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Characterization of Chemical Probes for Therapeutic Intervention of Innate Immune Signaling

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Characterization of Chemical Probes for Therapeutic
Intervention of Innate Immune Signaling

by

Christina (Smith) Boville

B.A., University of Colorado, 2009

A thesis submitted to the
Faculty of the Graduate School of the
University of Colorado in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
Department of Chemistry and Biochemistry
2017
This thesis entitled:
Characterization of Chemical Probes for Therapeutic Intervention of Innate Immune Signaling
written by Christina (Smith) Boville
has been approved for the Department of Chemistry and Biochemistry

Prof. Hang Yin

Dr. Johannes Rudolph

The final copy of this thesis has been examined by the signatories, and we find that both the content and the form meet acceptable presentation standards of scholarly work in the above mentioned discipline.
The innate immune system protects us from incessant attacks by microorganisms. However, immunity is a double-edged sword, and inflammation must be carefully regulated. Chemical biology offers a means to maintain healthy homeostasis by influencing the immune response. We have used small molecule and peptide chemistries to exploit Toll-like receptor, caspase, and antimicrobial peptide signaling for therapeutic purposes. These compounds may find applications treating sepsis and autoimmune disease, and can influence the design of next-generation antibiotics and vaccines.

Toll-like receptors are pattern recognition receptors that sense bacteria and viruses, and then induce inflammation to stave off infection. We have designed saccharin-derived small molecules capable of reducing inflammation by inhibiting JAK/STAT1 signaling. We have also explored Toll-like receptor specificity by designing TLR8-specific imidazole-derivatives that are promