most zebrafish drug screens use at least partially automated screening technologies, to enable higher throughput and avoid excessive manual manipulation of larvae (Peterson et al., 2011; Takaki et al., 2012; Wittmann et al., 2012). In an endeavour to incorporate an automated element into the screening process, it was carried out using a prototype automated plate reader designed by Ash Biotech. This was able to perform a rapid scan of each 96-well plate, capturing both bright field and fluorescent images of each individual well in just 7 minutes (Figure 3.6). Fluorescent images generated were of adequate resolution quality to enable positive hit identification by either manual or automated analysis (described later), and also allowed immediate recognition of drug toxicity. In comparison to the preliminary screening method, using the automated plate scanning equipment was more efficient than manually removing larvae from each well for examination by eye, and also eliminated the need to make a subjective decision regarding which larvae appeared to have fewer neutrophils at the site of injury than the DMSO control larvae.

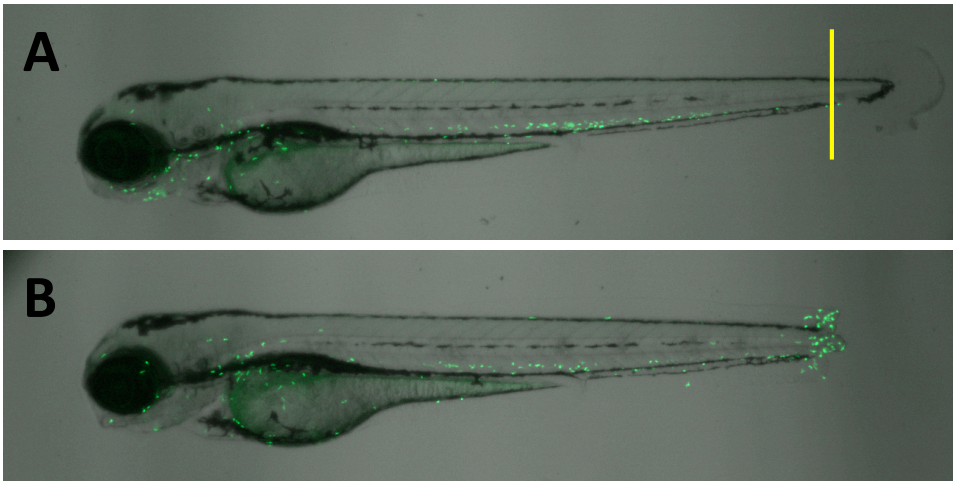

Figure 3.5: Selection of larvae that mount a substantial inflammatory response to tail fin injury.

(A) *mpx*:GFP larvae were injured consistently, as posterior as possible to the circulatory loop whilst remaining within the break in the pigment line, as indicated by the yellow line. (B) At 4 hpi, larvae were viewed under a fluorescent dissecting microscope and only those with approximately 25-30 neutrophils recruited to the site of injury (termed 'good responders') were selected for screening.

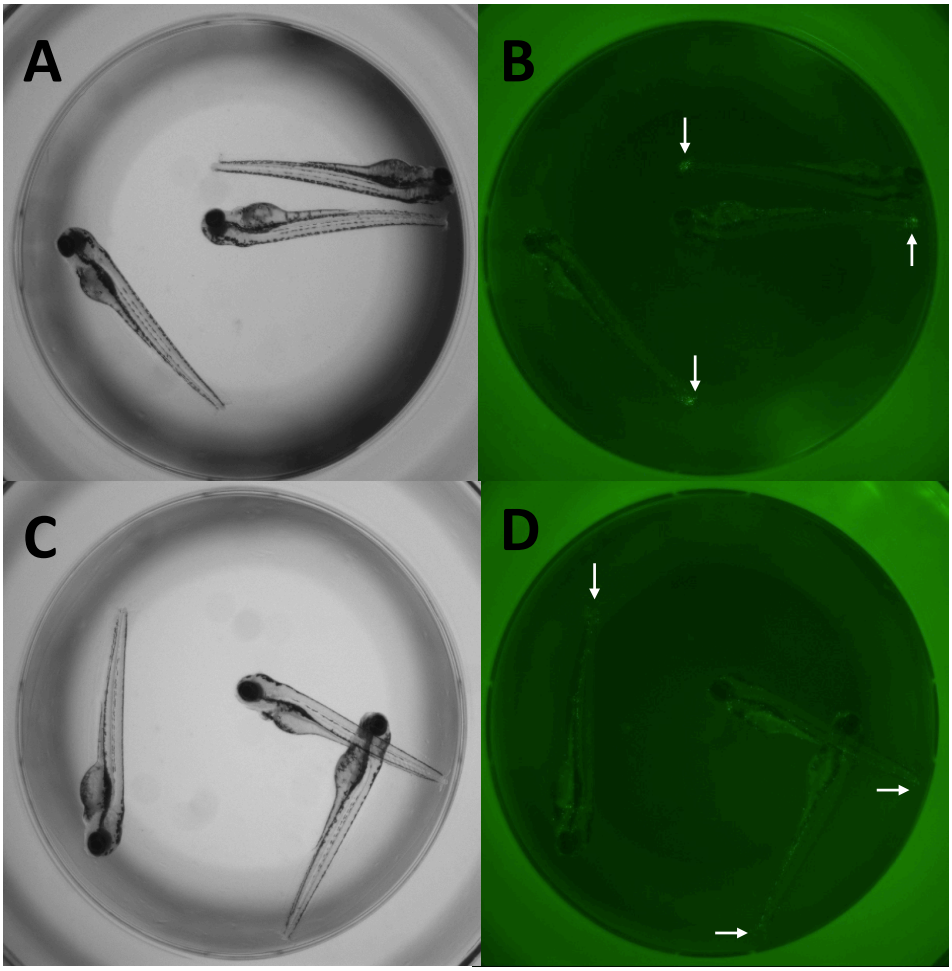

Figure 3.6: The Phenosight High-Content screening system generates whole well images to enable rapid identification of positive hits.

At 12 hpi, well plates were scanned on the Phenosight plate reader to generate bright field and fluorescent images of each individual well. Compounds that accelerate inflammation resolution could be immediately identified, for example in (A, B) DMSO control larvae have high numbers of neutrophils at the site of injury compared to (C, D), larvae treated with a compound that has caused a reduction in neutrophil numbers.

3.2.4 Determination of *n* number

In the preliminary screen, each compound was tested in a well containing 3 larvae. In general, increasing the number of fish per group (*n*) would decrease the inherent variability of the screening assay. However, this comes at the price of potentially reduced welfare and impaired imaging due to crowding of larvae in the wells. To establish the optimum number of larvae to incubate in each well of the screening plates so that their injured tail fins could be visualised for analysis, a test plate was set up containing 12 wells each of 3, 4 or 5 injured *mpx*:GFP larvae. At 12 hpi, the plate was scanned using the Phenosight system, and the images generated were viewed to calculate the number of wells in which the site of injury in all larvae could be seen clearly, without obstruction by other overlapping larvae or the edges of the well. As expected, the higher the number of larvae in each well, the lower the percentage frequency that the tails of all larvae in each well could be visualized (Figure 3.7). It was concluded that 3 larvae per well was optimum for screening using the Phenosight system, and as an additional measure to increase robustness it was determined that each compound would be screened on 2 independent occasions.

3.2.5 Development of a novel manual scoring method for analysis

Images generated by the automated plate reader were initially analysed using a manual scoring process. Scoring was designed such that each well containing 3 larvae was given a score between 0 and 3, dependent on the number of larvae within the well that appeared to have a reduced number of neutrophils at the site of injury compared to the DMSO control larvae (refer to Table 2.1).

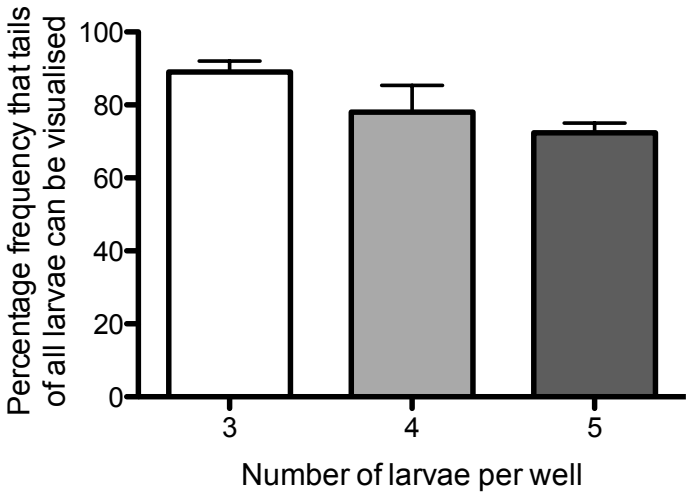

Figure 3.7: Determination of the optimum number of larvae per well.

At 12 hpi, a test plate containing 12 wells each of 3, 4 or 5 injured *mpx*:GFP larvae was scanned using the Phenosight system. The images were viewed to calculate the number of wells in which the site of injury in all larvae within each well could be visualised to allow accurate analysis. The higher the number of larvae in each well, the lower the percentage frequency that the tails of all larvae could be visualised, indicating that 3 larvae per well was the optimum number for screening ($n \geq 108$; performed on 3 independent occasions).

3.2.6 Identification of a reliable positive control

To identify a reliable positive control and ensure the manual scoring process was appropriate for the screening assay, compounds with known effects on neutrophil lifespan were tested. These included roscovitine, a CDK inhibitor that induces neutrophil apoptosis via down-regulation of Mcl-1 (Leitch et al., 2010), and pyocyanin, a phenazine pigment exotoxin produced by the bacteria *Pseudomonas aeruginosa*, which has been shown to induce neutrophil apoptosis *in vitro* and *in vivo* in both zebrafish and mouse models of inflammation (Usher et al., 2002; Allen et al., 2005; Loynes et al., 2009). GSK650394, an inhibitor of serum- and glucocorticoid-regulated kinase-1 (SGK-1) has been shown to drive inflammation resolution in the *mpx*:GFP zebrafish model (Burgon et al., in submission) and as such it was also considered as a potential positive control. The pan-caspase inhibitor zVAD.fmk delays inflammation resolution (Loynes et al., 2009) and was used to demonstrate the dynamic range of the system. Following the usual protocol, 3 dpf larvae were injured and good responders were selected at 4 hpi for treatment with the control compounds. At 12 hpi, the well plate was scanned on the Phenosight plate reader. The fluorescent images were analysed using the manual scoring method to assess reduction in neutrophil number at the site of injury, blinded to the experimental conditions (Figure 3.8).

Wells containing larvae treated with GSK650394 were given a significantly higher score compared to wells containing larvae treated with either E3 or DMSO, supporting its use as a positive control. Neither pyocyanin nor roscovitine were given significantly higher scores than the DMSO negative control, suggesting that they produced signals of insufficient amplitude to be reliably detected by this method. As expected, zVAD.fmk inhibited caspase-dependent neutrophil apoptosis, and no wells contained larvae with fewer neutrophils at the site of injury compared to larvae treated with DMSO.

To establish the optimum test dose, the experiment was repeated using varying concentrations of GSK650394. At both 9 and 10 μM, GSK650394 generated a significantly higher score than the E3 and DMSO vehicle controls (Figure 3.9). At doses higher than 10 μM, larvae began to display signs suggestive of toxicity.

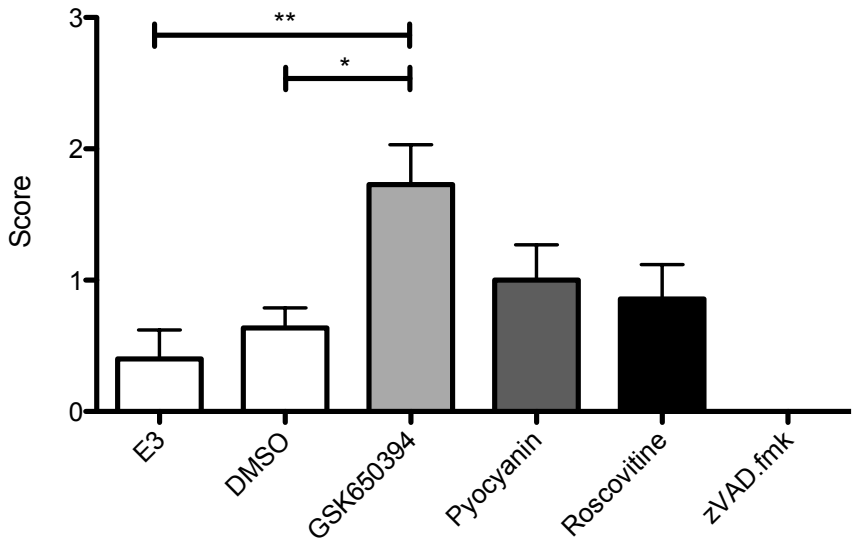

Figure 3.8: GSK650394 was the most effective positive control in the preliminary analysis performed using the manual scoring method.

At 3 dpf, larvae were injured and good responders were treated at 4 hpi with either E3, 1 % DMSO, 10 μM GSK650394, 75 μM pyocyanin, 40 μM roscovitine or 100 μM zVAD.fmk. At 12 hpi, larvae were imaged using the Phenosight system and each well was scored manually. Larvae treated with GSK650394 were given a significantly higher score (1.727 ± 0.304) than those treated with either E3 (0.400 ± 0.221) or DMSO (0.636 ± 0.152) (One-way ANOVA with Bonferroni's multiple comparison post-test to compare selected columns; where * $P < 0.05$ and ** $P < 0.01$; $n \geq 11$, performed on 3 independent occasions).

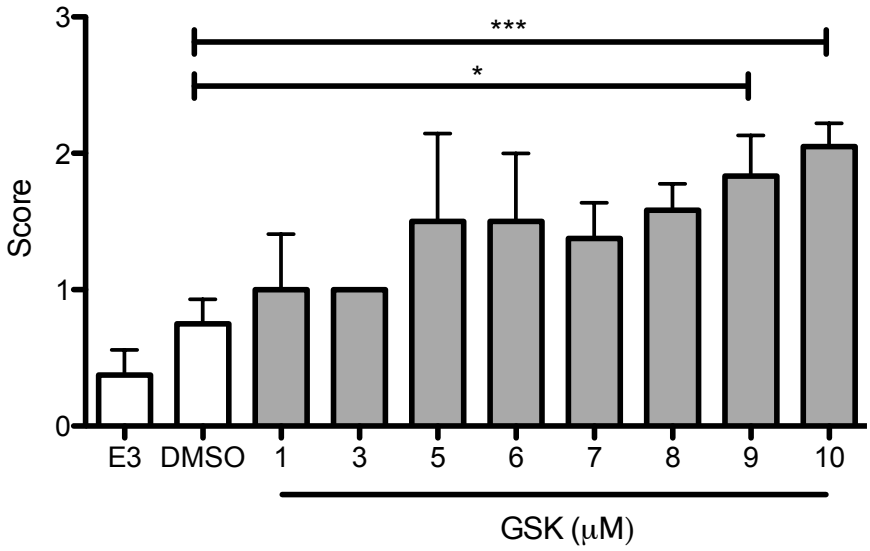

Figure 3.9: Wells containing larvae treated with GSK650394 score significantly higher than wells containing DMSO control treated larvae.

At 3 dpf, larvae were injured and good responders were treated at 4 hpi with E3, 1 % DMSO or varying concentrations of GSK650394. At 12 hpi, larvae were imaged using the Phenosight system, and each well was scored manually. Larvae treated with 9 and 10 μM GSK650394 scored significantly higher (1.833 ± 0.297 and 2.050 ± 0.170, respectively) than those treated with DMSO (0.750 ± 0.179) (One-way ANOVA with Bonferroni's multiple comparison post-test to compare selected columns; where * $P < 0.05$, and *** $P < 0.001$; $n \geq 12$, performed on 3 independent occasions).

3.2.7 Summary of screening assay conditions

In addition to the optimisation experiments described above, experiments to determine other conditions such as the volume of fluid per well and optimal type of well plate to use on the Phenosight system were also performed. The final assay conditions are summarised in Table 3.1. These experimental parameters, along with the implementation of the Phenosight screening platform to enable high-throughput data collection, aimed to improve the assay performance from that of the original preliminary screen, and the functionality of the novel screening method was tested using a small compound library, as described in the next section.

Table 3.1: Summary of optimised screening assay conditions.

Variable	Optimum condition determined by experiments
Method of embryo generation	Pairing or marbling
Age of larvae	3 dpf
Natural variability in numbers of neutrophils recruited to site of injury	Attempt to overcome by consistent tail fin injury and selection of 'good responders' only at 4 hpi
Time of compound treatment	Immediately following selection at 4 hpi
n number	3 larvae on 2 independent occasions
Positive control compound	10 µM GSK650394
Total volume per well	200 µl
Well plate	Clear plastic, flat-bottomed
Incubation temperature	28°C
Time point for image capture using Phenosight system	12 hpi

3.2.8 Evaluation of the novel screening protocol using the Merlion collection

To test the design, function and performance of the novel screening method, a small library of 98 natural products that were selected for induction of caspase-3 activity in a high-throughput *in vitro* assay was used for a pilot screen (Merlion Pharmaceuticals, Singapore). Plates of compounds were screened as described in the previous section and images generated by the Phenosight plate reader at 12 hpi were manually scored. The results from this pilot screen were also used as a sample dataset upon which to test an early version of automated analysis software, designed by Ash Biotech to complement the plate reader. This produced a read-out of fluorescence per unit area (FPU) at the site of injury in each larva, then for each compound the read-out was converted to the mean percentage FPU at the site of injury (refer to Figure 2.2).

Many compounds caused toxicity, likely due to widespread apoptosis caused by induction of caspase-3, but 9 compounds were identified as positive hits, with a mean score greater than or equal to 1.5, and a mean percentage fluorescence of less than or equal to 40%. The thresholds for positive hit selection were based on data obtained for the GSK650394 positive control, for which the mean (± SEM) manual score from all data combined was 1.625 (± 0.160) and the mean percentage specific FPU from the automated analysis was 36.03% (± 3.234) (Figure 3.10).

A significant correlation was observed between data generated by the Phenosight analysis software and the manual scoring method, as illustrated by data from Plate 2 of the Merlion collection (Figure 3.11). This suggested that both methods of analysis were adequate to produce valid and comparable results, however despite being partially automated, this version of the analysis software still required a large amount of manual user input and further development was necessary.

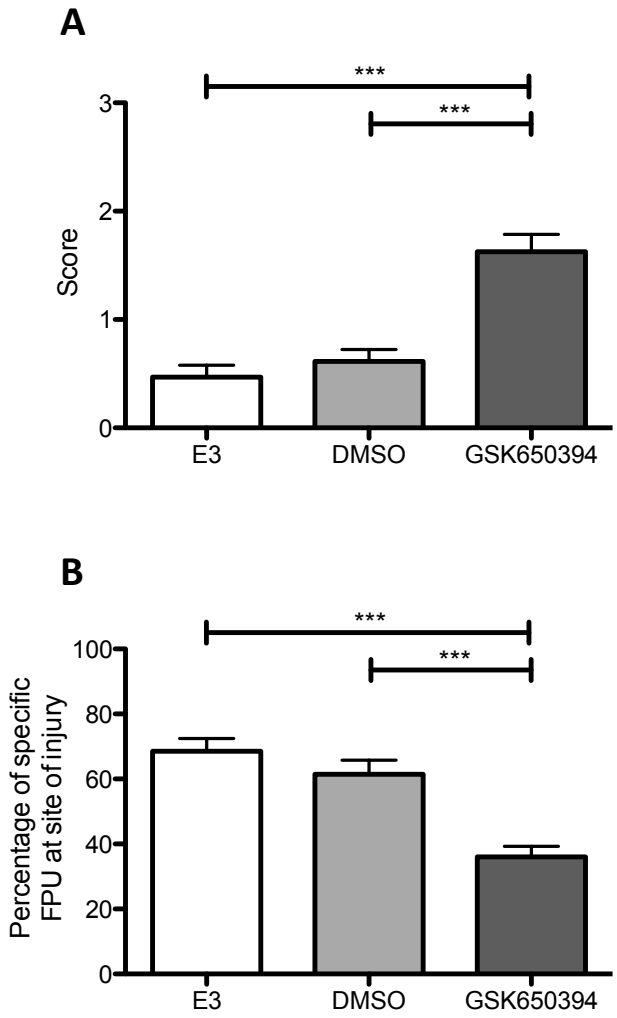

Figure 3.10: GSK650394 significantly increases manual scoring and significantly decreases percentage of specific FPU at the site of injury.

The Merlion natural product collection was screened using injured 3 dpf larvae that were selected as good responders at 4 hpi. Images generated by the Phenosight plate reader at 12 hpi were both manually scored and analysed using the automated Phenosight analysis software. The mean score (A) given to GSK treated larvae (1.625 ± 0.160) was significantly higher than both the E3 (0.469 ± 0.110) and DMSO (0.613 ± 0.111) negative controls, and the mean percentage specific FPU (B) of GSK treated larvae measured by the Phenosight analysis software (36.03 ± 3.234) was significantly lower than both the E3 (68.53 ± 3.927) and DMSO (61.49 ± 3.341) negative controls (One-way ANOVA with Bonferroni's multiple comparison post-test; $P < 0.0001$; $n = 32$ for (A), $n = 98$ for (B)).

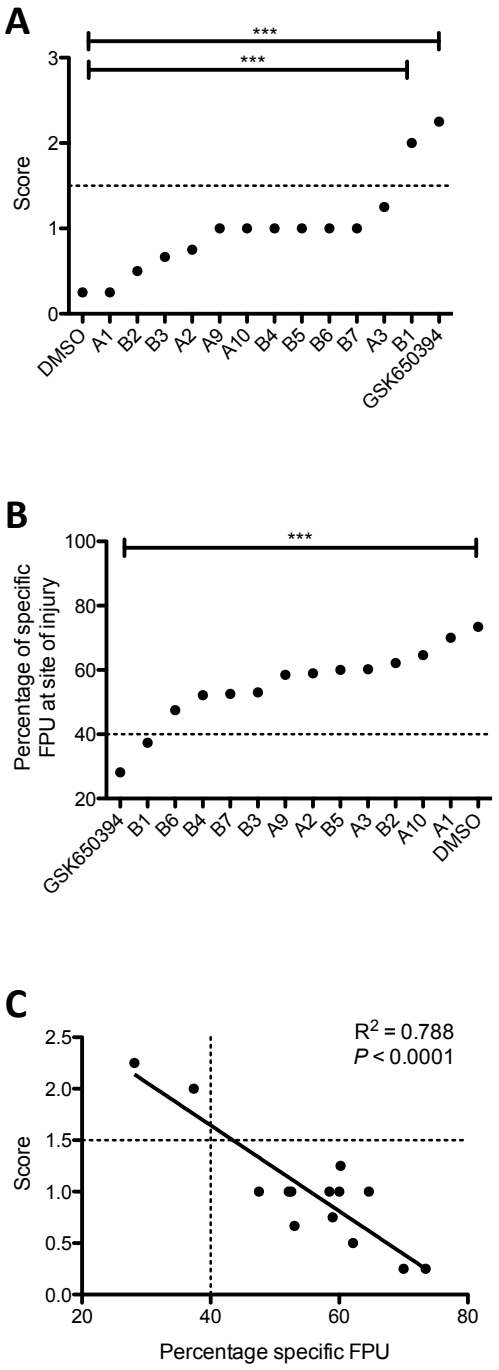

Figure 3.11: There is significant correlation between the manual scoring and automated analysis techniques.

Graphs show data obtained from Plate 2 of the Merlion collection. Dotted lines indicate threshold for positive hits. Compound B1 was identified as a hit by both the manual scoring method (A) and automated analysis software (B). B1 was given a significantly higher mean score (2.00 ± 0.41) than the DMSO control (0.25 ± 0.25), which was comparable to the score given to GSK650394 (2.25 ± 0.48) (One-way ANOVA with Dunnett's multiple comparison post-test; $P < 0.001$; $n = 12$). Linear regression analysis (C) showed a strong correlation between the two analysis techniques ($R^2 = 0.788$; $P < 0.0001$).

3.3 Identification and validation of novel compounds that can accelerate inflammation resolution

Following completion of the design and optimisation procedures, the novel screening method was used to perform an unbiased screen of the new 2010 version of the Spectrum Collection. This library was selected due to the broad biological activity and structural diversity of its contents. Alongside some known, predominantly synthetic drugs with confirmed targets and therapeutic action, it also contained some natural products with undefined biological activity, and other bioactive components such as enzyme inhibitors and receptor blockers. With screening of this type of chemical library, there is the potential to reveal novel applications for compounds that are already FDA-approved and have known toxicology profiles, as well as identifying key pathways involved in inflammation.

3.3.1 Primary screening of the Spectrum Collection

The Spectrum Collection screen was performed following the new protocol summarised in Table 3.1. Compounds were screened at 25 μM, a higher concentration than was used in the preliminary screen, to increase the potential to detect both pro-resolution activity of compounds and any toxic effects. Manual scoring results from a representative example plate of the Spectrum Collection are shown in Figure 3.12.

In some instances during the screen, the GSK650394 positive control compound was observed to form a precipitate, perhaps indicating an unstable nature, and it did not perform as effectively as in the pilot screen of the Merlion collection. As an alternative positive control, SP600125, an inhibitor of c-jun NH_2-terminal kinase (JNK), which has been suggested to affect TNFα-mediated neutrophil apoptosis (Hallett et al., 2008), was tested for its ability to drive inflammation resolution in the zebrafish. SP600125 significantly reduced neutrophil numbers at the site of injury at 12 hpi compared to the vehicle control (Figure 3.13 A), and replaced GSK650394 as the positive control in the screening assay. Although there was still a significant difference between the mean score of wells containing GSK650394 treated larvae compared to DMSO treated larvae, SP600125 scored significantly higher, making a more reliable control (Figure 3.13 B).

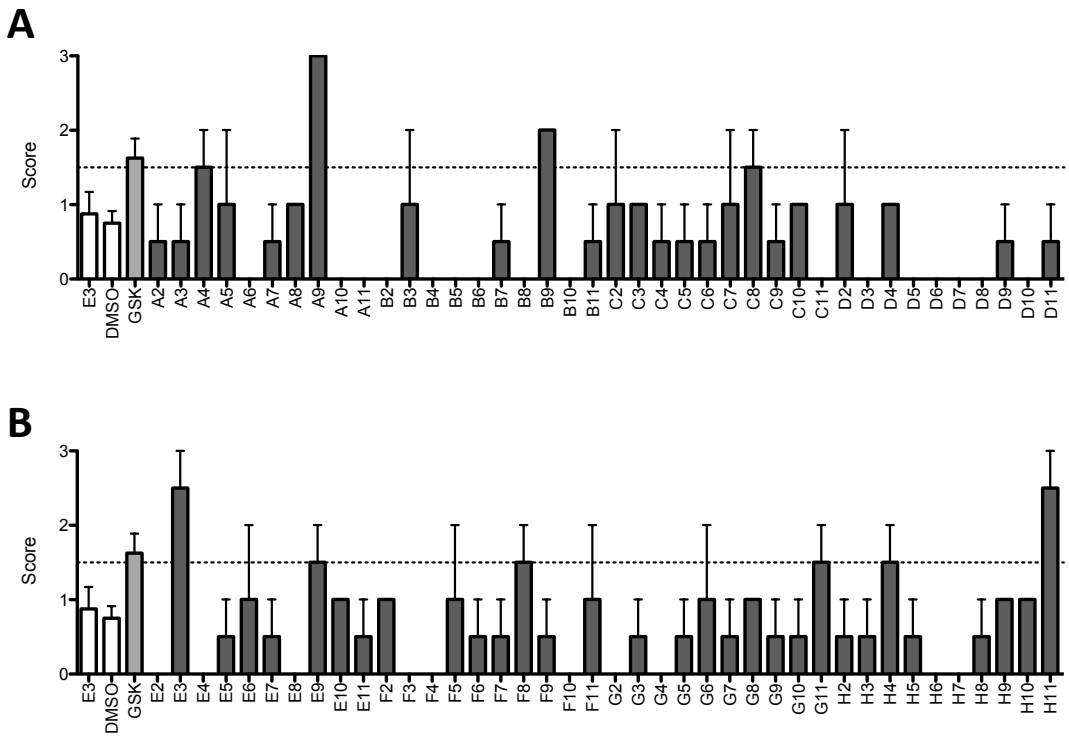

Figure 3.12: Manual scoring identifies 10 positive hits from primary screening of Plate 15 of the Spectrum Collection.

Compounds from Plate 15 were tested following the optimised screening protocol. At 12 hpi, the well plate was scanned using the Phenosight system and images generated were analysed by manual scoring. Graph (A) shows scores given to compounds in well plate rows A – D, and (B) shows those from rows E – H. With a threshold of 1.5, as indicated by the dotted line, 10 compounds were identified as positive hits for rescreening from this plate.

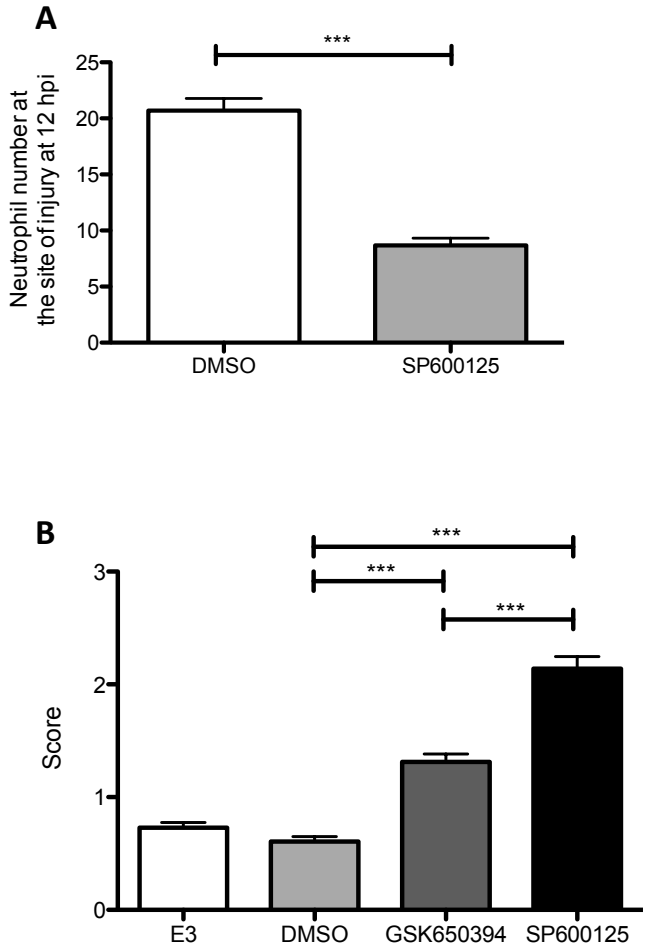

Figure 3.13: SP600125 is a more effective positive control than GSK650394.

(A) 3 dpf *mpx*:GFP larvae were injured and good responders were selected at 4 hpi for treatment with DMSO or 30 µM SP600125. At 12 hpi, neutrophils at the site of injury were counted by eye. SP600125 caused a significant reduction in neutrophil number (8.67 ± 0.65) compared to the DMSO control (20.70 ± 1.08) (Unpaired *t*-test; $P < 0.0001$; $n = 30$). (B) Graph shows combined scoring data from the Spectrum Collection screen. Although GSK650394 scored significantly higher (1.31 ± 0.07) than the DMSO control (0.61 ± 0.04), SP600125 scored significantly higher still (2.14 ± 011) than GSK650394 (One-way ANOVA with Bonferroni's multiple comparison post-test to compare selected columns; $P < 0.001$; $n \geq 129$).

It should be noted that SP600125 has also been reported to disrupt granulocyte chemotaxis (Zhang et al., 2008), and its inhibitory effect on neutrophil recruitment to damaged neuromasts in the lateral line system has since been demonstrated in a chemically induced inflammation assay in zebrafish (d'Alençon et al., 2010). The effects of SP600125 on neutrophil recruitment to the tail fin wound following injury will be discussed later.

In the initial screen, 95 compounds were identified as positive hits, defined as having a mean score equal to or greater than 1.5 (Figure 3.14). The threshold for positive hit selection was maintained at this level, as this resulted in a manageable number of compounds to retest in secondary screening. Due to various constraints encountered during development of an improved version of the automated analysis software, analysis of the screening results relied exclusively on manual scoring.

3.3.2 Secondary screening of the Spectrum Collection identifies 21 compounds with pro-resolution activity

To validate the 95 positive hits identified in the initial screen, these compounds were retested in the same manner, but each was screened on 4 independent occasions to generate a mean score from 12 larvae. Using the positive hit threshold of 1.5, 21 compounds were found to have reproducible effects, reducing neutrophil numbers at the site of injury to a level comparable to or even lower than SP600125 (Figure 3.15). The most efficacious compounds, those scoring greater than or equal to 2, were selected for further investigation of their effects on the neutrophilic inflammatory response. These included some compounds with known anti-inflammatory properties, alongside some with undefined biological activity (Table 3.2).

In conclusion, this novel screening platform successfully identified 21 compounds that appeared to accelerate the resolution of inflammation in a model of acute resolving inflammation in the zebrafish. The overall positive hit rate from screening of the Spectrum Collection was 1.05%.

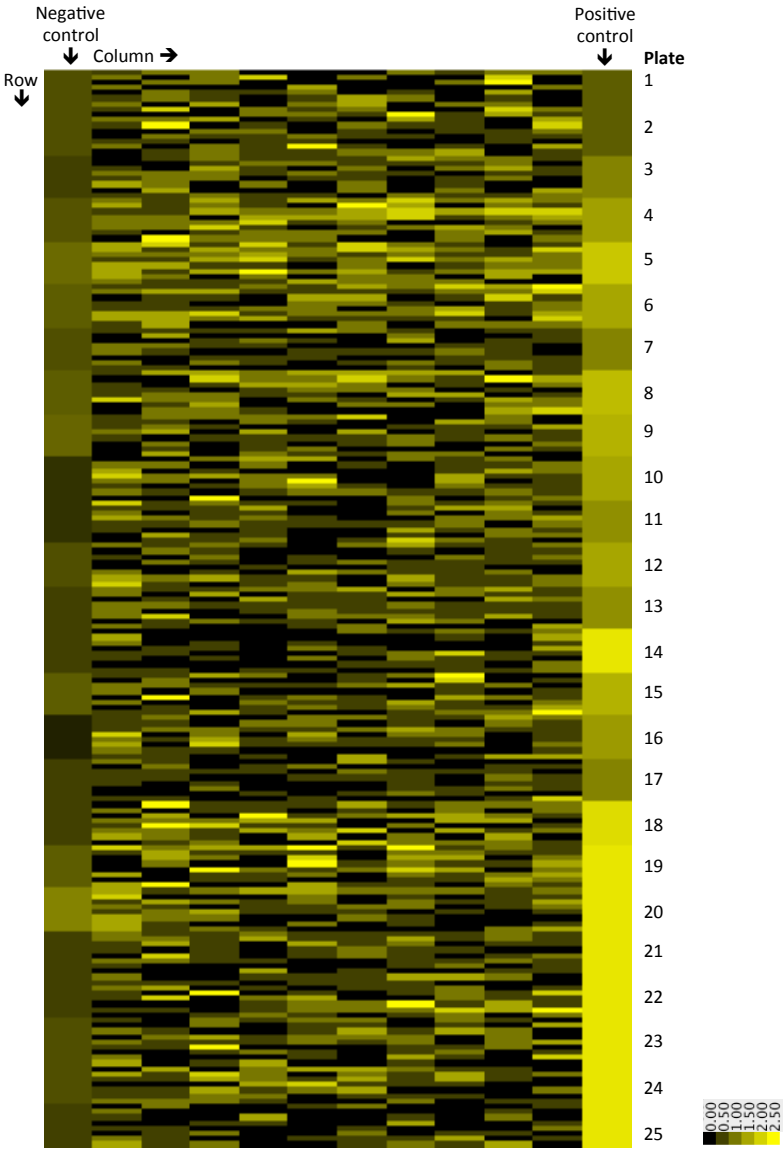

Figure 3.14: Primary screening of the Spectrum Collection identified 95 positive hits.

Representative heat map to display mean scores of all 2000 compounds from the Spectrum Collection. Mean negative control scores from each screening plate are shown down the left hand side, and mean positive control scores on the right. A total of 95 positive hits were identified in the primary screen, scoring equal to or greater than 1.5, as indicated by increasing colour ($n \geq 129$ for controls and $n = 6$ for screening compounds, performed on 2 independent occasions).

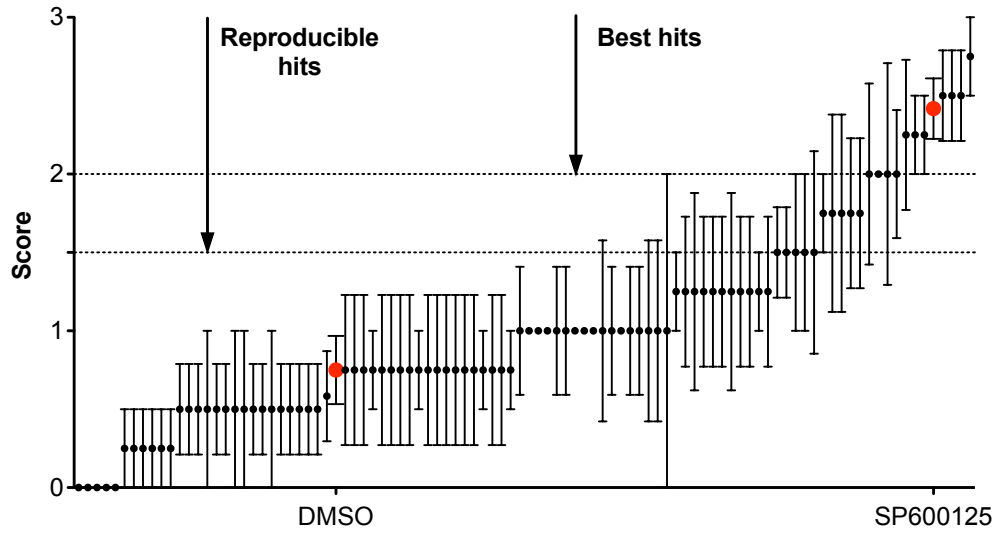

Figure 3.15: Secondary screening identifies 21 compounds with reproducible pro-resolution activity.

Positive hits identified in the primary screen were rescreened following the same protocol and 21 of these were found to be reproducibly positive, scoring greater than or equal to 1.5 (as indicated by the dotted line at y = 1.5). The most active compounds, those scoring greater than or equal to 2 (as indicated by the dotted line at y = 2), were defined as the best hits for further investigation (*n* = 36 for controls and *n* = 12 for screening compounds, performed on 4 independent occasions).

Table 3.2: Known anti-inflammatory properties of hit compounds.

Compound	Sources	Known anti-inflammatory properties
Cryptotanshinone	Diterpine quinone isolated from *Salvia miltiorrhiza* Traditional Chinese medicine used to treat cardiovascular and inflammatory disorders (Danshen)	Inhibition of iNOS and COX-2 expression in macrophages via NF-κB and AP-1 inhibition (Jeon et al., 2008; Li et al., 2011) Reduction of TNF-α and IL-6 via NF-κB and MAPK inhibition (Tang et al., 2011) Inhibition of macrophage chemotaxis via PI-3K inhibition (Don et al., 2007)
Deoxycholic acid	Secondary bile acid Traditional Chinese anti-inflammatory medicine (Niuhuang)	Inhibition of fMLP-induced neutrophil and monocyte chemotaxis (Chen et al., 2002)
Digitoxin	Cardiac glycoside isolated from *Digitalis lanata* and *Digitalis purpurea*	Inhibition of NF-κB, MCP-1 expression and monocyte migration in endothelial cells (Jagielska et al., 2009) Attenuation of IL-8 hypersecretion in cultured cystic fibrosis lung epithelial cells via NF-κB inhibition (Srivastava et al., 2004)
Erythromycin estolate	Macrolide antibiotic, derivative of erythromycin produced by *Streptomyces erythreus*	Inhibition of IL-8 production and neutrophil accumulation (Oishi et al., 1994) Inhibition of superoxide anion generation in neutrophils (Mitsuyama et al., 1995) Enhancement of phagocytosis of apoptotic neutrophils by macrophages (Yamaryo et al., 2003)
Gitoxigenin	Cardiac glycoside isolated from *Digitalis lanata* and *Digitalis purpurea*	No published data – likely to be similar to Digitoxin
Isopimpinellin	Furanocoumarin found in plants of the *Apiaceae* family e.g. carrot, celery, parsley	No published data
2-Benzoyl-5-methoxybenzoquinone	Synthetic	Derivatives shown to inhibit 5-LO (Titos et al., 2003)

3-O-Methylquercetin	Derivative of quercetin, flavonoid widespread in fruit and vegetables	Inhibition of degranulation and superoxide generation in neutrophils (Pečivová et al., 2012) Inhibition of iNOS protein and mRNA expression in macrophages via STAT1 and NF-κB inhibition (Hämäläinen et al., 2007) Inhibition of TNF-α and PDE3, reduction in neutrophil number (Ko et al., 2004) Reduction of neutrophil recruitment via inhibition of actin polymerisation (Souto et al., 2011)
Naproxen	Synthetic, available as prescription NSAID, used to treat chronic pain from arthritis, gout, menstrual cramps etc.	Inhibition of COX-1 and COX-2, reduction of prostaglandin and thromboxane production
Scopoletin	Naturally occurring coumarin widespread in flowering plants of the *Scopolia* genus	Reduction of NO, TNF-α and PGE_2 (Chang et al., 2012) Inhibition of NF-κB and MAPK in macrophages (Yao et al., 2012)
Tanshinone IIA	Diterpine quinone isolated from *Salvia miltiorrhiza* Traditional Chinese medicine used to treat cardiovascular and inflammatory disorders (Danshen)	Inhibition of NF-κB and AP-1 activation (Jang et al., 2006; Xu et al., 2009; Yang et al., 2010; An et al., 2011) Inhibition of iNOS, IL-1β, IL-6 and TNF- α expression (Fan et al., 2009) Inhibition of PLA_2 activity (Xu et al., 2009)
Xanthone	Naturally occurring in plants of the *Bonnetiaceae* and *Clusiaceae* families, particularly the mangosteen fruit	Inhibition of lysozyme release and superoxide generation in neutrophils (Lin et al., 1996)

3.4 Investigation into the effects of hit compounds on the neutrophilic inflammatory response

In order to characterise their specific effects on neutrophils, the highest scoring compounds from the Spectrum Collection screen were tested in a range of secondary assays in *mpx*:GFP zebrafish larvae. Cryptotanshinone, which obtained a mean score of 1.5, was also tested for interest due to its structural and potentially functional similarity to tanshinone IIA.

3.4.1 Effects on neutrophil recruitment

As described previously in the *Tg(mpx:GFP)i114* zebrafish model, neutrophil numbers at the injury site approach their peak at 4 hpi, but further recruitment can continue to occur up to approximately 6 hpi (Renshaw et al., 2006). In the screening assay, chemical treatment began shortly after selection of larvae at 4 hpi; therefore it was possible that the positive hits identified might have reduced neutrophil numbers at the injury site by preventing their recruitment later during the course of the inflammatory response. In order to more fully characterise the activity of the positive hits, specific assays were designed to distinguish between their effects on the recruitment and resolution phases of inflammation. For recruitment, larvae were treated immediately following tail fin injury, and the number of neutrophils at the site of injury was counted at 6 hpi. Each compound was tested at 3 different concentrations in order to investigate dose dependency.

Some, but not all, hit compounds caused a significant reduction in neutrophil recruitment to the wound compared to the DMSO vehicle control (Figure 3.16). At the highest concentration tested, the compounds cryptotanshinone and 2-benzoyl-5-methoxybenzoquinone caused mortality in almost all larvae. Signs of toxicity were also observed in larvae treated with digitoxin and deoxycholic acid.

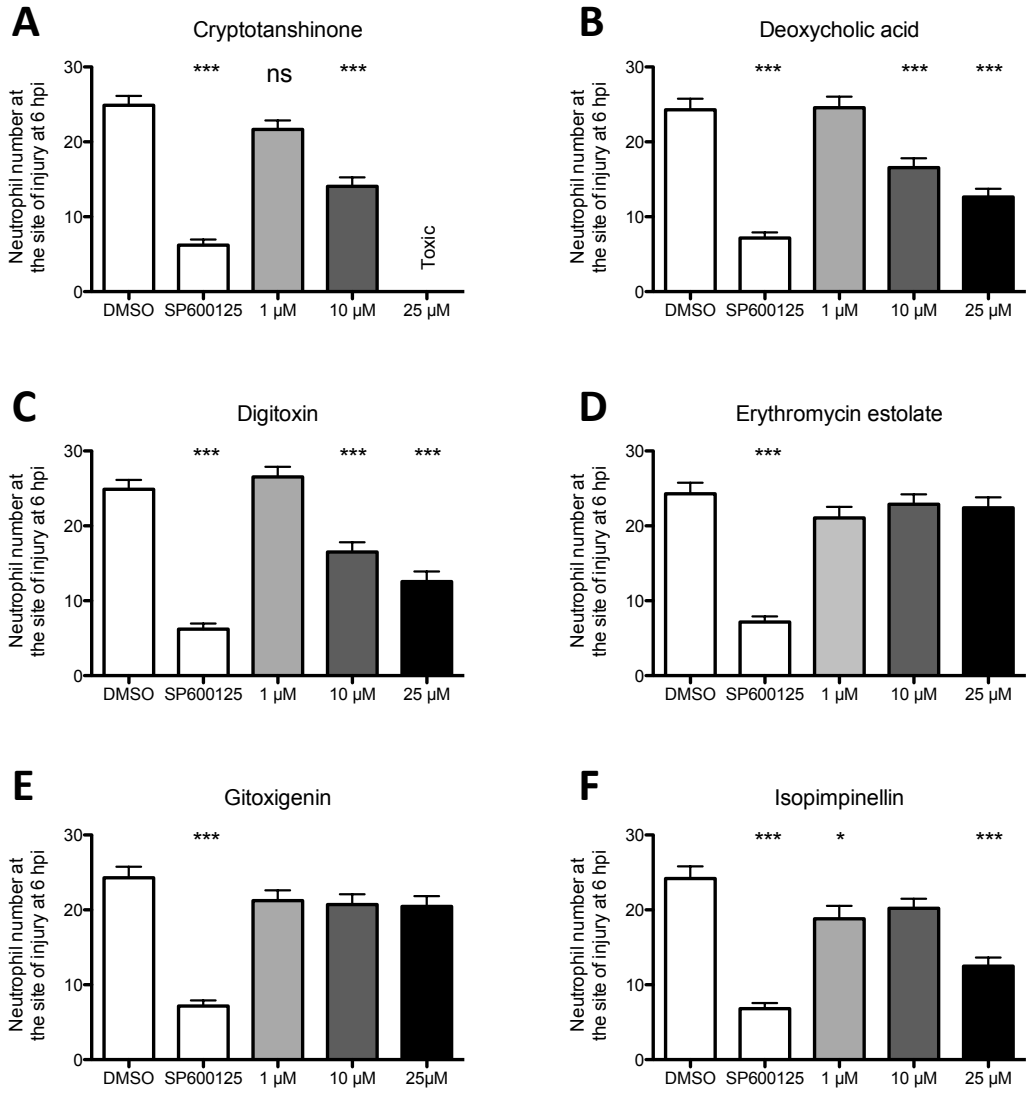

Figure 3.16: A subset of the positive hit compounds inhibit neutrophil recruitment.

At 3 dpf, *mpx*:GFP larvae were injured and treated immediately with compounds at 3 different doses. Neutrophil numbers at the site of injury were counted at 6 hpi. A) Cryptotanshinone B) Deoxycholic acid C) Digitoxin D) Erythromycin estolate E) Gitoxigenin F) Isopimpinellin (One-way ANOVA with Dunnett's multiple comparison post-test; where * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$; $n = 18$ performed as 3 independent experiments).

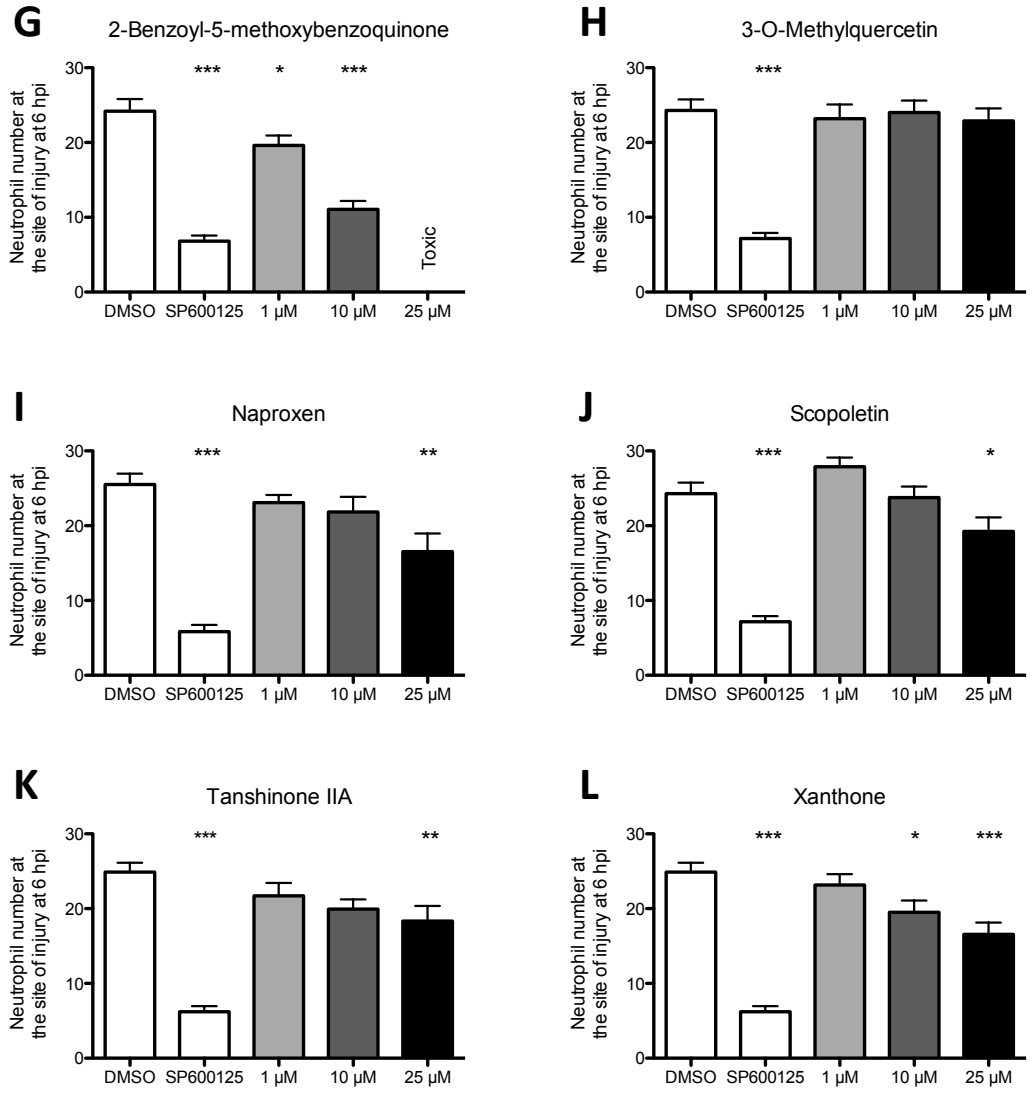

Figure 3.16: A subset of the positive hit compounds inhibit neutrophil recruitment.

At 3 dpf, *mpx*:GFP larvae were injured and treated immediately with compounds at 3 different doses. Neutrophil numbers at the site of injury were counted at 6 hpi. A) 2-Benzoyl-5-methoxybenzoquinone B) 3-O-Methylquercetin C) Naproxen D) Scopoletin E) Tanshinone IIA F) Xanthone (One-way ANOVA with Dunnett's multiple comparison post-test; where * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$; $n = 18$ performed as 3 independent experiments).

3.4.2 Effects on resolution of neutrophilic inflammation

To examine the effects of the positive hit compounds specifically on the resolution phase of the inflammatory response, larvae were injured and good responders were selected for treatment at 6 hpi, a time point at which most neutrophil recruitment has already occurred. Numbers of neutrophils remaining at the site of injury were counted at 12 hpi, to maintain consistency with the time point used to identify positive hits in the screening assay. All of the positive hits accelerated resolution by significantly reducing neutrophil numbers at the wound, and most of these appeared to act in a concentration-dependent manner (Figure 3.17). Toxicity was apparent in deoxycholic acid and 2-benzoyl-5-methoxybenzoquinone treated larvae and death occurred in those treated with the highest doses of cryptotanshinone and digitoxin.

3.4.3 Effects on total neutrophil number

To determine whether the hit compounds were acting specifically on activated neutrophils participating in the inflammatory response, uninjured larvae were incubated for 24 hours then mounted in agarose and imaged on an inverted compound fluorescence microscope using Volocity® software. Each compound was tested at the dose that caused the most significant effect on the resolution phase of the inflammatory response in the previous assay. Total neutrophil numbers were analysed using ImageJ software, which revealed that none of the hit compounds caused a significant reduction in the total neutrophil population compared to the vehicle control (Figure 3.18). Death was observed in all larvae treated with 25 μM deoxycholic acid and 10 μM digitoxin.

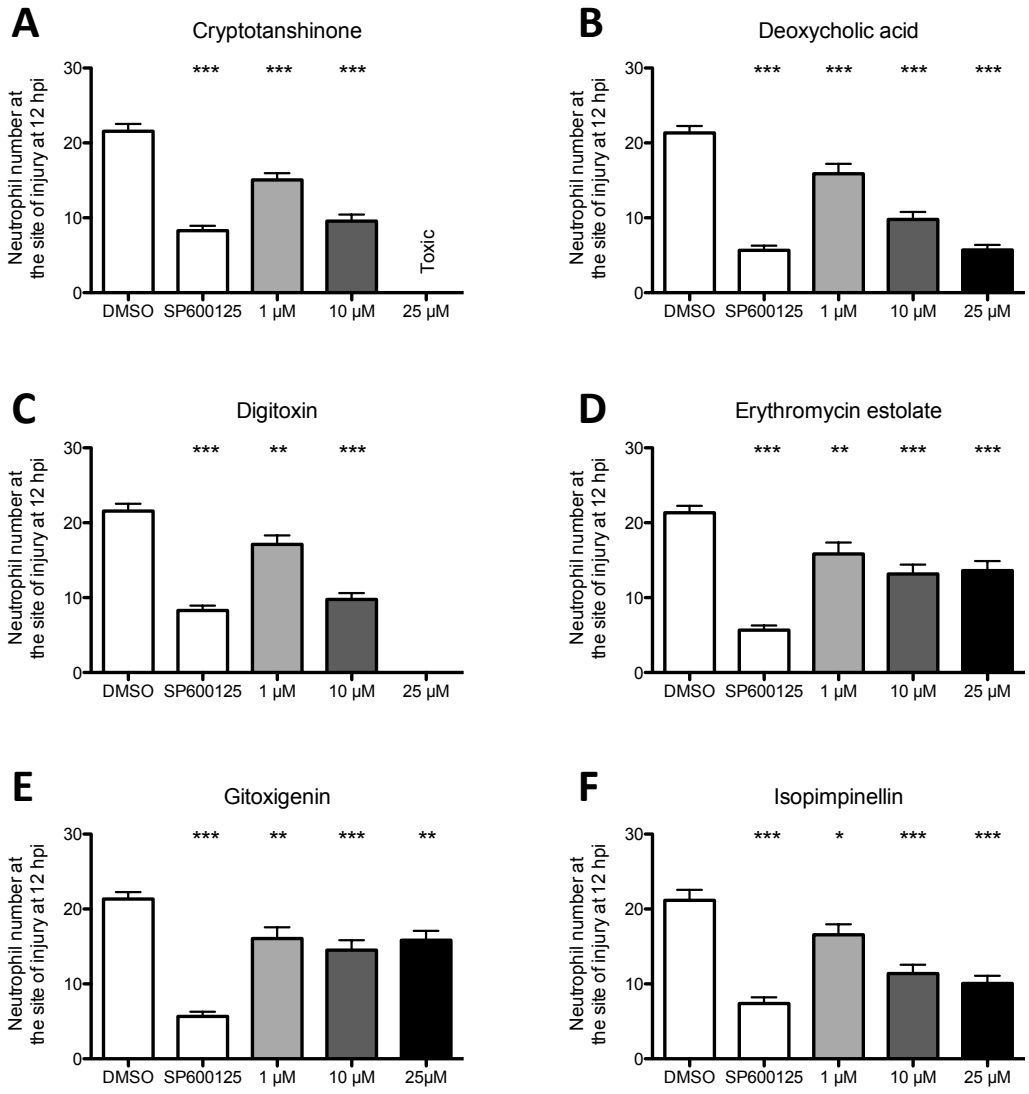

Figure 3.17: Positive hit compounds significantly reduce neutrophil numbers at 12 hpi to accelerate the resolution of inflammation.

At 3 dpf, *mpx*:GFP larvae were injured and treated with compounds at 3 different doses at 6 hpi. Neutrophil numbers at the site of injury were counted at 12 hpi. A) Cryptotanshinone B) Deoxycholic acid C) Digitoxin D) Erythromycin estolate E) Gitoxigenin F) Isopimpinellin (One-way ANOVA with Dunnett's multiple comparison post-test; where * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$; $n = 18$ performed as 3 independent experiments).

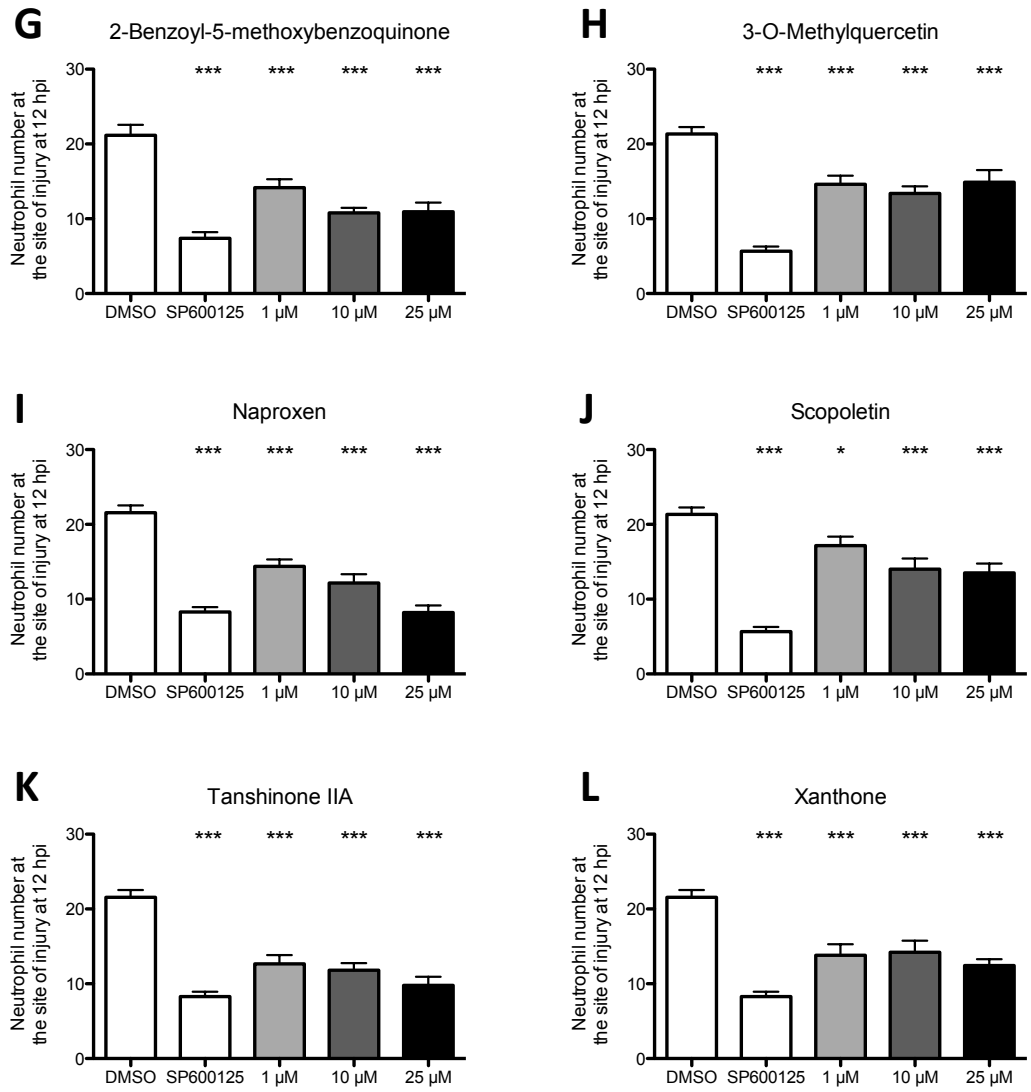

Figure 3.17: Positive hit compounds significantly reduce neutrophil numbers at 12 hpi to accelerate the resolution of inflammation.

At 3 dpf, *mpx*:GFP larvae were injured and treated with compounds at 3 different doses at 6 hpi. Neutrophil numbers at the site of injury were counted at 12 hpi. A) 2-Benzoyl-5-methoxybenzoquinone B) 3-O-Methylquercetin C) Naproxen D) Scopoletin E) Tanshinone IIA F) Xanthone (One-way ANOVA with Dunnett's multiple comparison post-test; where * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$; $n = 18$ performed as 3 independent experiments).

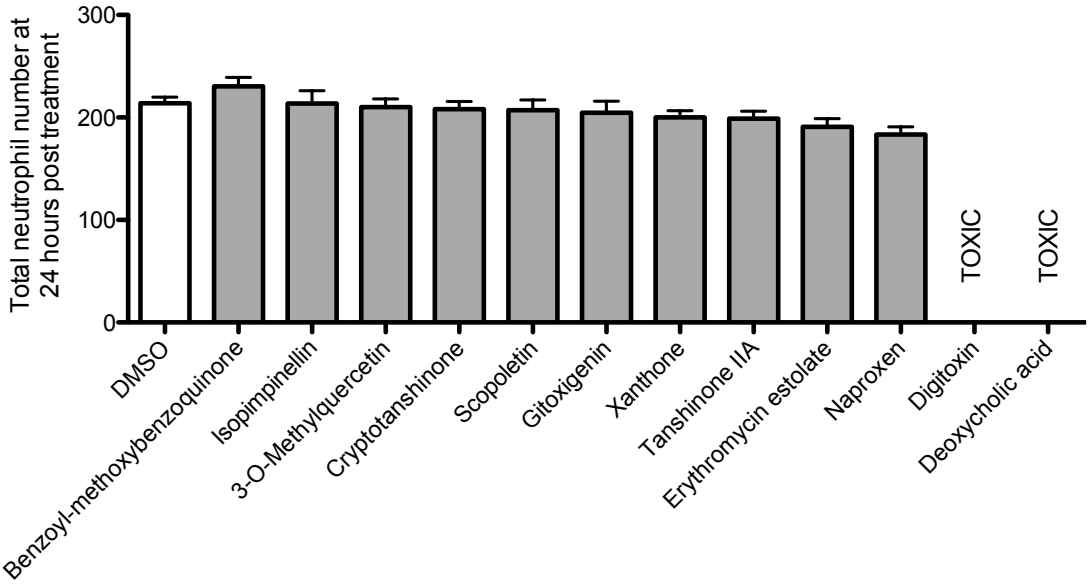

Figure 3.18: Assay positive compounds have no significant effects on total neutrophil number.

At 3 dpf, *mpx*:GFP larvae were incubated with hit compounds (using the dose that produced the maximum response in the resolution assay) for 24 hours. Larvae were then mounted and imaged on an inverted compound fluorescence microscope using Volocity® software and the total neutrophil number in each larva was analysed using ImageJ software. No significant differences were found between the mean total neutrophil number of DMSO control larvae and any of the hit compounds tested (One-way ANOVA with Dunnett's multiple comparison post-test; *n* = 18 performed as 3 independent experiments).

3.4.4 Summary of the effects of hit compounds on the neutrophilic inflammatory response

To summarise the data obtained in the secondary assays, hit compounds were ranked according to their maximum response, expressed as a percentage of the effect of the positive control, SP600125 (Figure 3.19). This enabled identification of the compounds that had the greatest effect on both neutrophil recruitment and resolution. Compounds that most significantly affected recruitment of neutrophils to the wound included isopimpinellin and 2-benzoyl-5-methoxybenzoquinone. Those with the most potent pro-resolution properties included tanshinone IIA, cryptotanshinone and naproxen. Digitoxin and deoxycholic acid were omitted from the ranking due to their toxicity in the total neutrophil number assays, as it was determined that their mechanisms of action would not be worthwhile pursuing.

3.4.5 Comparison of positive hits with known pharmacological regulators of inflammatory signalling pathways

Following investigation of the specific effects of each of the hit compounds on neutrophil recruitment, resolution and total neutrophil number, I chose to compare their activity in these assays with the activity profile of some known compounds with established anti-inflammatory activity, with the idea that this might provide clues towards identifying their mechanisms of action. A small, focused compound library was obtained from GlaxoSmithKline (GSK), containing known inhibitors of key inflammatory signalling pathway components including PI-3K, MAPK and PDE-4. Each of these compounds was tested in recruitment, resolution and total neutrophil number assays, performed in the same manner as described above. In this instance the recruitment and total neutrophil number assays were carried out by our technician, Aleksandra Bojarczuk.

To compare the compound activity profiles I used Cluster 3.0; clustering software originally designed for analysis of gene expression data, but which could be applied in a similar manner to the numerical data generated from each of my secondary assays.

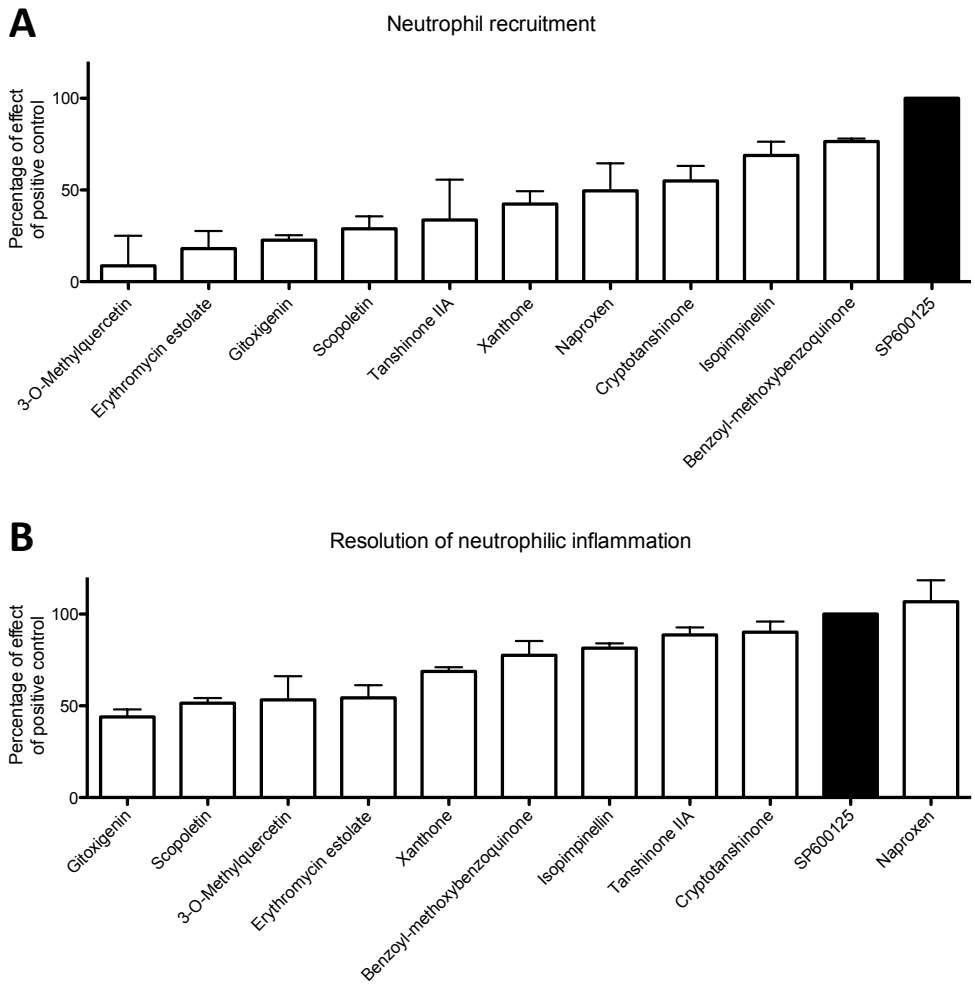

Figure 3.19: Summary and ranking of the effects of positive hit compounds on neutrophil recruitment and resolution of inflammation.

Compounds are ranked according to their maximum response, expressed as a percentage of the effect of SP600125. (A) Effect of hit compounds on neutrophil recruitment to the site of injury at 6 hpi, after treatment of injured 3 dpf *mpx*:GFP larvae immediately following injury. Doses shown are 1 µM for erythromycin estolate, 10 µM for cryptotanshinone and benzoyl-methoxybenzoquinone, and 25 µM for all other compounds. (B) Effect of hit compounds on resolution in terms of neutrophil number remaining at the site of injury at 12 hpi, following treatment of injured 3 dpf *mpx*:GFP larvae at 6 hpi. Doses shown are 25 µM for scopoletin, xanthone, isopimpinellin, tanshinone IIA and naproxen, and 10 µM for all other compounds.

For recruitment and resolution assays, data were normalised by expressing as a percentage of the effect of the positive control SP600125, and for total neutrophil number assays, data were expressed as the percentage change from the DMSO vehicle control. Data were clustered according to assay activity, using a hierarchical average-linkage clustering method, and the resulting heat map was generated using Java TreeView (Figure 3.20). Digitoxin and deoxycholic acid were omitted due to their toxicity in the total neutrophil number assays.

This cluster analysis revealed that the majority of the positive hits identified in my screening assay formed a group isolated from most of the known inhibitors of inflammatory signalling pathways that were tested in parallel. These compounds demonstrated potent pro-resolution activity with only moderate effects on neutrophil recruitment. Within this group, tanshinone IIA clustered with the PDE-4 inhibitor roflumilast.

Two of the positive hits, isopimpinellin and 2-benzoyl-5-methoxybenzoquinone, displayed an alternative phenotype, diverging from the main collection of pro-resolution compounds and clustering closely with the pan PI-3K inhibitor ZSTK474. In addition to accelerating inflammation resolution, these compounds caused a highly significant reduction in neutrophil recruitment.

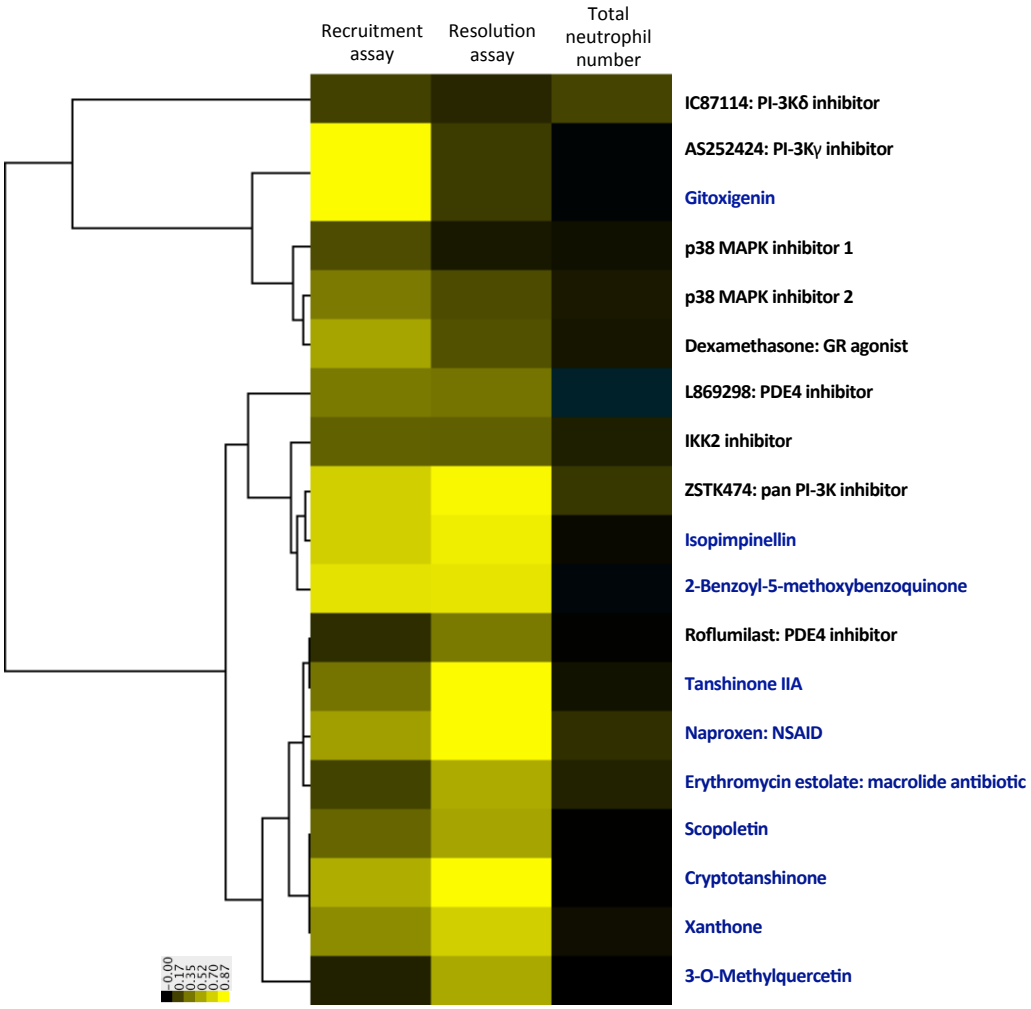

Figure 3.20: Hierarchical cluster analysis reveals hit compounds fall into distinct groups based on their activity profiles.

Clustergram represents secondary assay data for hit compounds (named in blue) and compounds obtained from GSK (named in black). Recruitment and resolution assay data are normalised by expressing as a percentage of the effect of SP600125, and total neutrophil number assay data are expressed as the percentage change from the DMSO control. Data were clustered according to assay activity using Cluster 3.0 and a hierarchical average-linkage method, and the heat map was generated using Java TreeView. Most hit compounds formed an isolated group, with potent pro-resolution activity and only moderate effects on neutrophil recruitment. Compounds outside of this cluster included isopimpinellin and 2-benzoyl-5-methoxybenzoquinone, which exhibit powerful effects on both recruitment and resolution, and gitoxigenin, which displays a recruitment only phenotype.

3.5 Discussion

In this chapter I have described the design, implementation and output of a novel drug screen for accelerators of inflammation resolution in a zebrafish model of acute inflammation. The screen identified 21 compounds that could reproducibly promote resolution by reducing neutrophil numbers at the injured tail fin. Further characterisation of the most active of these compounds has revealed a number which significantly enhance the resolution phase of inflammation in a dose-dependent manner, along with a subset which have both anti-recruitment and pro-resolution properties.

3.5.1 The *Tg(mpx:GFP)i114* zebrafish as a model for pro-resolution drug screening

As discussed in the introduction, the zebrafish immune system shares many of the features of mammalian systems, and in injured *Tg(mpx:GFP)i114* larvae, the onset and resolution phases of the acute inflammatory response proceed with comparable kinetics (Renshaw et al., 2006). It is well established that neutrophil clearance is a critical prerequisite for the resolution of acute inflammation in mammalian models (Gilroy et al., 2003; Rossi et al., 2006; Sawatzky et al., 2006), and this has likewise been demonstrated in zebrafish (Loynes et al., 2009). Simple analysis of neutrophil number at the tail fin wound thus provides a relevant read-out indicative of the degree of inflammation, enabling identification of pharmacological agents that modulate resolution. This approach has been validated by evidence showing that mediators known to promote neutrophil survival, such as caspase inhibitors and LPS, prevent resolution of inflammation by retaining neutrophils at the tail fin wound. In contrast, known inducers of apoptosis including pyocyanin and roscovitine, reduce inflammatory neutrophil numbers, accelerating resolution (Loynes et al., 2009). It must not be forgotten that the neutrophil is only one component of the complex system of cells and signalling molecules that contribute to the processes that define inflammation resolution.

Since the original investigation into granulocyte recruitment to an area of ‘minor trauma’ (Lieschke, 2001), tail fin wounding by either scalpel or needle injury has been used by a number of research groups as a model for studying the cellular and

molecular dynamics of inflammation (Renshaw et al., 2006; Mathias et al., 2006; Hall et al., 2007; Ellett et al., 2011). Similar research has also been undertaken by stimulating an inflammatory response using small, laser induced wounds (Feng et al., 2010; Yoo and Huttenlocher, 2011). The inflammatory response induced by tail fin injury can be genetically or pharmacologically manipulated to cause persistent inflammation, being of more relevance to the human chronic inflammatory disease state, and providing a useful tool to dissect the mechanisms involved (Mathias et al., 2007; Elks et al., 2011). Of course, the results obtained from screening of the Spectrum Collection are indicative of compounds that can promote the resolution phase of the acute inflammatory response, and these need to be validated in models of persistent inflammation, which will be discussed later. The preliminary compound screen discussed in this chapter has already been published, illustrating the potential impact of this model to identify novel lead candidates for inflammatory disease therapy (Loynes et al., 2009).

It is evident that as a model for high-throughput drug screening, the zebrafish has a number of advantages over mammalian systems, although it is important to consider alternative zebrafish inflammation models, developed with chemical screening in mind. One group have designed a chemically induced inflammation assay, termed 'ChIn' assay, with the idea to reduce manual manipulation of larvae and avoid physical fin injury (d'Alençon et al., 2010). Exposure of zebrafish larvae to copper sulphate, which induces oxidative stress and selective damage to sensory hair cells within the lateral line neuromasts (Olivari et al., 2008), initiates a rapid inflammatory response, with recruitment of leukocytes after just 15 minutes and resolution around 3 hours later. The response can be quantified in terms of cell numbers both manually and by automated processing and is susceptible to pharmacological manipulation by known anti-inflammatory compounds.

This model has been used to set up an automated screening platform with robotic liquid handling and automated microscopy. However, some steps remain labour-intensive, such as sorting of larvae, arraying them into well plates, and ensuring they are correctly oriented to enable reliable neuromast detection and analysis (Wittmann

et al., 2012). The authors screened a library of 640 compounds, initially identifying 45 with anti-inflammatory properties and 7 that appeared to promote resolution, however on retesting of these potential pro-resolution compounds, they were unable to confirm their findings. Although it is obvious when there are profound effects on leukocyte recruitment to the damaged neuromasts, perhaps this model is less appropriate for reliable detection of novel compounds with pro-resolving properties, possibly due to the transitory time course of the inflammatory response induced by copper sulphate treatment. This in addition makes it less analogous to the human acute inflammatory response than the simple tail fin wounding assay. Leukocyte recruitment to the damaged neuromast can be impaired genetically, for example in the zebrafish *was* mutant (d'Alençon et al., 2010). It can also be inhibited pharmacologically by known anti-inflammatory drugs, such as indomethacin, and by using diphenyleneiodonium (DPI) to inhibit NADPH oxidase, which implies that formation of a H_2O_2 gradient is important in copper-mediated wounding as well as the response induced by physical insult (Niethammer et al., 2009; d'Alençon et al., 2010). Looking at the resolution phase of inflammation, the authors suggest that by a reduction in prostaglandin synthesis, some NSAIDs cause leukocyte persistence in their assay, though this observation is made just 3 hours after copper removal. This model might be better applied to the identification of anti-recruitment compounds, although further characterisation is necessary, for example to determine why the sensory hair cells of the lateral line are selectively susceptible to copper damage.

To date, apart from those mentioned above, there are no other published anti-inflammatory or pro-resolution drug screens in zebrafish. This highlights the impact and novelty of this research and the demand to identify new therapeutic targets and lead compounds that might be translatable to the human patient. Discovery of new pro-resolution compounds that can specifically act on neutrophil clearance pathways is of particular importance, as there already exists a number of anti-inflammatory therapies that target neutrophil recruitment, but their effectiveness is limited.

3.5.2 Design and optimisation of the screening assay

The preliminary screen, implemented during my summer studentship, was performed in order to test the hypothesis that novel pharmacological drivers of inflammation resolution could be discovered using the *mpx*:GFP tail fin injury model. Almost 1000 compounds were screened, identifying 4 compounds that significantly accelerated resolution, including the known anti-inflammatories flumethasone and thalidomide. Though simple, the preliminary screen provided adequate enough evidence to demonstrate that the concept was feasible, and that with some careful design and optimisation, a relatively high-throughput and robust screening platform could be developed using this model.

The *mpx*:GFP model was initially characterised by manual counting of neutrophils at the site of injury at 6 and 12 hpi, to determine whether there were any differences in the numbers of neutrophils participating in the inflammatory response in various populations of larvae. These experiments also confirmed that inflammatory neutrophil numbers were normally distributed, allowing statistical analysis to be performed using standard parametric tests. It was concluded that 3 dpf larvae were most suitable for screening, with no apparent differences in neutrophil numbers at the wound in larvae drawn from any of the distinct populations tested. Though less variable, there were significantly fewer neutrophils in 2 dpf larvae, the use of which would likely result in a less robust assay, with increased probability of identifying false positives. It was evident that measures needed to be taken to overcome the inherent natural variability in neutrophil numbers observed in 3 dpf larvae. To approach this, I introduced a strict selection process, whereby larvae were only chosen for screening if they had recruited between approximately 25 and 30 neutrophils to the wound by 4 hpi. Larvae with lower or much higher numbers of neutrophils were discarded. A time point of 4 hpi was used for larvae selection, followed by immediate compound treatment, as this allowed sufficient time to collect the appropriate number of good responders and incubate them with the screening compounds well before the inflammatory response would begin to resolve naturally. It was probable that some of the compounds tested would have inhibitory, or even augmenting, effects on the later stages of neutrophil recruitment, however this could be dissected later in secondary assays, when the

experimental set up did not involve dealing with such large numbers of larvae to select and pipette into well plates. The 12 hpi time point was chosen as the end point of the assay, in concurrence with the preliminary screen, as this provided ample time for compounds to exert their effects and high enough numbers of neutrophils were remaining at the injury site in control larvae to reliably identify any active compounds that caused significant neutrophil removal. During the initial stages of the primary Spectrum Collection screen, one plate was screened at both the 8 hpi and 12 hpi time points (data not shown) to compare the identification of positive hits, and in this instance fewer hits were detected at 8 hpi, supporting use of the 12 hpi end point.

3.5.3 Automation of the screening method

The development of automated equipment and analysis techniques is considered essential to creating high-throughput, robust and successful screening platforms. Though there exist various technologies capable of aliquoting chemicals and zebrafish larvae into multi-well plates, there is currently no equipment that can perform tail fin transection on large numbers of zebrafish larvae in an automated manner. While this is theoretically possible, for this screening assay in particular, larvae would require further processing through an automated fluorescence sorter capable of identifying those with an above threshold level of GFP specifically at the tail fin wound. These steps, along with aliquoting of selected larvae into prepared screening plates, would likely remain relatively time consuming and realistically might not be beneficial in terms of increasing assay performance and throughput. Considering these factors, the chemically induced assay (d' Alençon et al., 2010) lends itself more readily to automated technologies, emphasising one particular advantage over the injury model.

As an alternative, to partially automate the screening assay the Phenosight High-Content plate reader was introduced, enabling rapid collection of bright field and fluorescent images of each individual well, along with immediate recognition of drug toxicity. This was an improvement from the preliminary screening method, which required manual observation of each well by eye and subjective identification of potential positive hits. One caveat of the plate reader was that it partially dictated the possible *n* number that could be used in screening experiments, with experiments

suggesting that 3 larvae per well was optimum to avoid the issue of data loss from obscured tail fins. Though it might have been preferable to increase this *n* number, larger numbers of larvae would need to be injured to generate enough good responders for each experiment. In an effort to resolve this, each compound was screened on 3 larvae on 2 independent occasions. For primary screening, testing of each compound on a total of 6 larvae is at least consistent with if not higher than the *n* numbers used in various published zebrafish screens (North et al., 2007; Tran et al., 2007; Namdaran et al., 2012; Ridges et al., 2012).

A manual scoring method was designed to enable simple and rapid analysis of the fluorescent images generated by the Phenosight system, which resulted in a basic yet accurate measure of pro-resolution activity, and this approach was first tested on a series of known compounds with the aim to identify a suitable positive control. The SGK-1 inhibitor GSK650394 scored significantly higher than the E3 and DMSO controls, the mechanism of which is at least in part due to neutrophil apoptosis (Burgon et al., in submission). Other known inducers of neutrophil apoptosis, roscovitine and pyocyanin (Usher et al., 2002; Allen et al., 2005; Rossi et al., 2006), did not reliably score significantly higher than the negative controls. Although previous data from our laboratory (Loynes et al., 2009) has demonstrated that both of these compounds can significantly drive resolution in zebrafish larvae, it is possible that in this particular assay the Phenosight system is not sensitive enough to distinguish less potent activity, and only those compounds that have a profound effect on reducing neutrophil numbers at the injured tail fin will be detected. This is not necessarily a disadvantage of the screening platform, but rather should ensure that only the most promising lead compounds are identified for further investigation.

Automated analysis software was designed to complement the plate reader and provide a quantitative readout of neutrophil persistence at the wound in terms of the level of fluorescence. The trial version of this software was unable to identify the tail fin region in an automated manner; instead the appropriate area needed to be defined by the user in each individual larva. By selecting the wound region and comparing this to a distal region of equal area in each larva to account for any background GFP, I

calculated the mean specific fluorescence at the tail fin. This was a somewhat labour-intensive and time consuming process and also involved making a subjective decision of where to define each region; not ideal attributes for an automated system. Despite this, when the Merlion test screen was performed, results from the automated analysis software correlated well with those from manual scoring, indicating that the software held potential but that further development was necessary. This pilot screen was a valuable test for the reliability and functionality of the novel screening protocol, ensuring it was sufficiently optimised to proceed. It also provided an opportunity to determine the appropriate sample size required to generate accurate results with statistical power.

At the time of writing, a fully automated version of the software was in the final stages of development. Progress has been made, with the current program able to accurately identify larvae and define their orientation, but no numerical data can be generated.

3.5.4 The Spectrum Collection screen

The screening assay was designed with the primary aim of identifying compounds that act to drive inflammation resolution, with translatable therapeutic potential. There are of course hundreds of existing chemical libraries that are commercially available for screening purposes. The Spectrum Collection was selected as it contained a range of compounds with both defined and unknown biological activity, including some FDA-approved drugs and a variety of natural products. Using such a diverse library with broad pharmacological potential seemed like a practical starting point for the screen, particularly as it was phenotype-driven and many of the precise mechanisms governing the resolution of inflammation remain to be discovered. Screening of more focused chemical libraries, such as those specifically targeting kinases, have since been carried out in our laboratory now that the screening platform has been established.

The novel screening method was designed to be relatively high-throughput, with the potential for one operator to screen up to 640 compounds per week. This performance cannot easily be compared to other inflammation-based zebrafish screens, as although it is claimed that the ChIn assay is amenable to high-throughput screening, evidence to

date only describes results from a small library, and there is no published record to indicate the actual rate at which compounds can be screened robustly (Wittmann et al., 2012). Primary screening of the entire Spectrum Collection, with each compound plate screened twice, was completed over the course of 24 weeks. This was somewhat slower than estimated, with the limiting factor being embryo availability.

A minor problem that arose intermittently during screening of the Spectrum Collection was precipitation of the positive control GSK650394, which in many cases correlated with its inactivity, resulting in a lower analysis score than expected. This is perhaps indicative of an unstable tendency, and there is little published information available relating to the stability of this compound. The precipitation seemed to occur inconsistently with no obvious logical cause, and this prompted the use of an alternative positive control, the JNK inhibitor SP600125. How JNK regulates neutrophil apoptosis is not well defined, and there is controversial evidence over its potential role during inflammation resolution (Nolan et al., 1999; Avdi et al., 2001; Kato et al., 2008), though SP600125 caused a highly significant reduction in neutrophil numbers at the tail fin wound at 12 hpi in my zebrafish resolution assay. It would be of interest to more fully examine the effect of this inhibitor on neutrophil apoptosis in zebrafish, for example by TSA/TUNEL staining, and in purified human neutrophils, though there is some evidence to suggest that freshly isolated, unstimulated neutrophils actually have increased viability in the presence of SP600125 (Geering et al., 2011).

The effect of SP600125 on neutrophil recruitment is better defined, and it has been shown to attenuate JNK-mediated recruitment of both neutrophils and monocytes to the lung in murine models of LPS-induced acute lung injury (Arndt, Young and Worthen, 2005; Arndt, Young, Lieber, et al., 2005; Young et al., 2009). Studies in zebrafish suggest that SP600125 inhibits macrophage migration to a tail fin wound and that this is at least in part via the inhibition of MMP13 (matrix metalloproteinase 13), a downstream target of the JNK - AP-1 signal transduction pathway, which plays a role in the degradation and remodelling of the extracellular matrix to assist chemotaxis (Zhang et al., 2008). This research was carried out using the transgenic line *Tg(zlyz:EGFP)*, which is not macrophage-specific (Pase et al., 2012), but rather is likely

to label a large population of neutrophils, indicating that the recruitment of these cells is also inhibited by SP600125. This is reflected in the data from my recruitment assays in *mpx*:GFP larvae, which show a highly significant reduction in neutrophil numbers at the wound at 6 hpi, and inhibition of neutrophil recruitment is also observed with SP600125 in the copper-induced inflammation assay (d'Alençon et al., 2010). Although the precise effects of SP600125 on the inflammatory response *in vivo* are not fully understood, this compound acted in a much more consistent and potent manner during screening experiments than GSK650394, and was a reliable positive control throughout the remainder of the Spectrum Collection screen and the secondary assays.

From the primary screen, 95 compounds were identified as positive hits using a threshold score of 1.5. This was slightly higher than the mean score of GSK650394, but lower than that of SP600125, and resulted in a manageable number of hits to rescreen. It was unfortunate that a completed version of the automated Phenosight software was not available to perform a more thorough analysis of the screening results. Images could have been processed using the early version of the software, however this would have been incredibly time consuming and not worthwhile, as the manual scoring technique was proven to be effective, with significant correlation to the automated analysis, in the Merlion pilot screen. The initial positives were retested on much larger numbers of larvae for validation, and 21 of the initial hits were found to be reproducibly positive, resulting in a positive hit rate of 1.1%, this being a comparable hit rate to that expected in robust phenotype-based zebrafish screens (Zhang & Yeh, 2012), and a false positive rate of 3.7%. A certain number of false positives are likely to be expected due to the natural variability in zebrafish larvae, though optimisation of the screening process successfully reduced the false positive rate by half that estimated in the preliminary proof-of-principle screen (Table 3.3).

Table 3.3: Comparison of the hit rates between the preliminary and optimised screening protocols.

	Preliminary screen	Final screen
Number of compounds screened	960	2000
Number of initial positives	79 (8.2%)	95 (4.8%)
Number of reproducible positives	8 (0.8%)	21 (1.1%)
Number of false positives	71 (7.4%)	74 (3.7%)

It is also possible that some pro-resolution compounds were not detected in the primary screen, and the false negative rate can be predicted using the positive control data. For instance, if I consider all of the screening assay data from wells containing larvae treated with SP600125, a score of less than 2 was given to 6 wells, from a total of 55 wells. This suggests there is a potential false negative rate of up to 10.9%. It is conceivable that this might explain why some compounds within the library, for example those with known anti-inflammatory properties such as indomethacin, ibuprofen and other NSAIDs were not identified in the screen, though there are of course many other explanations that could account for this, including inappropriate dosage, absorption deficiency, toxicity or lack of function in zebrafish.

For a more robust assay it would be beneficial to increase the *n* number in the primary screen, though as discussed previously it would be difficult to obtain accurate data from more than 3 fish per well. Increasing the number of replicates would be preferable, but at the expense of throughput, and although there is likely to be a number of potentially interesting candidates that were undetected, it is more important to focus on the compounds that were identified as positive hits and performed well in both primary and secondary screening. All of the compounds that were validated in the secondary screen and selected for further investigation significantly reduced neutrophil numbers at 12 hpi in the resolution assays, providing clear supporting evidence for the optimised screening protocol in the successful identification of accelerators of inflammation resolution.

3.5.5 Positive hits identified in the Spectrum Collection screen

A variety of hits were identified in the screen, including a number of compounds with known anti-inflammatory activity, as listed in Table 3.2. The properties of 12 of the best positive hits were validated through secondary assays in zebrafish larvae, which were designed to dissect each phase of the inflammatory response in order to observe the effect of compounds specifically on neutrophil recruitment or resolution. All of the selected compounds caused significant and dose-dependent acceleration of resolution, and a subset of these had some inhibitory effects on neutrophil recruitment. Total neutrophil number assays were also performed, which confirmed that all of the hit compounds, excluding those with toxic effects, acted specifically on activated neutrophils at the site of injury, rather than reducing neutrophil numbers throughout the entire larvae.

To investigate whether the hit compounds identified in the screen shared functional activity with some known pharmacological regulators of key inflammatory signalling pathways, a small panel of compounds was acquired from GSK. These were tested in each of the secondary assays and hierarchical cluster analysis was performed to compare their activity profiles with the positive hits, to identify any potential shared mechanisms of action. Most of the hit compounds formed a distinct cluster separate to almost all of the GSK compounds, due to their primarily pro-resolution and only moderate anti-recruitment activity. This outcome was a good indication of the success of the optimised screening assay, as it was designed with the primary aim to identify novel compounds that could drive resolution. This group includes two known drugs that have been used in clinical practice; the non-selective COX inhibitor naproxen and the macrolide antibiotic erythromycin estolate, which were not selected for further investigation due to their well-documented activity and known side effect profiles. Detection of an antibiotic prompted the concern that the anti-inflammatory effect observed might be secondary to anti-bacterial activity, so to investigate this, the resolution assay for erythromycin estolate was repeated alongside penicillin-streptomycin (Appendix 3), which confirmed that antibiotic activity alone was not sufficient to drive inflammation resolution and that there was no additive anti-bacterial effect responsible for the reduction in neutrophil number when larvae were

exposed to both erythromycin estolate and penicillin-streptomycin in combination. Recently the anti-inflammatory properties of macrolide antibiotics have been realised, which comprise modulation of a host of pro-inflammatory responses, particularly in neutrophils (Steel et al., 2012).

A number of naturally occurring compounds were also present in the pro-resolution cluster, including scopoletin, methylquercetin and xanthone. These compounds have some reported anti-inflammatory activity (as listed in Table 3.2) and have been associated with ameliorative effects in mouse models of carrageenan-induced paw oedema and arachidonic acid-induced ear oedema (Librowski et al., 2005; Kwon et al., 2011; Chang et al., 2012). In zebrafish, it has been suggested that a related flavonoid, quercetin, can down-regulate the overexpression of IL-1β, TNF-α and COX-2 in a model of dopaminergic neuron damage (Zhang et al., 2011).

Although cryptotanshinone was not included in the category of 'best hits', it was tested in the secondary assays due to its close structural similarity to tanshinone IIA. Both of these compounds clustered into the pro-resolution group, and tanshinone IIA was closely associated with the PDE4 inhibitor roflumilast, which has been reported to reduce neutrophil numbers in animal models of inflammation (Underwood et al., 1998; Bundschuh et al., 2001). As cryptotanshinone and tanshinone IIA were the most potent accelerators of resolution identified in the screen, surpassed only by clinically available naproxen (refer to Figure 3.19), these compounds were selected for further investigation, which will be described in Chapter 5.

Cluster analysis revealed a different activity profile for two of the positive hits, isopimpinellin and 2-benzoyl-5-methoxybenzoquinone, which shared both highly significant anti-recruitment and pro-resolution effects, and clustered closely with the pan PI-3K inhibitor ZSTK474. Due to a lack of published material describing any effect of isopimpinellin on the inflammatory response, this was an interesting lead compound to pursue further, and this will be approached in the next chapter.

Two of the positive hits, deoxycholic acid and the cardiac glycoside digitoxin, caused mortality in the total neutrophil number assays, and as such these were not included in the cluster analysis. Gitoxigenin is another cardiac glycoside closely related to digitoxin, and although no toxicity was observed in the presence of this compound, it was the least effective driver of resolution identified in the screen and hence was not considered worthwhile pursuing. The mortality caused by digitoxin might be explained by the well-documented cardiotoxicity profile of this class of compounds, which have also been described as neurotoxic in zebrafish (Sun et al., 2012), though despite this there is on-going research into their development as potential anti-cancer therapeutics (Elbaz et al., 2012).

3.5.6 Conclusions

In conclusion, I have established a novel, partially automated screening platform using a zebrafish model of acute inflammation, with the potential to discover lead compounds that could be advantageous in the development of pro-resolution therapies. From screening of a large, diverse chemical library, I have successfully identified 21 compounds that can reproducibly promote resolution *in vivo*, and a number of the most effective of these compounds have been selected for further investigation to determine their mechanisms of action.

Chapter 4: Investigation into the Structure-Activity Relationships of the Positive Hit Compounds

4.1 Introduction

In the previous chapter, the most promising lead compounds identified in the Spectrum Collection screen were compared against a panel of known inhibitors of key inflammatory signalling pathways, to indicate the potential mechanisms behind their activity. Based on their activity profiles, most of the hit compounds clustered together to form a pro-resolution group separate from the known inhibitors. One exception of particular interest, isopimpinellin, clustered closely with the pan-PI-3K inhibitor, ZSTK474. This is an ATP-competitive inhibitor, specific to class I PI-3K isoforms (Kong & Yamori, 2010). The anti-inflammatory properties of isopimpinellin are unknown, thus I reasoned that it might be interesting to pursue this compound as a potential lead and attempt to dissect the mechanisms responsible for its activity. Isopimpinellin is a naturally occurring furanocoumarin found in plants of the Apiaceae family such as carrot and parsley. Along with other similar compounds, it has been investigated for its potential anti-carcinogenic properties (Kleiner et al., 2002; Prince et al., 2006).

In the secondary assays performed following the screen, isopimpinellin appeared to have powerful effects on both the recruitment and resolution phases of the inflammatory response induced by tail fin wounding in zebrafish larvae. As the hierarchical clustering discussed in Chapter 3 paired this hit compound with a known inhibitor of PI-3K signalling, I hypothesised that isopimpinellin acts via PI-3K to inhibit neutrophil recruitment and potentially neutrophil survival during resolution.

4.1.1 The role of PI-3K in neutrophil recruitment

It is well established that PI-3K is functionally important in neutrophil chemotaxis, a role that was initially revealed using the pan-PI-3K inhibitor, wortmannin (Okada et al., 1994). Since then studies have indicated that PI-3Kγ is the principal class I isoform involved in leukocyte migration. Neutrophils from PI-3Kγ$^{-/-}$ mice are unable to produce PIP_3 and phosphorylate AKT upon stimulation with chemotactic agents signalling via GPCRs, such as IL-8, fMLP and C5a, resulting in an impaired motility and also reduced

respiratory burst (Hirsch, 2000). PI-3Kγ is important for multiple steps in the neutrophil migration response, enabling morphological changes to occur within the cell to permit polarisation and directional movement towards the chemotactic gradient. This is dependent on the tightly controlled spatial and temporal accumulation of PIP_3, AKT and F-actin at the leading edge, a process which is impaired in PI-3Kγ$^{-/-}$ neutrophils (Hannigan et al., 2002; Ferguson et al., 2007). Cytoskeletal rearrangements are regulated by Rho family GTPases and a positive feedback loop exists between the accumulation of PIP_3 and activation of Rac to amplify the chemotactic response, which mediates asymmetric F-actin assembly and specifies formation of the leading edge (Weiner & Neilsen, 2002; Costa et al., 2007). Another GTPase, Cdc42, is considered to be important for directionality in neutrophils and for preserving stability of the pseudopod (Srinivasan et al., 2003; Xu et al., 2003). There is evidence to suggest that an alternative isoform, PI-3Kδ, may also facilitate directional neutrophil migration. Its specific blockade using the pharmacological inhibitor IC87114 inhibits PIP_3 production and polarisation (Sadhu et al., 2003) and reduces recruitment of neutrophils to the lung in a mouse model of acute pulmonary inflammation (Puri et al., 2004).

The zebrafish has provided insight into the molecular events regulating the dynamics of neutrophil chemotaxis and is a particularly useful model due to the ease of visualising individual migrating cells *in vivo* in real time. It has been shown that inhibition of PI-3Kγ in zebrafish also disrupts neutrophil polarisation and directionality and that protrusion of the leading edge can be rescued using a photoactivatable form of Rac, however this is not sufficient to fully induce migration (Yoo et al., 2010). Using specific probes designed to detect both stable and dynamic F-actin, the authors demonstrated that PI-3K regulates the anteroposterior polarity of F-actin in parallel to activation of Rac at the leading edge, suggestive of a novel two-tiered regulation of neutrophil migration by PI-3K.

4.1.2 The role of PI-3K in neutrophil survival

Not only does PI-3K play a critical and complex role in neutrophil chemotaxis, but also in neutrophil survival, via the PI-3K/AKT/mTOR (mammalian target of rapamycin) signal transduction pathway. PI-3K mediates the anti-apoptotic effects of several

granulocyte survival signals through activation of AKT, which regulates a host of downstream target proteins including various members of the BCL-2 family, the Forkhead family of transcription factors, NF-κB and cAMP response element-binding protein (CREB) (Brazil et al., 2002; Hallett et al., 2008). For example, GM-CSF and TNF-α have been shown to promote neutrophil survival via PI-3K dependent phosphorylation and cytosolic translocation of BAD protein, in parallel with a reduction in transcription of *Bad* mRNA (Cowburn et al., 2002). AKT can activate NF-κB signalling by phosphorylation of the IKK complex (Datta et al., 1999) and it has been shown that NF-κB nuclear translocation is inhibited in neutrophils from PI-3Kγ$^{-/-}$ mice, leading to reduced levels of the survival protein Bcl-x(L) (Yang et al., 2003). Neutrophils from these mice also have diminished levels of Mcl-1, a key anti-apoptotic protein, due to reduced phosphorylation of CREB. Similarly, AKT can control the expression of pro-apoptotic factors, for example by the phosphorylation and inactivation of FKHR1, to stimulate nuclear export and inhibit transcriptional activity (Biggs et al., 1999).

PI-3K/AKT/mTOR signalling is tightly regulated to prevent uncontrolled cell proliferation and both PI-3K and AKT inhibitors can induce neutrophil death. Phosphatases act as endogenous regulators of AKT activity, for example survival is prolonged in phosphatase and tensin homologue (PTEN)-null neutrophils due to accumulation of PIP_3 at the plasma membrane, which leads to enhanced AKT signalling (Zhu et al., 2006). Depletion of PTEN and subsequent activation of AKT has also been shown to increase actin polymerisation and neutrophil responsiveness to chemoattractants and these properties are reflected *in vivo* by increased neutrophil recruitment in a mouse model of peritonitis (Subramanian et al., 2007).

4.1.3 Aims and hypotheses

The specific aim of this chapter is to investigate the effect of isopimpinellin on both the recruitment and resolution phases of inflammation, with the hypothesis that this activity can be attributed to an inhibition of PI-3K signalling. I will also examine and compare the chemical structures of the positive hit compounds identified in the Spectrum Collection screen, with the aim to identify any potential structure-activity relationships.

4.2 Isopimpinellin significantly inhibits neutrophil recruitment

With recruitment being the initial phase of the inflammatory response, it was logical to first explore the mechanism governing the inhibition of neutrophil recruitment observed with isopimpinellin treatment. To confirm these initial findings and investigate the dose dependency more thoroughly, 3 dpf *mpx*:GFP larvae were injured and treated immediately with a range of concentrations of isopimpinellin. Numbers of neutrophils at the site of injury at 6 hpi were counted as described in the previous recruitment assays. Isopimpinellin significantly inhibited neutrophil recruitment to the wound from a concentration of 10 µM up to 250 µM, though at the highest dose some signs of toxicity were observed (Figure 4.1).

A preliminary experiment was performed to further explore the effect of isopimpinellin on specific aspects of neutrophil migratory behaviour during the recruitment phase. At 3 dpf, *mpx*:GFP larvae were pre-treated with isopimpinellin for 2 hours, prior to injury and mounting in agarose for time-lapse imaging on an inverted compound fluorescence microscope. GFP-labelled neutrophils were tracked for one hour as they migrated towards the site of injury and Volocity® software was used to analyse the speed and meandering index of each individual cell track. Meandering index is calculated by dividing the displacement of each cell by the length of the path it has travelled. This gives an indication of how directly the cell moves, though does not take into account the actual direction in which it travels. A meandering index of 1 indicates movement in a straight line. The results obtained from one experiment suggested that isopimpinellin reduced both neutrophil speed and meandering index (Figure 4.2 A, B). Displacement towards the wound was significantly lower in neutrophils from isopimpinellin treated larvae (Figure 4.2 C). Despite this, neutrophils still appeared to move towards the wound, as indicated by the bearing, in which an angle of 0-180 degrees represents movement towards the wound and an angle of 180-360 degrees represents movement away (Figure 4.2 D). This gives a more accurate measure of directionality and suggested that even in the presence of isopimpinellin, the neutrophils were able to respond to the chemotactic gradient.

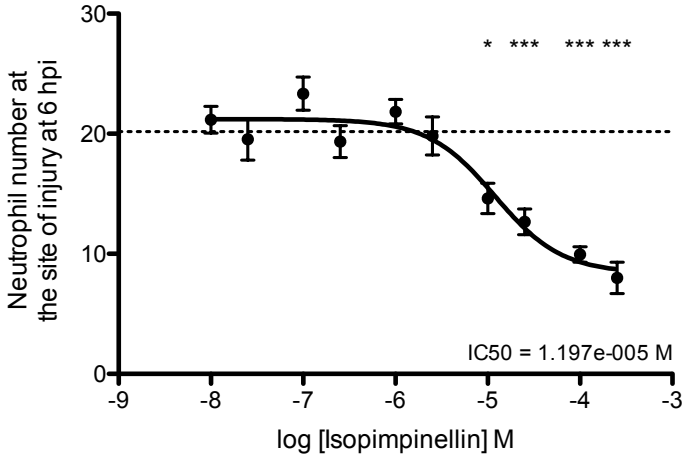

Figure 4.1: Isopimpinellin significantly inhibits neutrophil recruitment in a dose dependent manner.

At 3 dpf, *mpx*:GFP larvae were injured and treated immediately with varying doses of isopimpinellin. Neutrophil numbers at the site of injury were counted at 6 hpi. Isopimpinellin caused a dose-dependent reduction in neutrophil recruitment to the wound, with significantly lower neutrophil numbers present in larvae treated with isopimpinellin at a concentration of 10 µM and higher compared to the DMSO vehicle control (One-way ANOVA with Dunnett's multiple comparison post-test; where * $P < 0.05$ and *** $P < 0.001$; $n = 18$, performed as 3 independent experiments). Dotted line at y = 20.17 indicates mean neutrophil number at site of injury in control larvae.

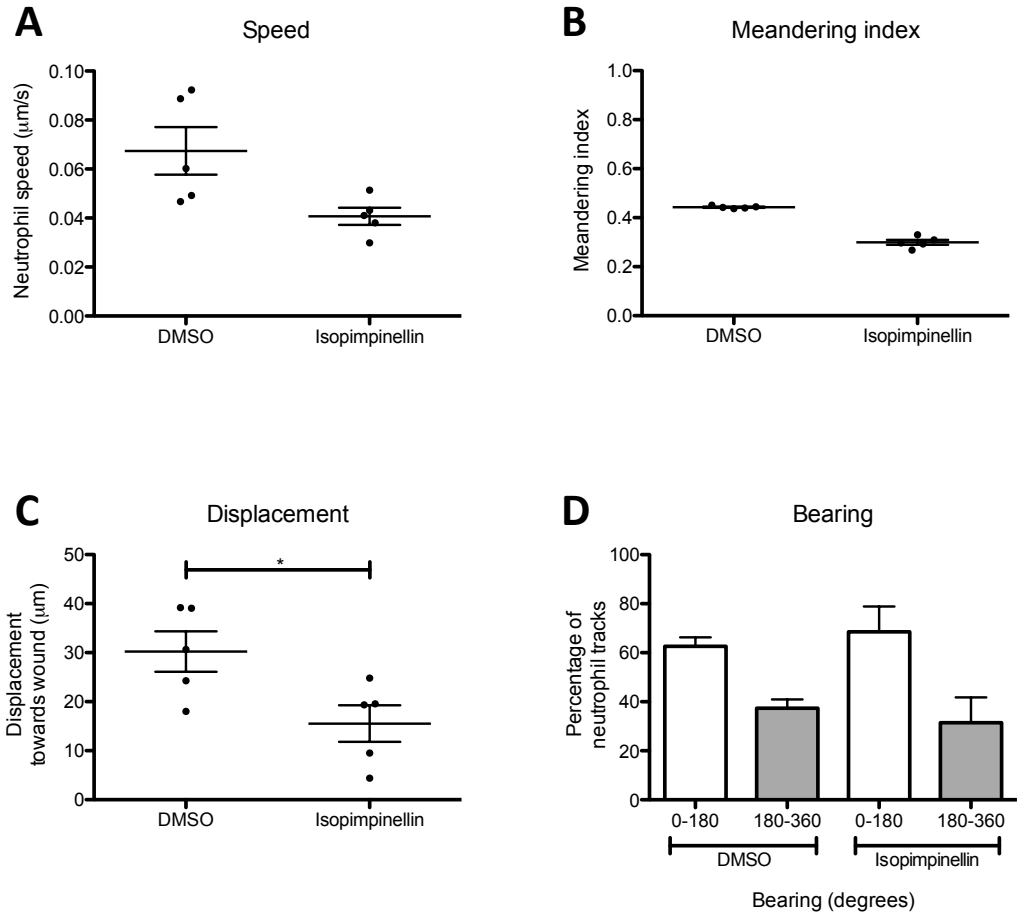

Figure 4.2: Isopimpinellin affects neutrophil chemotaxis but not directionality.

At 3 dpf, *mpx*:GFP larvae were pre-treated with DMSO or 25 μM isopimpinellin before injury and mounting. Larvae were imaged for 1 hour and individual GFP-labelled neutrophils were tracked and analysed using Volocity® software. Preliminary data suggested that isopimpinellin decreases (A) neutrophil speed, (B) meandering index and (C) displacement towards the wound (Unpaired *t*-test; $P < 0.05$; $n = 6$, performed as 1 experiment). For bearing (D), an angle of 0-180 degrees indicates movement towards the wound and 180-360 degrees indicates movement away. In both DMSO and isopimpinellin treated larvae, neutrophils appeared to move in a directed fashion towards the wound ($n = 6$, performed as 1 experiment).

4.3 Development of an *in vivo* assay for PI-3K activity

In order to determine whether the effect of isopimpinellin on neutrophil migration to the tail fin wound was occurring via an effect on PI-3K signalling, I sought to establish an assay similar to that described in (Yoo et al., 2010), which would allow me to observe cell-specific changes in PI-3K activity *in vivo*. I used the *Tg(lyz:PHAkt-EGFP)* zebrafish line, a kind gift obtained through collaboration with Dr Xingang Wang and Prof Philip Ingham (IMCB, Singapore), which expresses a mutant form of Akt with the Pleckstrin Homology (PH) domain fused to EGFP (PHAkt-EGFP), under the *lyz* promoter (Wang et al., in submission). This enables real time visualisation of the translocation of PHAkt-EGFP to the plasma membrane, which was originally demonstrated *in vitro* in HL-60 cells following stimulation with the chemoattractants fMLP and C5a (Servant et al., 2000). AKT binds directly to PIP_3 at the membrane via its PH domain, where once activated it can in turn activate a host of downstream targets required for neutrophil migration. Trafficking of PHAkt-GFP to the membrane is therefore dependent on the production of PIP_3 by activated Class I PI-3Ks and a loss of this localisation would indicate an inhibition of signalling either directly on or upstream of PI-3K.

I hypothesised that isopimpinellin would cause a reduction in the plasma membrane localisation of PHAkt-EGFP in neutrophils following tail fin injury, suggestive of an inhibitory effect on PI-3K signalling.

4.3.1 LY294002 is a potent inhibitor of neutrophil recruitment *in vivo*

To design a robust assay for PI-3K activity, a reliable positive control was required, ideally a known inhibitor of PI-3K signalling with functional activity in zebrafish. The readily available PI-3K inhibitor LY294002 had previously been shown to reduce neutrophil migration to a laser induced wound in the caudal haematopoietic tissue (Yoo et al., 2010). On testing this compound in a standard recruitment assay in 3 dpf *mpx*:GFP larvae, I found that it significantly reduced neutrophil numbers at the tail fin wound at 6 hpi (Figure 4.3 A). Interestingly, LY294002 also caused a reduction in neutrophil number during the resolution phase of inflammation (Figure 4.3 B).

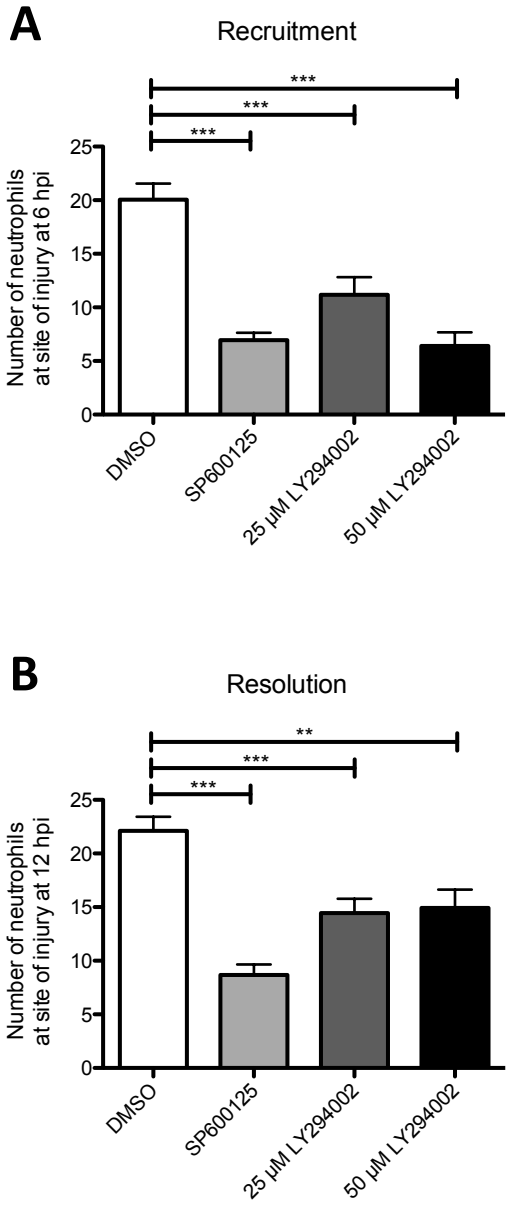

Figure 4.3: LY294002 significantly inhibits neutrophil recruitment and inflammation resolution.

Tail transection was performed on *mpx*:GFP larvae at 3 dpf. For the recruitment assay, larvae were treated immediately, as indicated, and numbers of neutrophils at the site of injury were counted at 6 hpi. For the resolution assay, larvae with a good inflammatory response were selected for treatment at 6 hpi and counts were performed at 12 hpi. SP600125 was used at 30 µM as a positive control. At both concentrations tested, LY294002 significantly inhibited neutrophil recruitment (A) and accelerated the resolution of inflammation (B) (One-way ANOVA with Dunnett's multiple comparison post-test; where ** $P < 0.01$ and *** $P < 0.001$; $n = 18$, performed as 3 independent experiments).

To more thoroughly investigate the effect of this inhibitor on neutrophil migration during the initial phase of neutrophil recruitment, 3 dpf *mpx*:GFP larvae were pre-treated with 50 µM LY294002 for 2 hours before tail fin transection was performed, then larvae were mounted in agarose for time-lapse imaging and tracking. During the first hour following injury, neutrophil speed was significantly reduced in the presence of LY294002, but meandering index was unchanged (Figure 4.4 A, B). The displacement was also significantly reduced in the LY294002 treated neutrophils (Figure 4.4 C), but those that did move, migrated directionally towards the wound (Figure 4.4 D). It seems that in the presence of LY294002, chemotaxis is partially inhibited such that most neutrophils either remain stationary or move only short distances slowly towards the wound. The bearing analysis reveals that significantly more of these neutrophils can detect the chemotactic gradient and move in the appropriate direction, compared to the number that appear to move away from the wound. It is possible that LY294002 does not fully inhibit PI-3K signalling in all of the neutrophils, or perhaps alternative signalling mechanisms are overriding this inhibition to promote directed migration.

4.3.2 LY294002 inhibits PI-3K activity and neutrophil polarisation

To investigate the effect of LY294002 in migrating neutrophils in the *Tg(lyz:PHAkt-EGFP)* line, subsequently referred to as *lyz:*PHAkt, larvae at 3 dpf were incubated with the inhibitor for 2 hours prior to tail fin transection, then mounted in agarose. Individual neutrophils in the region between the wound site and the posterior blood island were imaged on an Ultra*VIEW*VoX spinning disk confocal imaging system with an inverted Olympus IX81 microscope, at 60x magnification. It was immediately obvious through observation by eye that neutrophils in larvae treated with LY294002 displayed a very different pattern of PHAkt-EGFP localisation compared to the DMSO vehicle control larvae, with the EGFP signal dispersed over the whole cell rather than being accumulated at the cell membrane (Figure 4.5 A).

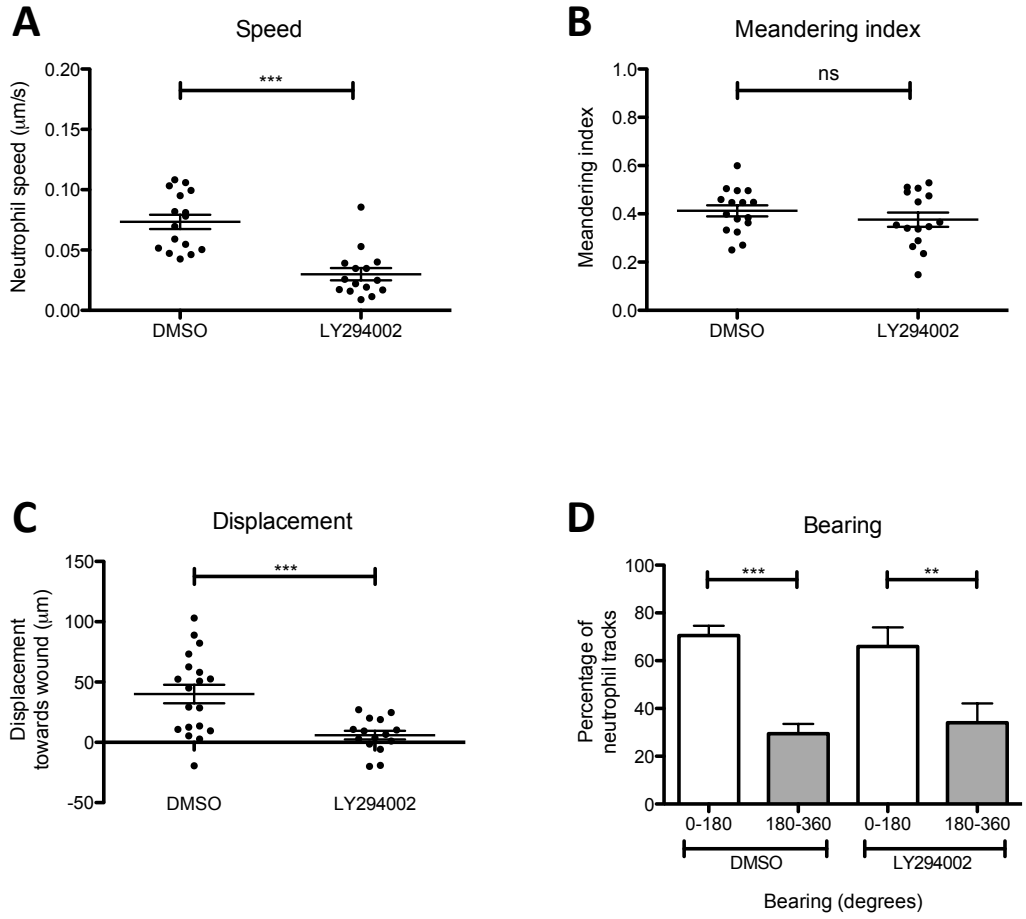

Figure 4.4: LY294002 significantly decreases neutrophil speed and displacement.

At 3 dpf, *mpx*:GFP larvae were pre-treated with DMSO or 50 μM LY294002 before injury, mounting and imaging for 1 hour. Individual GFP-labelled neutrophils were tracked and analysed using Volocity® software. LY294002 significantly decreased (A) neutrophil speed and (C) displacement towards the wound (Unpaired *t*-test; $P < 0.001$; $n \geq 16$ performed as 2 independent experiments). Meandering index was unchanged (B). For bearing, an angle of 0-180 degrees indicates movement towards the wound and 180-360 degrees indicates movement away. In both DMSO and LY294002 treated larvae, neutrophils move in a directed fashion towards the wound (One-way ANOVA with Bonferroni's multiple comparison post-test to compare selected columns; where ** $P < 0.01$ and *** $P < 0.001$; $n \geq 16$, performed as 2 independent experiments).

Neutrophils from DMSO vehicle control treated larvae appeared polarised, with particularly strong expression of EGFP in pseudopodia at their leading edge and only a faint EGFP signal within the cell body. When observed during short time-lapses of approximately 30 minutes, neutrophils from DMSO control larvae appeared to migrate normally to the site of injury whereas neutrophils in LY294002 treated larvae seemed mostly static.

In an attempt to quantify the images generated so that statistical analysis could be applied, a measurement termed the 'polarity index' was devised. This accounted for the difference in EGFP intensity at the leading edge of the cell compared to the trailing edge and also compared to the fluorescence within the cell. In ImageJ, a transecting line was drawn through each cell towards the leading edge; to create a quantitative readout of the fluorescence intensity per pixel (Figure 4.5 B, C). Using Microsoft Excel, the mean intensities along sections of this line (as defined in Figure 4.5 C and described in more detail in section 2.4.5) were entered into the equation:

$$polarity\ index = \left(log_{10}\, {}^{a}/_{b}\right) \times \frac{a+b}{c}$$

$$where\ a = trailing\ edge\ of\ cell;\ b = leading\ edge\ of\ cell; c = whole\ cell$$

Using this equation, LY294002 appeared to significantly reduce the polarity index of neutrophils compared to the DMSO control (Figure 4.5 D), suggesting reduced activation and translocation of PHAkt-EGFP to the plasma membrane and a loss of the cell polarity required to direct migration.

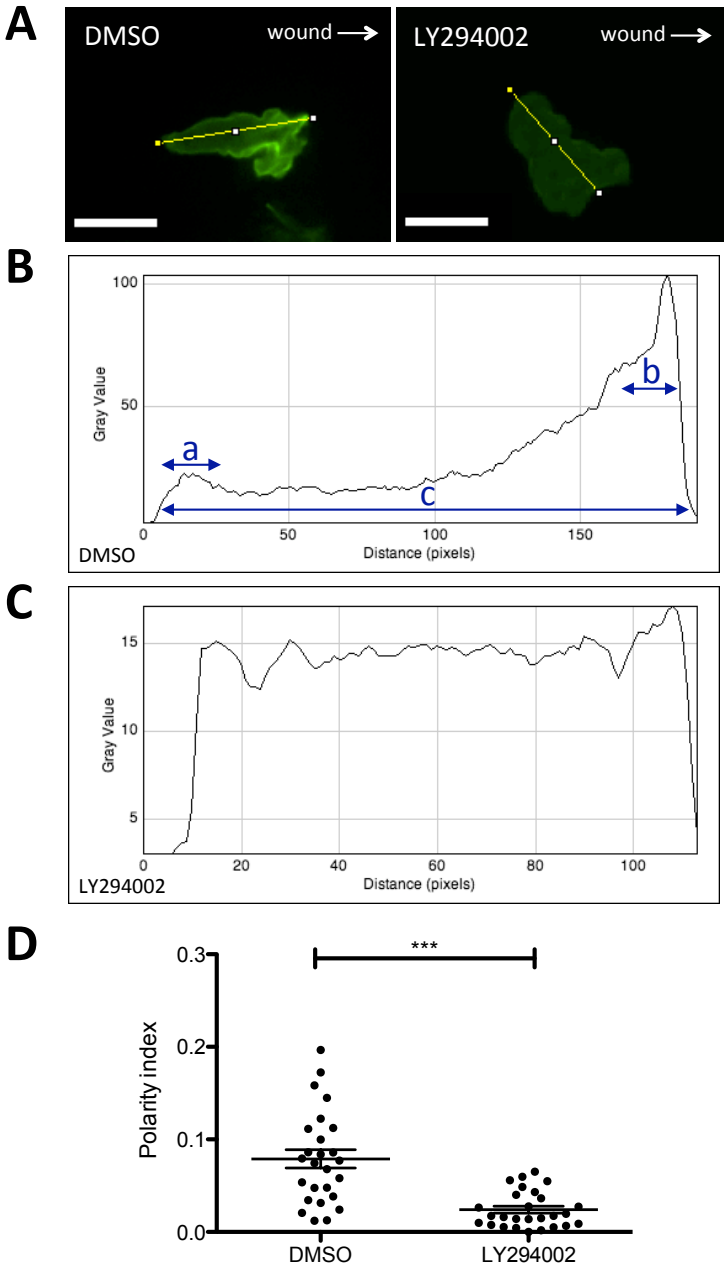

Figure 4.5: LY294002 significantly reduces neutrophil polarity index in *Tg(lyz:PHAkt-EGFP)* larvae.

At 3 dpf, *lyz*:PHAkt larvae were pre-treated with either 50 µM LY294002 or DMSO vehicle control for 2 hours before tail fin injury and mounting for observation on a confocal spinning disk imaging system. Representative images of individual neutrophils in the region between the wound site and the posterior blood island are shown in (A). Scale bar represents 11 µm. To analyse polarity, the fluorescence intensity in a transection of each neutrophil was measured in ImageJ to create intensity profiles as exampled in (B) and (C). Polarity index was calculated using the equation described above and the parameters illustrated in (B). LY294002 significantly reduced neutrophil polarity index compared to the DMSO control (D) (Unpaired *t*-test; $P < 0.0001$; $n = 27$, performed as 3 independent experiments).

4.3.3 Isopimpinellin significantly reduces neutrophil polarity index

Having designed an assay with the potential to identify compounds that inhibited PI-3K activity *in vivo*, I tested isopimpinellin in the same manner and found that after 2 hours, neutrophils from isopimpinellin treated larvae did not migrate as readily to the site of injury as neutrophils from vehicle only control treated larvae. Most of these neutrophils did not appear to have a defined leading edge, comparable to those from larvae treated with the positive control LY294002, and they displayed a similar pattern of dispersed EGFP signal over the whole cell (Figure 4.6). Using the polarity index equation, isopimpinellin significantly reduced neutrophil polarisation, to a level almost equal to that of LY294002 (mean ± SEM of isopimpinellin polarity index = 0.02684 ± 0.004, compared to 0.02404 ± 0.004 for LY294002). These results suggest that isopimpinellin acts either directly on or upstream of PI-3K, to disrupt Akt activation, neutrophil polarisation and directional migration, which may explain the inhibitory effect of this compound on neutrophil recruitment.

To test whether the effects observed on PHAkt-EGFP localisation and neutrophil polarity were specific to the individual compounds tested and not simply a general consequence of exposing larvae to compounds that have been shown to affect neutrophilic inflammation, I chose to investigate the effects of some of the other hit compounds in this PI-3K assay. Xanthone and tanshinone IIA were tested in the same manner, alongside LY294002. In xanthone treated *lyz:*PHAkt larvae, neutrophils adopted a similar phenotype to that observed in LY294002 and isopimpinellin treated larvae, which was reflected in a significantly reduced polarity index compared to the DMSO control (Figure 4.7 D). Tanshinone IIA however, did not appear to have any effect on the membrane localisation of PHAkt-EGFP and polarity index remained unchanged (Figure 4.7 E).

These results suggested that the loss of membrane localisation of PHAkt-EGFP was not a universal property of the positive hits identified in the Spectrum Collection screen, but a specific effect of some compounds, which could be explained by inhibition of PI-3K signalling upstream of Akt activation.

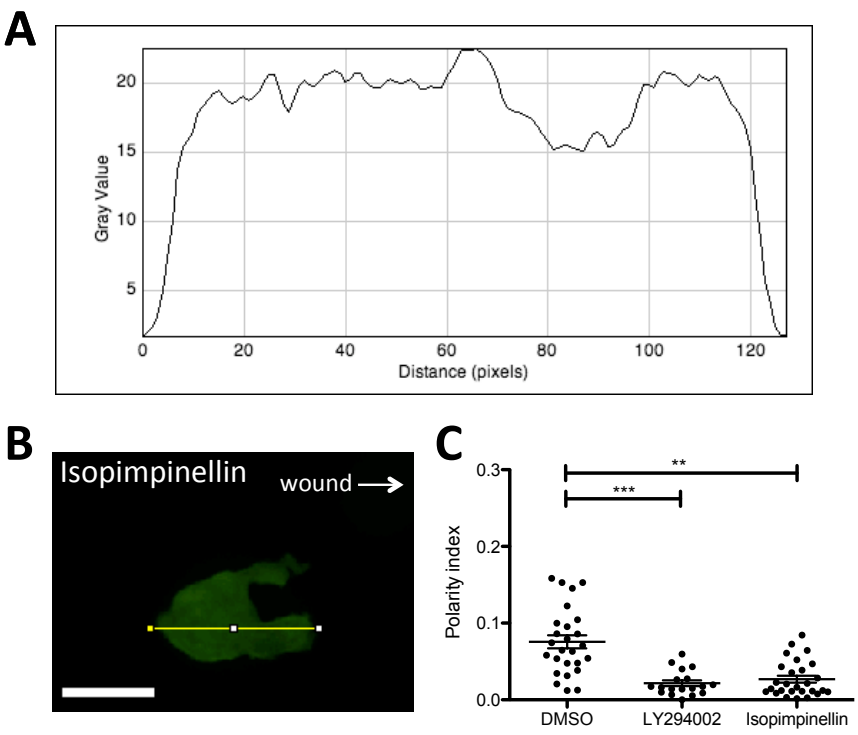

Figure 4.6: Isopimpinellin significantly reduces neutrophil polarity index in *Tg(lyz:PHAkt-EGFP)* larvae.

At 3 dpf, *lyz*:PHAkt larvae were pre-treated with either 25 µM isopimpinellin, 50 µM LY294002 or DMSO vehicle control for 2 hours before tail fin injury and imaging as described previously. Neutrophils in isopimpinellin treated larvae exhibited similar fluorescence intensity profiles as neutrophils from LY294002 treated larvae. A representative intensity plot is shown in (A), matched to the neutrophil in (B). Scale bar represents 11 µm. Isopimpinellin significantly reduced neutrophil polarity index compared to the DMSO control (One-way ANOVA with Dunnett's multiple comparison post-test; where ** $P < 0.01$ and *** $P < 0.001$; $n = 27$, performed as 3 independent experiments).

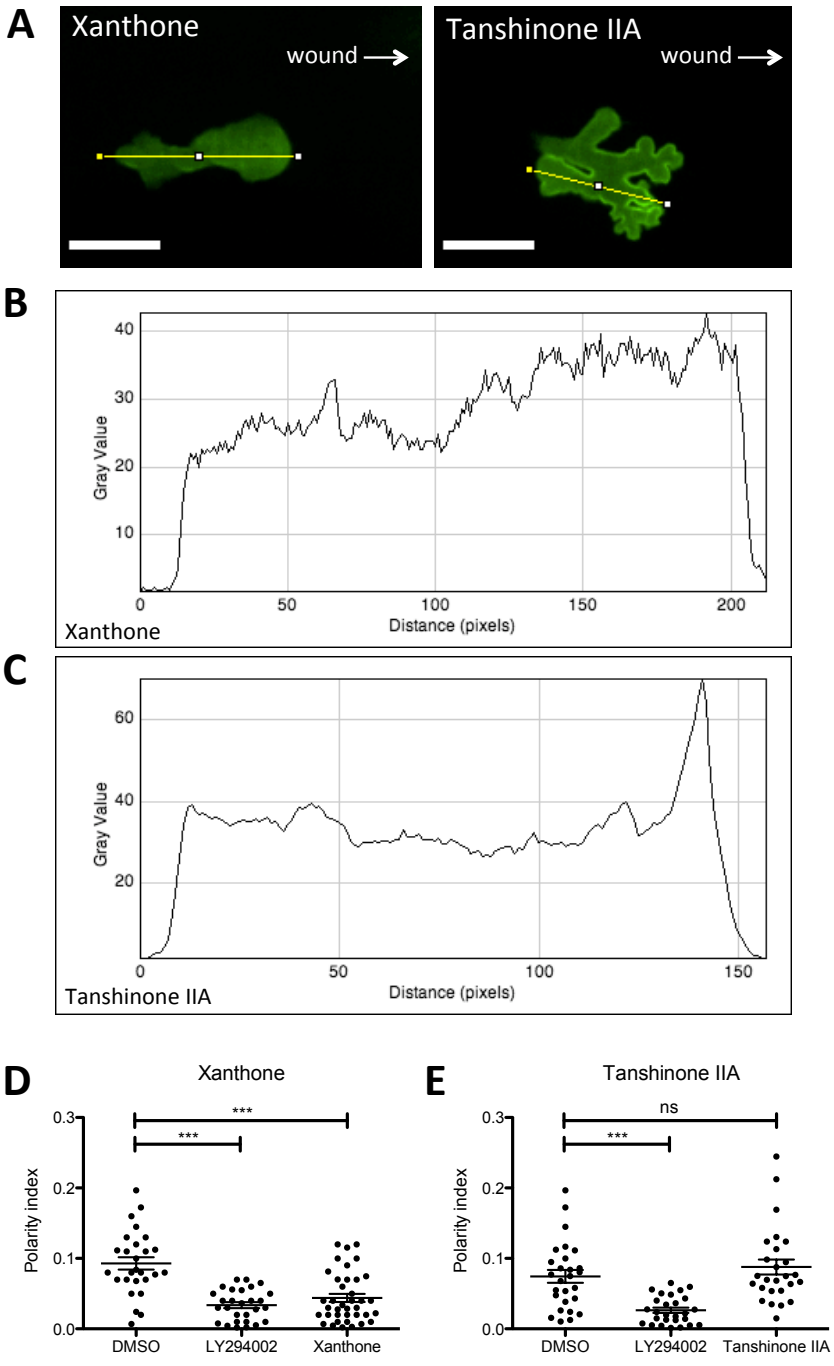

Figure 4.7: Xanthone but not tanshinone IIA reduces neutrophil polarity index.

At 3 dpf, *lyz*:PHAkt larvae were pre-treated with either 25 µM xanthone, 25 µM tanshinone IIA, 50 µM LY294002 or DMSO vehicle control for 2 hours before tail fin injury and imaging as described previously. Representative images of neutrophils are shown in (A) along with matched fluorescence intensity profiles for (B) xanthone and (C) tanshinone IIA. Scale bar represents 11 µm. Xanthone significantly reduced neutrophil polarity index compared to the DMSO control (One-way ANOVA with Dunnett's multiple comparison post-test; $P < 0.001$) whereas tanshinone IIA had no significant effect ($n \geq 27$, performed as 3 independent experiments).

4.4 Chemical structural comparison reveals a common 'chromone' group

In *lyz*:PHAkt larvae, both isopimpinellin and xanthone appeared to inhibit recruitment of Akt to the plasma membrane and reduced the neutrophil polarity index to a level comparable to that of the known PI-3K inhibitor, LY294002. With the idea that these compounds might be exerting their effects on the inflammatory response by acting on a common pathway or pathways, I hypothesised that their activity might be due to shared structural similarity. The molecular structures of the positive hits were identified using their unique Chemical Abstracts Service (CAS) numbers as provided in the Spectrum Collection database. By simple visual comparison, it was obvious that there was some structural similarity. A particular chemical group was common to a subset of the hits, consisting of fused benzene and pyran rings with an attached carbonyl group. Technically termed 1,4-benzopyrone and more commonly referred to as 'chromone', this group or its isomer 'coumarin' (1-benzopyran-2-one) is present in 4 of the best positive hits identified in the screen and interestingly also in LY294002 (Figure 4.8). From the other 10 reproducible positive hits that weren't tested in secondary assays (those that scored greater than or equal to 1.5 but less than 2 in the second round of screening), a further 3 compounds were derivatives of chromone (Figure 4.9).

4.4.1 Investigation into the effects of chromone on the inflammatory response

As the chromone group was shared by a number of the positive hits and the PI-3K inhibitor LY294002, I hypothesised that this structure might be responsible for the activity of these compounds, particularly on neutrophil recruitment. Notably the two compounds that had similar effects to LY294002 in the PI-3K assay described above, isopimpinellin and xanthone, both contain a chromone group, whereas tanshinone IIA, which did not reduce neutrophil polarity index, does not share this structural similarity. To test whether the effects of the chromone-derived positive hits on neutrophilic inflammation were specifically due to the presence of this structural moiety, chromone alone was tested in each of the standard secondary assays.

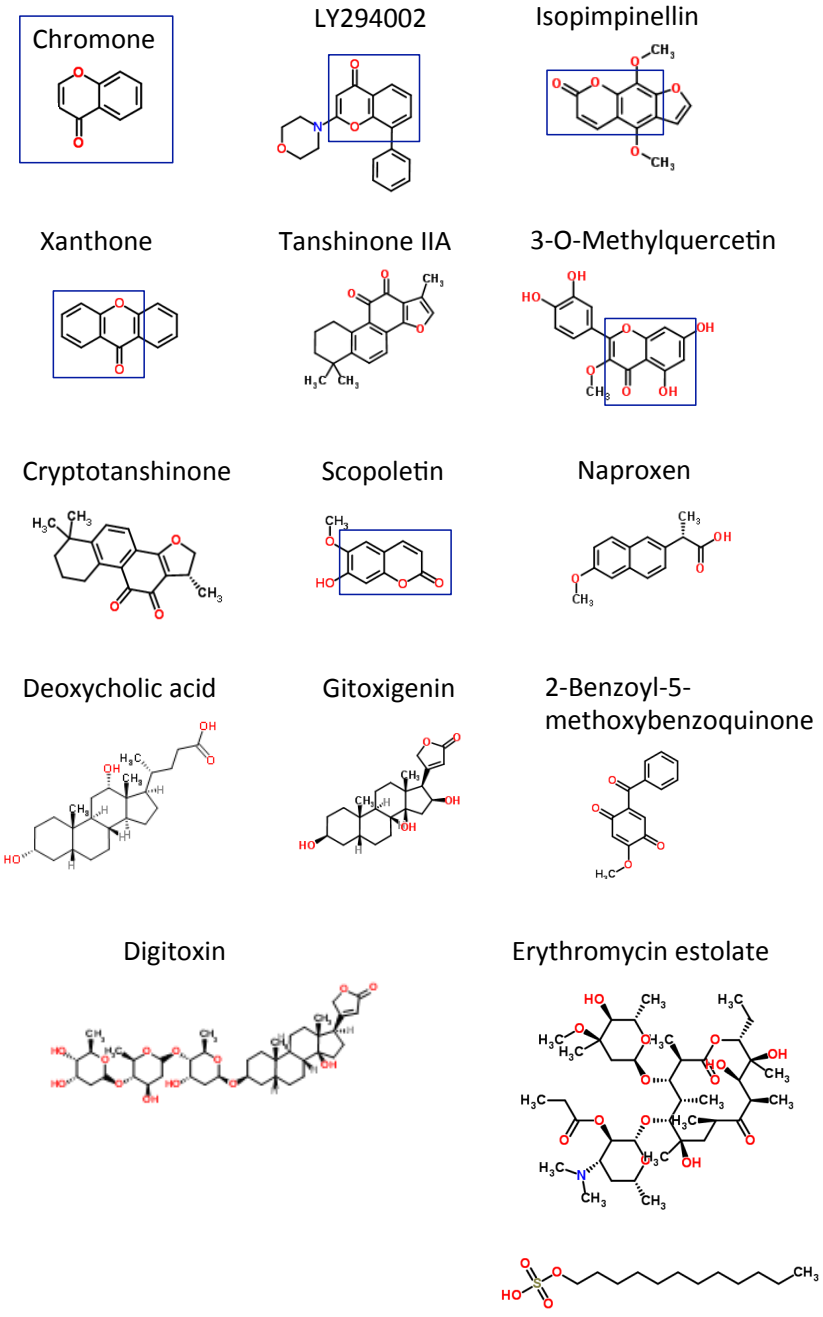

Figure 4.8: Structural similarity searching reveals a common chromone structure.

Figure shows the chemical structures of 12 of the highest scoring hits from the Spectrum Collection screen, in comparison to LY294002 and chromone. LY294002 and 4 of the hit compounds share the chromone group, as indicated by the blue box.

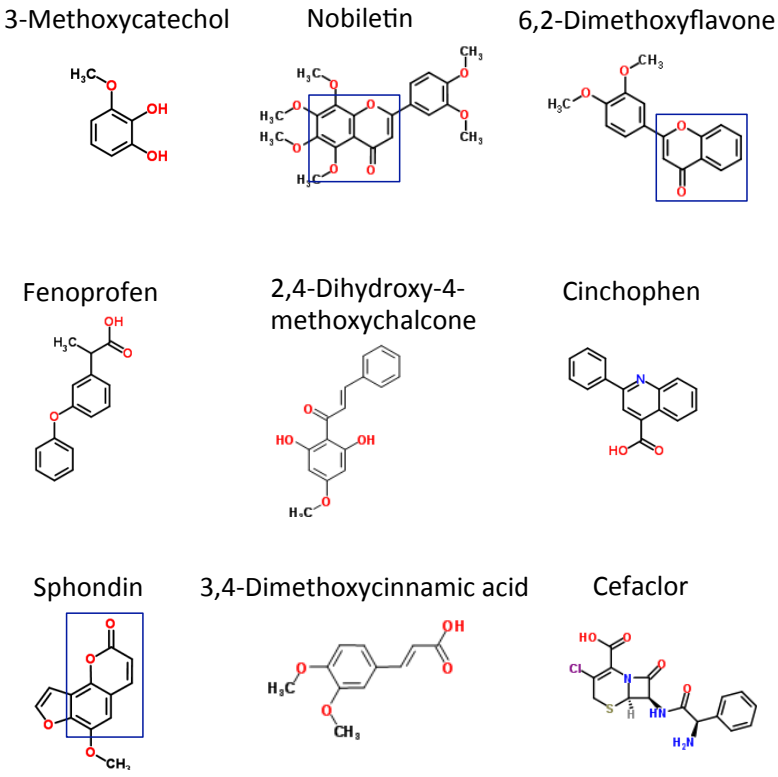

Figure 4.9: Chemical structure comparison of the remaining positive hits reveals three additional chromone-derivatives.

Figure shows the chemical structures of the remaining hits from the Spectrum Collection screen, which scored greater than 1.5 but less than 2 and subsequently were not selected for further investigation in secondary assays. Three of these hit compounds also share the chromone group, as indicated by the blue box.

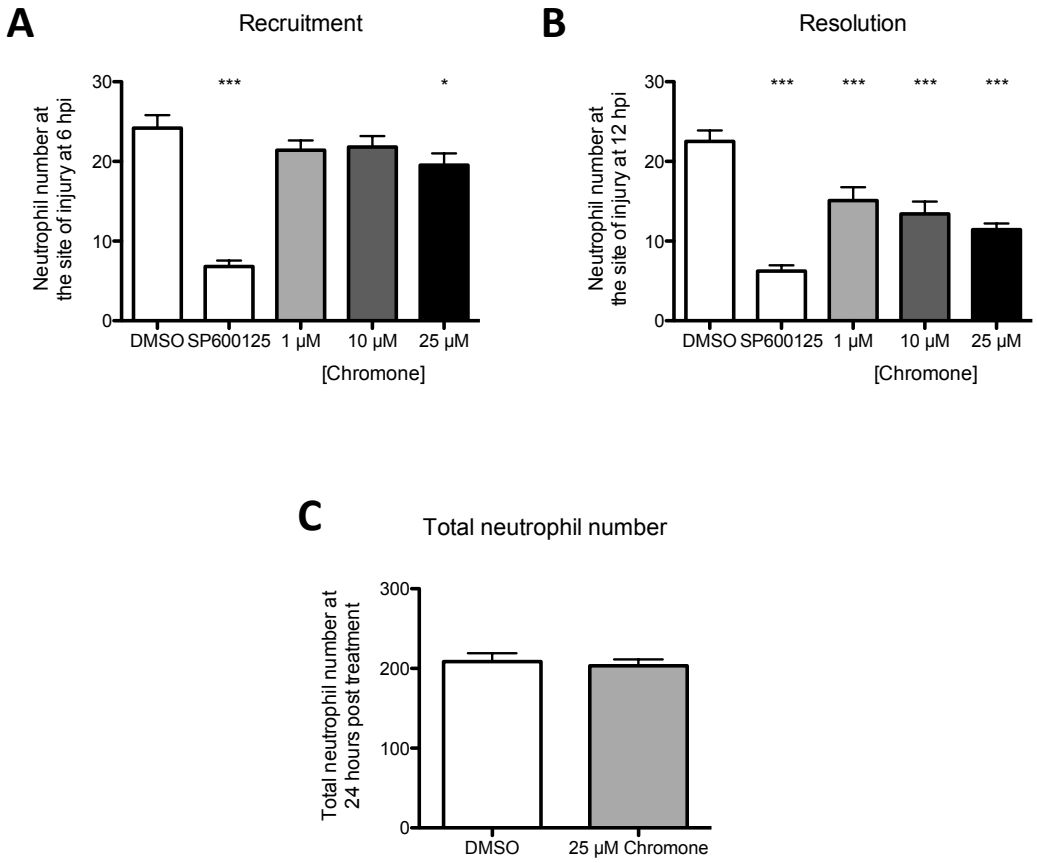

Figure 4.10: Chromone has a more significant effect on the resolution of inflammation than on neutrophil recruitment.

Chromone was tested in 3 dpf *mpx*:GFP larvae for its effects on (A) neutrophil recruitment at 6 hpi, (B) resolution of neutrophilic inflammation at 12 hpi and (C) total neutrophil number after 24 hours of pre-treatment as previously described. Chromone caused a small but significant reduction in neutrophil recruitment at the highest dose tested and significantly accelerated resolution in terms of neutrophil numbers at 12 hpi at all doses tested (One-way ANOVA with Dunnett's multiple comparison post-test; where * $P < 0.05$ and *** $P < 0.001$; $n = 18$, performed as 3 independent experiments). Chromone had no effect on total neutrophil number (Unpaired *t*-test; $P = 0.6977$; $n = 12$, performed as 2 independent experiments).

Unexpectedly, chromone only had a small effect on neutrophil recruitment, but had a more significant effect on resolution (Figure 4.10). There was no effect on total neutrophil number, confirming that chromone was acting specifically to reduce numbers of activated neutrophils at the site of injury. These results suggested it was unlikely that the chromone group was solely responsible for the effect of the chromone-derived hit compounds on neutrophil recruitment, but that it might in fact play an important role in accelerating the resolution of inflammation. This could explain the pro-resolution activity of some of the hit compounds by acting on a currently unidentified target.

4.4.2 Investigation into the effects of chromone analogues on inflammation resolution

With the idea that chromone might be an interesting lead candidate, I formed a collaboration with Dr Simon Jones and Marco Meloni from the Chemistry Department at the University of Sheffield, to further investigate the structure-activity relationships of chromone and its derivatives. The chemists initially synthesised a set of seven chromone analogues, which I tested, blinded to chemical structure, on injured *mpx*:GFP larvae to examine their effects on resolution. Most of the analogues significantly reduced neutrophil numbers at the site of injury at 12 hpi, with some indication of dose-dependency (Figure 4.11). By coincidence, one of the synthesised analogues was xanthone, and its effect on resolution was comparable to the data obtained previously (refer to Figure 3.17). The compounds were ranked in order based on their efficacy and their chemical structures were compared to identify any potential trends that might explain their relative activity (Figure 4.12). Xanthone and anthrone reduced neutrophil numbers most significantly, suggestive that the addition of a third hydrocarbon ring might increase potency, but with the exception of xanthene, which might be explained by the removal of the ketone group from the pyrone ring.

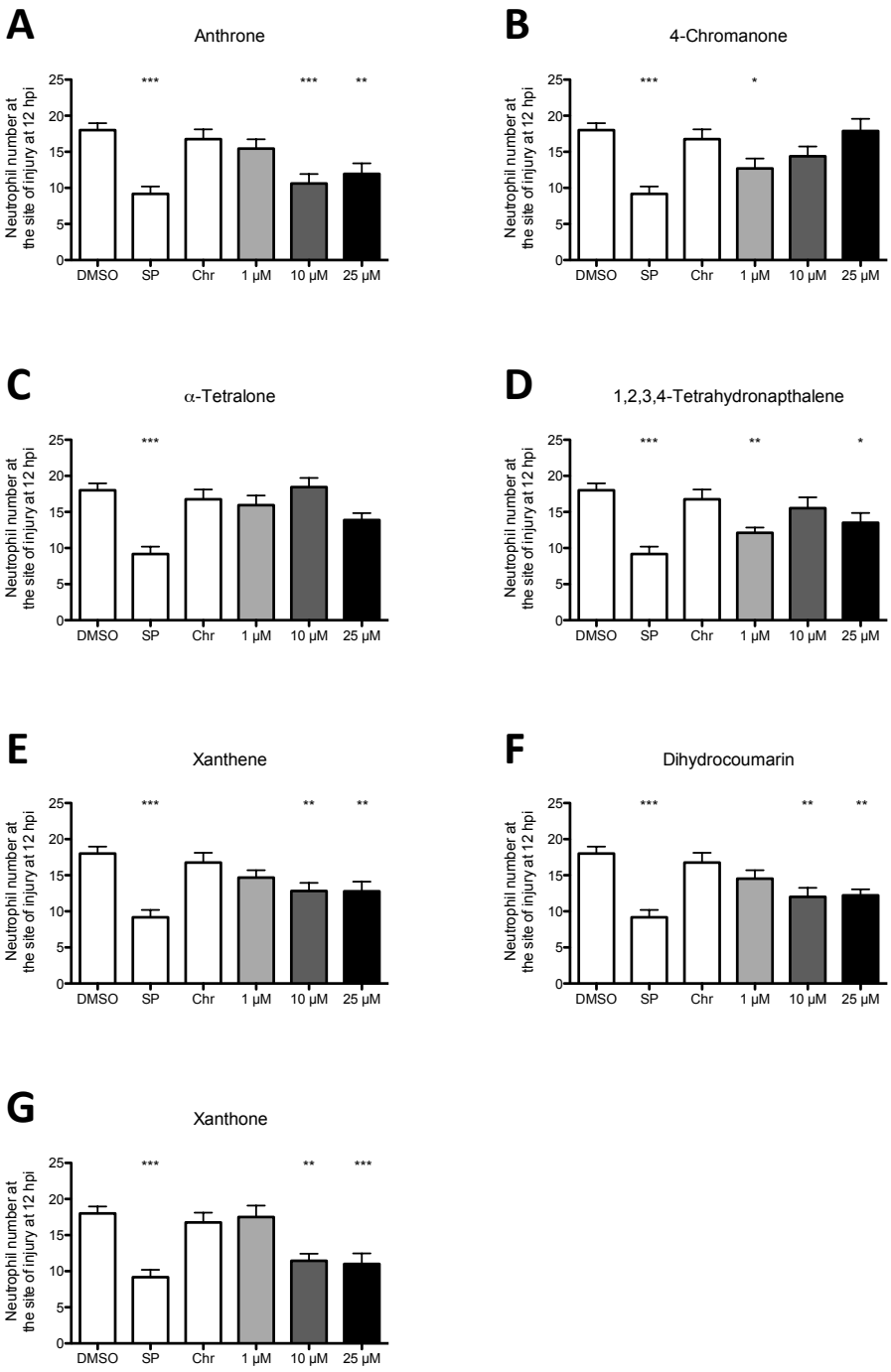

Figure 4.11: A subset of the synthesised chromone analogues accelerate resolution.

Chromone analogues were tested at varying doses in the standard resolution assay using *mpx*:GFP larvae, with treatment at 6 hpi and neutrophil counts at the site of injury performed at 12 hpi. All compounds except α-tetralone caused a significant reduction in neutrophil number at one or more of the doses tested (One-way ANOVA with Dunnett's multiple comparison post-test; where * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$; $n = 18$, performed as 3 independent experiments). SP refers to 30 μM SP600125 and Chr refers to 25 μM chromone, tested alongside as positive controls.

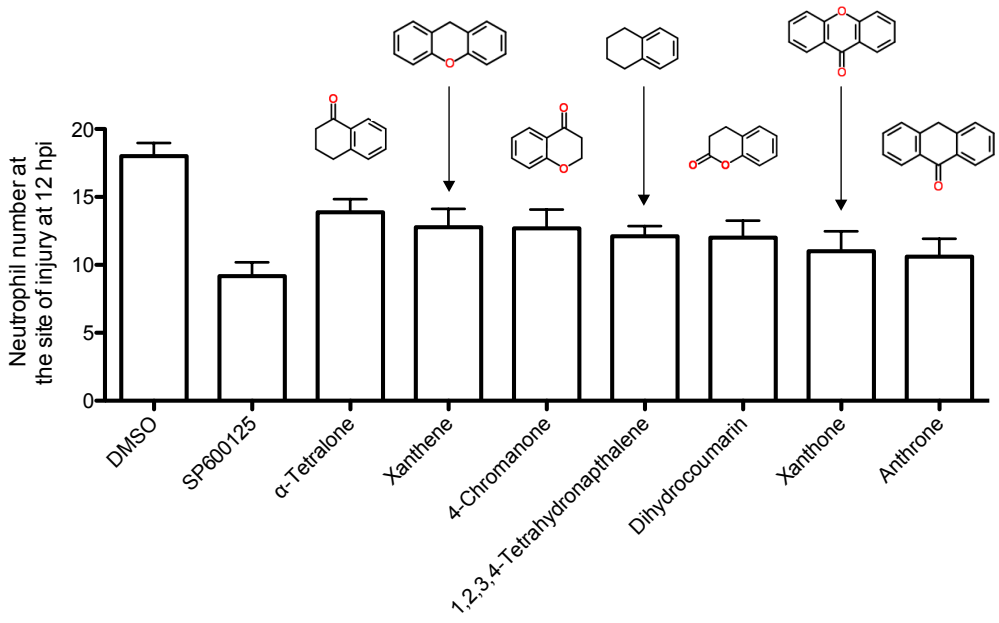

Figure 4.12: Summary and ranking of the effects of synthesised chromone analogues on the resolution of inflammation.

Compounds are ranked according to their maximum response in the previous resolution assay. Doses shown are 1 µM for 4-chromanone and 1,2,3,4-tetrahydronapthalene, 10 µM for dihydrocoumarin and 25 µM for all other compounds. Chemical structures of compounds are illustrated above their respective data bar.

Though these were intriguing results, it was evident when carrying out the resolution assays that chromone, which was tested alongside as a control, was not functioning as effectively as in the previous experiments. With the hypothesis that chromone might be sensitive to degradation over time, a resolution assay was performed to compare the effectiveness of the original, 'old' chromone stock compared to a 'new' stock solution prepared in fresh DMSO at the beginning of each experiment. Anthrone was also tested in the same manner and the data obtained suggested that both compounds became less potent with age (Figure 4.13).

Based on the hypothesis that the presence of a third hydrocarbon ring increased pro-resolution activity of the chromones, a set of tricyclic analogues were synthesised to test whether their activity could be improved by the addition of other chemical groups. Two of these significantly reduced neutrophil numbers at the highest dose tested, but these effects were much smaller than those observed with the previous tricyclic analogues, suggesting that the addition of these particular moieties was not functionally advantageous (Figure 4.14). In these experiments, freshly prepared stock solutions of chromone and anthrone were used as controls each time. Activity appeared to be preserved with anthrone only.

It was hypothesised that the instability of chromone could be due to a tendency to be oxidised and subsequently rapidly degraded. However as phenolic compounds typically have an antioxidant nature, it was also possible that the functional activity of the chromones might be at least partially attributed to an ability to scavenge reactive oxygen species. To investigate this, a third series of analogues was synthesised, with increasing ability to undergo oxidation (Figure 4.15). Compound MMM115, which has the highest oxidation level and is therefore least likely to undergo oxidation, significantly reduced neutrophil numbers at all doses tested (Figure 4.15 A). Coincidentally MMM115, composed of the chromone backbone with an attached phenyl group, is identical to the naturally occurring flavone. This is a plant secondary metabolite, forms of which are found commonly in fruit, vegetables and traditional herbal medicines (Havsteen, 2002). Flavones identified as reproducible hits in the Spectrum Collection screen include methylquercetin and nobiletin.

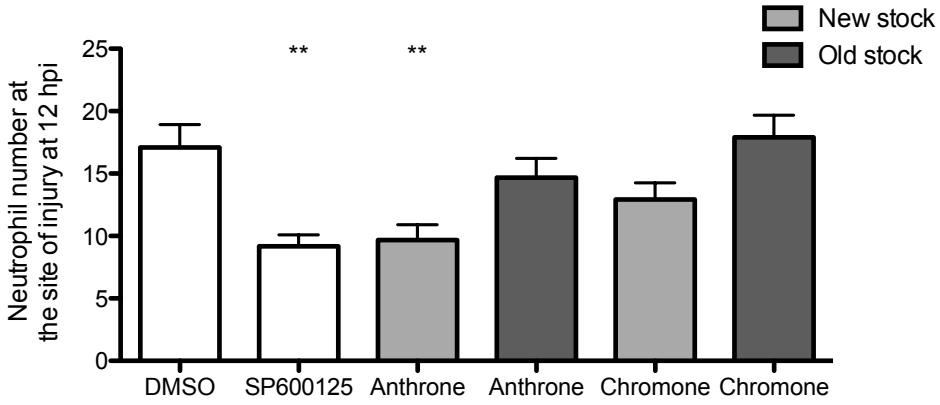

Figure 4.13: Chromone and anthrone become less effective with age.

Anthrone (10 µM) and chromone (25 µM) were tested in the standard resolution assay using *mpx*:GFP larvae, with treatment at 6 hpi and neutrophil counts at the site of injury performed at 12 hpi. New stock refers to fresh compounds prepared shortly before treatment; old stock refers to frozen aliquots of compound prepared previously. Fresh anthrone significantly reduced neutrophil numbers at the site of injury compared to the DMSO vehicle control, but this effect was not maintained with the old stock. Neither fresh nor old chromone stock significantly reduced neutrophil numbers (One-way ANOVA with Bonferroni's multiple comparison post-test; $P < 0.01$; $n = 12$, performed as 2 independent experiments). SP600125 was used as a positive control at 30 µM.

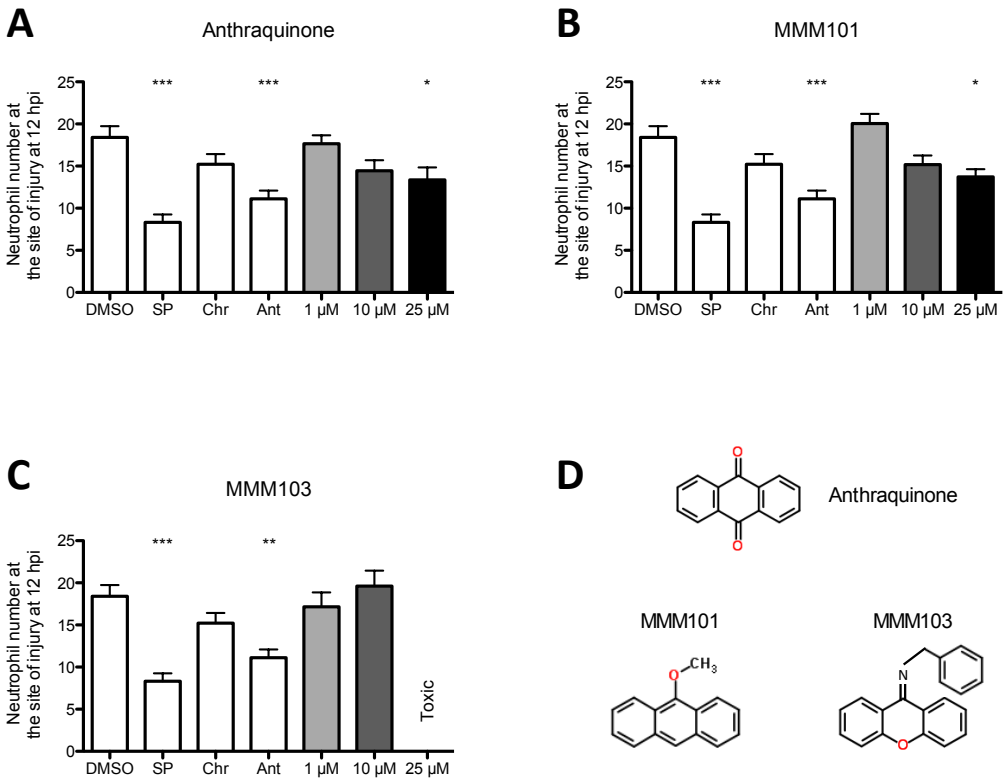

Figure 4.14: Some tricyclic chromone analogues significantly accelerate inflammation resolution.

Tricyclic chromone analogues were tested at varying doses in the standard resolution assay using *mpx*:GFP larvae, with treatment at 6 hpi and neutrophil counts at the site of injury performed at 12 hpi. (A) Anthraquinone and (B) MMM101 caused a significant reduction in neutrophil number at the highest dose tested, whereas (C) MMM103 had no effect (One-way ANOVA with Dunnett's multiple comparison post-test; $P < 0.05$; $n = 18$, performed as 3 independent experiments). SP refers to 30 µM SP600125, Chr refers to 25 µM chromone and Ant refers to 10 µM anthrone, tested alongside as positive controls and to investigate their reliability. Chemical structures of tricyclic chromone analogues are shown in (D).

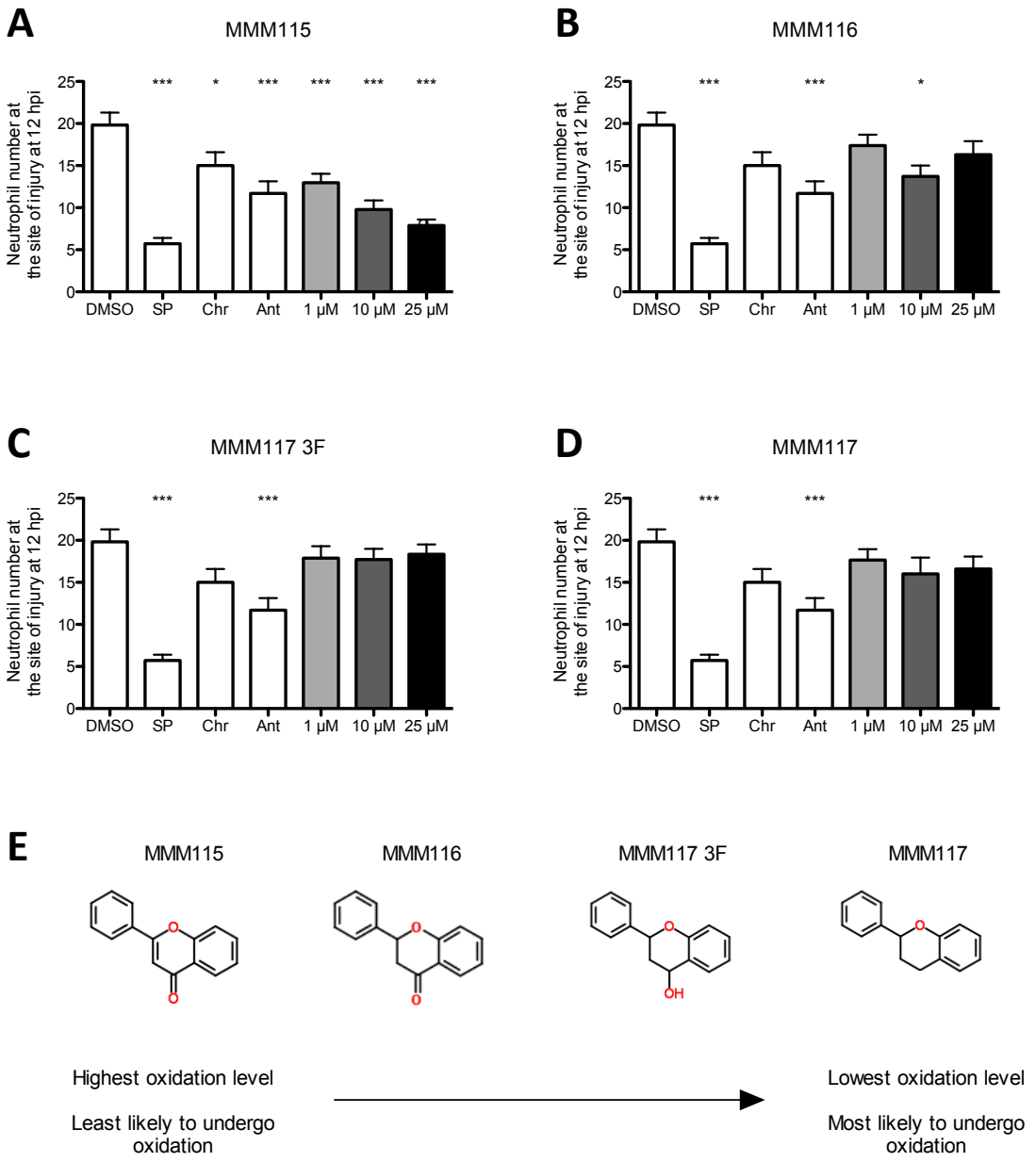

Figure 4.15: Pro-resolution activity of chromone derivatives does not correlate with their potential antioxidant activity.

Chromone analogues with increasing ability to be oxidised were tested at varying doses in the standard resolution assay using *mpx*:GFP larvae, with treatment at 6 hpi and neutrophil counts at the site of injury performed at 12 hpi. (A) MMM115 caused a significant reduction in neutrophil number and appeared to act in a dose-dependent manner. (B) MMM116 significantly reduced neutrophil number at 10 µM, but no effect was seen with either (C) MMM117 3F or (D) MMM117 (One-way ANOVA with Dunnett's multiple comparison post-test; * $P < 0.05$ and *** $P < 0.001$; $n = 18$, performed as 3 independent experiments). SP refers to 30 µM SP600125, Chr refers to 25 µM chromone and Ant refers to 10 µM anthrone, tested alongside as positive controls and to investigate their reliability. (E) Chemical structures are illustrated, arrow indicates highest to lowest oxidation state.

Compounds MMM117 3F and MMM117, which have the lowest oxidation levels due to removal of the ketone group, did not have any effect on inflammation resolution (Figure 4.15 C, D). This might be explained by their increased potential to be oxidised, which perhaps results in loss of their functional activity and subsequent degradation. These data indicated that the active chromone analogues were unlikely to be driving resolution by antioxidant mechanisms. However, these experiments did not help to clarify why chromone, in which the ketone is present, was unreliable. Perhaps in such simple form, chromone is a particularly reactive molecule.

Due to the unstable nature of chromone I decided not to pursue the synthesis of new chromone analogues with the Chemistry Department. I returned the focus of my investigation to the positive hits identified in the Spectrum Collection screen, specifically isopimpinellin, which, out of all of the chromone-related compounds identified in the screen, had the greatest effect on inflammation resolution.

4.5 Investigation into the pro-resolution activity of isopimpinellin

Having previously established that the mechanism responsible for the effect of isopimpinellin on neutrophil recruitment in zebrafish larvae was at least in part due to an inhibition of PI-3K signalling, I sought to investigate the effect of this compound on the resolution phase of the acute inflammatory response. Data obtained in the secondary assays performed following the Spectrum Collection screen indicated that isopimpinellin significantly accelerated resolution. To confirm these findings, *mpx*:GFP larvae were injured at 3 dpf and those which mounted a good response were selected for treatment with a range of concentrations of isopimpinellin at 6 hpi. Neutrophil numbers at the site of injury were counted at 12 hpi. Isopimpinellin significantly accelerated the resolution of inflammation at 25 and 100 μM, though some toxicity was observed at the latter dose (Figure 4.16).

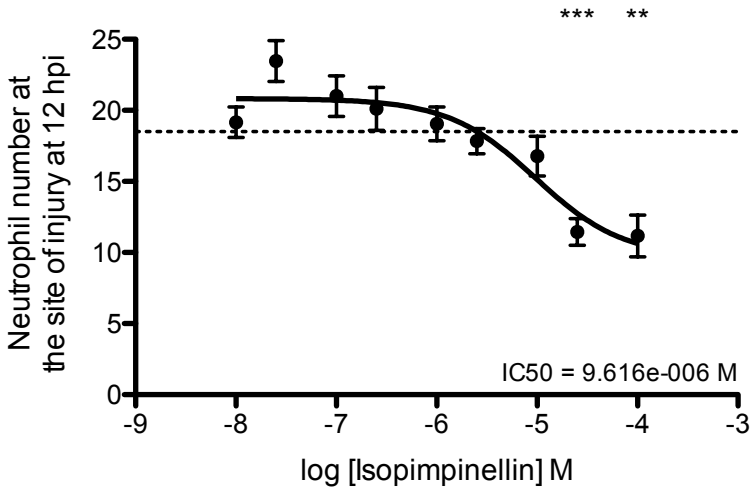

Figure 4.16: Isopimpinellin significantly accelerates inflammation resolution in a dose dependent manner.

At 3 dpf, *mpx*:GFP larvae were injured and those which mounted a good neutrophilic response were treated at 6 hpi with varying doses of isopimpinellin. Neutrophil numbers at the site of injury were counted at 12 hpi. Isopimpinellin caused a dose-dependent reduction in neutrophil number at the wound, with significantly lower neutrophil numbers present in larvae treated with isopimpinellin at a concentration of 25 μM and higher compared to the DMSO vehicle control (One-way ANOVA with Dunnett's multiple comparison post-test; where ** $P < 0.01$ and *** $P < 0.001$; $n = 18$, performed as 3 independent experiments). Dotted line at y = 18.5 indicates mean neutrophil number at site of injury in control larvae.

4.5.1 Isopimpinellin enhances resolution *in vivo* by causing neutrophil apoptosis

Neutrophil apoptosis at sites of inflammation, followed by phagocytic uptake of corpses by macrophages, is the best described process by which neutrophils are removed during resolution (Savill et al., 1989). This has been shown to play a particularly important role in mammalian models of inflammation (Gilroy et al., 2003; Rossi et al., 2006; Sawatzky et al., 2006). More recent evidence has indicated that neutrophil apoptosis is also one of the mechanisms responsible for driving inflammation resolution in the zebrafish (Renshaw et al., 2006; Loynes et al., 2009; Elks et al., 2011).

I hypothesised that isopimpinellin was accelerating resolution by causing an increase in neutrophil apoptosis and investigated this in zebrafish larvae using dual TSA/TUNEL staining. Following the standard resolution assay protocol, 3 dpf *mpx*:GFP larvae were injured and treated with isopimpinellin at 6 hpi. At 12 hpi, rather than counting neutrophil numbers at the site of injury, larvae were fixed in 4% PFA overnight in preparation for fluorescence labelling. A TSA stain was performed for endogenous neutrophil-specific myeloperoxidase activity, followed by TUNEL staining for double stranded DNA breaks as an indicator of apoptosis. Injured tail fins were imaged on an Ultra*VIEW*VoX spinning disk confocal imaging system with an inverted Olympus IX81 microscope, at 20x magnification (Figure 4.17). The total neutrophil number at the site of injury and number of dual-labelled apoptotic neutrophils was analysed in each larva, to calculate the mean percentage of neutrophil apoptosis in each treatment group. Isopimpinellin significantly increased the percentage of neutrophil apoptosis at the site of injury compared to the DMSO vehicle control (Figure 4.18 A). These data were consistent with the rates of apoptosis expected in both age-matched control larvae and larvae treated with a known inducer of neutrophil apoptosis, GSK650394 (Burgon et al., in submission).

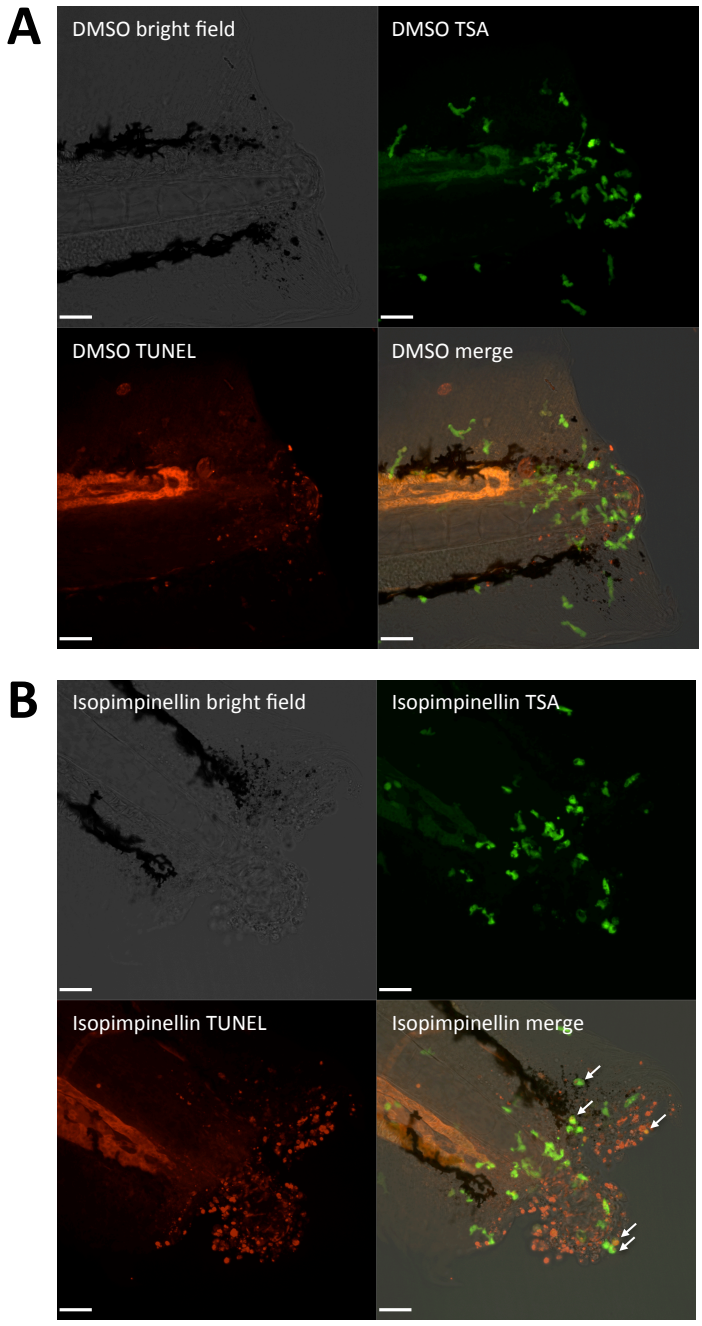

Figure 4.17: TSA/TUNEL staining indicates that isopimpinellin increases neutrophil apoptosis during inflammation resolution.

Tail transection was performed on 3 dpf *mpx*:GFP larvae and good responders were treated with 25 µM isopimpinellin or DMSO at 6 hpi. Larvae were fixed in 4% PFA at 12 hpi and neutrophils were stained with TSA, followed by TUNEL staining for apoptosis. Tail fins were mounted and imaged on a confocal spinning disk imaging system at 20x magnification. Images from (A) a DMSO control larva and (B) an isopimpinellin treated larva are shown. Apoptotic neutrophils are indicated by the white arrows. Scale bar represents 70 µm.

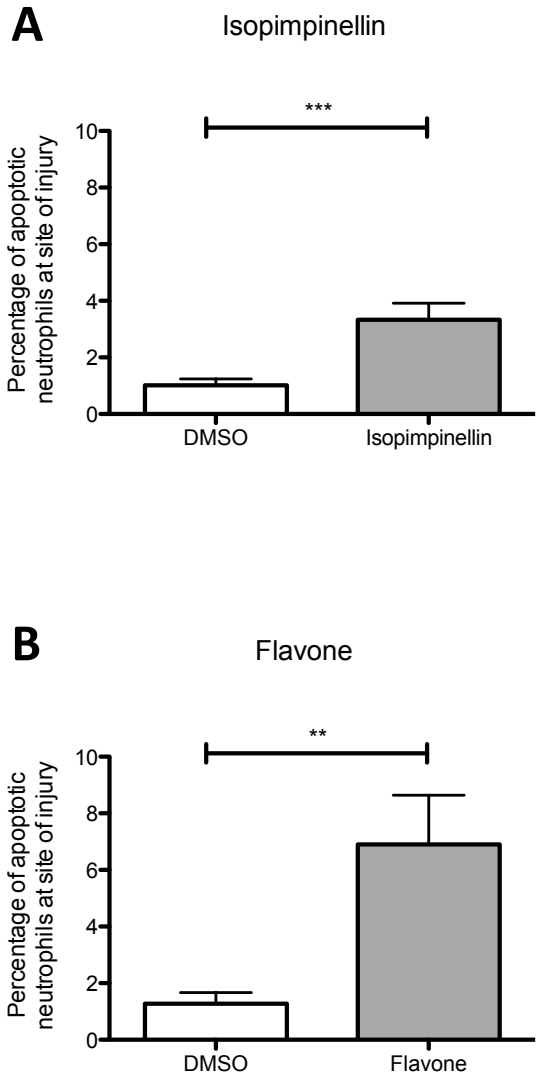

Figure 4.18: Isopimpinellin and flavone significantly increase neutrophil apoptosis during inflammation resolution.

Tail transection was performed on 3 dpf *mpx*:GFP larvae and good responders were treated with 25 µM isopimpinellin or DMSO at 6 hpi. Larvae were fixed in 4% PFA at 12 hpi and neutrophils were stained with TSA, followed by TUNEL staining for apoptosis. Tail fins were mounted and imaged on a confocal spinning disk imaging system at 20x magnification. The total number of neutrophils at the site of injury and the number of apoptotic neutrophils were counted to calculate the percentage apoptosis. (A) Isopimpinellin caused a significant increase in neutrophil apoptosis (Unpaired *t*-test; $P < 0.001$; $n \geq 111$, performed as 2 independent experiments). (B) Preliminary data indicated that flavone also causes a significant increase in neutrophil apoptosis (Unpaired *t*-test; $P < 0.01$; $n \geq 55$, performed as 1 experiment).

Interestingly, preliminary data from a TSA/TUNEL assay performed on larvae treated with flavone (compound MMM115 as synthesised by Marco Meloni) also showed a highly significant increase in neutrophil apoptosis (Figure 4.18 B), suggesting that this might be a common mechanism of action by which these chromone-related compounds drive inflammation resolution. In support of this hypothesis, another research group has since reported that a series of flavones can induce apoptosis in isolated human neutrophils, even in the presence of various neutrophil survival factors (Lucas et al., 2012). They also show that one of these flavones, wogonin, can drive inflammation resolution in the *mpx*:GFP zebrafish, in a caspase-dependent manner.

4.5.2 Isopimpinellin does not increase the reverse migratory behaviour of neutrophils during inflammation resolution *in vivo*

Having determined that isopimpinellin accelerated inflammation resolution by causing a significant increase in neutrophil apoptosis, I wished to explore an alternative mechanism by which neutrophils might also be removed from the site of injury. There is a growing body of evidence to suggest that neutrophils can be cleared from sites of inflammation by reverse migration, a phenomenon which has also been reported to occur in mammalian systems (Hughes et al., 1997; Buckley et al., 2006; Nourshargh et al., 2011). This can be visualised more readily and in real time using transparent zebrafish larvae, in which various research groups have observed large numbers of fluorescently-labelled neutrophils leaving a wound site (Mathias et al., 2006; Hall et al., 2007; Elks et al., 2011; Yoo & Huttenlocher, 2011).

In order to investigate the migratory behaviour of neutrophils during the resolution phase of inflammation in the presence of isopimpinellin, I performed a reverse migration assay using larvae raised from the double transgenic line *Tg(mpx:Gal4;UAS:Kaede)i222*, subsequently referred to as *mpx*/Kaede. Neutrophils in these larvae express photoactivatable Kaede, a fluorescent protein originally found in stony coral. This converts from green to red fluorescence upon exposure to certain wavelengths of light, enabling the photoconversion and tracking of distinct populations of neutrophils. To examine the effects of isopimpinellin on neutrophil reverse migration, tail fin transection was performed on *mpx*/Kaede larvae at 3 dpf. At

4 hpi, those that mounted a good inflammatory response were incubated with either isopimpinellin or DMSO as a vehicle control. At 6 hpi, larvae were mounted in a chamber slide, in agarose containing the appropriate treatment to ensure their continued exposure. Larvae were viewed on the Ultra*VIEW*VoX spinning disk confocal imaging system and Kaede-labelled neutrophils at the site of injury were converted to red fluorescence using an Ultra*VIEW*PhotoKinesis^{TM} device, with the 405 nm laser line at 40% laser energy (optimum parameters defined by a former BMedSci student, Giles Dixon). Larvae were then transferred to an inverted compound fluorescence microscope and neutrophils in both the red and green channels were imaged at 2.5 minute intervals for approximately 300 minutes. To analyse reverse migration, red neutrophils that migrated away from the site of injury into a defined distal region (as illustrated in Figure 4.19 A) were quantified over the course of the time-lapse.

In DMSO control larvae, neutrophils gradually migrated away from the site of injury, with some movement back and forth within the injured region, characteristic of patrolling behaviour. Fewer neutrophils appeared to reverse migrate in isopimpinellin treated larvae and linear regression analysis indicated that there was a highly significant difference in neutrophil movement compared to the controls (Figure 4.19 B). On observation of the time-lapse movies it became apparent that in the majority of cases, in preference to leaving the site of injury via reverse migration, neutrophils in isopimpinellin treated larvae remained static and adopted a rounded morphology, characteristic of the apoptotic phenotype that has been described previously (Loynes et al., 2009). This occurred in both red-labelled neutrophils at the site of injury and green-labelled neutrophils that migrated into the wound region after photoconversion was performed. Apoptosis and the subsequent loss of fluorescence appeared to occur more frequently, or at least earlier in the course of the time-lapse, in red-labelled cells compared to green ones (Figure 4.20).

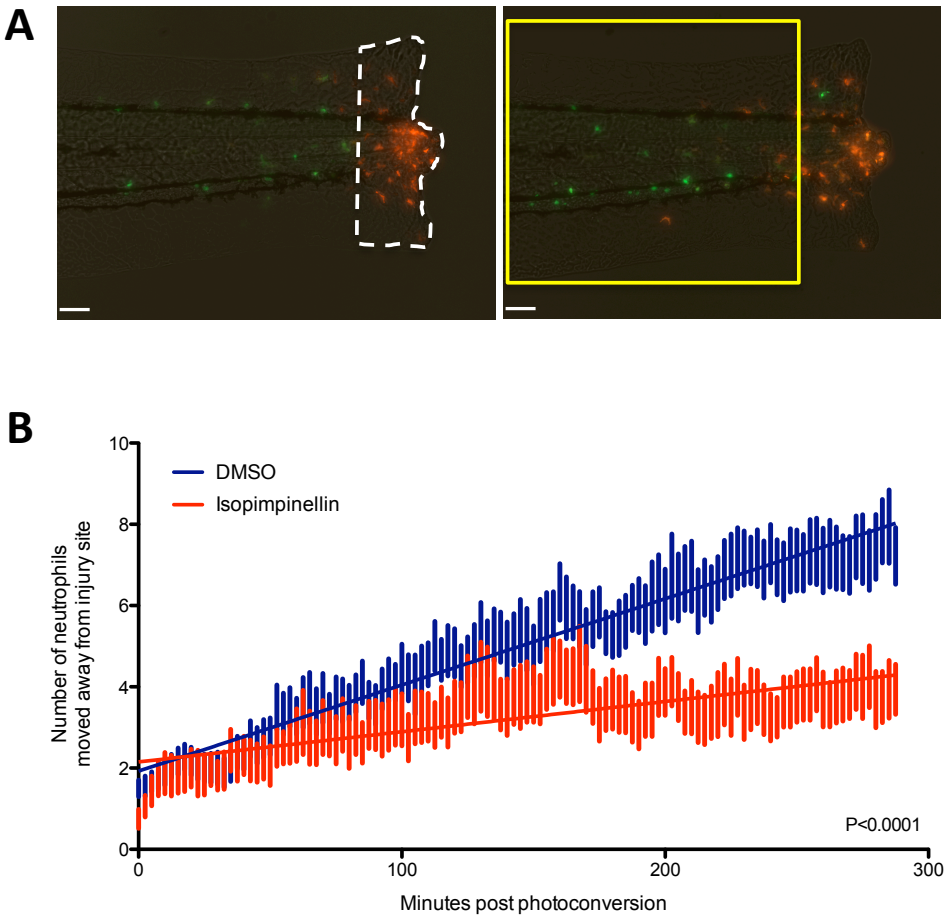

Figure 4.19: Isopimpinellin does not increase reverse migration of neutrophils during inflammation resolution.

Tail fin transection was performed on *mpx*/Kaede larvae at 3 dpf, and those that mounted a good neutrophilic response were selected for treatment with 25 µM isopimpinellin or the DMSO vehicle control at 4 hpi. At 6 hpi, larvae were mounted and neutrophils at the site of injury were photoconverted from green to red fluorescence as illustrated in (A, left), on a confocal spinning disk system using an Ultra*VIEW*PhotoKinesis™ device, with the 405 nm laser line at 40% laser energy. Scale bar represents 60 µm. Larvae were then time-lapsed on an inverted compound fluorescence microscope for 3.5 hours and the number of red cells that migrated away from the site of injury into a region defined as illustrated in (A, right) were quantified over the time-course. Isopimpinellin caused a significant change in neutrophil reverse migration compared to the DMSO vehicle control (B), with fewer neutrophils migrating away from the wound over time (*n* = 18, performed as 3 independent experiments, data expressed as mean ± SEM).

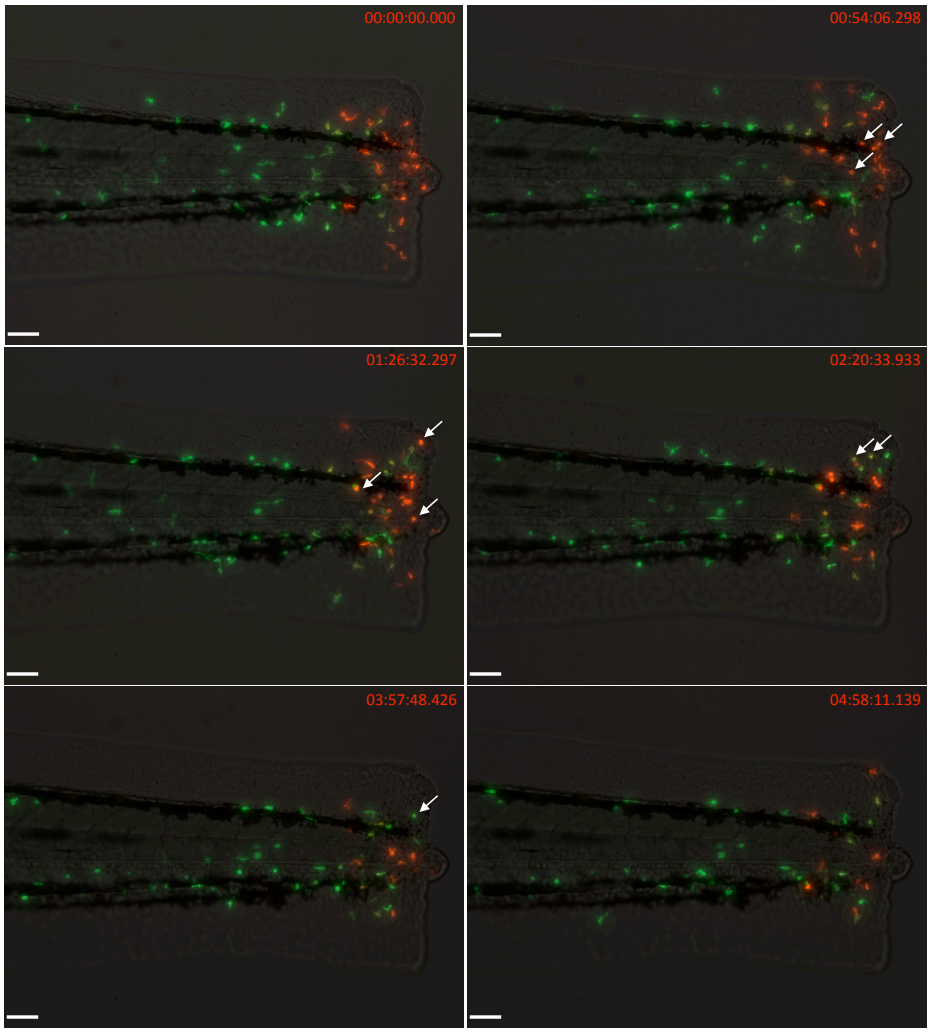

Figure 4.20: Morphological changes characteristic of neutrophil apoptosis can be observed in the presence of isopimpinellin during inflammation resolution.

Tail fin transection was performed on *mpx*/Kaede larvae at 3 dpf and good responders were selected for treatment with 25 µM isopimpinellin at 4 hpi. At 6 hpi, larvae were mounted and neutrophils at the site of injury were photoconverted from green to red fluorescence on a confocal spinning disk system using an Ultra*VIEW*PhotoKinesis™ device, with the 405 nm laser line at 40% laser energy. Larvae were then time-lapsed on an inverted compound fluorescence microscope for up to 5 hours. Neutrophils are observed to adopt a rounded, apoptotic morphology within 1 hour, as indicated by the white arrows. Multiple apoptotic events can be visualised over the course of the time-lapse (note not all apoptotic neutrophils are indicated), with few red neutrophils remaining at the final time point. Scale bar represents 60 µm.

4.5.3 Conclusions on the pro-resolution activity of isopimpinellin *in vivo*

The TSA/TUNEL staining revealed a significant increase in neutrophil apoptosis in isopimpinellin treated larvae. This, combined with the striking observation of multiple apoptotic events occurring at the site of injury during the time-lapse movies generated in the reverse migration assay, indicated that the primary mechanism by which isopimpinellin could drive inflammation resolution in zebrafish larvae was by promoting neutrophil apoptosis. On searching through the literature, I discovered that isopimpinellin is a structural isomer of khellin (Figure 4.21 A), an extract of *Ammi visnaga* from the Apiaceae family of plants, various species of which are also sources of isopimpinellin. This was of particular interest, as a series of drugs that are now used clinically to treat allergic conditions were originally developed based on khellin and I realised that these are in fact termed the 'cromolyns' or 'cromones'. The mast cell stabilising properties of this group of compounds are well known, although it is believed that they have additional anti-inflammatory properties, the mechanisms of which remain elusive. I sought to investigate the activity of these compounds, with the idea that isopimpinellin might share some of their functional activity due to their common chromone-derived origin. As isopimpinellin had such a profound effect on neutrophil apoptosis, I hypothesised that this might be a previously unidentified mechanism of action of the cromones, which might lead to interesting implications for their therapeutic use in inflammatory disease.

For clarity, the clinically available drugs originally developed from khellin, including sodium cromoglycate and nedocromil, are collectively referred to as the 'cromones'. Use of the word 'chromone' refers to any compound with a structural formula based on the 1,4-benzopyrone backbone, for example the hits isopimpinellin and methylquercetin. It should be noted that the cromones are derivatives of chromone, therefore in circumstances when experiments were carried out on both these and the hit compounds together, all of the compounds are collectively referred to as 'chromones' or 'chromone-derivatives'.

4.6 Investigation into the effects of cromones on neutrophil apoptosis

4.6.1 History and development of the cromones

The ancient herb 'khella' was originally used in Egyptian folk medicine as a remedy for renal colic due to its diuretic and smooth muscle relaxant properties (Edwards & Howell, 2000), but it was not until 1947 that it was discovered that the isolated component khellin was highly effective in the treatment of bronchial asthma (Anrep & Barsoum, 1947). This triggered the synthesis and development of more soluble and efficacious analogues of khellin, one of the first being K18, which was shown to be protective against the potentially fatal bronchoconstriction induced by albumin in pre-sensitised 'Herxheimer' guinea pigs (Herxheimer, 1952; Edwards & Howell, 2000). It was also discovered that another chromone analogue, sodium cromoglycate, possessed an additional mechanism of action alongside its bronchodilator effects; inhibition of the allergic reaction mediators histamine and SRS-A (slow releasing substance of anaphylaxis) (Sheard & Blair, 1970). However, the significance of these findings was not fully realised until the now famous research carried out by Dr Roger Altounyan in the late 1950s. Altounyan suffered from severe chronic asthma, and used himself as a human model to test the preventative action of the newly synthesised khellin analogues on asthma attacks following inhaled bronchial antigen challenge. Throughout his lifetime, Altounyan self-tested over 200 compounds, some of his work being carried out in secret due to the chromone project being temporarily shut down as it was considered 'quite useless and unproven' (Edwards & Howell, 2000). Despite this minor setback, Altounyan was able to recognise the structure-activity relationships emerging from the series of compounds he tested and the most protective against antigen challenge were used in several small patient trials. Disodium cromoglycate was one of the first bis-chromones (Figure 4.21 B) to be developed, and was launched by Fisons Pharmaceuticals as Intal® for inhaled asthma treatment in the late 1960s (Cox, 1967). The related nedocromil sodium followed a few years later and was found to have greater potency than sodium cromoglycate, being particularly effective in patients with moderate asthma (Edwards & Stevens, 1993). Due to their limited side effects, the cromones began to be used in the treatment of other allergic conditions including conjunctivitis and intestinal allergies (Yazid, Ayoub, et al., 2010).

4.6.2 Known anti-inflammatory properties of the cromones

The cromones were originally defined as 'mast cell stabilisers' due to their ability to inhibit mast cell degranulation and histamine release (Cox, 1967). It is now widely accepted that this is not the only clinically relevant mechanism of action of these drugs but that their therapeutic benefit in asthma patients can be attributed to their additional anti-inflammatory activities. Evidence indicates that disodium cromoglycate can inhibit leukocyte activation (Kay et al., 1987) and neutrophil chemotaxis (Szkudlińska et al., 1996). Nedocromil sodium has been reported to inhibit cytokine and eicosanoid release from bronchial epithelial cells, eosinophils and macrophages (Mattoli et al., 1990; Radeau et al., 1993; Devalia et al., 1996) and down-regulate expression of the adhesion molecule ICAM-1 (Sacco et al., 1999).

The molecular mechanisms by which the cromones modulate their anti-inflammatory effects remained elusive until the last few years, during which developments have been made towards understanding the complex activity of these compounds. Although their effects on neutrophil trafficking could be secondary to the inhibition of pro-inflammatory mediator release from mast cells (Kubes & Granger, 1996), evidence now indicates that cromones have a more direct effect on neutrophils. One line of research suggests this may be mediated via the extracellular protein Annexin-A1 (Anx-A1), which is released from neutrophils and other cell types upon stimulation with glucocorticoids (Goulding et al., 1990; Ahluwalia et al., 1994). Secretion of Anx-A1 is dependent upon its phosphorylation at Ser^{27} by protein kinase C (PKC). Its subsequent translocation to the cell membrane can be prevented by PI-3K and MAPK inhibitors, suggestive of a role for these kinases in Anx-A1 trafficking (Solito, 2003; Solito et al., 2006). Once secreted, Anx-A1 signals through receptors of the formyl peptide receptor family, most likely ALX, in an autocrine or paracrine fashion, to inhibit neutrophil activation (Perretti & Flower, 2004; Brancaleone et al., 2011).

Cromones were initially reported to exert their activity through an Anx-A1 dependent mechanism in the U937 human monocytic cell line, in which activation of PKC combined with the phosphorylation, translocation and release of Anx-A1 is triggered by glucocorticoids (Yazid et al., 2009). Disodium cromoglycate and sodium nedocromil

alone had only a weak effect in these cells, however in combination with a fixed concentration of dexamethasone these activities were greatly enhanced. They also caused a more pronounced inhibition in the generation of the eicosanoid TxB_2, to levels above those recorded with dexamethasone alone. Importantly, the cromones were found to directly inhibit protein phosphatase 2A (PP2A), which limits the activity of PKC, leading to the conclusion that this might be the main mechanism by which they act to promote Anx-A1 release. It is likely that this occurs in a synergistic manner with the activation of PKC by glucocorticoids. These effects have also been demonstrated in neutrophils, in which the inhibition of Anx-A1 release by cromones is associated with reduced neutrophil adhesion and emigration (Yazid, Leoni, et al., 2010). As further evidence to support the Anx-A1 dependent mechanism of action of the cromone drugs, nedocromil was found to inhibit neutrophil migration in a murine model of acute peritonitis, having no effect in Anx-$A1^{-/-}$ mice (Yazid, Leoni, et al., 2010).

4.6.3 Disodium cromoglycate accelerates the resolution of inflammation *in vivo*

To begin to explore the function of the cromone drugs on the resolution of inflammation *in vivo*, I investigated the activity of disodium cromoglycate in a standard resolution assay. Tail transection was performed on *mpx*:GFP larvae at 3 dpf, and those that mounted a good response to injury at 6 hpi were selected for treatment with disodium cromoglycate. Preliminary data suggested that low doses of this compound, in the range of 0.01 to 10 µM were ineffective, with mean neutrophil numbers at the site of injury not significantly lower than in control larvae. This was in keeping with published *in vivo* data, in which the doses of cromones used were higher than those estimated from *in vitro* studies, likely due to poor absorption, rapid degradation or other pharmacokinetic properties of these compounds (Yazid, Leoni, et al., 2010). In an effort to negate the possibility that the compound might be metabolised into inactive components too quickly, larvae were re-dosed with disodium cromoglycate every hour after 6 hpi. At 12 hpi, a significant reduction in neutrophil number at the injury site was observed in treated larvae compared to the controls (Figure 4.21 C).

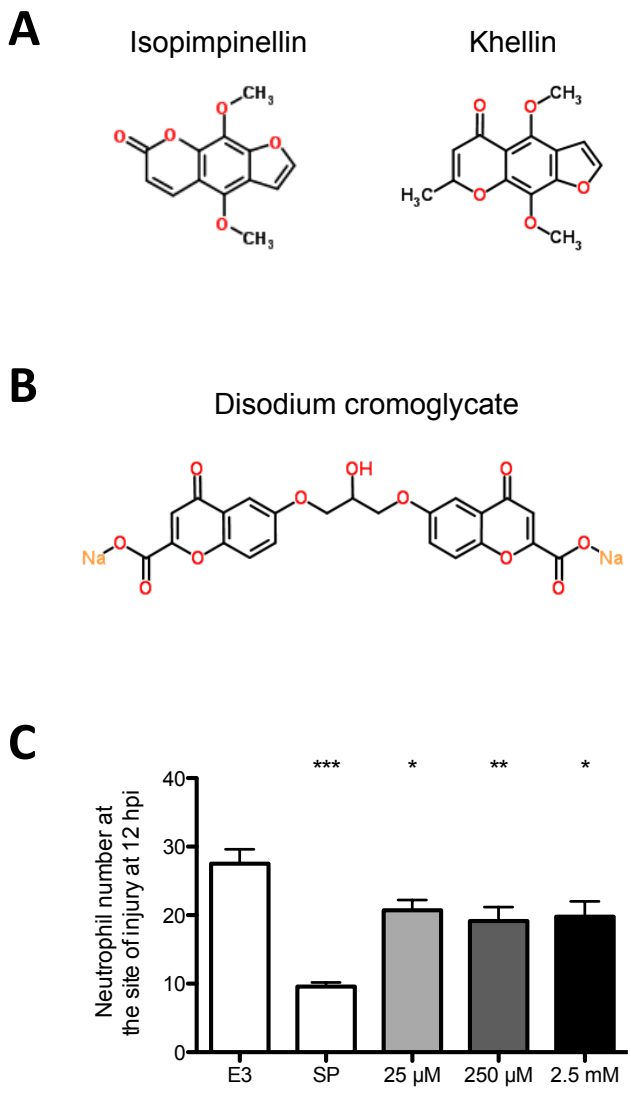

Figure 4.21: The khellin derivate disodium cromoglycate significantly accelerates inflammation resolution *in vivo*.

(A) Isopimpinellin is a structural isomer of khellin, the compound from which a group of anti-allergic drugs were developed, including disodium cromoglycate (B). To investigate its effect *in vivo*, 3 dpf *mpx*:GFP larvae were injured and treated with varying concentrations of disodium cromoglycate at 6 hpi. Neutrophil counts were performed at 12 hpi as per the standard resolution assay protocol, using 30 µM SP600125 as a positive control (referred to as SP). (C) Disodium cromoglycate significantly reduced neutrophil numbers at the site of injury at 12 hpi compared to the E3 vehicle control (One-way ANOVA with Dunnett's multiple comparison post-test; where * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$; $n = 18$, performed as 3 independent experiments).

4.6.4 Disodium cromoglycate and other chromone-derived compounds do not cause neutrophil apoptosis *in vitro*

Cromones have been shown to increase Anx-A1 phosphorylation and release by directly inhibiting PP2A in neutrophils (Yazid, Leoni, et al., 2010). In these studies, similar levels of PKC and Anx-A1 phosphorylation were observed with a pharmacological inhibitor of PP2A, okadaic acid. Disodium cromoglycate, nedocromil and okadaic acid caused enhanced Anx-A1 release compared to dexamethasone alone. These findings were of particular interest, as PP2A inhibition by okadaic acid has been shown to induce hyper-phosphorylation of the anti-apoptotic protein Mcl-1 in neutrophils (Derouet et al., 2004). This leads to instability and accelerated turnover of Mcl-1, resulting in increased neutrophil apoptosis.

As mentioned previously, a potential structure-activity relationship exists between the known cromone drugs and the chromone-derived hit compounds identified in the Spectrum Collection screen. These include isopimpinellin, which I have shown to increase neutrophil apoptosis in zebrafish. Additionally, disodium cromoglycate appeared to accelerate resolution of inflammation in the zebrafish tail fin injury model. In light of these data, I hypothesised that the cromones might drive resolution by inducing neutrophil apoptosis via inhibition of PP2A.

To investigate this hypothesis, I initially tested disodium cromoglycate *in vitro*, in order to investigate its effect on human neutrophil apoptosis. Neutrophils were isolated from peripheral whole blood by either the Optiprep™ or Percoll® method of purification, as described, and were treated with multiple doses of disodium cromoglycate. After 8 hours in culture, two cytospins were acquired per condition and the percentage of neutrophil apoptosis was calculated based on nuclear morphology. No significant difference in constitutive apoptosis was found in neutrophils treated with disodium cromoglycate compared to the vehicle control, however in these particular experiments there was high variation between donors (Figure 4.22 A).

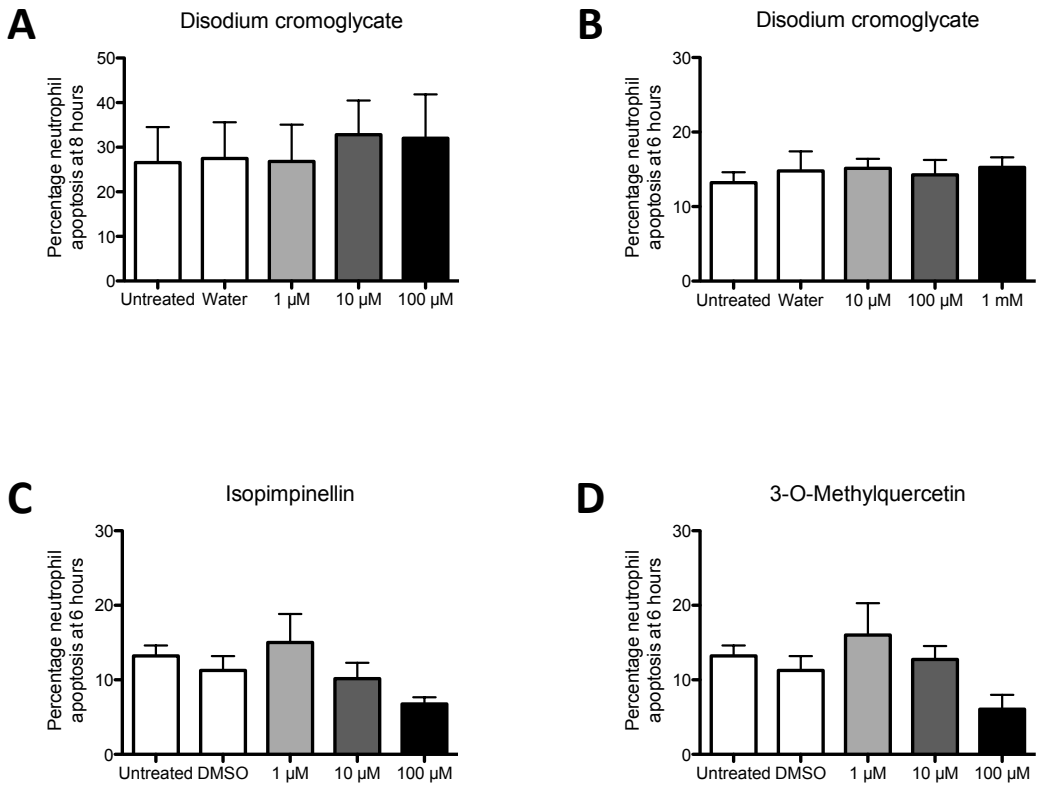

Figure 4.22: Chromone-derived compounds do not induce human neutrophil apoptosis *in vitro*.

Neutrophils were isolated from whole blood using either the Optiprep™ or Percoll® method of separation and were incubated with (A) disodium cromoglycate for 8 hours, (B) disodium cromoglycate for 6 hours, (C) isopimpinellin for 6 hours or (D) methylquercetin for 6 hours. In each case, 2 cytospins were acquired and percentage neutrophil apoptosis calculated based on nuclear morphology. No significant change in neutrophil apoptosis was observed in any condition, although there appeared to be a trend towards neutrophil survival with (C) isopimpinellin and (D) methylquercetin (One-way ANOVA with Dunnett's multiple comparison post-test; $n = 2$).

It was possible that the concentration of disodium cromoglycate used was not high enough to cause an effect on neutrophil apoptosis, therefore the previous experiment was repeated using doses up to 1 mM. Another factor to consider was that any effect on neutrophil apoptosis could be occurring very rapidly; within the first few hours of culture, and that the disodium cromoglycate might become less effective over time due to degradation. By 8 hours, the normal levels of constitutive apoptosis occurring in the controls might mask any early effect on apoptosis caused by the cromone. For this experiment, cytospins were acquired after 6 hours of culture, as at this time point the levels of constitutive apoptosis in untreated neutrophils are expected to be approximately 15% lower than at 8 hours (personal communication, Joseph Burgon and Dr Stephen Renshaw). However, even at 6 hours and with the maximum dose of 1 mM, no increase in neutrophil apoptosis was observed in the presence of disodium cromoglycate compared to the vehicle control (Figure 4.22 B). To investigate the effects of other chromone-derived compounds on neutrophil apoptosis *in vitro*, the hit compounds isopimpinellin and methylquercetin were also tested in this assay. There was no significant increase in neutrophil apoptosis with either compound and somewhat surprisingly there appeared to be a trend towards survival with increasing dose (Figure 4.22 C, D).

4.6.5 Chromone-derived compounds do not affect GM-CSF induced survival signalling

As no effect on constitutive apoptosis was observed in the presence of disodium cromoglycate, isopimpinellin or methylquercetin, I hypothesised that these compounds might interfere with neutrophil survival signalling, for example by the endogenous survival signal, granulocyte-macrophage colony-stimulating factor (GM-CSF). GM-CSF is known to activate multiple signalling pathways including the PI-3K and JAK/STAT pathways (Epling-Burnette et al., 2001) and delays neutrophil apoptosis by regulation of Mcl-1 (Moulding et al., 1998). At this point I was also able to obtain some nedocromil (a kind gift from Prof Rod Flower) and this was tested alongside the other chromones.

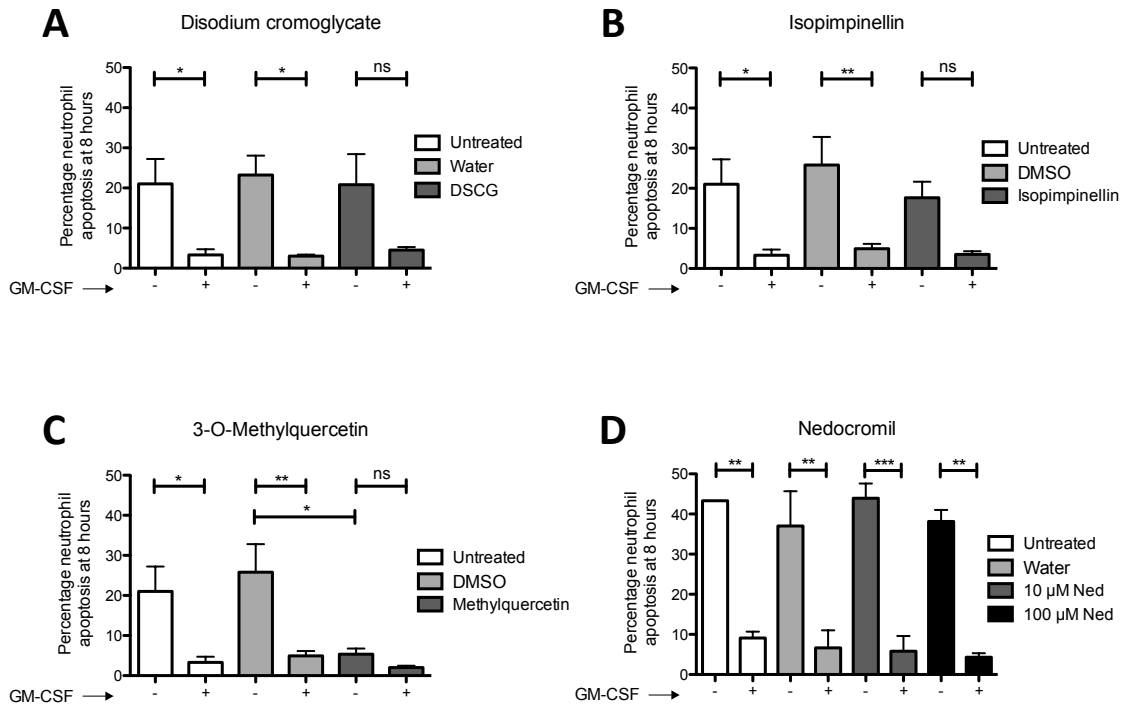

Figure 4.23: Chromone-derived compounds do not affect GM-CSF induced neutrophil survival.

Neutrophils were isolated from whole blood using either the Optiprep™ or Percoll® method of separation and were incubated for 8 hours with (+) and without (-) GM-CSF as indicated and with (A) 10 µM disodium cromoglycate (DSCG), (B) 10 µM isopimpinellin, (C) 10 µM methylquercetin or (D) nedocromil at the dose indicated. In each case, 2 cytospins were acquired and the percentage of neutrophil apoptosis was calculated based on nuclear morphology. GM-CSF significantly reduced apoptosis in control groups and this survival did not appear to be inhibited in the presence of any of the chromone derivatives (One-way ANOVA with Bonferroni's multiple comparison post-test to compare selected columns; where * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$; $n = 2$). A significant reduction in constitutive neutrophil apoptosis was observed with methylquercetin (C).

For these experiments, neutrophils were isolated from peripheral whole blood, as previously described, and treated with each compound at the dose indicated, in the presence and absence of GM-CSF. After 8 hours in culture, two cytospins were acquired per condition and the percentage of neutrophil apoptosis was calculated based on nuclear morphology. In all cases, GM-CSF caused a significant reduction in neutrophil apoptosis in untreated and vehicle control neutrophils, as expected (Figure 4.23). The level of GM-CSF induced survival in the presence of all chromones tested was comparable to the appropriate vehicle controls, suggesting that these compounds did not interfere with GM-CSF survival signalling pathways. Although not statistically significant, there was a clear reduction in the percentage of neutrophil apoptosis when neutrophils were treated with GM-CSF in combination with disodium cromoglycate, isopimpinellin and methylquercetin (Figure 4.23 A-C). This lack of significance was most likely due to their slightly lower rates of constitutive apoptosis compared to the vehicle controls. In contrast to the other chromones, methylquercetin caused significant neutrophil survival (Figure 4.23 C). Nedocromil did not have any effect on constitutive neutrophil apoptosis or GM-CSF induced survival at either dose tested (Figure 4.23 D). These results indicated that the chromones were not affecting either constitutive neutrophil apoptosis or GM-CSF induced survival pathways, with the exception of methylquercetin, which in contrast to the original hypothesis appeared to promote neutrophil survival.

4.6.6 Chromone-derived compounds do not induce neutrophil apoptosis in the presence of dexamethasone

As an alternative mechanism by which the chromones might affect neutrophil apoptosis, I chose to investigate their effects in the presence of another activator of neutrophil survival signalling, dexamethasone. Glucocorticoids such as dexamethasone are not particularly effective in the treatment of inflammatory diseases characterised by neutrophilic inflammation (Barnes, 2000; Green et al., 2002). This is believed to be due to their inhibitory effect on neutrophil apoptosis (Cox, 1995; Kato et al., 1995; Liles et al., 1995) in contrast to their apoptosis-inducing properties in other cell types, including eosinophils and T cells (Smoak & Cidlowski, 2004). Although the precise mechanism by which dexamethasone promotes neutrophil survival is not fully defined,

it is thought to be associated with up-regulation of Mcl-1 (Sivertson et al., 2007). Other anti-apoptotic proteins such as XIAP may also be involved (Saffar et al., 2011).

As discussed previously, studies have indicated that cromones can potentiate the effect of glucocorticoids, resulting in enhanced Anx-A1 release (Yazid et al., 2009; Yazid, Leoni, et al., 2010). Despite the fact that glucocorticoids maintain neutrophil survival, there is evidence to support a pro-apoptotic role for Anx-A1 in human neutrophils (Solito et al., 2003), which is dependent on calcium entry and caspase-3 activation (Perretti & Solito, 2004). I hypothesised that by treating human neutrophils in combination with dexamethasone and cromones, there would be increased Anx-A1 release and this might be sufficient to override the survival effect of dexamethasone to induce neutrophil apoptosis.

To investigate this hypothesis, freshly isolated neutrophils were treated with each compound at the dose indicated, in the presence and absence of dexamethasone at 1 µM. This concentration was selected based on published data, in which 1 µM is sufficient to cause a significant increase in neutrophil survival (Cox, 1995; Liles et al., 1995). Methylquercetin was not tested in these experiments due to its significant effect on neutrophil survival in the previous assay. Ketotifen, a histamine receptor antagonist, was tested instead, as this compound has been shown to exhibit similar pharmacological properties to disodium cromoglycate (Kubes & Granger, 1996; Cook et al., 2002). Studies indicate that, like both the cromones and glucocorticoids, ketotifen triggers phosphorylation of Anx-A1 at Ser^{27} and its translocation to the plasma membrane (Yazid, Leoni, et al., 2010).

Following 8 hours of culture, two cytospins were acquired for each condition and the percentage of neutrophil apoptosis was calculated. Dexamethasone induced neutrophil survival in untreated, vehicle control and compound treated neutrophils with the exception of nedocromil (Figure 4.24). In this case there was still a clear reduction in apoptosis in the presence of dexamethasone and the lack of significance could most likely be attributed to the high level of variability between donors (Figure 4.24 D).

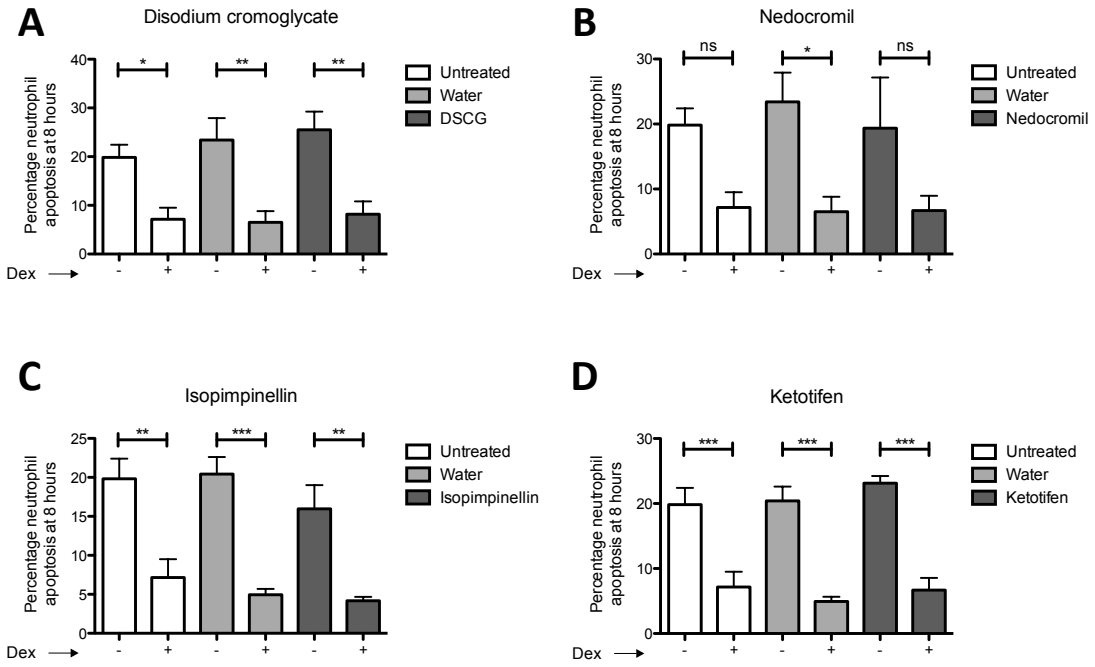

Figure 4.24: Chromone-derived compounds do not override the survival effect of dexamethasone.

Neutrophils were isolated from whole blood using either the Optiprep™ or Percoll® method of separation and were incubated for 8 hours with (+) and without (-) 1 µM dexamethasone (Dex) as indicated and with (A) 10 µM disodium cromoglycate (DSCG), (B) 10 µM nedocromil, (C) 10 µM isopimpinellin or (D) 10 µM ketotifen. In each case, 2 cytospins were acquired and the percentage of neutrophil apoptosis was calculated based on nuclear morphology. Dexamethasone significantly reduced apoptosis in control groups and this survival did not appear to be inhibited in the presence of any of the chromone derivatives or ketotifen (One-way ANOVA with Bonferroni's multiple comparison post-test to compare selected columns; where * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$; $n = 2$).

The results from these experiments indicated that neither the clinically available cromones nor isopimpinellin caused apoptosis of isolated neutrophils *in vitro*. If there was an increase in release of Anx-A1 in the combined presence of dexamethasone and the cromones, this was either not a pro-apoptotic signal or not sufficient to override the survival signalling activated by glucocorticoid-receptor binding.

4.6.7 Disodium cromoglycate might potentiate the effect of glucocorticoids *in vivo*

Data obtained in the *in vitro* human neutrophil experiments strongly suggested that the chromone-derived compounds tested did not have a direct effect on neutrophil survival. I had previously shown that disodium cromoglycate could accelerate inflammation resolution in zebrafish. It was possible that either this effect was not a consequence of neutrophil apoptosis but rather an alternative resolution mechanism, or that some necessary stimulus was missing from the *in vitro* system. To investigate whether the effect of disodium cromoglycate *in vivo* was likely to be Anx-A1 dependent, a resolution assay was performed on 3 dpf *mpx*:GFP larvae with the hypothesis that there would be a greater reduction in neutrophil number in the combined presence of disodium cromoglycate and a glucocorticoid compared to either alone. Betamethasone was used in these experiments, as this glucocorticoid was known to be functionally active in other zebrafish assays, implying that there would be no issues with its absorption or metabolism (personal communication, Dr Freek van Eeden). Larvae were injured and those that mounted a good response to injury were selected for treatment at 6 hpi. Larvae were re-dosed every hour until 12 hpi, when neutrophil numbers at the site of injury were counted. Betamethasone alone did not significantly reduce the neutrophil count compared to the vehicle control (Figure 4.25). In these experiments, disodium cromoglycate alone did not cause a significant reduction in neutrophil number at either dose tested, although there were significantly fewer neutrophils at the site of injury in the larvae treated with both betamethasone and 2.5 mM disodium cromoglycate in combination (Figure 4.25). Despite this, the results were inconclusive, as the combined effect was not significantly different to either betamethasone or 2.5 mM disodium cromoglycate alone. From these experiments, I was unable to determine whether or not disodium cromoglycate could potentiate the effect of betamethasone.

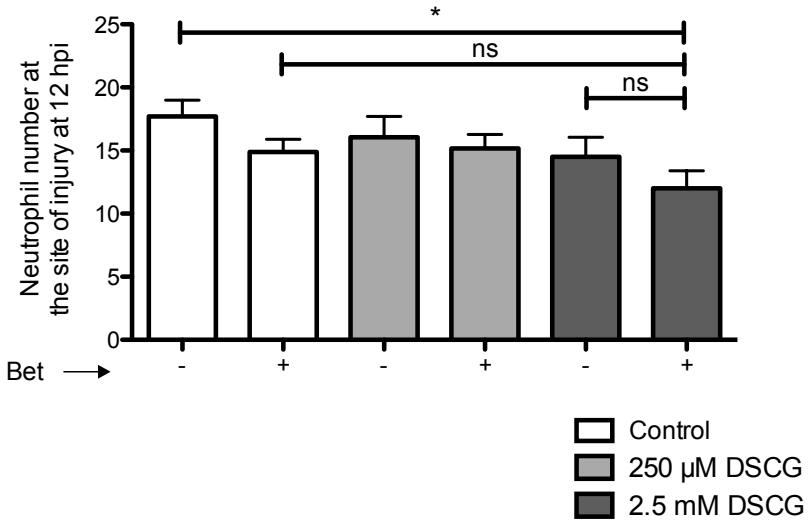

Figure 4.25: Resolution of inflammation is accelerated *in vivo* in the combined presence of disodium cromoglycate and a glucocorticoid.

At 3 dpf, *mpx*:GFP larvae were injured and treated in the presence (+) or absence (-) of 10 µM betamethasone (Bet) and with either 250 µM or 2.5 mM disodium cromoglycate (DSCG) as indicated at 6 hpi. Neutrophil counts were performed at 12 hpi. There was a significant reduction in neutrophil number at the site of injury at 12 hpi in larvae treated with disodium cromoglycate and betamethasone in combination compared to the vehicle control, but not compared to either drug alone (One-way ANOVA with Bonferroni's multiple comparison post-test; $P < 0.05$; $n = 18$, performed as 3 independent experiments).

4.7 Discussion

In this chapter I have investigated the activity of a subset of the hit compounds identified in the Spectrum Collection screen. Work has primarily focused on isopimpinellin, a naturally occurring furanocoumarin with both anti-inflammatory and pro-resolving properties. An assay designed to measure PI-3K activity *in vivo* indicated that the inhibitory properties of isopimpinellin on neutrophil recruitment were at least in part due to inhibition of the PI-3K signalling pathway. This mechanism of action was shared by another hit compound, xanthone, which prompted a comparison of the chemical structures of the hit compounds. Structural similarity was identified within a subset of the hits, in the form of a shared 1,4-benzopyrone group, more commonly referred to as chromone. Neutrophil apoptosis was identified as the mechanism by which isopimpinellin could drive inflammation resolution and this led to an investigation of the apoptotic potential of chromone derivatives.

4.7.1 An *in vivo* assay for PI-3K assay

Isopimpinellin is a naturally occurring furanocoumarin, the anti-inflammatory properties of which have not yet been defined. Similar compounds such as imperatorin and byakangelicin have been shown to inhibit PGE_2 and COX-2 production (Ban et al., 2003). Isopimpinellin was found to both significantly reduce neutrophil recruitment and accelerate resolution during the acute inflammatory response initiated by tail fin transection in zebrafish larvae. These properties were identified in the previous chapter and further investigation confirmed that they occurred in a dose dependent manner. Based on the hierarchical cluster analysis performed in Chapter 3, which revealed that isopimpinellin shared a similar activity profile to the pan-PI-3K inhibitor ZSTK474, it was hypothesised that this compound acted via inhibition of PI-3K signalling. To explore this, an *in vivo* assay was designed to measure PI-3K activity in neutrophils during recruitment to the wound. A similar assay has been described by (Yoo et al., 2010), in which a double transgenic zebrafish line was created expressing the PHAkt-EGFP construct under the *mpx* promoter, alongside an mCherry construct to measure cytoplasmic volume. This enabled ratiometric-imaging analysis to account for any background signal caused by changes in cell volume. In these studies, polarisation of PHAkt-EGFP at the leading edge of neutrophils was visualised during recruitment to

a small laser or needle induced wound, in a similar manner to the localisation of GFP observed in neutrophils following tail fin injury of the *lyz*/PHAkt larvae used here. The authors reported loss of this polarity and a reduction in neutrophil migration in the presence of the PI-3K inhibitor LY294002. I tested this compound in *mpx*:GFP larvae using similar concentrations and also found a significant reduction in the number of neutrophils recruited to the tail fin following wounding. Tracking analysis of individual neutrophils during the first hour of recruitment revealed that LY294002 treated neutrophils migrated more slowly than those from control larvae, in keeping with a loss of polarity and ability to undergo chemotaxis. As directed neutrophil migration relies on cell polarisation, a loss of directionality might be expected when PI-3K signalling is inhibited. However, in the presence of LY294002, most neutrophils that did undergo migration appeared to move preferentially towards the wound. It has been proposed that during neutrophil migration, PI-3K signalling is activated by intermediary chemoattractants such as IL-8, but that end-target chemoattractants such as fMLP and C5a signalling via p38 MAPK can take precedence (Heit et al., 2002). Thus, perhaps some neutrophils are able to respond to the latter chemotactic signalling gradients and migrate towards the wound even in the presence of LY294002. A similar observation has been made in PI-3Kγ $^{-/-}$ mice, in which some neutrophils are still able to undergo chemotaxis following fMLP or C5a stimulation (Hirsch, 2000).

In *lyz*/PHAkt larvae, LY294002 treatment resulted in a loss of plasma membrane localisation of PHAkt-EGFP and a rounded tail phenotype as previously observed (Yoo et al., 2010). I wished to quantify the distribution of EGFP signal in order to make accurate and statistically valid comparisons between treatment groups. The polarity index equation was devised, to enable quantitative measurement of the fluorescence at the leading edge of the cell compared to the trailing edge and also compared to the fluorescence within the cell. Though basic, this equation was seemingly effective, as in all experiments the mean polarity index of neutrophils from LY294002 treated larvae was significantly lower than the mean from DMSO vehicle control treated larvae.

However, there were a number of caveats associated with this assay. Within the 3D tissue environment neutrophils are frequently traversed over multiple Z planes,

making identification of the dominant leading edge quite difficult. In some cases this issue could be overcome by observing the cell movements over a short period of time to make a more informed estimate as to which was the leading pseudopodium. On occasions where it was still unclear where to transect the cell in ImageJ, the line was drawn across the most longitudinal section. The nature of this assay made it difficult to avoid bias, particularly when deciding which cells to image within the larvae. To account for this, experiments were performed blind to treatment conditions and for each replicate experiment, 3 neutrophils from each of 3 larvae were imaged per group to generate high enough *n* numbers for reliability. Care was also taken to confirm that only neutrophils situated between the wound site and the posterior blood island were imaged, not those directly at the wound, as the behaviour of these cells would be somewhat different to the migrating ones. Despite the limitations of this assay and the polarity index equation, there was an obvious phenotypic difference between the intracellular distribution of PHAkt-EGFP in neutrophils from LY294002 treated larvae compared to the controls and this was reflected in the statistically lower mean polarity index. It was apparent that in some instances, neutrophils from the DMSO control larvae displayed a low polarity index, more comparable to the value expected in neutrophils exposed to LY294002. It might be that at the time of imaging, these cells had not yet detected the chemokine gradient and subsequently had not begun to polarise. There might have been less variability in the assay if imaging took place an hour or two after injury rather than within the first 30 minutes, however this might have made imaging of individual cells more tricky as there would have been larger numbers of neutrophils closer to the wound, particularly in the control larvae.

LY294002 acts as a competitive inhibitor of PI-3K by binding to the ATP-binding site (Walker et al., 2000) resulting in reduced phosphorylation of AKT. Although initially developed as a specific PI-3K inhibitor, there is some evidence to suggest that in some cell types LY294002 may inhibit other protein kinases such as mTOR (Brunn et al., 1996) or reduce the DNA-binding activity of NF-κB (Kim et al., 2005). These potential off-target effects must be considered when interpreting the data. In addition, LY294002 has broad inhibitory activity spanning throughout the different classes of PI-3K and in hindsight it might have been more relevant to use a γ-specific PI-3K inhibitor,

as this is considered to be the most important isoform in mammalian neutrophil recruitment (Hirsch, 2000; Hannigan et al., 2002; Ferguson et al., 2007). Morpholino knockdown of PI-3Kγ has demonstrated that this isoform is also required for neutrophil chemotaxis in zebrafish (Yoo et al., 2010).

Evidence revealed that PI-3K is also important during reverse migration of neutrophils, which can be observed to repolarise and migrate away from a wound (Yoo et al., 2010). In these studies, inflammation is initiated by only a very small incision in the tissue, rather than complete transection of the tail fin. Although it would be interesting to investigate PI-3K signalling during resolution in my assay, it would be very difficult to image individual cells due to the large numbers of neutrophils recruited to the tail fin in this model, and combining the PHAkt-EGFP transgenic with the *mpx*/Kaede line would be technically highly complex.

4.7.2 Isopimpinellin inhibits neutrophil recruitment by disruption of PI-3K signalling

In this *in vivo* PI-3K assay, isopimpinellin was found to reduce neutrophil polarity as determined by the polarity index equation discussed above. In these neutrophils, the cellular localisation of PHAkt-EGFP was dispersed with lack of any defined leading edge, similar to the phenotype observed in neutrophils from larvae treated with LY294002. These data suggested that the inhibitory effect of isopimpinellin on neutrophil recruitment might be explained by reduced phosphorylation and activation of Akt and loss of the downstream signalling events required for cell polarisation and directed migration. Notably, the preliminary tracking experiments performed to analyse the migratory behaviour of neutrophils during the recruitment phase indicated that isopimpinellin altered the chemotactic behaviour in a similar manner to LY294002. Both compounds significantly reduced neutrophil speed and displacement, however to be certain of the effects of isopimpinellin, this experiment would need to be repeated.

It has not been determined whether isopimpinellin acts directly on PI-3K, for example by competitive binding to the ATP-binding site in a similar manner to LY294002, or whether the inhibition occurs upstream of PI-3K, for example at the cell surface

receptor level. It is theoretically possible that rather than acting in an inhibitory fashion, isopimpinellin might be activating a phosphatase such as PP2A, which can directly dephosphorylate AKT, or PTEN, which converts PIP_3 to PIP_2 (Liao & Hung, 2010). Both might result in inactivation of Akt and impaired chemotaxis in zebrafish. It is also possible that the effect observed is not neutrophil specific and that isopimpinellin could be inhibiting PI-3K in other cell types, or that it could be affecting a wide range of other protein kinases.

Another hit compound, xanthone, also significantly reduced polarity index but this was not found to be a universal effect of exposure of zebrafish larvae to pharmacological agents, as tanshinone IIA had no effect in this assay. Interestingly, in the presence of xanthone, neutrophils sometimes appeared to distribute PHAkt-EGFP at the cell membrane, but this was at a universal intensity rather than localised to a defined leading edge. This could indicate that xanthone was acting further downstream in the PI-3K/Akt signalling pathway, enabling Akt to be recruited to the membrane but then perhaps blocking further downstream signalling associated with leading edge specification. It is also possible that xanthone could be inhibiting another component of the PI-3K/Akt signalling pathway, for example phosphoinositide-dependent kinase-1 (PDK1) (Alessi et al., 1997) or mTOR complex 2 (mTORC2) (Sarbassov et al., 2005), which function downstream of PI-3K to phosphorylate and activate AKT once it has been recruited to the plasma membrane. Indeed, inhibition of PDK1 by either pharmacological or siRNA knockdown is associated with impaired neutrophil migration (Yagi et al., 2009).

4.7.3 Structure-activity relationships between hit compounds

The discovery of a potential shared mechanism of action between two of the hit compounds, xanthone and isopimpinellin, prompted an investigation to determine whether any structure-activity relationships existed between the compounds identified in the Spectrum Collection screen. Structural similarity in the form of a shared 1,4-benzopyrone group, more commonly referred to as chromone, was found in 7 out of the 21 reproducible positive hits. An interesting observation was the presence of the chromone group in LY294002, which is in fact a derivative of quercetin

and was originally synthesised alongside a number of other chromones in a search for more specific PI-3K inhibitors (Vlahos et al., 1994). Quercetin is suspected to directly inhibit PI-3K by associating with the ATP-binding site (Matter et al., 1992) and evidence also suggests that it can disrupt neutrophil recruitment by inhibiting actin polymerisation (Souto et al., 2011). It might have been interesting to explore the activity of the positive hit methylquercetin in the PI-3K assay, although if the assumption were made that it would act similarly to its analogue quercetin, then it is possible that it might have more potent inhibitory activity against PI-3Kα and δ isoforms, as has been demonstrated for similar flavonoids (Kong et al., 2011).

As the chromone group was common to a number of the positive hit compounds and LY294002, it was hypothesised that this structure might be responsible for their inhibitory effects on neutrophil recruitment. However, when tested alone, chromone itself was actually found to have a more potent effect on the resolution phase of inflammation. These data led to the hypothesis that the chromone-derived compounds had been identified in the screen due to a shared and novel pro-resolution mechanism of action. With the idea that the chromone group was responsible for driving resolution, a number of chromone analogues were synthesised and tested in the zebrafish. These compounds were found to have varying degrees of pro-resolution activity, allowing the prediction to be made that the addition of a third hydrocarbon ring might promote their effects. It was reassuring that one of the most potent compounds synthesised by the chemists turned out to be xanthone, emphasising the robustness and reproducibility of the zebrafish resolution assay.

Unfortunately, issues arose over the course of these experiments, in that chromone appeared to lose its effectiveness over time, although it could not be determined whether this was due to a tendency to be oxidised and degraded or a result of the inherent reactivity of this compound. The effect of chromone on the resolution of inflammation was greater when the stock solution was prepared from new, unopened purified solid, suggesting that a reaction took place once the compound was exposed to air, although this seems rather extreme. It is also possible that there were small

differences in batches of chromone obtained from the supplier. Regardless of the underlying cause, it was impractical to purchase new chromone for each experiment.

As no promising lead compounds had been generated from the chemical synthesis, other than the already naturally occurring xanthone and flavone, this project was deprioritised in favour of other hits identified in the Spectrum Collection screen. The identification of flavone as a chromone analogue led to the realisation that many compounds, particularly those that can be isolated from plant species, may be chromone-derived. Flavones are just one of the classes of plant secondary metabolites known as the flavonoids. It may be that the correlation found here between chromone-derived compounds and pro-resolution activity is simply circumstantial and that the functional activity of this subset of hit compounds is principally due to the other structural moieties attached to the chromone backbone, which might dictate target binding and affinity. Flavones are particularly known for their antioxidant activity, although the series of analogues tested here indicated that the most effective drivers of resolution were the least likely to act via antioxidant mechanisms. However only a handful of compounds were tested and their potency in the resolution assay was by no means a direct measure of their antioxidant capacity.

4.7.4 Isopimpinellin drives neutrophil apoptosis during inflammation resolution

The hit compound isopimpinellin significantly accelerated the resolution of inflammation *in vivo*, alongside its inhibitory action on neutrophil recruitment. Using TSA/TUNEL staining in fixed larvae, it was revealed that there was a significant increase in the rate of neutrophil apoptosis at the site of injury in the presence of this compound. Experiments performed in the previous chapter showed that there was no change in total neutrophil number following 24 hours of exposure to isopimpinellin, suggesting that the effect on neutrophil apoptosis was specific to activated neutrophils participating in the inflammatory response. However, what cannot be determined from these data is whether the apoptosis-inducing properties of isopimpinellin are targeted solely to neutrophils, or if other cell types such as macrophages are also affected.

Although the endogenous rates of apoptosis identifiable at any one time during inflammation resolution in very young zebrafish larvae are low (Elks et al., 2011, Burgon et al., in submission), the data obtained here indicate that it is pharmacologically possible to drive resolution via this mechanism, as has been previously described (Loynes et al., 2009). It is difficult to measure precisely how much neutrophil apoptosis takes place using this assay, as larvae are fixed at a specific time point, illustrating only a snapshot of the entire resolution phase. It would be useful and informative to observe apoptosis occurring in real time. To this end, a novel *Tg(mpx:FRET)sh237* reporter line has been developed, in which the FRET signal is lost following caspase-3 mediated cleavage of the FRET reporter (Dr Nikolay Ogryzko and Dr Stephen Renshaw, personal communication). Preliminary experiments performed using this line revealed a significant increase in apoptotic events at the site of injury in isopimpinellin treated larvae, associated with a loss of the FRET signal. These data not only provide supportive evidence for the apoptotic potential of isopimpinellin in driving inflammation resolution but also suggest that this is occurring in a caspase-3 dependent manner.

It has been proposed that neutrophil apoptosis does not contribute to inflammation resolution in zebrafish (Mathias et al., 2006). In these studies, wounding consisted of only a small incision in the ventral fin, resulting in relatively low numbers of neutrophils recruited compared to the tail fin transection model. With fewer cells present it would therefore be even less likely to observe apoptosis, but this does not necessarily mean that it does not occur at all. The authors claim that the main mechanism responsible for driving resolution in zebrafish is by their migration away from the wound, which has also been reported by others (Hall et al., 2007; Elks et al., 2011; Yoo & Huttenlocher, 2011). In the presence of isopimpinellin, reverse migration was inhibited and in fact during time-lapse microscopy neutrophils at the site of injury could be observed to round up and undergo apoptosis in real time. These data provided further evidence that apoptosis was the main mechanism of neutrophil clearance by isopimpinellin. Preliminary TSA/TUNEL data indicated that flavone might also be a potent inducer of neutrophil apoptosis. Related compounds have been

shown to drive inflammation resolution in zebrafish and isolated human neutrophils in a caspase-dependent manner (Lucas et al., 2012).

4.7.5 Investigation into the effect of chromone derivatives on neutrophil apoptosis

Isopimpinellin was discovered to be an isomer of khellin, a naturally occurring compound from which a series of clinically available anti-histamine drugs collectively termed the cromones was developed. The anti-inflammatory activity of these compounds has not yet been fully defined. Due to the structural similarity between isopimpinellin and khellin, I hypothesised that the anti-inflammatory potential of the cromones might be associated with neutrophil apoptosis, which would support the use of these already clinically available drugs in the treatment of neutrophilic inflammatory diseases. The archetypal cromone, disodium cromoglycate, significantly accelerated inflammation resolution in the zebrafish model. To observe an effect, relatively high doses of the drug were required compared to those used in most zebrafish assays and it was necessary to re-dose larvae with fresh drug solution every hour. This may be a result of the poor pharmacokinetic properties of the cromones, which has been suggested in other systems (Yazid, Leoni, et al., 2010). This observation might also explain the variable effects observed previously in the resolution assays carried out using chromone. In hindsight, perhaps its activity would have been maintained by re-dosing the larvae. Much of the early work that was carried out to develop more stable and effective khellin analogues indicated that tricyclic chromone derivatives were more active, one of these being nedocromil sodium (Edwards & Howell, 2000). In light of this, it is interesting that the chromone derivatives synthesised earlier for this project displayed more significant effects on inflammation resolution with the addition of a third hydrocarbon ring.

The cromones directly inhibit the phosphatase PP2A in neutrophils to promote release of the protein Anx-A1, which may at least in part mediate their effects on the inflammatory response (Yazid, Leoni, et al., 2010). PP2A inhibition can result in hyper-phosphorylation and instability of Mcl-1, accelerating neutrophil apoptosis (Derouet et al., 2004). This supports the hypothesis that chromone derivatives might promote inflammation resolution via this mechanism. Disodium cromoglycate, nedocromil

sodium and two of the hit compounds, isopimpinellin and methylquercetin, were investigated for their effects on apoptosis in freshly isolated human neutrophils. No increase in constitutive neutrophil apoptosis was observed with any of the compounds tested, disputing the original hypothesis. It was possible that rather than influencing constitutive apoptosis the chromones might interfere with neutrophil survival signalling, for example by inhibiting some component of the MAPK, PI-3K or JAK/STAT pathways. However there was no inhibition of GM-CSF induced neutrophil survival in the presence of the chromones tested. Interestingly, but in even greater contrast to the original hypothesis, methylquercetin appeared to promote neutrophil survival. This also does not correlate with published data, which describe Mcl-1 down-regulation and induction of neutrophil apoptosis *in vitro* by highly similar flavones (Lucas et al., 2012). In these studies, the flavones tested are also shown to override survival factors including GM-CSF and dexamethasone. It may be that subtle differences in the chemical structure of the various chromone derivatives affect target binding, or even the ability to permeate the cell membrane.

The data obtained in these *in vitro* experiments did not support a direct pro-apoptotic effect caused by inhibition of PP2A and Mcl-1 down-regulation by chromones. Although disodium cromoglycate and nedocromil are associated with PP2A inhibition, this did not appear to correlate with the instability of Mcl-1 as described by the pharmacological inhibitor of PP2A, okadaic acid (Derouet et al., 2004). In this particular study, neutrophils were treated with okadaic acid in combination with the *de novo* protein synthesis inhibitor cyclohexamide and no control data are presented to show the effect of okadaic acid alone, making it difficult to interpret the results. Experiments performed using alternative PP2A inhibitors suggest that PP2A can promote neutrophil apoptosis (Alvarado-Kristensson & Andersson, 2005). Indeed, PP2A is known to play a role in survival and can directly inhibit the PI-3K/AKT signalling pathway by dephosphorylating and inactivating AKT (Liao & Hung, 2010). In neutrophils, AKT promotes survival in multiple ways, for example by phosphorylation and inhibition of the pro-apoptotic BCL-2 family members BAX and BAD (Datta et al., 1999; Gardai et al., 2004). Based on this evidence, it is perhaps more likely that if the mechanism of action

of chromones is associated with PP2A inhibition, then increased neutrophil survival would be expected, as observed here.

4.7.6 The effects of chromones on glucocorticoid induced neutrophil survival

Studies suggest that cromones can potentiate the effect of glucocorticoids via enhanced release of Anx-A1 (Yazid et al., 2009; Yazid, Leoni, et al., 2010), which has a pro-apoptotic effect in human neutrophils (Solito et al., 2003). This is somewhat contradictory, as glucocorticoids are known to directly promote neutrophil survival (Cox, 1995; Kato et al., 1995; Liles et al., 1995). I hypothesised that the combined activity of dexamethasone and cromones might enhance Anx-A1 release to a level sufficient to override the anti-apoptotic effect of the glucocorticoid. However, the data obtained from isolated human neutrophils revealed that dexamethasone induced neutrophil survival was maintained in the presence of all the compounds tested. The histamine receptor antagonist ketotifen was also investigated, which although not a chromone derivative, is believed to function via the same, Anx-A1 dependent mechanism (Yazid, Leoni, et al., 2010). No increase in apoptosis was observed in the presence of ketotifen, suggesting that either under these experimental conditions Anx-A1 is not pro-apoptotic, or that the concentration of ketotifen used was not optimal. It may be that much higher doses of the chromone derivatives were required to induce sufficient Anx-A1 release to override the survival effect of dexamethasone.

From data obtained in these *in vitro* experiments, it was concluded as unlikely that the chromone derivatives had a direct effect on neutrophil apoptosis as a result of enhanced Anx-A1 release. It is possible that some necessary stimulus was missing from the *in vitro* system, which might explain the significant effect observed on inflammation resolution in the presence of disodium cromoglycate and other chromone derivatives *in vivo*. Indeed, the lack of neutrophil apoptosis in isopimpinellin treated neutrophils did not correlate with the significant increase in neutrophil apoptosis revealed by the *in vivo* data, suggesting that it does not cause neutrophil apoptosis directly but that another cell type or stimulus is involved. In particular, it would be interesting to assess whether isopimpinellin can still cause neutrophil apoptosis in macrophage-ablated zebrafish larvae. Experiments performed to

investigate the combined effect of glucocorticoids and disodium cromoglycate on inflammation resolution *in vivo* were inconclusive, as in this instance the effect of disodium cromoglycate alone was not significant. This may have been a result of excessive degradation.

Despite their ability to promote neutrophil survival, glucocorticoids can function in a pro-resolution manner, for example by enhancing the non-phlogistic phagocytic clearance of apoptotic neutrophils by macrophages (Liu et al., 1999). It has been proposed that one of the mechanisms regulating this activity is Anx-A1 dependent as macrophages release Anx-A1 in the presence of dexamethasone (Maderna et al., 2005). Anx-A1 enhances phagocytosis of apoptotic neutrophils by binding to ALX receptors (Scannell et al., 2007). The phagocytic capacity can be abrogated using the receptor antagonist Boc2, an annexin blocking antibody, or by removal of Anx-A1 from the supernatant of apoptotic neutrophils (Maderna et al., 2005; Scannell et al., 2007). Thus, it may be that in the *in vitro* experiments performed here, the lack of phagocytic macrophages prevented an increase in Anx-A1 mediated neutrophil apoptosis. This would also fit with the *in vivo* data obtained for isopimpinellin and it would be interesting to investigate the effect of both this compound and disodium cromoglycate *in vivo* following macrophage ablation. However, there is evidence to suggest that endogenous Anx-A1 is not only released by phagocytic macrophages but also by apoptotic cells. For example, in apoptotic Jurkat T cells, Anx-1 is exported and co-localises with phosphatidylserine on the cell membrane to function as an engulfment ligand (Arur et al., 2003). This is also believed to occur in neutrophils, in a caspase-dependent manner (Scannell et al., 2007). It is possible that Anx-A1 acts via two distinct but complementary mechanisms, being released by both the phagocyte and target cell for pro-phagocytic activity.

4.7.7 Conclusions

Much of the work presented in this chapter focuses on the structure-activity relationship between the chromone-derived hit compounds identified in the Spectrum Collection screen and the group of clinically available cromones. It is possible that this shared chemical moiety may be responsible for the pro-resolution activity of these

compounds, although the precise mechanism could not be defined here. Their activity does not appear to be due to increased neutrophil apoptosis, or the enhanced release of Anx-A1, although this was not measured directly. One of the most interesting hit compounds, isopimpinellin, was investigated due to the similarity of its activity profile with a known PI-3K inhibitor. The activity of isopimpinellin *in vivo* is two-fold; recruitment of neutrophils to the site of injury is reduced, which occurs at least in part by PI-3K inhibition, and resolution of inflammation is accelerated by enhanced neutrophil apoptosis. Due to the lack of apoptosis observed *in vitro* in the presence of isopimpinellin, it is my hypothesis that the effect seen *in vivo* is mediated via another cell type.

Chapter 5: Identification of a Novel Mechanism to Pharmacologically Accelerate Inflammation Resolution

5.1 Introduction

Screening of the Spectrum Collection identified 21 compounds with the ability to drive inflammation resolution in the zebrafish model. The best of these were further investigated to examine their specific effects on neutrophil number at the site of injury during both the recruitment and resolution phases of inflammation. When comparing their activity against a panel of known inflammatory signalling pathway inhibitors from GlaxoSmithKline, most of the hit compounds formed a distinct cluster, with greater effects on resolution rather than neutrophil recruitment. Aside from the chromone derivatives discussed in the previous chapter, two other closely structurally related compounds were present within the pro-resolution cluster, cryptotanshinone and tanshinone IIA. These were the most potent accelerators of inflammation resolution identified in the screen, surpassed only by the NSAID naproxen (refer to Figure 3.19).

Tanshinone IIA and cryptotanshinone are both natural extracts from *Salvia miltiorrhiza*, more commonly referred to as Danshen, a Chinese medicinal herb that is traditionally used to treat cardiovascular disorders (Zhou et al., 2005). These compounds are known to possess some anti-inflammatory activity, for instance tanshinone IIA has protective effects against LPS-induced lung injury in mammalian systems (Shi et al., 2007; Xu et al., 2009). These reports, along with evidence from other mammalian models of inflammation, suggest that the anti-inflammatory effects of tanshinone IIA are due to an inhibition of NF-κB activation, however the precise mechanisms are not well defined (Bai et al., 2008; Xu et al., 2008; An et al., 2011; Yin et al., 2012). This is supported by evidence from *in vitro* systems. In the RAW264.7 mouse macrophage-like cell line, tanshinone IIA inhibits NF-κB activation following LPS stimulation, the downstream effects of which include reduction of COX-2 and inducible nitric oxide synthase (iNOS) activity (Jang et al., 2006; Jeon et al., 2008). Inhibition of MAPK signalling in LPS treated RAW264.7 cells by both tanshinone IIA and cryptotanshinone has also been reported (Jeon et al., 2008; Tang et al., 2011). The

downstream effects of cryptotanshinone are similar to those described for tanshinone IIA, such as down-regulation of iNOS and COX-2 (Jeon et al., 2008) and in addition, reduced production of TNF-α and IL-6 (Tang et al., 2011). It has also been suggested that cryptotanshinone can directly inhibit COX-2 enzymatic activity, without affecting mRNA or protein expression (Jin et al., 2006). Aside from NF-κB, tanshinones have been implicated in the inhibition of other transcription factors such as activator protein-1 (AP-1) and STAT1 (Kogut et al., 2008; Yang et al., 2010). In the secondary assays performed in Chapter 3, there appeared to be some toxicity in the presence of cryptotanshinone and subsequently the majority of the work described here was carried out using tanshinone IIA.

Evidence indicates that tanshinone IIA can induce apoptosis in human leukaemia cell lines by activation of caspase-3 (Sung et al., 1999). It has also been shown to cause apoptosis in other cell types, including multiple cancer cell lines (Won et al., 2010; Jiao et al., 2011; Chen et al., 2012; Wei et al., 2012). A pro-apoptotic effect of tanshinone IIA on human peripheral blood neutrophils has not been reported. Given that neutrophil apoptosis is known to be one of the main mechanisms by which neutrophils are removed during inflammation resolution (Savill et al., 1989), it was possible that this could explain the acceleration in resolution observed in the presence of tanshinone IIA in the zebrafish model.

In the hierarchical cluster analysis performed in Chapter 3, tanshinone IIA was closely associated with roflumilast. This PDE4 inhibitor is clinically effective in the treatment of COPD, in part by reducing neutrophil numbers in the lung (Grootendorst et al., 2007). Another PDE4 inhibitor, rolipram, has been shown to enhance resolution in a murine model of LPS-induced pleurisy by promoting neutrophil apoptosis (Sousa et al., 2010). This appeared to be partially mediated via inhibition of the PI-3K/AKT signalling pathway and was dependent on caspase activation and down-regulation of Mcl-1. This provides supportive evidence for a pro-apoptotic role of tanshinone IIA, considering that this compound might function in a similar manner to the PDE4 inhibitors to drive inflammation resolution.

5.1.1 Aims and hypotheses

The aim of this chapter is to investigate the profound effect of tanshinone IIA on the resolution of inflammation, with the hypothesis that this action might be due to enhanced neutrophil apoptosis. This chapter also aims to investigate alternative mechanisms by which tanshinone IIA might drive resolution.

5.2 Tanshinone IIA does not significantly affect neutrophil recruitment

In the secondary assays performed following the Spectrum Collection screen, tanshinone IIA was found to have only a small effect on neutrophil recruitment. To confirm this observation, 3 dpf *mpx*:GFP larvae were injured and treated immediately with a range of concentrations of tanshinone IIA until 6 hpi, when neutrophil numbers at the site of injury were recorded. In these experiments, tanshinone IIA did not significantly affect neutrophil recruitment at 6 hpi, although there was a trend towards a reduction in neutrophil numbers at the site of injury with increasing dose (Figure 5.1).

The effect of tanshinone IIA on neutrophil migratory behaviour during recruitment was investigated further by pre-treatment of *mpx*:GFP larvae with tanshinone IIA for 2 hours, followed by injury and time-lapse imaging on an inverted compound fluorescence microscope. GFP-labelled neutrophils were tracked for one hour as they migrated towards the site of injury and Volocity® software was used to analyse the speed and meandering index of each individual cell track, as previously described. There was no difference in either neutrophil speed or meandering index in the presence of tanshinone IIA compared to the DMSO vehicle control (Figure 5.2 A, B). There was also no change in displacement towards the wound (Figure 5.2 C), indicating that neutrophils in tanshinone IIA treated larvae moved a comparable distance in the direction of the wound to those from DMSO treated larvae.

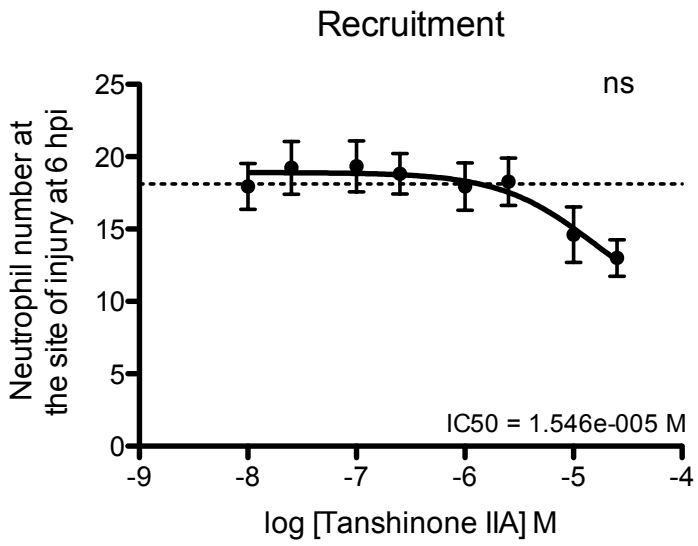

Figure 5.1: Tanshinone IIA does not significantly reduce neutrophil recruitment.

At 3 dpf, *mpx*:GFP larvae were injured and treated immediately with varying doses of tanshinone IIA. Neutrophil numbers at the site of injury were counted at 6 hpi. Tanshinone IIA did not cause a significant reduction in neutrophil recruitment to the wound, although there was a trend towards lower neutrophil numbers with increasing dose (One-way ANOVA with Dunnett's multiple comparison post-test; *n* = 18, performed as 3 independent experiments). Dotted line at y = 18.11 indicates mean neutrophil number at site of injury in control larvae.

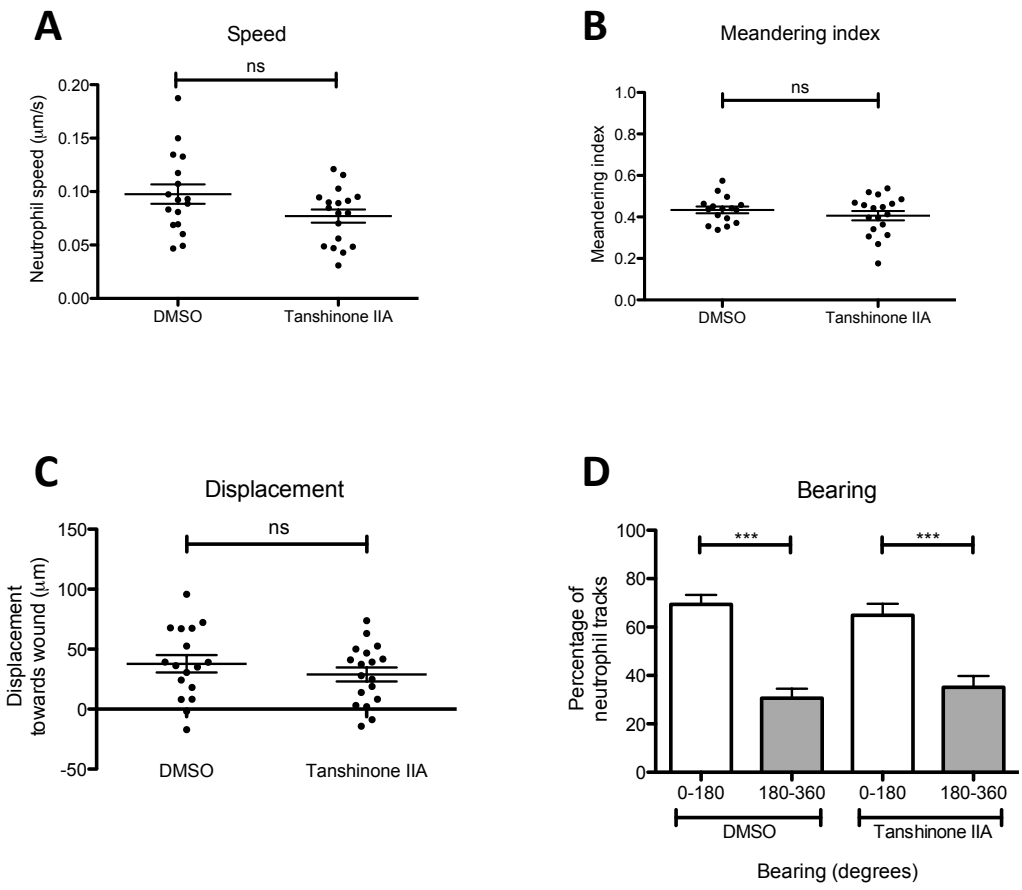

Figure 5.2: Tanshinone IIA has no effect on neutrophil chemotaxis.

At 3 dpf, *mpx*:GFP larvae were pre-treated with DMSO or 25 μM tanshinone IIA before injury and mounting. Larvae were imaged for 1 hour and individual GFP-labelled neutrophils were tracked and analysed using Volocity® software. Tanshinone IIA had no significant effect on (A) neutrophil speed, (B) meandering index or (C) displacement towards the wound (Unpaired *t*-test; *n* = 18, performed as 3 independent experiments). For bearing, an angle of 0-180 degrees indicates movement towards the wound and 180-360 degrees indicates movement away. In both DMSO and tanshinone IIA treated larvae, neutrophils move in a directed fashion towards the wound (One-way ANOVA with Bonferroni's multiple comparison post-test to compare selected columns; where *** $P < 0.001$; *n* = 18, performed as 3 independent experiments).

Neutrophil bearing also remained unchanged, which suggested that the cells remained able to respond to directional chemotactic cues in the presence of tanshinone IIA (Figure 5.2 D). These data were consistent with the PI-3K assay described in Chapter 4, in which tanshinone IIA appeared to have no effect on PHAkt-EGFP localisation or directed migration and did not alter neutrophil polarity index during the recruitment phase of inflammation.

5.3 Tanshinone IIA significantly accelerates inflammation resolution *in vivo*

To confirm the pro-resolution activity of tanshinone IIA before embarking on a more thorough investigation into the potential mechanisms involved, concentration-response experiments were carried out to examine its effects on the resolution phase of inflammation. Tail fin transection was performed on 3 dpf *mpx*:GFP larvae and those larvae which recruited high numbers of neutrophils at 6 hpi were selected for treatment with varying doses of tanshinone IIA. Neutrophil numbers remaining at the wound were assessed at 12 hpi. Tanshinone IIA significantly accelerated inflammation resolution by reducing neutrophil numbers at the site of injury, from a concentration of 1 µM up to 25 µM (Figure 5.3). Toxicity was observed at 100 µM and 250 µM.

5.4 Tanshinone IIA and cryptotanshinone induce human neutrophil apoptosis

An important prerequisite for successful resolution of inflammation is the constitutive apoptosis of neutrophils and their subsequent recognition and clearance by phagocytic macrophages (Savill et al., 1989). I first hypothesised that the mechanism responsible for the reduction in neutrophil numbers observed during the resolution of inflammation *in vivo* might be due to an increase in neutrophil apoptosis. To investigate this hypothesis I initially tested tanshinone IIA in an *in vitro* setting, in order to look at its direct effect on neutrophils without the possible involvement of other cell types or factors within the inflammatory environment. Despite its toxicity at high doses in zebrafish larvae, cryptotanshinone was also tested for completeness.

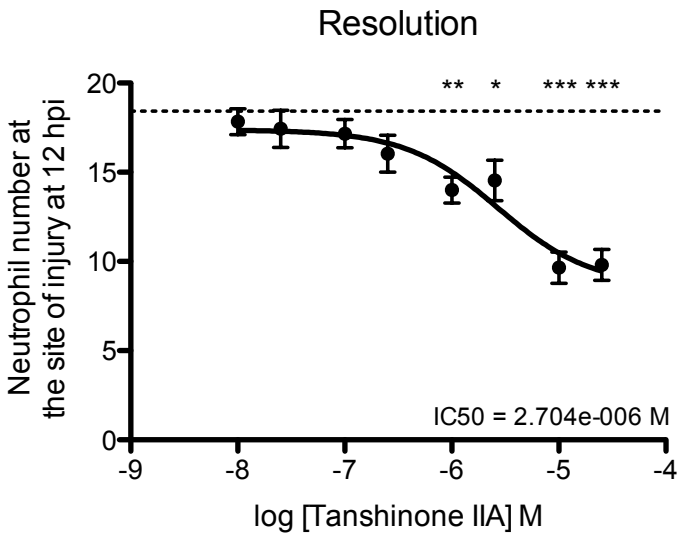

Figure 5.3: Tanshinone IIA significantly accelerates inflammation resolution.

At 3 dpf, tail transection was performed on *mpx*:GFP larvae and those which mounted a good neutrophilic response at 6 hpi were treated with varying doses of tanshinone IIA. Neutrophil numbers at the site of injury were counted at 12 hpi. At doses of 1 µM and higher, tanshinone IIA significantly reduced neutrophil numbers at the wound compared to the DMSO vehicle control (One-way ANOVA with Dunnett's multiple comparison post-test; where * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$; $n = 24$, performed as 4 independent experiments). Dotted line at y = 18.42 indicates mean neutrophil number at site of injury in control larvae.

Human neutrophils were isolated from peripheral whole blood by either the Optiprep™ or Percoll® method of purification, as described, and were treated with multiple doses of tanshinone IIA or cryptotanshinone. After 8 hours in culture at 37°C, two cytospins per condition were prepared and the percentage of neutrophil apoptosis was calculated based on nuclear morphology. A small but significant increase in constitutive apoptosis was found in neutrophils treated with 10 μM and 100 μM tanshinone IIA compared to the DMSO vehicle control (Figure 5.4 A). Cryptotanshinone had a more profound effect on neutrophil apoptosis, with a highly significant increase at both 10 μM and 100 μM (Figure 5.4 B).

5.4.1 Tanshinones abrogate GM-CSF induced neutrophil survival *in vitro*

The previous data indicated that both tanshinone IIA and cryptotanshinone directly increased the rate of constitutive neutrophil apoptosis *in vitro*. At sites of inflammation *in vivo*, a number of host-derived pro-survival factors are thought to delay neutrophil apoptosis, such as GM-CSF, ATP and hypoxia, the functions of which have been demonstrated in culture (Lee et al., 1993; Hannah et al., 1995; Vaughan et al., 2007). Novel anti-inflammatory therapies designed to drive inflammation resolution must be able to override these survival signals, which persist during chronic inflammation. To examine the potential of the tanshinones to promote neutrophil apoptosis in the presence of survival signalling, their effects were investigated in combination with GM-CSF. Exposure of neutrophils to GM-CSF results in prolonged neutrophil survival due to stabilisation of the anti-apoptotic protein Mcl-1 (Moulding et al., 1998).

Neutrophils were isolated from peripheral whole blood, as previously described, and treated with 10 μM of either tanshinone IIA or cryptotanshinone, in the presence and absence of GM-CSF. After 8 hours, two cytospins were acquired per condition and the percentage of neutrophil apoptosis was calculated based on nuclear morphology. GM-CSF caused a significant reduction in neutrophil apoptosis in untreated and vehicle control neutrophils, but this survival was lost in the presence of either tanshinone IIA or cryptotanshinone (Figure 5.5). These data suggested that these compounds could directly override the neutrophil survival induced by signalling downstream of GM-CSF.

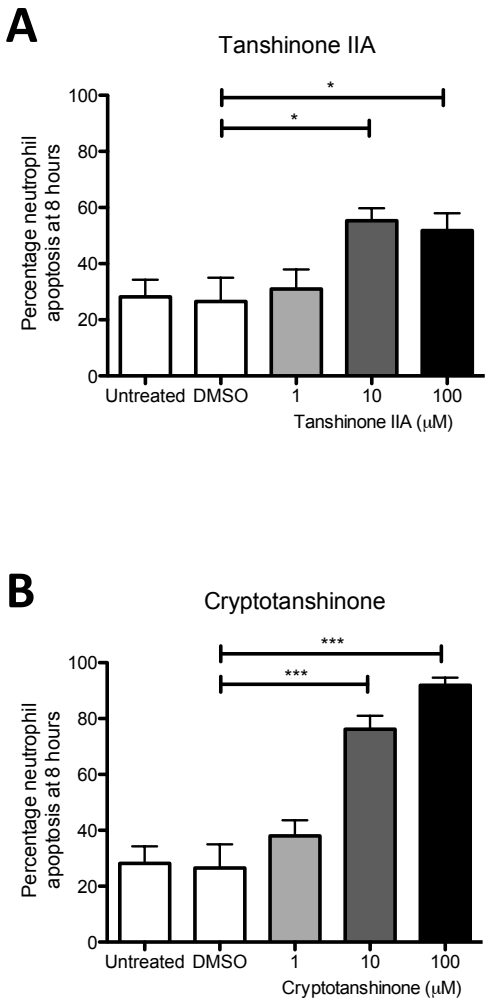

Figure 5.4: Tanshinones increase constitutive neutrophil apoptosis *in vitro*.

Neutrophils were isolated from whole blood using either the Optiprep™ or Percoll® method of separation and were incubated with (A) tanshinone IIA or (B) cryptotanshinone for 8 hours at 37°C. In each case, 2 cytospins were acquired and percentage neutrophil apoptosis calculated based on nuclear morphology. Both compounds significantly increased neutrophil apoptosis at doses of 10 μM and 100 μM (One-way ANOVA with Dunnett's multiple comparison post-test; where * $P < 0.05$ and *** $P < 0.001$; $n = 4$).

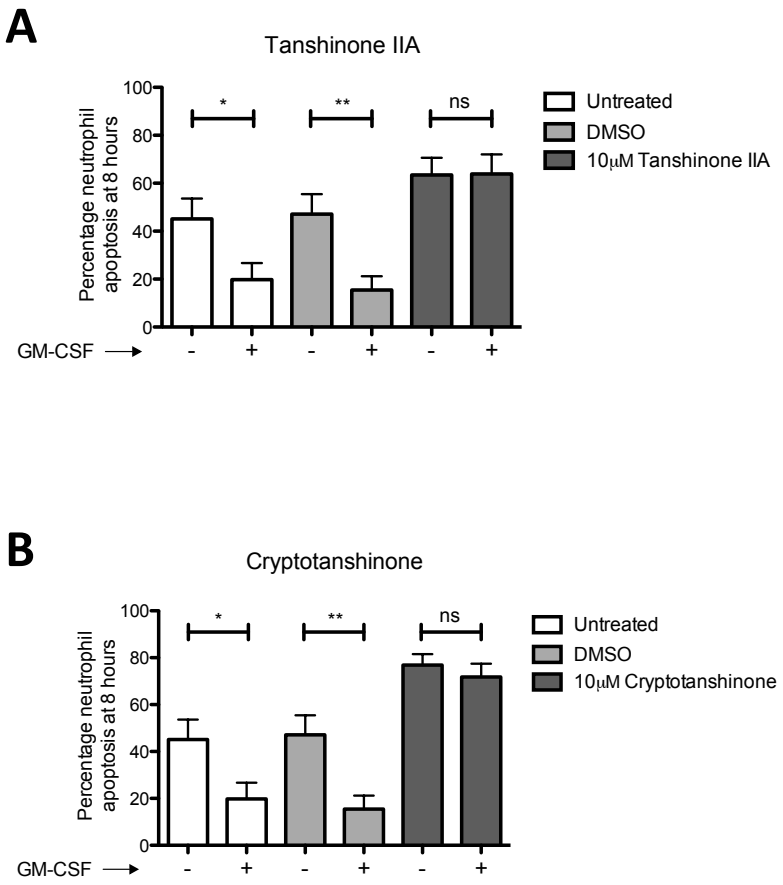

Figure 5.5: Tanshinones abrogate GM-CSF induced neutrophil survival *in vitro*.

Neutrophils were isolated from whole blood using either the Optiprep™ or Percoll® method of separation and were incubated for 8 hours at 37°C with (+) and without (-) GM-CSF as indicated and with (A) 10 μM tanshinone IIA or (B) 10 μM cryptotanshinone. In each case, 2 cytospins were acquired and the percentage of neutrophil apoptosis was calculated based on nuclear morphology. GM-CSF significantly reduced apoptosis in control groups but this survival was lost in the presence of either tanshinone IIA or cryptotanshinone (One-way ANOVA with Bonferroni's multiple comparison post-test to compare selected columns; where * $P < 0.05$ and ** $P < 0.01$; $n = 3$).

5.4.2 Tanshinone IIA increases neutrophil apoptosis *in vivo*

Having established that tanshinone IIA could promote both constitutive neutrophil apoptosis and apoptosis in the presence of GM-CSF survival signalling *in vitro*, I hypothesised that this compound would also cause apoptosis *in vivo*, to reduce neutrophil numbers at the site of injury and drive inflammation resolution in the zebrafish model. To investigate this, tail transection was performed on 3 dpf *mpx*:GFP larvae and those that mounted a good inflammatory response were treated with 25 µM tanshinone IIA at 6 hpi. At 12 hpi, larvae were fixed in 4% PFA prior to TSA staining to label neutrophil-specific myeloperoxidase activity, followed by TUNEL staining to label apoptosis. The tail fins were imaged on an Ultra*VIEW*VoX spinning disk confocal imaging system at 20x magnification. The total neutrophil number at the site of injury and number of dual-labelled apoptotic neutrophils was assessed in individual larvae, to calculate the mean percentage of neutrophil apoptosis. This revealed that tanshinone IIA significantly increased the percentage of neutrophil apoptosis at the site of injury compared to the DMSO vehicle control (Figure 5.6). The rate of apoptosis in the control larvae was comparable to that observed in the experiments performed previously, although tanshinone IIA was a less potent inducer of apoptosis than isopimpinellin (refer to Figure 4.18).

It was evident that tanshinone IIA could drive inflammation resolution by promoting neutrophil apoptosis. However, its effect in the TUNEL assay appeared relatively small given that this compound was one of the best hits identified in the Spectrum Collection screen. Due to the nature of this assay, neutrophil apoptosis can only be measured at one time point, which might not necessarily be representative of the entire resolution phase, as discussed previously. The resolution assay data indicate that there is a highly significant reduction in neutrophil number at the wound in tanshinone IIA larvae at 12 hpi compared to control larvae. This suggests that either neutrophil apoptosis is occurring much earlier and apoptotic corpses cannot be observed by TSA/TUNEL staining as they have already been removed by macrophages by 12 hpi, or that there is an additional pro-resolution mechanism responsible for the reduction in neutrophil numbers caused by tanshinone IIA.

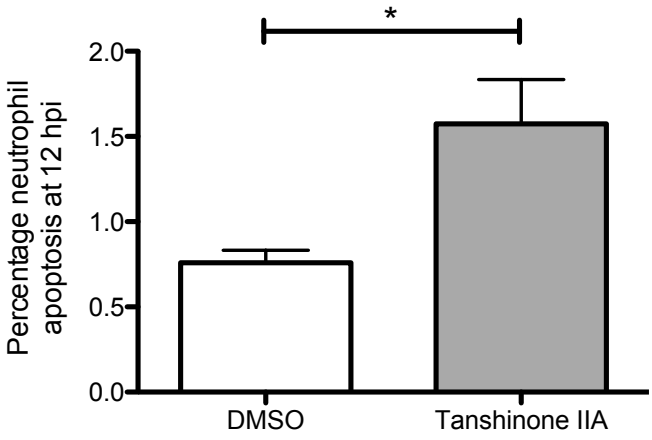

Figure 5.6: Tanshinone IIA significantly increases neutrophil apoptosis during inflammation resolution *in vivo*.

Tail transection was performed on 3 dpf *mpx*:GFP larvae and good responders were treated with 25 µM tanshinone IIA or DMSO at 6 hpi. Larvae were fixed in 4% PFA at 12 hpi and neutrophils were stained with TSA, followed by TUNEL staining for apoptosis. Tail fins were mounted and imaged on a confocal spinning disk imaging system at 20x magnification. The total number of neutrophils at the site of injury and the number of apoptotic neutrophils were counted to calculate the percentage apoptosis. Tanshinone IIA caused a significant increase in neutrophil apoptosis (Unpaired *t*-test; $P < 0.05$; $n \geq 161$, performed as 3 independent experiments).

5.5 Tanshinone IIA increases neutrophil reverse migration

Recently is has become accepted that apoptosis is not the only mechanism responsible for neutrophil clearance, but that potentially viable neutrophils can leave sites of inflammation by reverse migration. This has been reported in mammalian systems (Buckley & Ross, 2006; Woodfin et al., 2011) and in zebrafish (Mathias et al., 2006; Hall et al., 2007; Elks et al., 2011; Yoo & Huttenlocher, 2011), but the precise signalling pathways involved remain undefined. I hypothesised that an increase in neutrophil reverse migration might be an additional mechanism to explain the significant pro-resolution effect of tanshinone IIA in zebrafish.

To investigate this hypothesis, reverse migration assays were performed using 3 dpf *mpx*/Kaede larvae, as described in the previous chapter. At 4 hpi, larvae that mounted a good inflammatory response were treated with either 25 µM tanshinone IIA or DMSO vehicle control. At 6 hpi, Kaede-labelled neutrophils at the site of injury were photoconverted from green to red fluorescence using an Ultra*VIEW*PhotoKinesis™ device, with the 405 nm laser line at 40% laser energy. Larvae were imaged for approximately 300 minutes and the number of red-labelled neutrophils that migrated away from the site of injury into a defined distal region in each larva was quantified (refer to Figure 4.19 A). Strikingly, this analysis revealed that neutrophils in tanshinone IIA treated larvae moved away from the site of injury more quickly than in DMSO control larvae (Figure 5.7 A). The most rapid movement appeared to occur within the first 2 hours following photoconversion, as illustrated in (Figure 5.7 B). Neutrophils tended to migrate away through the tissue, rather than re-entering the vasculature.

These results suggested that tanshinone IIA exerts its predominant pro-resolution effect by accelerating the endogenous reverse migratory behaviour of neutrophils *in vivo*, promoting neutrophil clearance from the wound. This, combined with the apoptosis-inducing properties of tanshinone IIA, may account for its highly significant pro-resolution activity in the zebrafish model.

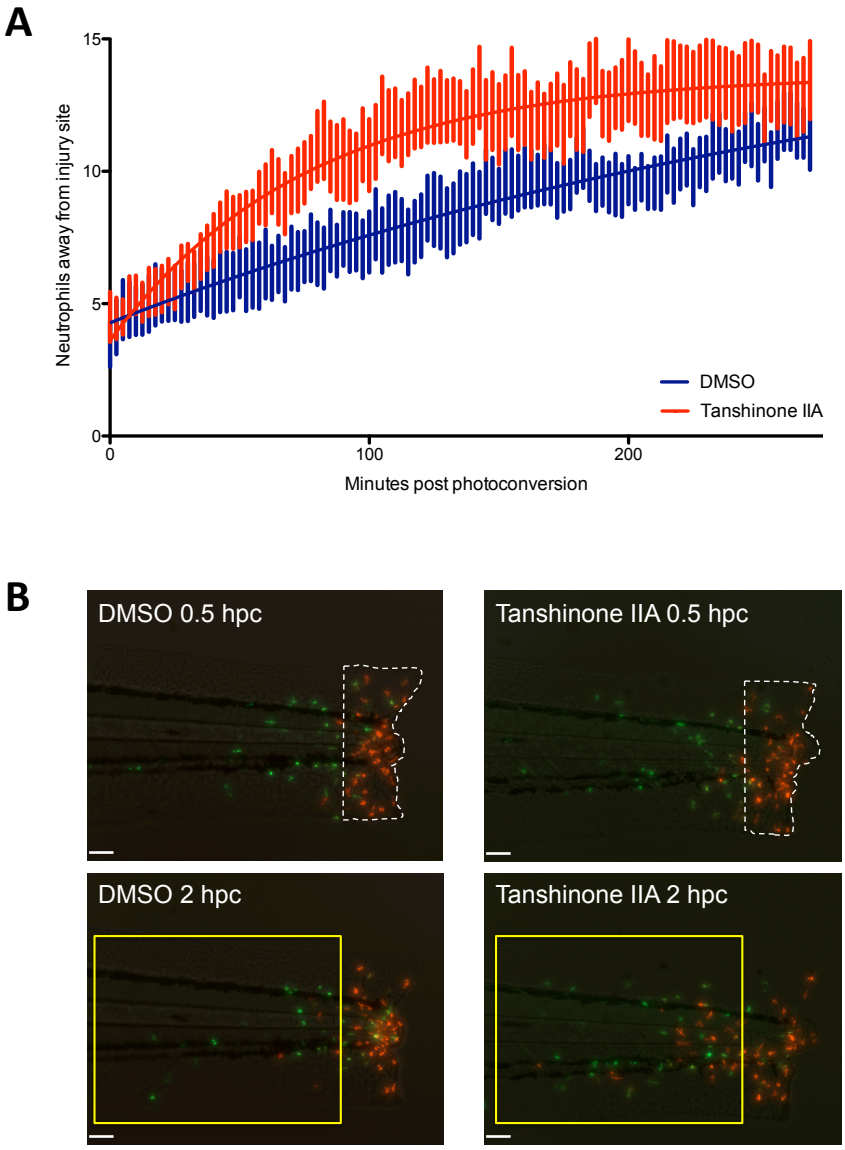

Figure 5.7: Tanshinone IIA accelerates reverse migration of neutrophils during inflammation resolution.

Tail fin transection was performed on *mpx*/Kaede larvae at 3 dpf, and those that mounted a good neutrophilic response were selected for treatment with 25 µM tanshinone IIA or the DMSO vehicle control at 4 hpi. At 6 hpi, larvae were mounted and neutrophils at the site of injury were photoconverted from green to red fluorescence on a confocal spinning disk system using an Ultra*VIEW*PhotoKinesis™ device, with the 405 nm laser line at 40% laser energy. Larvae were imaged on an inverted compound fluorescence microscope for 4.5 hours and the number of red cells that migrated away from the site of injury into the defined region was quantified over the time-course. Tanshinone IIA accelerated neutrophil reverse migration compared to the DMSO vehicle control (A), with the most rapid movement occurring within the first 2 hours following photoconversion, as illustrated in (B) (n = 18, performed as 3 independent experiments, data expressed as mean ± SEM). Dotted line indicates photoconverted region and yellow box indicates region in which neutrophil number were quantified. Scale bar represents 60 µm.

5.5.1 Tanshinone IIA overrides hypoxic signalling *in vivo*

Having already shown that tanshinone IIA could override pro-inflammatory stimuli and drive neutrophil apoptosis *in vitro*, I hypothesised that it might also drive resolution *in vivo* by promoting reverse migration even under prolonged hypoxic conditions, which would more accurately reflect the inflammatory environment of the persistent neutrophilic inflammation characteristic of disease states. It has been shown previously that reverse migration in zebrafish is regulated by Hypoxia-inducible factor (Hif) and that dominant-active forms of Hif-1α can delay resolution of inflammation by causing a reduction in reverse migration and increased neutrophil retention at the wound (Elks et al., 2011).

To investigate whether tanshinone IIA could accelerate reverse migration in the presence of dominant-active Hif signalling, *mpx*/Kaede larvae were injected with dominant-active *hif-1αb* RNA at the one-cell stage. Tail transection was performed at 2 dpf and larvae that mounted a good response were selected at 4 hpi. Following 1 hour of pre-treatment with either 25 μM tanshinone IIA or the DMSO vehicle control, Kaede-labelled neutrophils at the site of injury were photoconverted from green to red fluorescence using an Ultra*VIEW*PhotoKinesis™ device, as described. Larvae were imaged for approximately 5 hours and the number of red-labelled neutrophils that migrated away from the site of injury was quantified. Tanshinone IIA appeared to promote reverse migration even in the presence of hypoxia signalling, whereas most neutrophils were retained at the wound in the DMSO treated larvae, in a manner consistent with published data (Elks et al., 2011) (Figure 5.8 A). Control experiments were also performed by microinjection of phenol red instead of the RNA, which showed that the effect of tanshinone IIA was maintained and that there was more reverse migration in the DMSO treated larvae injected with phenol red compared to those injected with dominant-active *hif-1αb* RNA, which increased neutrophil retention (Figure 5.8 B).

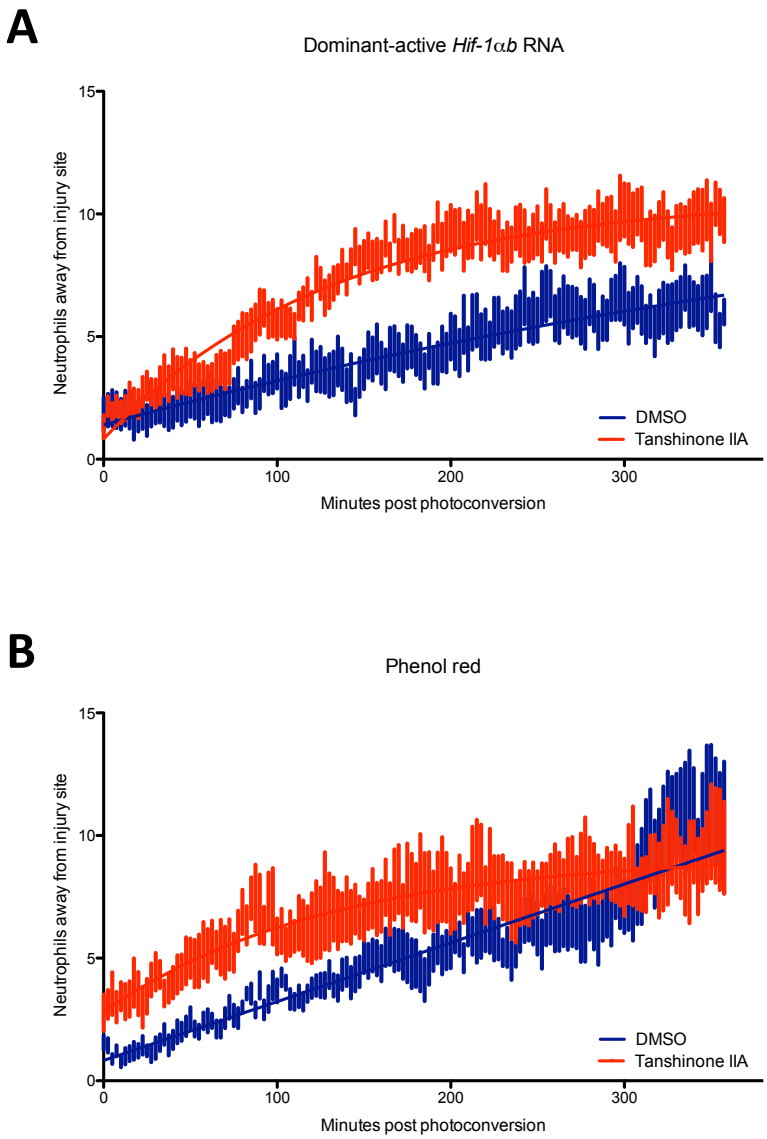

Figure 5.8: Tanshinone IIA overrides hypoxia signalling to accelerate reverse migration of neutrophils during inflammation resolution *in vivo*.

At the one-cell stage, *mpx*/Kaede larvae were injected with either (A) dominant-active *hif-1αb* RNA or (B) phenol red. Tail fin transection was performed at 2 dpf and larvae that mounted a good neutrophilic response were selected for treatment with 25 µM tanshinone IIA or the DMSO vehicle control at 4 hpi. At 5 hpi, larvae were mounted and neutrophils at the site of injury were photoconverted from green to red fluorescence on a confocal spinning disk system using an Ultra*VIEW*PhotoKinesis^{TM} device, with the 405 nm laser line at 40% laser energy. Larvae were time-lapsed on an inverted compound fluorescence microscope for approximately 5 hours and the number of red cells that migrated away from the site of injury into the defined region was quantified over the time-course. Tanshinone IIA accelerated neutrophil reverse migration compared to the DMSO vehicle control in the presence of both (A) dominant-active *hif-1αb* RNA and (B) phenol red (n = 18, performed as 3 independent experiments, data expressed as mean ± SEM).

These results indicated that tanshinone IIA could not only drive inflammation resolution in the model of acute inflammation, but also in a model with attributes more comparable to the persistent inflammatory environment found in disease states, by overriding the effects of hypoxic signalling.

The ability of tanshinone IIA to increase reverse migration of neutrophils away from the site of inflammation was both an interesting and novel observation. Although there is some knowledge of the mechanisms governing neutrophil chemotaxis during recruitment to sites of inflammation, those regulating the reverse of this process are largely undefined. In zebrafish, the migration of neutrophils away from a wound has been termed 'retrograde chemotaxis' or 'fugetaxis', implying that it is an active process, in which the cells are driven away by pro-resolution chemorepellents (Mathias et al., 2006). It has been demonstrated that high concentrations of the chemokine IL-8 can repel neutrophils both *in vitro* and *in vivo* (Tharp et al., 2006). This phenomenon has also been observed in other leukocytes, for example high concentrations of the CXCR4 ligand SDF-1 can repel T cells (Poznansky et al., 2000). Alternatively, it has been suggested that neutrophils respond to competing chemoattractant signals emanating from the vasculature, which might draw them away from the wound (Starnes et al., 2012). However, in the experiments performed here, neutrophils were not observed to preferentially undergo transendothelial reverse migration rather than migrate away via the tissues, in either tanshinone IIA or DMSO treated larvae.

Other evidence directly disputes the theory of active reverse migration, instead describing it as a process of passive redistribution (Holmes, Dixon, et al., 2012; Holmes, Anderson, et al., 2012). In these studies, a thorough analysis of neutrophil migratory behaviour during the resolution of inflammation was performed using time-lapse data generated from reverse migration assays carried out on injured *mpx*/Kaede larvae, in the same manner as described here. Using both drift-diffusion analysis and Approximate Bayesian Computation Sequential Monte Carlo (ABC SMC) techniques, it was revealed that the process of reverse migration is best fitted to a model in which neutrophils redistribute in a stochastic fashion, termed the 'pure-diffusion' model. This

is in contrast to the 'drift-diffusion' model, in which an element of 'drift' would imply directed movement away from the wound, characteristic of chemorepulsion.

As tanshinone IIA appeared to promote reverse migration away from the wound, it was hypothesised that this compound might directly activate fugetaxis. To examine this, dynamic modelling of the neutrophils in tanshinone IIA treated larvae was performed, in order to characterise their migration dynamics.

5.5.2 Neutrophils migrate by 'pure-diffusion' in the presence of tanshinone IIA

To investigate the migratory behaviour of neutrophils undergoing reverse migration in the presence of tanshinone IIA, the data obtained in the reverse migration assays described above were analysed using the dynamic modelling methods outlined in (Holmes, Anderson, et al., 2012). The results described here are generated from data analysis that was performed by Geoffrey Holmes (Automatic Control and Systems Engineering, University of Sheffield). It was hypothesised that the increased neutrophil reverse migration observed in tanshinone IIA treated larvae might be explained by the production of a repelling gradient at the wound, which would drive the neutrophils away more rapidly.

To give an indication of how far the neutrophils travelled over the course of the time-lapse, the co-ordinates of each neutrophil centroid were identified in Volocity®. The distance from the wound of each neutrophil in each individual time frame was measured and used to calculate the mean squared distance (MSD). When plotted against time, it was clear that neutrophils in tanshinone IIA treated larvae moved further over time than those from DMSO control larvae (Figure 5.9). This indicated that not only did more neutrophils move away from the wound in the presence of tanshinone IIA, but those that did move away also travelled further over time. These analyses appeared to show a linear increase in MSD, consistent with a pure-diffusion model. There was a clear difference in the slope of the line generated from the tanshinone IIA treated larvae data compared to the control data, indicating that the diffusivity constants were different, as will be further explained below.

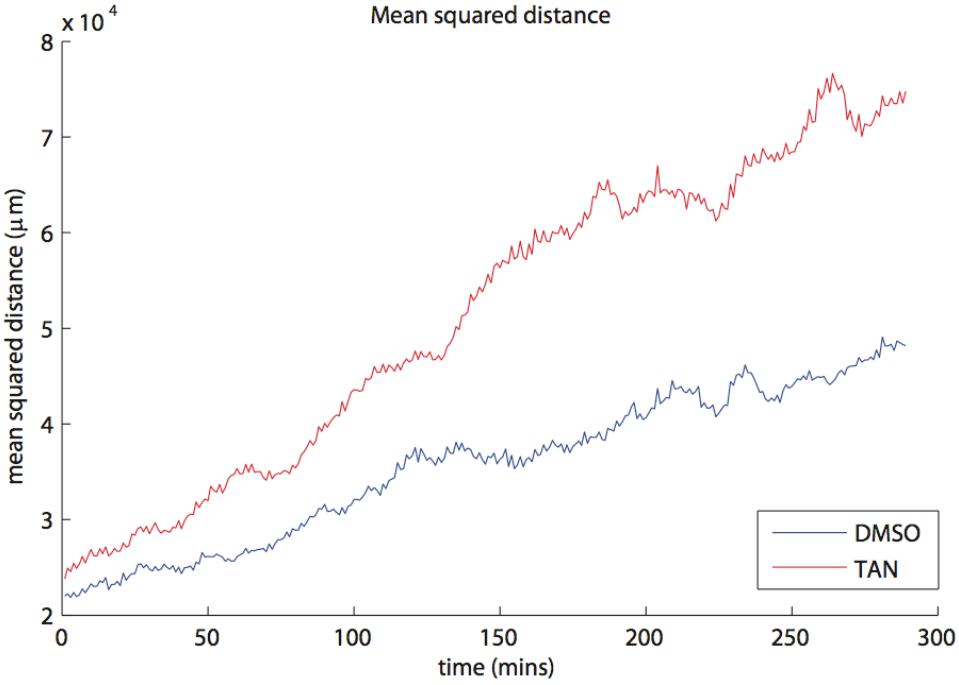

Figure 5.9: The mean squared distance of neutrophils from the wound is increased in the presence of tanshinone IIA.

Data analysis and generation of this figure was performed by Geoffrey Holmes (Automatic Control and Systems Engineering, University of Sheffield), as described in (Holmes, Anderson, et al., 2012). In brief, the distance from the wound of each neutrophil in each individual time frame was measured using the co-ordinates of each neutrophil centroid, as identified in Volocity®. This was used to calculate the mean squared distance over time. Neutrophils in tanshinone IIA treated larvae appeared to move further over time than those from DMSO control larvae.

As an additional method to investigate the neutrophil migration dynamics, a simulation based ABC SMC modelling method was used (Toni et al., 2009). This approach can be applied to dynamic biological systems to compare the data to a set of candidate models, in order to select the model that best represents the data. In this case, models with varying degrees of diffusivity and drift were initially simulated at random and compared to the experimental observations. Those that adequately represented the data within a certain threshold were saved as a sample of all possible models. At each subsequent iteration the parameters of plausible models were adapted slightly to reduce the error threshold until only those close to the experimental observations remained. In both DMSO and tanshinone IIA treated larvae neutrophil reverse migration was best described by the pure-diffusion model (model 1), indicative of undirected migration (Figure 5.10 A).

Interestingly, the diffusivity was much higher in the presence of tanshinone IIA (Figure 5.10 B). When comparing the combined distribution of drift and diffusivity in the drift-diffusion models, it was clear that there was no significant difference in drift between tanshinone IIA and the controls (Figure 5.10 C, D).

These analyses indicated that tanshinone IIA does not actively drive neutrophils away from the wound, but rather they move away in a passive, diffusion-like manner, comparable to the neutrophils in the control larvae. These results suggested that the increase in reverse migration was not due to a repelling gradient at the wound, but rather an increase in random movement. This was reflected by the measurement of diffusivity, which was approximately doubled in the presence of tanshinone IIA.

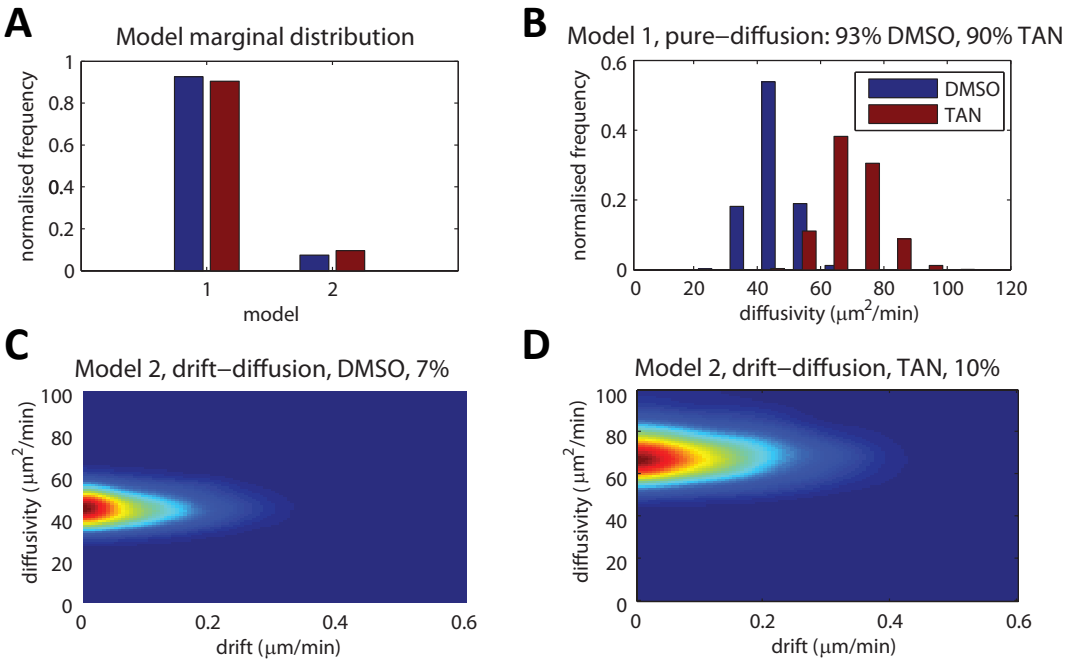

Figure 5.10: Neutrophil migration dynamics are best described by the pure-diffusion model under control conditions and in the presence of tanshinone IIA.

Data analysis and generation of this figure was performed by Geoffrey Holmes (Automatic Control and Systems Engineering, University of Sheffield), using simulation based ABC SMC modelling as described in (Holmes, Anderson, et al., 2012). In brief, models with varying degrees of diffusivity and drift were initially simulated at random, compared to the experimental observations, and those that adequately represented the data within a certain threshold were saved as a sample of all possible models. At each subsequent iteration, the parameters of plausible models were adapted to reduce the error threshold until only those close to the experimental observations remained. (A) In both DMSO and tanshinone IIA treated larvae, pure-diffusion was the preferred model (model 1). The element of diffusivity was higher in tanshinone IIA treated larvae (B), but there was no significant difference in drift between these and the controls (C, D).

5.5.3 Tanshinone IIA does not increase neutrophil speed during inflammation resolution

It was possible that the increase in neutrophil diffusivity observed in the presence of tanshinone IIA could be a consequence of altered directional preference or enhanced speed, which might result in the neutrophils moving away from the wound more rapidly. To investigate this hypothesis, the individual tracks of the reverse migrating neutrophils were analysed using Volocity® software. There was no difference in neutrophil speed or meandering index between tanshinone IIA and DMSO treated larvae (Figure 5.11).

These results suggested that a change in neutrophil speed was not responsible for the increase in reverse migration, but that an alternative mechanism must be involved. The pure-diffusion model describes migration as a form of stochastic redistribution without directional bias. As the neutrophils in tanshinone IIA treated larvae appeared to move away from the wound in an undirected manner, it was hypothesised that perhaps they were unable to detect and respond to the chemotactic gradient at the wound. This would enable them to 'diffuse' away more readily and perhaps gradually move away from the wound rather than favouring migration along the injury site, characteristic of patrolling behaviour.

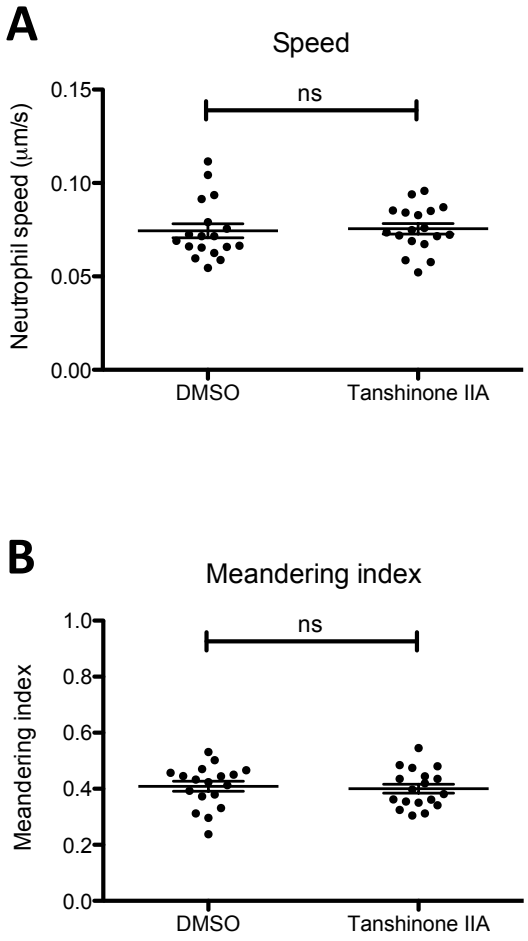

Figure 5.11: Tanshinone IIA does not affect neutrophil speed or meandering index during inflammation resolution.

Tail fin transection was performed on *mpx*/Kaede larvae at 3 dpf, and those that mounted a good neutrophilic response were selected for treatment with 25 μM tanshinone IIA or the DMSO vehicle control at 4 hpi. At 6 hpi, larvae were mounted and neutrophils at the site of injury were photoconverted from green to red fluorescence on a confocal spinning disk system using an Ultra*VIEW*PhotoKinesis™ device, with the 405 nm laser line at 40% laser energy. Larvae were time-lapsed on an inverted compound fluorescence microscope for 3.5 hours and red-labelled cells were tracked over the course of the time-lapse using Volocity®. There was no difference in (A) neutrophil speed or (B) meandering index in tanshinone IIA treated larvae compared to the DMSO vehicle controls (Unpaired *t*-test; $P = 0.8196$ for speed and $P = 0.7244$ for meandering index; $n = 18$, performed as 3 independent experiments).

5.6 Investigation into the effect of tanshinone IIA on chemokine receptor surface expression

Little is known about the mechanisms governing neutrophil retention at sites of inflammation and what signals, or lack of, might regulate reverse migration. This may be partly due to the technical difficulties associated with the investigation of this process in mammalian models. Despite this, reverse migration has been observed *in vivo* in a mouse model of ischemia-reperfusion injury. Here, fluorescently-labelled neutrophils were reported to undergo reverse transendothelial migration more frequently following genetic or pharmacological inhibition of the junctional adhesion molecule JAM-C (Woodfin et al., 2011). In this study, the reverse migrated neutrophils displayed elevated cell surface expression of Intercellular Adhesion Molecule-1 (ICAM-1; also known as CD54). This is a cell surface glycoprotein that can be expressed on both leukocytes and endothelial cells, which binds to integrin molecules to facilitate cell migration (Zimmerman et al., 1992). Its up-regulation was consistent with studies in human neutrophils, which have revealed that reverse migrated neutrophils display a unique cell-surface receptor phenotype, with elevated expression of CD11b and ICAM-1 and reduced expression of the key neutrophil chemokine receptors CXCR1 and CXCR2 (Buckley et al., 2006).

The modelling analysis performed on the reverse migration data suggested that the neutrophils in tanshinone IIA treated larvae might be unable to detect a chemokine gradient. Given the unique cell surface receptor expression reported in reverse migrated neutrophils, it was hypothesised that tanshinone IIA might induce this altered phenotype, for example by causing down-regulation of CXCR1 and CXCR2. Down-regulation of these chemokine receptors might result in the neutrophils being unable to respond to a retention signal at the wound, causing them to diffuse away, which would correlate with the modelling data. In support of this hypothesis, it has recently been demonstrated that the putative zebrafish homologue of IL-8, zCxcl8, mediates neutrophil migration and retention *in vivo* (Sarris et al., 2012).

5.6.1 Investigation into the effect of tanshinone IIA on CXCR1 and CXCR2 surface expression in unstimulated human neutrophils

To begin investigating the effect of tanshinone IIA on CXCR1 and CXCR2, I first sought to establish the basal surface expression levels of these chemokine receptors on freshly isolated human neutrophils. Neutrophils were separated from peripheral whole blood as described previously, followed by staining with either CXCR1-fluorescein isothiocyanate (CXCR1-FITC) or CXCR2-phycoerythrin (CXCR2-PE) monoclonal antibodies (MAbs), which were directly conjugated to the fluorochromes. Cells were stained for 30 minutes at 4^{o}C, alongside the appropriate isotype controls, and cell surface expression of CXCR1-FITC and CXCR2-PE was assessed using a FACSCalibur flow cytometer.

To isolate the neutrophil population from cell debris and other cell types that may have been contaminating the neutrophil suspension, a neutrophil gate was applied to an unstained control sample based on forward and side scatter, using FlowJo software (Figure 5.12 A). The same gating was applied to all samples, ensuring that neutrophils were not lost from the gate. Single-parameter histograms were generated to display the level of fluorescence in the FL-1 channel for CXCR1-FITC (Figure 5.12 B) and the FL-2 channel for CXCR2-PE (Figure 5.12 C). The geometric mean (also known as mean fluorescence intensity (MFI)) was measured for each sample. Under basal conditions, neutrophils expressed both CXCR1 and CXCR2, with mean fluorescence intensities of 82.3 and 78.1, respectively.

To investigate the effect of tanshinone IIA on CXCR1 and CXCR2 surface expression, neutrophils were isolated as described, followed by treatment with either 10 µM tanshinone IIA or DMSO as a vehicle control for 2 hours at 37°C. Neutrophils were re-suspended for staining with either CXCR1-FITC, CXCR2-PE or the appropriate isotype controls, for 30 minutes at 4^{o}C. Cell surface expression of CXCR1 and CXCR2 was assessed by flow cytometry in the same manner as described for the previous experiment. Based on the MFI values, there was no significant difference in CXCR1 or CXCR2 neutrophil surface expression in the presence of tanshinone IIA compared to the DMSO control (Figure 5.13).

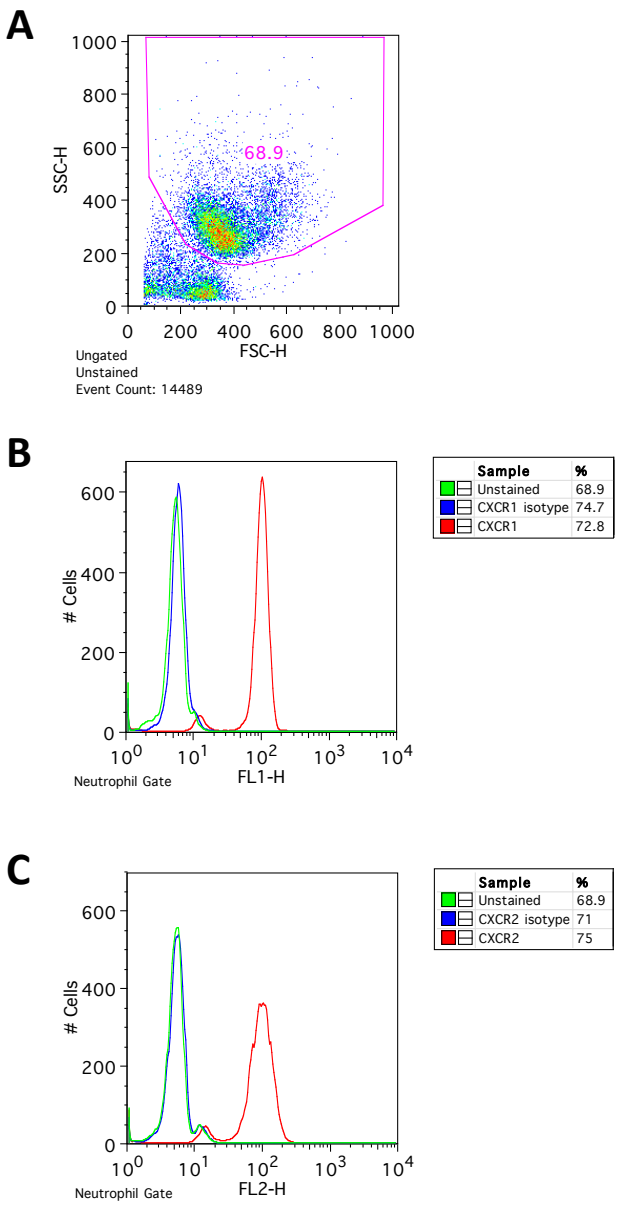

Figure 5.12: Human neutrophils express CXCR1 and CXCR2 under basal conditions.

Human neutrophils were isolated from whole blood, as described, and stained with either human CXCR1-FITC, human CXCR2-PE, mouse IgG2A-FITC or mouse IgG2A-PE at a 1:5 dilution, for 30 minutes at 4°C. After washing and fixing, sample fluorescence was measured using a FACSCalibur flow cytometer and the results were analysed using FlowJo. An unstained control was used to isolate the neutrophil population (A), and this gating was applied to all subsequent samples. Single parameter histograms were generated to measure the MFI of (B) CXCR1-FITC in the FL-1 channel (82.3) and (C) CXCR2-PE in the FL-2 channel (78.1) (*n* = 1).

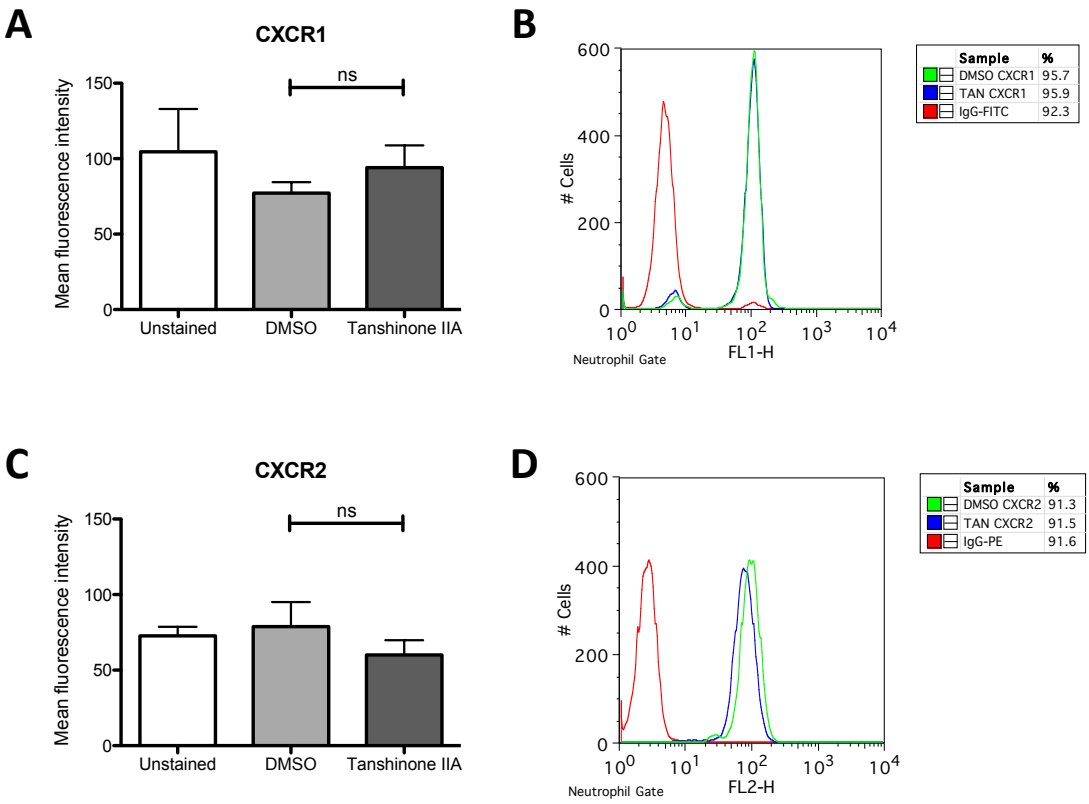

Figure 5.13: Tanshinone IIA does not affect neutrophil surface expression of CXCR1 or CXCR2.

Human neutrophils were isolated from whole blood, as described, and treated with either 10 µM tanshinone IIA or DMSO as a vehicle control, for 2 hours at 37 ^{o}C. After washing and re-suspending, neutrophils were stained with either human CXCR1-FITC, human CXCR2-PE, mouse IgG2A-FITC or mouse IgG2A-PE at a 1:5 dilution, for 30 minutes at 4^{o}C. After washing and fixing, sample fluorescence was measured using a FACSCalibur flow cytometer and the results were analysed using FlowJo. Neutrophil gating was applied as previously indicated (Figure 5.12 A). There was no significant difference in the MFI of (A) CXCR1-FITC or (C) CXCR2-PE between tanshinone IIA and the DMSO vehicle control (One-way ANOVA with Bonferroni's multiple comparison post-test to compare all columns; *P* = 0.6443 for CXCR1, *P* = 0.4729 for CXCR2; *n* = 3). Representative single-parameter histograms are shown in (B) and (D).

5.6.2 Investigation into the effect of tanshinone IIA on CXCR1 and CXCR2 surface expression in human neutrophils following IL-8 stimulation

The results described above suggested that tanshinone IIA had no effect on CXCR1 and CXCR2 surface expression. However, these experiments were performed on unstimulated neutrophils and perhaps the lack of stimulus could explain the absence of any effect. Upon stimulation with IL-8 (CXCL8), CXCR1 and CXCR2 are rapidly internalised, IL-8 is degraded by lysosomal enzymes, and the receptors are recycled back to the neutrophil surface membrane (Samanta et al., 1990). Based on the hypothesis that tanshinone IIA could prevent neutrophils from detecting the chemokine gradient, it was perhaps more likely that rather than affecting basal levels of cell surface expression, tanshinone IIA might inhibit recycling of the receptors once they had been internalised.

To investigate the hypothesis that tanshinone IIA could reduce chemokine receptor recycling, freshly isolated human neutrophils were stimulated with varying doses of IL-8, in combination with either 10 μM tanshinone IIA or DMSO as a vehicle control, for 30 minutes at 37°C. The concentrations of IL-8 required to stimulate CXCR1 and CXCR2 internalisation were selected based on published data (Sabroe et al., 1997). Following stimulation, neutrophils were transferred to ice and washed multiple times to remove all traces of the chemokine. At this stage, some samples were used to measure receptor internalisation, and were stained with either CXCR1-FITC or CXCR2-PE as described above. To measure receptor recycling, the appropriate samples were re-suspended in the corresponding buffer containing either tanshinone IIA or DMSO and were returned to 37°C. At various time intervals, as indicated, the neutrophils were transferred to ice, washed and re-suspended, followed by staining for CXCR1-FITC or CXCR2-PE.

Flow cytometry and neutrophil gating were performed as previously described and the mean fluorescence intensity in the appropriate channel was measured for each sample. The data were expressed as a percentage of the basal fluorescence for each

chemokine receptor, which was measured using an unstimulated, untreated control sample.

The results indicated that tanshinone IIA did not significantly affect internalisation of either CXCR1 (Figure 5.14) or CXCR2 (Figure 5.15) in neutrophils compared to the DMSO vehicle control. For each receptor, there was a clear dose response in the presence of both tanshinone IIA and DMSO, with more internalisation occurring with increasing concentration of IL-8 (illustrated in Figure 5.14 B, C and Figure 5.15 B, C). It was also apparent that a higher ligand concentration was required for internalisation of CXCR1 than CXCR2, which was in keeping with published data (Richardson et al., 1998; Rose et al., 2004).

As described above, receptor recycling could be investigated in the same experiments, such that the percentage of fluorescence measured following receptor internalisation could be used as a baseline, or the 0 minute time point. Re-expression of the receptors could then be measured following removal of the IL-8 ligand and re-incubation of the neutrophils at 37°C to allow the receptors to recycle back to the membrane. For CXCR1, re-expression was measured after 30 minutes and there was no significant difference between the surface membrane levels of CXCR1 between tanshinone IIA and DMSO treated neutrophils, stimulated with either 10 or 100 nM IL-8 (Figure 5.16). At the 10 nM concentration, there actually appeared to be down-regulation of CXCR1 surface expression in the DMSO treated neutrophils between 0 and 30 minutes, although this was likely due to random noise and the fact that there was only a small level of internalisation to begin with.

For CXCR2, re-expression was measured at both 10 and 30 minute intervals following receptor internalisation and removal of IL-8. Again, no significant differences in the surface level expression of CXCR2 were observed between tanshinone IIA and DMSO treated neutrophils, stimulated with either dose of IL-8, at either 10 or 30 minutes. In comparison to CXCR1, the levels of CXCR2 in neutrophils stimulated with 100 nM IL-8 were lower at 30 minutes, suggesting that recycling of CXCR2 occurred more slowly. This was consistent with published evidence (Rose et al., 2004).

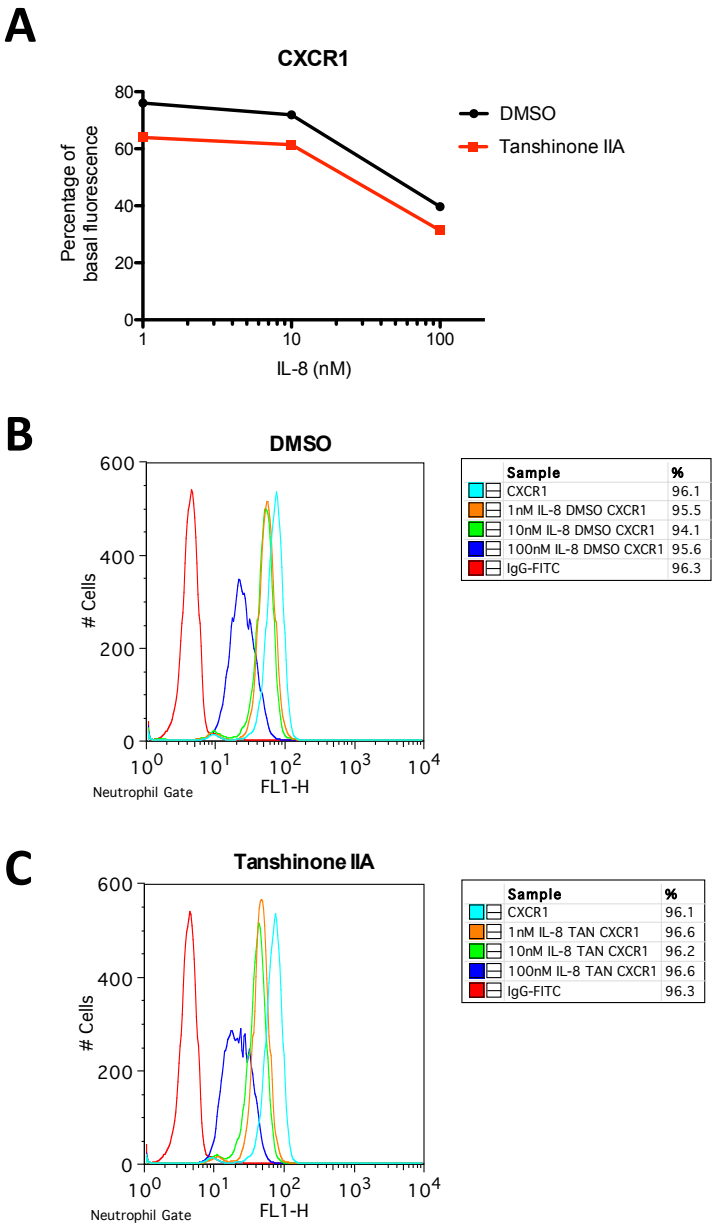

Figure 5.14: Tanshinone IIA does not increase internalisation of CXCR1.

Human neutrophils were isolated from whole blood, as described, and stimulated with IL-8 at the dose indicated, alongside either 10 µM tanshinone IIA or DMSO for 30 minutes at 37 °C. After washing and re-suspending, neutrophils were stained with either human CXCR1-FITC or mouse IgG2A-FITC at a 1:5 dilution, for 30 minutes at 4°C. After washing and fixing, sample fluorescence was measured using a FACSCalibur flow cytometer and the results were analysed using FlowJo. Neutrophil gating was applied as previously indicated (Figure 5.12 A). Data were expressed as a percentage of basal CXCR1-FITC fluorescence for each dose of IL-8 (A). There was no significant difference in internalisation of CXCR1 between tanshinone IIA and the DMSO vehicle control at any of the IL-8 doses tested (Two-way ANOVA with Bonferroni's multiple comparison post-test; $P > 0.05$; $n = 1$). Representative single-parameter histograms are shown for (B) DMSO and (C) tanshinone IIA.

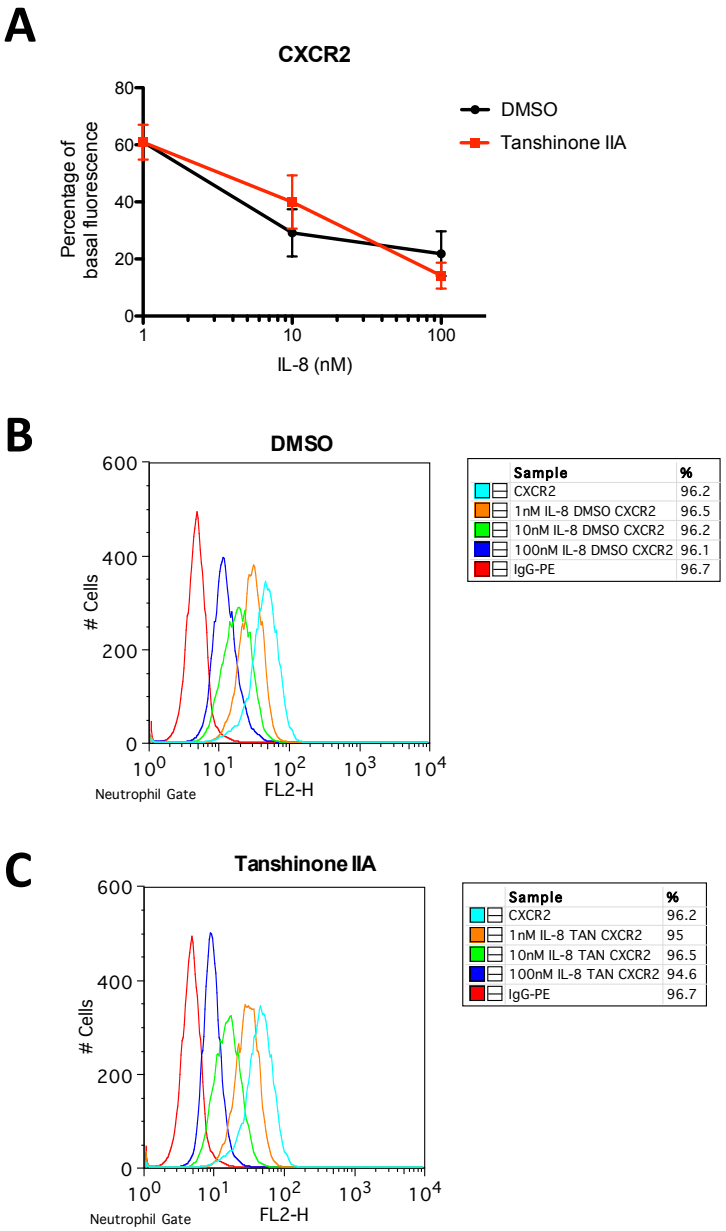

Figure 5.15: Tanshinone IIA does not increase internalisation of CXCR2.

Human neutrophils were isolated from whole blood, as described, and stimulated with IL-8 at the dose indicated, alongside either 10 µM tanshinone IIA or DMSO for 30 minutes at 37 °C. After washing and re-suspending, neutrophils were stained with either human CXCR2-PE or mouse IgG2A-PE at a 1:5 dilution, for 30 minutes at 4°C. After washing and fixing, sample fluorescence was measured using a FACSCalibur flow cytometer and the results were analysed using FlowJo. Neutrophil gating was applied as previously indicated (Figure 5.12 A). Data were expressed as a percentage of basal CXCR2-PE fluorescence for each dose of IL-8 (A). There was no significant difference in internalisation of CXCR2 between tanshinone IIA and the DMSO vehicle control at any of the IL-8 doses tested (Two-way ANOVA with Bonferroni's multiple comparison post-test; $P > 0.05$; $n = 3$). Representative single-parameter histograms are shown for (B) DMSO and (C) tanshinone IIA.

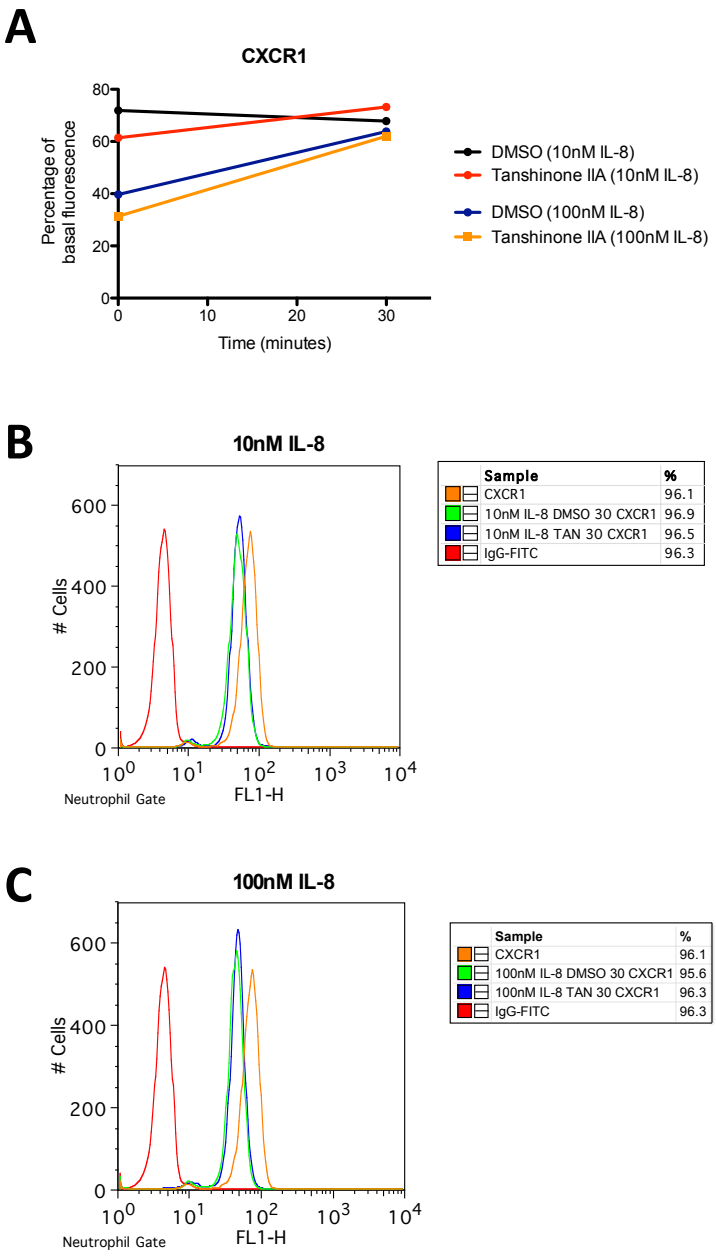

Figure 5.16: Tanshinone IIA does not reduce surface re-expression of CXCR1.

Human neutrophils were isolated from whole blood, as described, and stimulated with IL-8 at the dose indicated, alongside either 10 µM tanshinone IIA or DMSO for 30 minutes at 37 °C. After washing, neutrophils were re-suspended and incubated with either 10 µM tanshinone IIA or DMSO at 37 °C for 30 minutes. Neutrophils were then washed and stained with either human CXCR1-FITC or mouse IgG2A-FITC at a 1:5 dilution, for 30 minutes at 4°C. After washing and fixing, sample fluorescence was measured using a FACSCalibur flow cytometer and the results were analysed using FlowJo. Neutrophil gating was applied and data were expressed as a percentage of basal CXCR1-FITC fluorescence for each dose of IL-8. (A) Data are plotted alongside the percentage fluorescence obtained in the internalisation assay (0 minutes) to represent receptor recycling. There was no significant difference in re-expression of CXCR1 between tanshinone IIA and the DMSO vehicle control at either of the IL-8 doses tested (Two-way ANOVA with Bonferroni's multiple comparison post-test; $P > 0.05$; $n = 1$). Representative single-parameter histograms are shown for re-expression with (B) 10 nM IL-8 and (C) 100 nM IL-8.

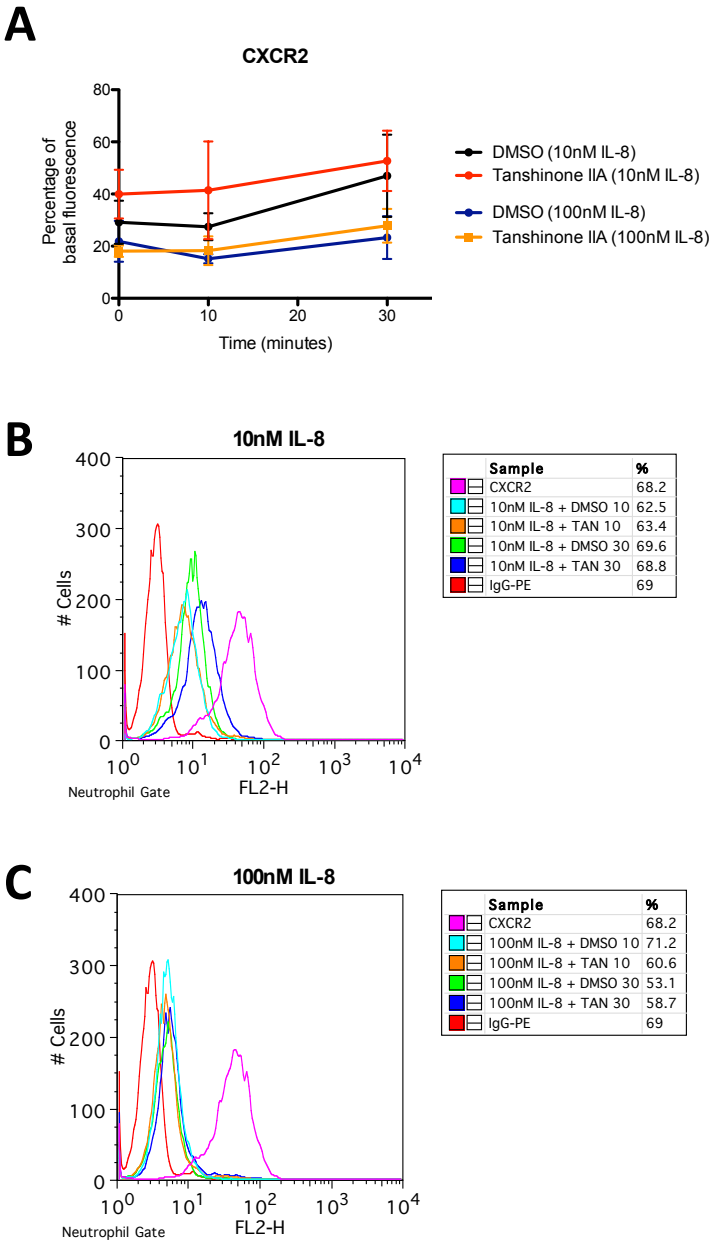

Figure 5.17: Tanshinone IIA does not reduce surface re-expression of CXCR2.

Human neutrophils were isolated from whole blood, as described, and stimulated with IL-8 at the dose indicated, alongside either 10 µM tanshinone IIA or DMSO for 30 minutes at 37 °C. After washing, neutrophils were re-suspended and incubated with either 10 µM tanshinone IIA or DMSO at 37 °C for 30 minutes. Neutrophils were then washed and stained with either human CXCR2-PE or mouse IgG2A-PE at a 1:5 dilution, for either 10 or 30 minutes at 4°C. After washing and fixing, sample fluorescence was measured using a FACSCalibur flow cytometer and the results were analysed using FlowJo. Neutrophil gating was applied and data were expressed as a percentage of basal CXCR2-PE fluorescence for each dose of IL-8. (A) Data are plotted alongside the percentage fluorescence obtained in the internalisation assay (0 minutes) to represent receptor recycling. There was no significant difference in re-expression of CXCR2 between tanshinone IIA and the DMSO vehicle control at either of the IL-8 doses tested or at either time point (Two-way ANOVA with Bonferroni's multiple comparison post-test; $P > 0.05$; $n = 3$ for 0 and 30 minute time points, $n = 2$ for 10 minute time point). Representative single-parameter histograms are shown for re-expression with (B) 10 nM IL-8 and (C) 100 nM IL-8.

5.6.3 The CXCR2 antagonist SB225002 has no effect on neutrophil reverse migration

Although there did not appear to be any effect on CXCR2 internalisation or recycling in human neutrophils stimulated with IL-8 in the presence of tanshinone IIA *in vitro*, this did not rule out an effect that might be occurring downstream of the receptor to cause the neutrophil reverse migration observed *in vivo*. To determine whether the effect of tanshinone IIA was likely to be downstream of CXCR2, reverse migration assays were performed to test whether the effect could be recapitulated using a pharmacological inhibitor of human CXCR2, SB225002. This is a selective, non-peptide antagonist that has ameliorative effects in mammalian models of inflammation, primarily by reducing neutrophil influx (White et al., 1998; Bento et al., 2008).

Initially, a preliminary experiment was performed to investigate the effect of SB225002 specifically on the resolution phase of inflammation. At 3 dpf, *mpx*:GFP larvae were injured and those that mounted a good neutrophilic response were treated with varying doses of SB225002 at 6 hpi. Neutrophil numbers remaining at the site of injury were recorded at 12 hpi. Although not significant, there was a clear reduction in neutrophil number at the wound in larvae treated with 3 µM SB225002 compared to those treated with the DMSO vehicle control (Figure 5.18 A). It is likely that this result would become significant if the assay was repeated, however the outcome from this single experiment was sufficient to indicate an appropriate concentration of SB225002 to use to examine reverse migration. Toxicity was apparent at higher doses.

Reverse migration assays were performed using 3 dpf *mpx*/Kaede larvae, as previously described, and the numbers of neutrophils that migrated away from the wound were quantified over time. There was no difference in reverse migration in the presence of SB225002 compared to the DMSO vehicle control (Figure 5.18 B). These data suggested it was unlikely that tanshinone IIA was exerting its effect by blocking a component of the signalling pathway downstream of Cxcr2. However, it is possible that the dose of SB225002 used in these experiments was not high enough to induce an observable effect.

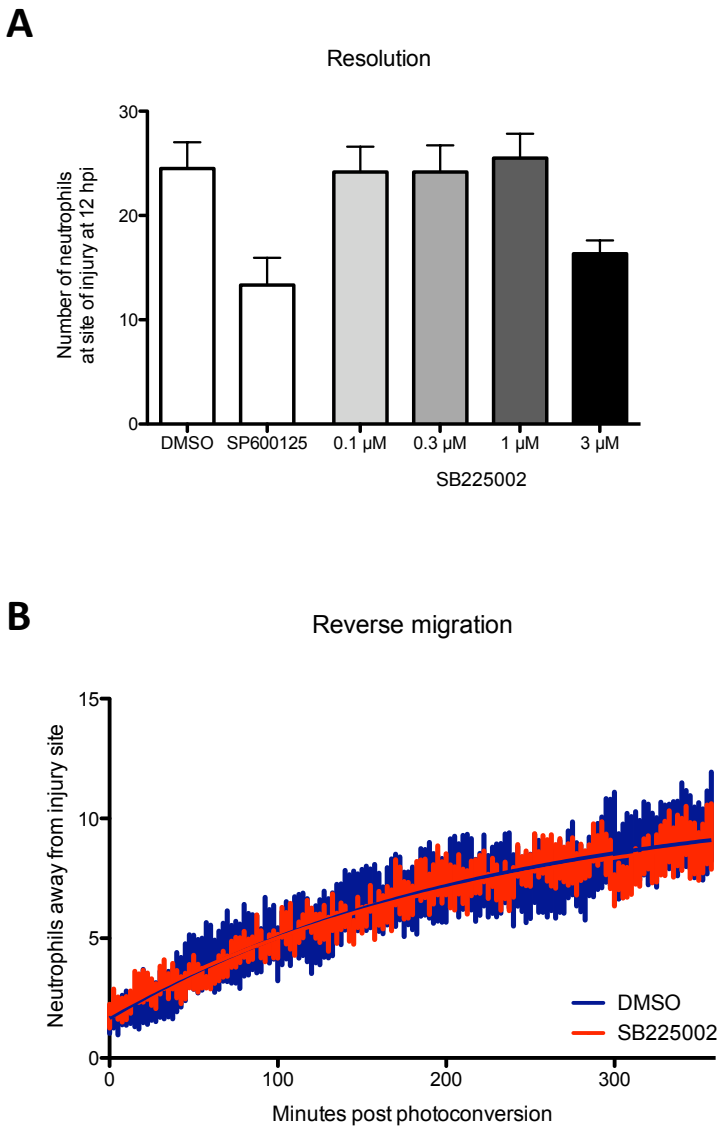

Figure 5.18: The human CXCR2 inhibitor SB225002 does not increase the reverse migration of neutrophils in zebrafish larvae.

(A) As a preliminary experiment, tail transection was performed on *mpx*:GFP larvae at 3 dpf and those that mounted a good neutrophilic response at 6 hpi were treated with varying doses of SB225002. Neutrophil numbers at the site of injury were counted at 12 hpi and there appeared to be a reduction in neutrophil number with 3 µM SB225002, though this was not significant (One-way ANOVA with Dunnett's multiple comparison post-test; *n* = 6, performed as 1 experiment). Toxicity was observed at higher concentrations. (B) Tail fin transection was performed on *mpx*/Kaede larvae at 3 dpf, and those that mounted a good neutrophilic response were selected for treatment with 3 µM SB225002 or the DMSO vehicle control at 4 hpi. At 6 hpi, larvae were mounted and neutrophils at the site of injury were photoconverted from green to red fluorescence on a confocal spinning disk system using an Ultra*VIEW*PhotoKinesis™ device, with the 405 nm laser line at 40% laser energy. Larvae were time-lapsed on an inverted compound fluorescence microscope for 4.5 hours and the number of red cells that migrated away from the site of injury into a region was quantified over the time-course. There was no difference in neutrophil reverse migration between SB225002 treated larvae and the DMSO control treated larvae (*n* = 18, performed as 3 independent experiments, data expressed as mean ± SEM).

5.6.4 Preliminary investigation into the effect of tanshinone IIA on CXCR4

The results obtained in the previous experiments implied that the effect of tanshinone IIA on neutrophil reverse migration was not mediated by changes in the surface expression of CXCR1 or CXCR2, or by an inhibition of the downstream signalling pathways. I hypothesised that regulation of an alternative chemokine receptor might be responsible for the activity, as although neutrophils predominantly express CXCR1 and CXCR2 (Baggiolini, 1993), there is some evidence to suggest that under inflammatory conditions, neutrophils may alter their chemokine receptor expression phenotype (Johnston et al., 1999; Yamashiro et al., 2001).

Of particular interest was the finding that neutrophils from the bronchoalveolar lavage fluid of chronic lung disease patients and the synovial fluid of arthritis sufferers express high levels of CXCR4 in comparison to peripheral blood neutrophils (Brühl et al., 2001; Hartl et al., 2008). The association between CXCR4 and its ligand, SDF-1, is responsible for the retention of neutrophils in the bone marrow and disruption of this interaction, for example by CXCR4 antagonists, induces neutrophil release (Devine et al., 2008). It was hypothesised that a similar mechanism might retain neutrophils at sites of inflammation. In support of this, evidence from both human patients and mouse models of inflammatory disease indicates that SDF-1 expression is increased at sites of inflammation and promotes neutrophil recruitment, as migration is abrogated by antibody blocking of SDF-1 (Struyf et al., 2005; Petty et al., 2007). It is suggested that SDF-1 mediated migration is secondary to the initial wave of leukocyte recruitment regulated by other chemokines and that SDF-1 is released primarily by epithelial cells to coordinate a sustained response (Petty et al., 2007).

Zebrafish possess two Cxcr4 receptors, Cxcr4a and Cxcr4b, the latter of which is the predominantly expressed subtype on neutrophils (Walters et al., 2010). There also exist two Sdf-1 ligands, of which Sdf-1a appears to be highly expressed in regions of neutrophil production such as the CHT (Walters et al., 2010). In zebrafish, the Cxcr4-Sdf-1 signalling axis is believed to function in a similar manner to that identified in humans. Cxcr4-Sdf-1 signalling retains neutrophils and a transgenic zebrafish line has been generated to model WHIM syndrome (Walters et al., 2010). This expresses a

mutant form of Cxcr4 with a truncated C-terminal tail, which might prevent internalisation of the receptor, causing sustained signalling and neutrophil retention in the CHT.

Based on the hypothesis that Cxcr4-Sdf-1 binding might function as a retention signal for neutrophils at sites of inflammation, I initially tested the human CXCR4 inhibitor, AMD3100, to investigate its effect during the resolution phase. Tail transection was performed on 3 dpf *mpx*:GFP larvae and good responders were exposed to AMD3100 at 6 hpi. Neutrophils at the site of injury were counted at 12 hpi, which revealed that there was no significant difference in neutrophil number in the AMD3100 treated larvae compared to the DMSO control treated larvae (Figure 5.19 A). SP600125, which significantly reduced neutrophil numbers at the wound, was used as a positive control. I also explored the effect of AMD3100 on neutrophil recruitment, by treating 3 dpf *mpx*:GFP larvae immediately following tail fin injury and counting neutrophil numbers at the wound at 6 hpi. There was no significant difference between AMD3100 and the DMSO control groups, however there was a significant increase in neutrophil recruitment between 10 and 25 µM AMD3100 (Figure 5.19 B). This suggested that inhibition of Cxcr4 might increase neutrophil recruitment, potentially by promoting release of neutrophils from the CHT.

From these experiments, it seemed unlikely that antagonism of Cxcr4 would replicate the increase in reverse migration observed in the presence of tanshinone IIA. However, it is possible that blocking Cxcr4 with AMD3100 would cause multiple off-target effects, particularly as it would inhibit both forms of Cxcr4 in zebrafish, therefore it could not be ruled out that Cxcr4 signalling in neutrophils might mediate retention at sites of inflammation. As an alternative approach to investigate my hypothesis, I performed whole mount *in situ* hybridisation (WISH) to determine the expression of *sdf-1a* during the resolution phase of inflammation in injured *mpx*:GFP zebrafish larvae exposed to either tanshinone IIA or DMSO alone. I hypothesised that there might be an Sdf-1 gradient at the wound, which would be reduced in the presence of tanshinone IIA, in favour of neutrophil reverse migration.

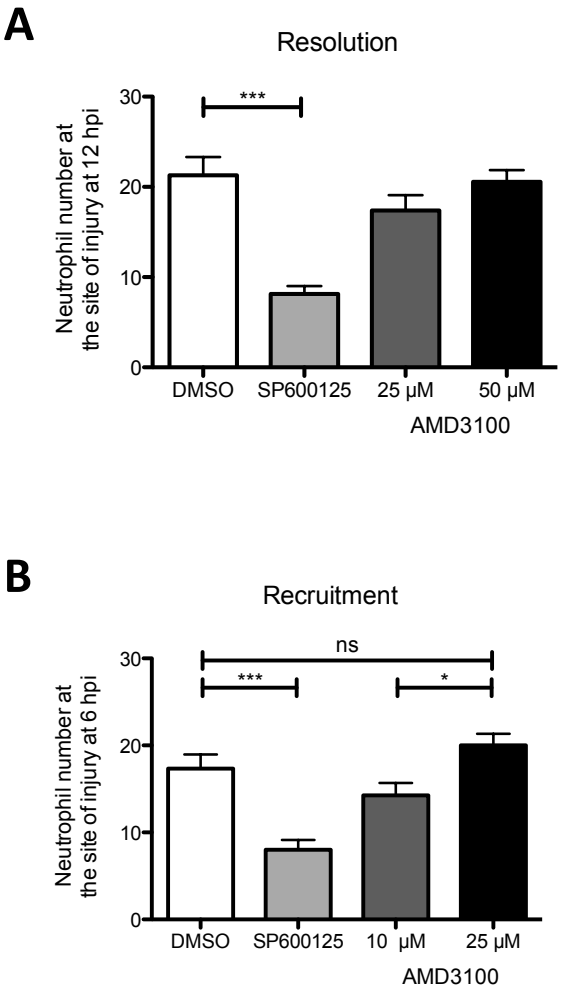

Figure 5.19: The CXCR4 antagonist AMD3100 does not accelerate inflammation resolution in zebrafish.

(A) To investigate the effect of AMD3100 on inflammation resolution, tail transection was performed on *mpx*:GFP larvae at 3 dpf and those that mounted a good neutrophilic response at 6 hpi were treated with varying doses of AMD3100. Neutrophil numbers at the site of injury were counted at 12 hpi. There was no difference in neutrophil number in the presence of AMD3100 compared to the DMSO vehicle control (One-way ANOVA with Dunnett's multiple comparison post-test; where *** $P < 0.001$; $n = 18$, performed as 3 independent experiments). (B) To investigate the effect of AMD3100 on neutrophil recruitment, 3 dpf *mpx*:GFP larvae were injured and treated immediately with varying doses of AMD3100. Neutrophil numbers at the site of injury were counted at 6 hpi. There was no difference in neutrophil number in the presence of AMD3100 compared to the DMSO vehicle control, however significantly more neutrophils were recruited with a dose of 25 µM AMD3100 compared to 10 µM. (One-way ANOVA with Bonferroni's multiple comparison post-test; where * $P < 0.05$ and *** $P < 0.001$; $n = 12$, performed as 2 independent experiments).

A resolution assay was carried out on *mpx*:GFP zebrafish larvae at 3 dpf following the usual protocol, but rather than counting neutrophil number at 12 hpi, larvae were fixed in 4% PFA. WISH was performed, as described, using an *in situ* RNA probe generated from a plasmid vector containing the zebrafish *sdf-1a* coding sequence, obtained as a kind gift from Dr Stone Elworthy (University of Sheffield). Strikingly, there appeared to be an increase in *sdf-1a* expression in the injured tail fins of tanshinone IIA treated larvae compared to the DMSO control treated larvae (Figure 5.20). This was unexpected given that fewer neutrophils are retained at the wound in the presence of tanshinone IIA. To date, this experiment has been performed once only and repetition is required to confirm this preliminary, yet interesting, observation.

Finally, I also carried out a preliminary experiment to investigate the effect of tanshinone IIA on CXCR4 surface expression in human neutrophils, in a similar manner to the experiments performed on CXCR1 and CXCR2. Freshly isolated human neutrophils express only low levels of CXCR4, but this is up-regulated with ageing, a mechanism that is believed to mediate neutrophil clearance by the homing of senescent neutrophils back to the bone marrow (Martin et al., 2003). In order to measure the effect of tanshinone IIA on CXCR4, neutrophils were isolated from peripheral blood, followed by culture in media at 37°C for 12 hours. Flow cytometry was performed as previously described, using a CXCR4-FITC MAb. This revealed that 12 hours was sufficient to observe an increase in CXCR4 expression compared to a control neutrophil sample, which was incubated at 4°C immediately after the separation process (Figure 5.21). After 12 hours, neutrophils were incubated with either tanshinone IIA or DMSO as a vehicle control for 2 hours, followed by stimulation with varying concentrations of SDF-1α ligand for 30 minutes. CXCR4 internalisation was assessed by flow cytometry as previously described, and the preliminary data obtained suggested that there was a lack of CXCR4 internalisation in the presence of tanshinone IIA, at all doses of SDF-1α tested (Figure 5.22). The significance of this remains unclear, and requires further experimentation in order to determine whether this is a real effect.

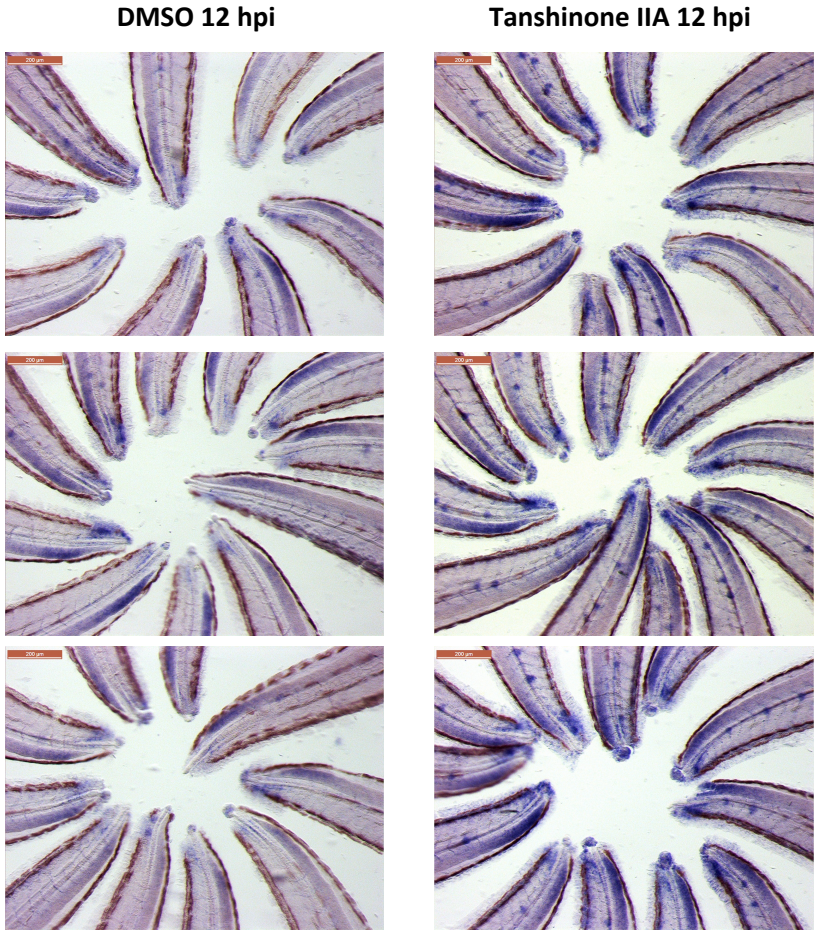

Figure 5.20: WISH reveals that tanshinone IIA up-regulates *sdf-1a* expression at the site of injury in 3 dpf larvae.

Tail transection was performed on *mpx*:GFP larvae at 3 dpf and those that mounted a good neutrophilic response at 6 hpi were treated with either 25 µM tanshinone IIA or DMSO. Larvae were fixed in 4% PFA at 12 hpi and WISH was performed, as described, to investigate *sdf-1a* expression. Larvae were imaged using a fluorescent dissecting microscope with a Leica DFC310 camera, which suggested that there was an up-regulation of *sdf-1a* at the site of injury in tanshinone IIA treated larvae. Increased staining was also evident in the neuromasts ($n \geq 30$, performed as 1 experiment).

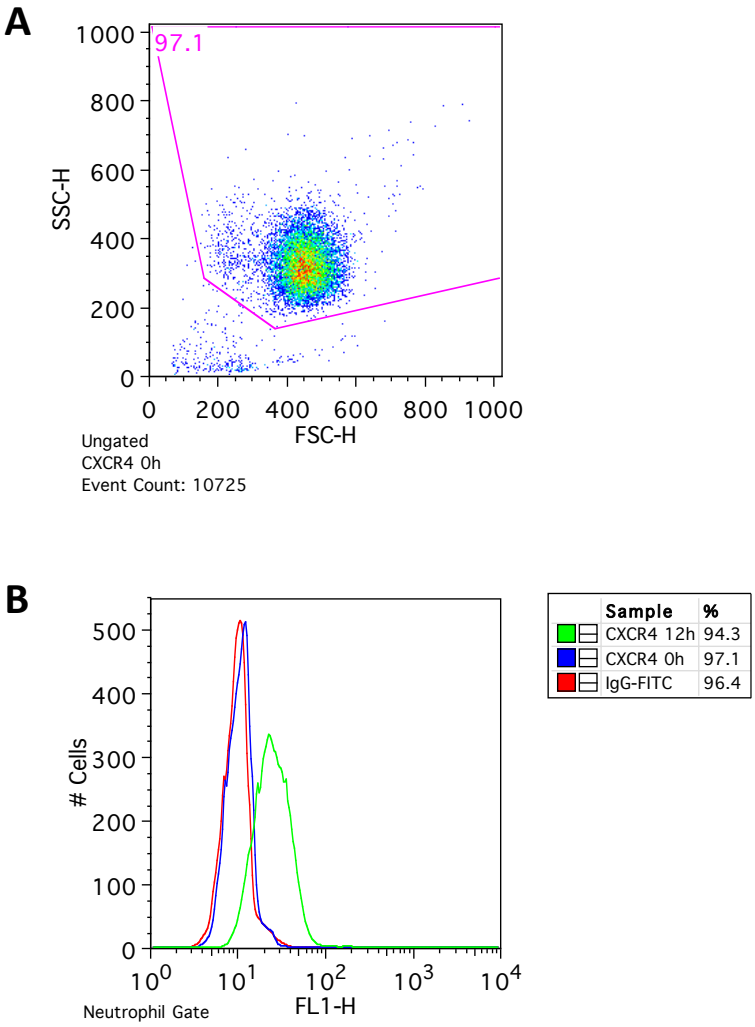

Figure 5.21: CXCR4 expression in human neutrophils increases with senescence.

Human neutrophils were isolated from whole blood, as described, and cultured in media for 12 hours prior to staining with either human CXCR4-FITC or mouse IgG2A-FITC at a 1:10 dilution, for 30 minutes at 4°C. After washing and fixing, sample fluorescence was measured using a FACSCalibur flow cytometer and the results were analysed using FlowJo. The neutrophil population was gated as illustrated in (A), and this gating was applied to all subsequent samples. A single parameter histogram was generated to measure the mean fluorescence intensity in the FL-1 channel (B), which indicated that CXCR4 expression increased over time (*n* = 1).

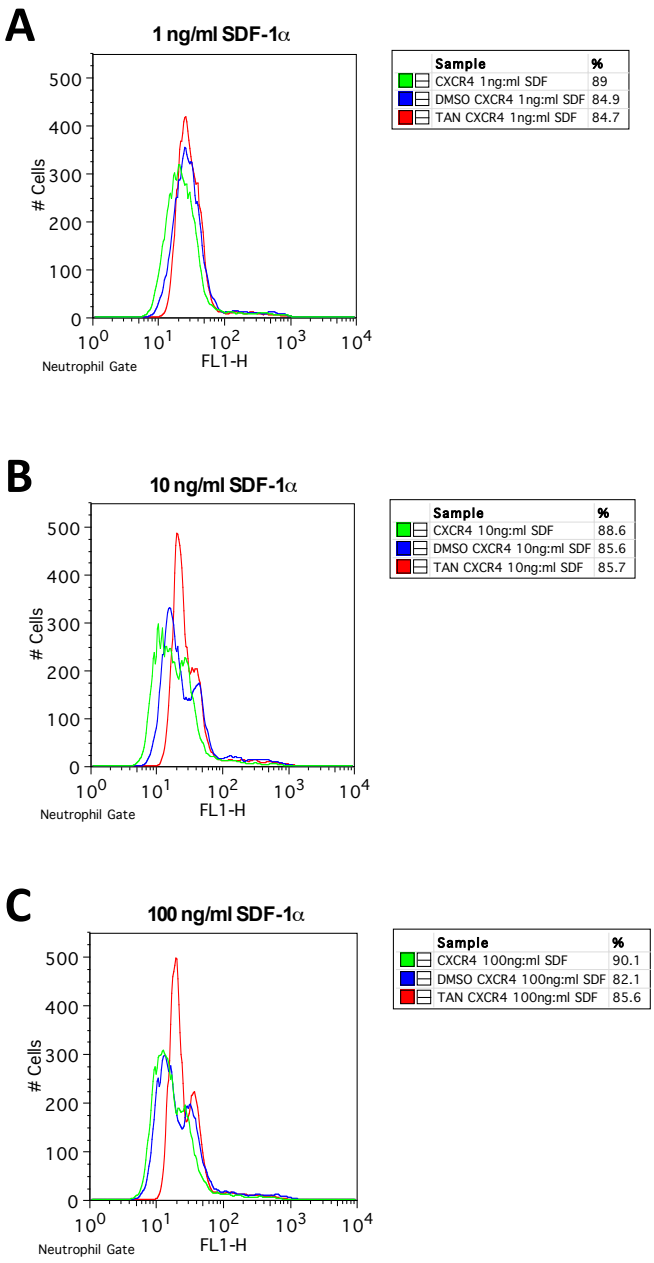

Figure 5.22: Preliminary evidence suggests that tanshinone IIA might prevent internalisation of CXCR4 in human neutrophils.

Human neutrophils were isolated from whole blood, as described, and cultured in media for 12 hours. Neutrophils were then incubated with either 10 µM tanshinone IIA or DMSO for 2 hours, followed by stimulation with varying doses of SDF-1α, as indicated. After washing and re-suspending, neutrophils were stained with either human CXCR4-FITC or mouse IgG2A-FITC at a 1:10 dilution, for 30 minutes at 4°C. After washing and fixing, sample fluorescence was measured using a FACSCalibur flow cytometer and the results were analysed using FlowJo. Neutrophil gating was applied and single parameter histograms were generated to assess the levels of CXCR4-FITC expression. Internalisation of CXCR4 did not appear to occur in the presence of tanshinone IIA, at all doses of SDF-1α tested, judged by a lack of left shift in the histograms (*n* = 1).

5.7 Discussion

In this chapter I have investigated the pro-resolution properties of tanshinone IIA, which was one of the most potent accelerators of inflammation resolution identified in the Spectrum Collection screen. Tanshinone IIA and the closely related cryptotanshinone possess known anti-inflammatory activity and their use in traditional remedies to treat cardiovascular and inflammatory disorders provides some evidence that they are functionally active and can be tolerated in humans. I found that both compounds could induce neutrophil apoptosis *in vitro* and that tanshinone IIA blocked the effects of pro-survival stimuli. Tanshinone IIA also acted via an additional mechanism *in vivo*; by promoting reverse migration of neutrophils away from the wound. This is the first evidence to demonstrate that it is possible to pharmacologically drive inflammation resolution in this manner and suggests a potential strategy by which the neutrophil could be targeted in the development of more effective inflammatory disease therapy.

5.7.1 Tanshinone IIA does not affect neutrophil recruitment

Initially, the effects of tanshinone IIA on neutrophil recruitment to the site of injury were examined in zebrafish larvae. No significant reduction in neutrophil number was found in tanshinone IIA treated larvae compared to those treated with DMSO alone, suggesting that this compound acted specifically on the resolution phase of inflammation. These findings were slightly contradictory to those observed in the secondary assays performed immediately following the Spectrum Collection screen described in Chapter 3, which indicated that there was a reduction in neutrophil recruitment with tanshinone IIA at the highest dose tested. This discrepancy could likely be attributed to the inherent variability between zebrafish larvae, along with the nature of this assay. In contrast to the resolution assay in which larvae that mount a good response to injury are selected for compound treatment, in the recruitment assay larvae are treated immediately following injury, therefore it is not possible to select those that should recruit the expected mean number of neutrophils by 6 hpi. This is perhaps reflected in the higher standard deviation calculated in tanshinone IIA treated larvae in the original recruitment assay (8.63 compared to 5.16 calculated in the more recent dose response experiment). Another possible explanation might be

that in some zebrafish larvae, the inflammatory response was beginning to resolve at 6 hpi and some of the neutrophils that were recruited early to the site of injury were either undergoing apoptosis or had begun to migrate away. As an extra measure to ensure that the data generated in the dose response experiment were valid, the assay was repeated four times, still finding no significant difference in neutrophil recruitment between the DMSO control treated larvae and those treated with tanshinone IIA.

These data were further supported by the lack of effect on neutrophil chemotactic behaviour in the presence of tanshinone IIA. There were no significant differences in neutrophil speed, meandering index or displacement between treatment groups and analysis of neutrophil bearing indicated that neutrophils in tanshinone IIA treated larvae migrated preferentially towards the wound. In the previous chapter, tanshinone IIA was found to have no effect on PI-3K activity during recruitment and the neutrophil polarity index remained unchanged. This provides additional evidence that this compound has no effect on neutrophil recruitment in the zebrafish model. The activity of cryptotanshinone was not further tested in recruitment assays, although interestingly it has been reported that macrophage migration is reduced in the presence of cryptotanshinone and this is partly mediated by inhibition of PI-3K signalling (Don et al., 2007). Whether this is specific to macrophages is unknown, but it would be possible to investigate the effect of cryptotanshinone on PI-3K activation in neutrophils using the PI-3K assay described in Chapter 4.

5.7.2 Tanshinone IIA causes neutrophil apoptosis

Tanshinone IIA significantly accelerated inflammation resolution in zebrafish by reducing neutrophil numbers at the wound in a dose-dependent manner. Apoptosis followed by phagocytic uptake by macrophages is the best described process by which neutrophils are removed from sites of inflammation (Savill et al., 1989) and due to the known apoptotic potential of tanshinone IIA in other cell types (Sung et al., 1999; Won et al., 2010; Jiao et al., 2011; Chen et al., 2012; Wei et al., 2012), I hypothesised that this mechanism might be responsible for its pro-resolution activity. Both tanshinone IIA and cryptotanshinone were found to increase constitutive neutrophil apoptosis *in*

vitro, with the latter having the greatest effect. It is possible that the higher level of apoptosis observed in the presence of cryptotanshinone might be due to toxicity, as this compound caused some death in zebrafish larvae. Although, by simple visual observation of the cytospins obtained from both the tanshinone IIA and cryptotanshinone conditions, the neutrophils appeared apoptotic rather than necrotic in morphology. To be certain, it would be informative to investigate whether this effect is abrogated in the presence of a pan-caspase inhibitor such as Q-VD-OPh. There is currently no published evidence to describe a pro-apoptotic role for the tanshinones in neutrophils, although evidence does indicate that both compounds are associated with caspase activation and down-regulation of Mcl-1 in other cell types (Sung et al., 1999; Won et al., 2010; Wei et al., 2012), which might explain their mechanistic activity.

One of the most interesting findings from these *in vitro* studies was that the pro-apoptotic effect of the tanshinones was not lost in the presence of GM-CSF, a potent neutrophil survival factor (Lee et al., 1993). Thus, it is possible that these compounds are able to block the intracellular signalling downstream of GM-CSF receptor binding that delays apoptosis, such as the PI-3K, MAPK or JAK/STAT pathways (Epling-Burnette et al., 2001). Intriguingly, tanshinone IIA and cryptotanshinone have been reported to inhibit activity of STAT1 and STAT3, respectively (Xu et al., 2008; Shin et al., 2009). However, as these compounds also appeared to accelerate constitutive death, it is perhaps more likely that they are acting further downstream, for example by inhibiting anti-apoptotic BCL-2 family proteins such as Mcl-1.

The apoptotic potential of tanshinone IIA was also investigated *in vivo* by TSA/TUNEL staining, which revealed an increase in neutrophil apoptosis at the site of injury in 3 dpf zebrafish larvae. The caveats associated with this assay have already been discussed in the previous chapter; so will not be dwelled on further here. However, it was clear that the effect on neutrophil apoptosis in the presence of tanshinone IIA was only small (1.57%, compared to 3.33% for isopimpinellin and 6.90% for flavone), which prompted the investigation into an additional mechanism to explain the significant pro-resolution effect of tanshinone IIA observed *in vivo*.

5.7.3 Tanshinone IIA increases neutrophil reverse migration

Reverse migration is becoming increasingly accepted as a mechanism for neutrophil clearance from sites of inflammation and resolution of the inflammatory response. Numerous research groups have observed this phenomenon in zebrafish larvae (Mathias et al., 2006; Hall et al., 2007; Elks et al., 2011; Yoo & Huttenlocher, 2011). Although more technically challenging to investigate, neutrophil reverse migration has also been reported to occur in mammalian systems (Buckley et al., 2006; Woodfin et al., 2011). As such, it is possible that targeting the mechanisms responsible for driving neutrophil migration might provide some therapeutic benefit in the chronic inflammatory disease state.

Using zebrafish larvae expressing the photoconvertible protein Kaede specifically in neutrophils, I was able to label the neutrophil population that had recruited to the tail fin wound at 6 hpi and track their migration during the resolution phase. Interestingly, I found that in larvae pre-treated with tanshinone IIA, the reverse migration of neutrophils away from the wound was enhanced compared to the controls. This is the first evidence to demonstrate that it is possible to pharmacologically drive the resolution of neutrophilic inflammation by promoting reverse migration. The most rapid movement occurred during the first 2 hours following photoconversion and neutrophils appeared to preferentially migrate through the tissues rather than undergoing transendothelial reverse migration back into the vasculature. This is in direct contrast to other reports, which suggest that a large proportion of neutrophils migrate away from a wound via the bloodstream (Mathias et al., 2006; Yoo & Huttenlocher, 2011), however it is possible that these differences are due to both the size and location of the wound.

The data obtained here, along with other published evidence, suggest that during inflammation resolution in the zebrafish, the majority of neutrophils undergo reverse migration rather than detectable apoptosis (Mathias et al., 2006; Yoo & Huttenlocher, 2011). In a rat model of glomerulonephritis, it was demonstrated that neutrophils were cleared from the inflamed glomeruli by reverse migration in preference to apoptosis *in situ* (Hughes et al., 1997). However, this is contradictory to evidence from

other mammalian systems, which suggest that the rate of neutrophil reverse migration is much lower (Buckley et al., 2006; Woodfin et al., 2011). It is possible that these differences are species specific, depend on the type of inflammatory stimulus involved, or that reverse migration plays a more important role in zebrafish at the larval stage than it does in adult animals. The relative contribution of reverse migration compared to apoptosis towards neutrophil clearance and inflammation resolution remains unclear.

The precise mechanisms involved in neutrophil reverse migration are not well understood, although it has been revealed that this process can be delayed in zebrafish by activation of Hif-1α, a component of the hypoxia signalling pathway (Elks et al., 2011). As I had already shown that tanshinone IIA could override GM-CSF induced survival signalling *in vitro*, I wished to investigate whether this might be reflected by an alternative survival factor *in vivo*. Using the techniques established in (Elks et al., 2011), I performed reverse migration assays on larvae injected with dominant-active *hif-1αb* RNA at the one-cell stage and found that the reverse migration effect of tanshinone IIA was maintained even in the presence of hypoxia signalling. This was an important finding given that a prerequisite for novel therapies is the ability to override host survival signals, in order to promote resolution of inflammation in the chronic disease state. Recent data obtained in our laboratory (Joseph Burgon and Dr Stephen Renshaw, personal communication) and in collaboration with others (Joseph Wilson and Dr Sarah Walmsley, personal communication) indicate that tanshinone IIA might also abrogate hypoxic survival signalling *in vitro* to promote neutrophil apoptosis. It would be interesting to determine the contribution of tanshinone IIA induced neutrophil apoptosis towards the resolution of inflammation under hypoxic conditions in the zebrafish, compared to its effect on reverse migration.

5.7.4 Reverse migration is a passive rather than active process

Given that little is known about the mechanisms involved in neutrophil reverse migration, it is difficult to speculate whether it functions as an active process to drive neutrophils away from sites of inflammation, or if it is more characteristic of a passive

redistribution-like process, whereby neutrophils gradually diffuse away once they have completed their anti-inflammatory tasks. These ideas are subject to some controversy, with some zebrafish research groups claiming that it is an active process (Mathias et al., 2006; Starnes et al., 2012), whilst others are in dispute (Holmes, Dixon, et al., 2012; Holmes, Anderson, et al., 2012). The latter evidence is based on mathematical modelling of the whole neutrophil population involved in the inflammatory response induced by tail fin injury. Analysis in this manner should avoid any possible bias towards identification of an active component, particularly as neutrophils are known to display some directionality over short distances due to their inherent behaviour (Guyader et al., 2008). In addition, differences in the size and location of the wound may dictate the nature of the neutrophil migration dynamics.

In order to determine whether the neutrophil reverse migration occurring in the presence of tanshinone IIA was characteristic of an active or passive process, the data were analysed by Geoffrey Holmes from the Automatic Control and Systems Engineering department at the University of Sheffield, using dynamic modelling techniques previously described in (Holmes, Anderson, et al., 2012). This analysis indicated that under both control and tanshinone IIA treated conditions, neutrophil reverse migration during inflammation resolution was most accurately described by a pure-diffusion model, without the influence of an attractive or repulsive chemokine gradient. Analysis of the mean squared distance from the wound indicated that over time, neutrophils moved further away in tanshinone IIA treated larvae compared to the controls, in support of the data obtained by quantification of neutrophil number in the region away from the wound. This was reflected in the increased neutrophil diffusivity measured in the presence of tanshinone IIA. Interestingly, the enhanced reverse migration observed in tanshinone IIA treated larvae could not be explained by an increase in neutrophil speed, suggesting that an alternative mechanism was involved, such as a change in directional preference.

5.7.5 How does tanshinone IIA promote neutrophil reverse migration?

Reverse migrated neutrophils are reported to have a unique cell surface receptor phenotype, which includes down-regulation of the key chemokine receptors CXCR1

and CXCR2 (Buckley et al., 2006). Based on this evidence, along with the observation that tanshinone IIA caused an increase in the passive redistribution of neutrophils, it was hypothesised that this compound might cause down-regulation of these receptors to limit the gradient sensing capacity of neutrophils.

In the initial flow cytometry experiments, no effect on the basal surface expression of CXCR1 or CXCR2 was found in freshly isolated neutrophils treated with tanshinone IIA. Although it was possible that this was due to issues relating to dosing or duration of treatment, it was more accurate to investigate the effect of the compound on neutrophils stimulated with the CXCR ligand IL-8, this being more relevant to physiological conditions. However, even following IL-8 stimulation, no effect was seen on either CXCR1 or CXCR2 internalisation, or receptor recycling, in the presence of tanshinone IIA. There did appear to be a small shift towards lower expression in the internalisation curve for CXCR1 from neutrophils treated with tanshinone IIA, however this was not significant and as there was no effect on receptor recycling, it was unlikely that this was a real effect.

The effect of tanshinone IIA on CXCR2 was also investigated *in vivo*, as although no effect was observed *in vitro*, it could not be determined from these assays whether the compound was affecting intracellular signalling downstream of ligand-receptor binding. CXCR2 was prioritised, as it is believed to play a more significant role in neutrophil recruitment than CXCR1 (Chuntharapai et al., 1995; Nasser et al., 2009). Recently, a putative zebrafish homologue of IL-8, zCxcl8, has been identified in zebrafish and has been shown to mediate both neutrophil recruitment and, most interestingly, neutrophil retention (Sarris et al., 2012). This study indicated that the interaction of zCxcl8 with extracellular heparan sulphate proteoglycans (HSPGs) formed tissue localised chemokine gradients that restricted leukocyte motility at sites of infection. This effect was lost when the interaction was impaired using either a mutant zCxcl8 that was unable to bind to HSPGs or by injection of heparinase to degrade the extracellular matrix. A second zebrafish *Cxcl8* gene has been identified and both ligands have been shown to be important for neutrophil recruitment to the injured tail fin, with *cxcl8-l2* being up-regulated to higher levels than *cxcl8-l1* at the

wound (de Oliveira et al., 2013). Using the human CXCR2 antagonist SB225002, this study also demonstrated that neutrophil recruitment to a wound was mediated by zebrafish Cxcr2. If there were a high zCxcl8 gradient at the site of inflammation, promoting neutrophil retention, then this would fit with my hypothesis that tanshinone IIA reduces the ability of neutrophils to respond to this gradient, potentially via an inhibition of Cxcr2 signalling. However, I was unable to replicate the effect of tanshinone IIA using SB225002, suggesting that inhibition of Cxcl8-Cxcr2 signalling was not sufficient to promote neutrophil reverse migration during resolution. It could be that the concentration of SB225002 used in these experiments was too low to produce an effect and more thorough dosing to establish the optimum concentration required for Cxcr2 inhibition during both recruitment and resolution might be valuable. Some toxicity was observed with this compound at higher doses, so care must be taken to ensure that effects on neutrophil number are not simply due to increased and non-specific cell death.

Although Cxcr2 is believed to be the predominant receptor mediating neutrophil recruitment in zebrafish (Sarris et al., 2012; Deng et al., 2013; de Oliveira et al., 2013), in the presence of SB225002, signalling will still be occurring via the interaction of Cxcl8 with Cxcr1. The association between CXCR1 and CXCR2 is complex; receptor cross-talk exists and ligand-binding to either receptor can influence the internalisation of the other by heterologous desensitisation (Stillie et al., 2009). It has also been proposed that the high level of IL-8 at sites of inflammation promotes global inactivation and internalisation of CXCR1 and CXCR2, which functions as a stop signal for human neutrophils (Rose et al., 2004). It is possible that the downstream signalling pathways mediated by IL-8 are differentially regulated during neutrophil recruitment and retention. For example, perhaps PI-3K signalling is predominantly activated during neutrophil recruitment, but a different pathway is activated to promote neutrophil retention. Under normal conditions in which the inflammatory response resolves, there must be a switch in chemokine signalling or a gradual loss of the retention gradient, permitting neutrophils to migrate away. Whether tanshinone IIA is acting on a component of this signalling cascade in order to accelerate the process, or whether an entirely different chemokine receptor pathway is being affected, remains unclear.

Perhaps signalling downstream of end-target chemoattractants such as fMLP and C5a (Heit et al., 2002) is more likely to mediate neutrophil retention.

5.7.6 Does preliminary evidence suggest that tanshinone IIA affects the CXCR4-SDF-1 signalling pathway?

It is believed that under certain inflammatory conditions, neutrophils may alter their chemokine receptor expression phenotype (Johnston et al., 1999; Yamashiro et al., 2001). In particular, up-regulation of CXCR4 has been reported in chronic inflammatory disease patients (Brühl et al., 2001; Hartl et al., 2008). As the interaction between CXCR4 and its ligand, SDF-1, functions as a retention signal for neutrophils in the bone marrow (Devine et al., 2008), I hypothesised that a similar mechanism might be important for neutrophil retention at sites of inflammation and that tanshinone IIA might disrupt this signalling axis.

I first explored the effect of Cxcr4 inhibition *in vivo* using AMD3100, a mammalian CXCR4 antagonist that has been shown to induce neutrophil mobilisation in both mice and humans (Liles et al., 2003; Broxmeyer et al., 2005). Data obtained from both recruitment and resolution assays suggested that Cxcr4 inhibition did not prevent neutrophil retention at the wound. In fact, in both assays there appeared to be higher numbers of neutrophils at the site of injury with increasing dose of AMD3100. Cxcr4-Sdf-1 signalling has been shown to retain neutrophils in the CHT in zebrafish (Walters et al., 2010), thus inhibition of this retention signal to augment neutrophil release might explain the increase in neutrophil number observed at the wound in the presence of AMD3100. It could be that a Cxcr4-Sdf-1 axis does exist at the wound, but perhaps the dose of inhibitor used was not sufficient to overcome the excess of neutrophil release from the CHT and cause a detectable effect on inflammation resolution. Thorough dosing and reverse migration assays need to be performed to establish whether or not there is any effect. Interestingly, Cxcl8-Cxcr2 signalling has recently been shown to play an important role in neutrophil recruitment and mobilisation from the CHT in response to infection (Deng et al., 2013). This may indicate that Cxcr4 and Cxcr2 signal in an antagonistic manner to regulate neutrophil retention in zebrafish, as has been reported in other systems (Eash et al., 2010).

In contrast to my hypothesis that tanshinone IIA might reduce an Sdf-1 gradient at the wound, preliminary data obtained by WISH suggested that there were actually higher levels of *sdf-1a* expression at the site of injury in the presence of tanshinone IIA. This assay needs to be repeated, with the appropriate sense probe control, in order to confirm the observation. It would be useful to fully characterise the gradient of *sdf-1a* expression during both the recruitment and resolution phases of inflammation. Some evidence suggests that neutrophils can be repelled by high levels of chemokines, including IL-8 (Tharp et al., 2006). It could be hypothesised that tanshinone IIA increases the level of Sdf-1a and this acts as a chemorepellent to drive neutrophils away from the wound, although this would not correlate with the mathematical modelling data. Interestingly, high levels of SDF-1 have been shown to repel T cells (Poznansky et al., 2000). WISH could also be performed to investigate the expression of *cxcr4b* in injured zebrafish larvae, although the technique may not be sensitive enough to detect changes in its expression specifically on neutrophils.

Preliminary *in vitro* experiments performed on freshly isolated human neutrophils revealed that tanshinone IIA might prevent internalisation of CXCR4 in response to SDF-1α stimulation. However, care needs to be taken when interpreting these data, as CXCR4 is known to be up-regulated in neutrophils when they become apoptotic (Hartl et al., 2008; Rankin, 2010). It is possible that the lack of CXCR4 internalisation observed here is simply due to a higher level of surface expression on tanshinone IIA treated neutrophils compared to the controls, as my data indicate that tanshinone IIA does cause an increase in neutrophil apoptosis. Repeating these experiments in the presence of a caspase inhibitor might help to determine whether the reduced internalisation of CXCR4 is occurring independently of apoptosis.

5.7.7 Conclusions

Tanshinone IIA was identified as a potent pro-resolution compound and its effects have been investigated in both zebrafish and isolated human neutrophils. It promotes neutrophil apoptosis both *in vitro* and *in vivo*, but also drives resolution *in vivo* by enhancing the reverse migration of neutrophils away from a site of inflammation. Currently, no other pharmacological agents have been reported to function via this

mechanism. Importantly, the effects of tanshinone IIA on both neutrophil apoptosis and reverse migration appear to be maintained in the presence of survival factors that are expressed at sites of inflammation. It has been established by dynamic modelling techniques that in zebrafish, reverse migration occurs as a passive rather than active process, to clear neutrophils from the wound. Tanshinone IIA accelerates this, which I hypothesise is due to a reduction in the chemokine gradient sensing ability of the neutrophils. This does not appear to be CXCR1- or CXCR2-dependent.

Although the precise mechanistic activity of tanshinone IIA has not been fully determined, the identification of this compound has provided some insight into the nature of reverse migration in zebrafish, showing that it is possible to pharmacologically manipulate this process to promote inflammation resolution. Tanshinone IIA may provide a useful tool for further dissection of the molecular pathways involved. The preliminary data suggest that tanshinone IIA may disrupt CXCR4-SDF-1 signalling, although further experimentation is needed to fully establish whether or not this plays a role in neutrophil retention at a site of inflammation in zebrafish.

Chapter 6: Final Discussion and Future Work

The resolution of inflammation is an active and regulated process, which is essential for maintaining normal homeostasis. A prerequisite for successful inflammation resolution is the clearance of extravasated neutrophils, key inflammatory granulocytes that are equipped with an array of anti-microbial mediators to eliminate infectious agents at sites of inflammation. Neutrophils are highly specialised for host defence, but their powerful effector functions are not specific to invading pathogens and have the potential to cause extensive damage to host cells. If the mechanisms governing inflammation resolution are not precisely regulated, persistent neutrophilic inflammation may ensue and this can lead to extensive host tissue damage, which contributes to the pathogenesis of many chronic inflammatory diseases. Current treatments are unable to resolve existing inflammation. Therefore, there is a demand for novel therapies that specifically target neutrophil clearance mechanisms to drive inflammation resolution. High-throughput screening of diverse compound collections is an approach towards the discovery of new lead compounds that might provide therapeutic benefit. The zebrafish model is ideal for research such as this, filling a niche between *in vitro* cell based studies and mammalian disease models, which would be inappropriate for the testing of large numbers of compounds. It is comparatively simple to perform phenotypic-based assays on zebrafish larvae, enabling the rapid, straightforward and unbiased identification of potential lead compounds that induce the desired effect.

6.1 An *in vivo* screen for accelerators of inflammation resolution

Using a zebrafish model of acute resolving inflammation, I designed a screening assay with the aim to identify compounds that could drive the resolution of inflammation. This approach was based on the output of a preliminary screen, which illustrated the potential of this model to discover novel candidate compounds for inflammatory disease therapy (Loynes et al., 2009). To date, one other inflammation based zebrafish drug screen has been published, the chemically-induced inflammation assay, in which copper sulphate is used to cause oxidative stress and selective damage to sensory hair cells within neuromasts of the lateral line (d'Alençon et al., 2010). Although this model has provided some promising results in terms of the identification of anti-

inflammatory compounds that may inhibit neutrophil recruitment, there remained the need to perform a more focused drug screen in order to identify agents that acted predominantly on the resolution phase of inflammation.

The drug screen performed here was optimised according to multiple experimental variables, including the method of embryo generation, age of larvae, temperature of incubation and time point of final screening for positive hit identification. The assay was partially automated using the Phenosight High-Content plate reader, which enabled rapid data collection and analysis by manual scoring of the images generated. The screening assay may have benefited from the implementation of automated analysis software to validate the results. However, the test screen performed using the Merlion compound collection indicated that the manual scoring technique was sufficient to identify positive hits, based on comparison with a trial version of automated analysis software. Screening of the Spectrum Collection identified 21 compounds that reproducibly promoted inflammation resolution *in vivo* and all of those that were selected for further testing significantly reduced neutrophil numbers in the resolution assay, supporting the robustness of the screening assay to identify drivers of inflammation resolution. The functionality of the screen was illustrated further by cluster analysis of the hit compounds with a collection of known anti-inflammatory compounds provided by a collaboration with GSK, based on their activity in the secondary assays. This revealed that the majority of the hits formed a distinct cluster separate to almost all of the known compounds, due to their primarily pro-resolution and minor anti-recruitment activity; the desired effects for lead compounds from which to design novel inflammatory disease therapies. Despite their effectiveness in the zebrafish model however, it must be appreciated that this does not necessarily directly translate to effectiveness in mammalian models and human disease, in which different mechanisms may be important for regulating inflammation resolution. Testing of the most promising compounds in mammalian models of inflammatory disease is required.

6.2 An *in vivo* assay to measure PI-3K activity

From the hierarchical cluster analysis performed in Chapter 3, I was able to hypothesise that the hit compound isopimpinellin acted via an inhibition of PI-3K activity. This prompted the development of an *in vivo* assay to investigate PI-3K in neutrophils during the recruitment phase of inflammation. Using simple imaging and analysis techniques, I designed a method to assess the polarity of migrating neutrophils in lyz/PHAkt larvae, comparable to published techniques (Yoo et al., 2010). Activation of PI-3K triggers translocation of PHAkt-EGFP to the plasma membrane and formation of the leading edge to enable cell polarisation and directional movement, which can be observed *in vivo*. My assay accurately identified a loss of neutrophil polarity induced by the PI-3K inhibitor, LY294002. This effect was replicated in the presence of isopimpinellin, suggesting that the inhibitory activity of this compound on neutrophil recruitment is at least in part due to blockade of PI-3K signalling. Further work would be required to investigate whether isopimpinellin acts specifically on the PI-3K pathway, or whether other key neutrophil recruitment pathways, such as MAPK signalling, are also affected.

6.3 Did the screen identify a common chemical structure that is responsible for driving inflammation resolution?

Chemical structure comparison revealed that a number of the positive hits identified in the screen contained a 1,4-benzopyrone group, commonly referred to as chromone. To investigate the relevance of this, I tested chromone alone in each of the secondary assays, which indicated that this structure might play a role in driving inflammation resolution, without affecting neutrophil recruitment or total neutrophil number. A series of chromone analogues were synthesised and tested, with varying degrees of activity, however due to issues with the instability of these compounds, I was unsure of their reliability and indeed usefulness in terms of drug development. The realisation that a number of chromone-derived drugs are used in clinical practice as mast cell stabilisers prompted further investigation into the activity of these compounds, as their anti-inflammatory is only partially defined (Yazid, Leoni, et al., 2010). Interestingly, I had discovered that isopimpinellin was a potent inducer of neutrophil apoptosis *in vivo* and I hypothesised that this might be a mechanism to explain the

anti-inflammatory potential of the currently available cromones. In support of this hypothesis, there is evidence to suggest that these drugs promote release of Anx-A1 by directly inhibiting PP2A (Yazid, Leoni, et al., 2010), a phosphatase that has been associated with the destabilisation of Mcl-1 and acceleration of neutrophil apoptosis (Derouet et al., 2004).

However, on testing of chromone derivatives including disodium cromoglycate, nedocromil and isopimpinellin on human neutrophils *in vitro*, I found no effect on either constitutive apoptosis or survival induced by GM-CSF or dexamethasone. Surprisingly, in contrast to my original hypothesis, I found that another chromone-derived hit compound, methylquercetin, appeared to promote neutrophil survival. This disputes published evidence that reports an acceleration of neutrophil apoptosis by similar flavones (Lucas et al., 2012). It is highly likely that the addition of different structural groups onto the chromone backbone influences the function of each compound, which may, perhaps in combination with variations in dosing or experimental technique, explain the differences observed.

The research presented here does not provide conclusive evidence that the presence of the chromone group is responsible for the pro-resolution activity of the hits identified. It does however raise some interesting questions relating to the role of Anx-A1 in neutrophil apoptosis. Although I did not observe an increase in neutrophil apoptosis *in vitro* in the presence of the chromone derivatives tested, some of these did appear to promote inflammation resolution *in vivo*. This might suggest that some necessary stimulus or cell type was missing from the *in vitro* system. A potential candidate is the macrophage, in which the release of Anx-A1 has been shown to enhance the phagocytic uptake of apoptotic neutrophils (Scannell et al., 2007). Further studies could be performed in zebrafish following macrophage ablation, to investigate whether the pro-resolution effects of chromone derivatives such as isopimpinellin and disodium cromoglycate are maintained in the absence of this cell type. It might also be interesting to further explore the interaction between these compounds and glucocorticoids, as the results obtained here were inconclusive. This may help to decipher the role of Anx-A1 in driving inflammation resolution.

6.4 Tanshinone IIA as a potent inducer of inflammation resolution

Perhaps the most interesting finding from this project is the identification of a compound that can drive inflammation resolution *in vivo* by increasing the reverse migration of neutrophils away from the wound. There are currently no other published reports to describe the pharmacological acceleration of this process, highlighting the potential impact of this finding for research into the mechanisms governing inflammation resolution. Tanshinone IIA might provide a useful tool to dissect the currently unknown mechanisms involved in mediating neutrophil reverse migration. As this compound has already been tested in multiple mammalian models of disease and has even been used clinically without any reports of adverse effects (Zhou et al., 2005), it represents a good candidate for inflammatory disease therapy.

The pro-resolution effect of tanshinone IIA is two-fold. Not only does this compound promote reverse migration but it also appears to induce neutrophil apoptosis both *in vitro* and *in vivo*. Further work is required to determine the signalling pathway or pathways that are affected. A potential target might be MAPK, as tanshinone IIA has been reported to induce apoptosis in other cell types by the inhibition of this pathway (Jiao & Wen, 2011; Pan & Wang, 2012). One of the most important properties of tanshinone IIA is its ability to induce neutrophil apoptosis even in the presence of pro-survival stimuli, including GM-CSF and hypoxia. This is key for a potential therapeutic, which must be able to predominate persistent host survival signalling at sites of chronic inflammation. The potential of tanshinone IIA to override pro-inflammatory stimuli is also reflected *in vivo*. The reverse migration of neutrophils away from the site of injury is not only increased by tanshinone IIA during resolution of the acute inflammatory response, but this effect is also maintained in the presence of hypoxia signalling, induced by the injection of dominant-active *hif-1αb* RNA. Further work could be performed to confirm this effect by testing tanshinone IIA in a stable transgenic line expressing dominant-active Hif-1αb specifically in neutrophils.

Whether tanshinone IIA can affect other immune cells, such as macrophages, has not yet been investigated *in vivo*. There are reports that describe inhibition of MAPK signalling in LPS-treated RAW264.7 cells by tanshinone IIA (Jeon et al., 2008; Tang et

al., 2011), thus it is possible there may be an indirect effect on neutrophils due to a change in macrophage function. Performing experiments to assess macrophage numbers during inflammation in the presence of tanshinone IIA might be informative, along with investigating whether the effect on reverse migration and apoptosis is maintained following macrophage ablation.

6.5 What has this research revealed about neutrophil reverse migration?

The work presented here has provided some insight into neutrophil reverse migration and has identified some potentially interesting candidates that may be involved in the regulation of this process. In accordance with published data (Holmes, Anderson, et al., 2012), the results from the modelling analyses provide further evidence that neutrophil reverse migration is a passive redistribution-like process, in contrast to an active fugetaxis. Tanshinone IIA appeared to enhance this activity, without the introduction of an active, or 'drift' component. As there was no change in neutrophil speed compared to the controls, yet there was an increase in diffusivity, this strongly indicated that tanshinone IIA alters the directional preference of neutrophils. This may be related to a change in neutrophil chemokine receptor expression or function, or changes in the extracellular chemokine gradients. The latter seems unlikely given that neutrophil-specific overexpression of dominant-active *hif-1αb* delays reverse migration (Elks et al., 2011), however, it is possible that something much more complex is occurring. Data obtained from the preliminary WISH experiments suggested that there might be an up-regulation in Sdf-1a expression at the site of injury in zebrafish larvae treated with tanshinone IIA. Further work is required to determine the role of the Cxcr4-Sdf-1 signalling axis in zebrafish during inflammation and the potential impact of tanshinone IIA on this pathway.

The investigation into the effect of tanshinone IIA on CXCR1 and CXCR2 expression both *in vitro* and *in vivo* provided reasonably conclusive evidence that a down-regulation in surface expression of these receptors does not contribute to the enhanced neutrophil reverse migration observed in the presence of tanshinone IIA. However, it cannot be ruled out that this compound may affect intracellular signalling, downstream of ligand-receptor binding. There is evidence to suggest that Cxcl8 may

play a role in neutrophil retention at sites of inflammation (Sarris et al., 2012), thus it is logical that Cxcr1 or Cxcr2 may be important for transducing this signal, however this requires further investigation.

6.6 What are the consequences of neutrophil reverse migration?

The clearance of neutrophils from sites of inflammation is required for successful inflammation resolution. In zebrafish, neutrophil removal has been shown to contribute to rapid and efficient tissue repair (Li et al., 2012; de Oliveira et al., 2013). It seems likely that reverse migration is a pro-resolution process and there is only limited evidence to suggest that reverse migrated neutrophils may contribute to the dissemination of inflammation in a pathophysiological manner (Buckley et al., 2006; Woodfin et al., 2011). Research carried out in the Renshaw lab and by others has indicated that zebrafish neutrophils can survive for prolonged periods, with a potential half-life of up to 5 days, and they do not appear to localise to specific tissues, even after leaving a wound (Yoo and Huttenlocher, 2011; Dixon et al., 2012). Further work is required in order to establish the role of reverse migrated neutrophils and their function and viability. An interesting way to investigate this would be to adapt the reverse migration assay by inducing a second injury in zebrafish larvae after peak inflammation, perhaps at a site anterior to the tail fin wound, to determine whether neutrophils were able to reverse migrate away from the original wound and detect and respond to the chemokine gradient at the second wound. It would be particularly useful to investigate the effect of tanshinone IIA in this assay, which might provide further clues towards its mechanism of action.

In the reverse migration data presented here, the majority of neutrophils appeared to preferentially migrate away from the wound through the tissues rather than undergoing transendothelial reverse migration back into the blood vessels. This is in contrast to mammalian systems, in which neutrophils are reported to return to the vasculature (Woodfin et al., 2011). Thus, it is possible that there are limitations in the use of zebrafish for investigating neutrophil reverse migration, as the molecular mechanisms regulating this process in zebrafish may be different to those in mammals. Likewise, there may be a distinct set of signalling pathways that are active in zebrafish

at the larval stage but the function of which are not maintained in adulthood. The effect of tanshinone IIA must be explored in mammalian systems to determine whether its ability to accelerate neutrophil reverse migration is zebrafish specific, or if this mechanism may have translatable therapeutic potential to promote inflammation resolution in mammalian inflammatory disease models. This research is currently underway.

Despite being originally considered as a cell of the innate immune system, it is becoming increasing evident that neutrophils may interact with cells of the adaptive immune system and have the capacity to alter their functional response. For example, neutrophils can release a host of DAMPs during inflammation or infection, which can induce the proliferation and maturation of T cells and dendritic cells (Kumar et al., 2010). Neutrophils have also been reported to act as antigen-presenting cells and migrate to lymph nodes following infection (Chtanova et al., 2008), thus it is possible that reverse migration may in fact be a pro-inflammatory mechanism that functions to activate adaptive immune responses. Investigating the effect of tanshinone IIA on neutrophil migration in mammalian models of inflammation will help to elucidate the mechanisms and consequences of its activity.

6.7 Thesis summary

To summarise, in this thesis I have described the design, optimisation and implementation of an inflammation drug screen performed in transgenic zebrafish larvae. I aimed to screen a diverse compound collection with the hypothesis that novel compounds with the ability to drive inflammation resolution would be identified. My approach successfully detected 21 active compounds, acting via distinct anti-inflammatory and pro-resolution mechanisms, and the techniques that I developed have since been employed to screen large numbers of compounds within our laboratory, generating some promising results. Hierarchical cluster analysis of these compounds with known anti-inflammatories enabled me to accurately predict that the hit compound isopimpinellin inhibits PI-3K activity during neutrophil recruitment. I also identified a group of compounds with specific effects on the resolution phase of

inflammation. The most interesting of these, tanshinone IIA, induces apoptosis of neutrophils both *in vivo* and *in vitro*, even in the presence of survival signals. It also drives inflammation resolution *in vivo* by promoting neutrophil clearance from the wound by reverse migration and this effect is maintained in the presence of survival signalling.

Much work is required to determine the mechanisms governing neutrophil reverse migration and its importance in driving the resolution of inflammation. The zebrafish is an ideal model for the study of this complex process and tanshinone IIA may be a particular useful tool to dissect the mechanisms involved. Further investigation is required in order to establish the precise chemotactic signals, inflammatory mediators and signalling proteins that are important throughout the different phases of the inflammatory response. Expanding our knowledge in this area of research will enable the appropriate targeting of key components in neutrophils and advance the development of more effective treatments for chronic inflammatory diseases.

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Appendix 1

Transgenic line	Abbreviation	Application	Reference
Tg(mpx:GFP)i114	*mpx*:GFP	Drug screening Recruitment, resolution and total neutrophil number assays TUNEL assays WISH	(Renshaw et al., 2006)
Tg(lyz:PHAkt-EGFP)	*lyz*/PHAkt	PI-3K assays	(Wang, Li, Robertson et al., in submission)
Tg(mpx:Gal4;UAS:Kaede)i222	*mpx*/Kaede	Reverse migration assays	(Robertson et al., manuscript in preparation)

Appendix 2

1. E3 medium

To make 1 L of 60x E3 stock:
5 mM NaCl
0.17 mM KCl
0.33 mM $CaCl_2$
0.33 mM $MgSO_4$
dH_2O up to 1 L

To make 1 L of 1x E3 stock:
16.6 ml 60x E3 stock
1% methylene blue
dH_2O up to 1 L

In all cases, the 60x E3 stock was autoclaved prior to dilution and use in *in vivo* inflammation assays, to avoid contamination.

2. 20x Saline sodium citrate buffer (SSC)

To make 200 ml:
35.06 g NaCl
17.64 g NaCitrate
dH_2O up to 200 ml
pH7 using citric acid

3. Hybridisation wash buffer (HybWash)

To make 50 ml:
25 ml 50% formamide
12.5 ml 20x SSC
460 µl citric acid
50 µl Tween 20

4. Pre-hybridisation buffer (PreHyb)

As HybWash, with:
50 µg/ml heparin
500 µg/ml tRNA

5. Probe-hybridisation buffer (ProbeHyb)

As PreHyb, with:
1:200 dilution of probe in formamide

6. Blocking solution

PBT, with:
2% sheep serum
2 mg/ml BSA

7. Staining wash

To make 50 ml: 5 ml 100 mM trisHCl (pH 9.5)
2.5 ml 50 mM $MgCl_2$
5 ml 100 mM NaCl
dH_2O up to 50 ml

8. Staining solution

As staining wash, with: 100 mg/ml NBT
50 mg/ml BCIP

9. Fluorescence-activated cell sorting (FACS) buffer 1

To make 50 ml: 0.5 ml 1 M HEPES
0.125 g BSA
PBS up to 50 ml

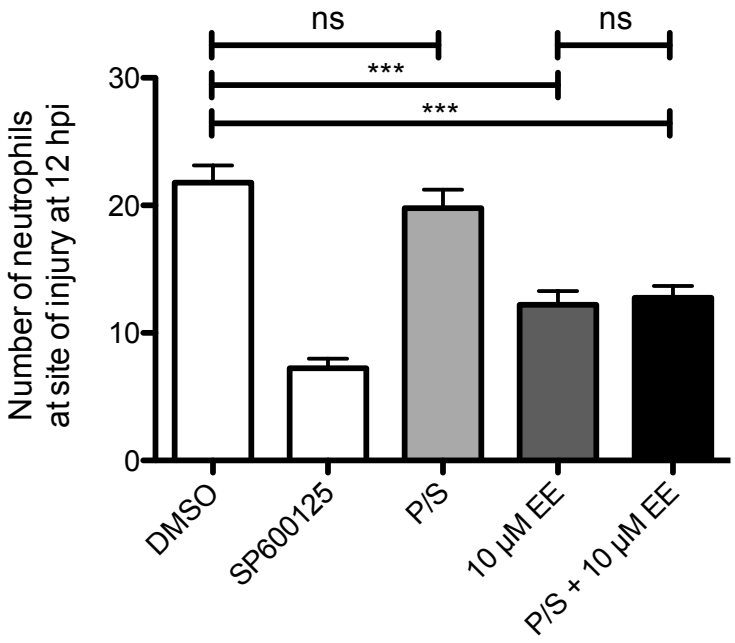
FACS buffer 1 was kept at 4°C at all times.

10. Fluorescence-activated cell sorting (FACS) buffer 2

To make 50 ml: 0.5 ml 1 M HEPES
0.125 g BSA
HBSS up to 50 ml

FACS buffer 2 was kept at 4°C at all times.

Appendix 3



Appendix 2: Antibiotic activity alone is not sufficient to drive inflammation resolution *in vivo*.

At 3 dpf, *mpx*:GFP larvae were injured and good responders were treated at 6 hpi with either the DMSO vehicle control, 30 µM SP600125, penicillin streptomycin at a final concentration of 50 U/ml penicillin and 50 µg/ml streptomycin (P/S), 10 µM erythromycin estolate (EE) or P/S and EE in combination. Neutrophil numbers at the site of injury were counted at 12 hpi. There was no difference in neutrophil number in the presence of P/S compared to the DMSO vehicle control but there were significantly fewer neutrophils at the site of injury in larvae treated with either EE or EE and P/S combined (One-way ANOVA with Bonferroni's multiple comparison post-test; where *** $P < 0.001$; $n = 12$, performed as 2 independent experiments). These results indicated that antibiotic activity alone was not sufficient to drive inflammation resolution and that there was no additive anti-bacterial effect responsible for the reduction in neutrophil number when larvae were exposed to both erythromycin estolate and penicillin-streptomycin in combination.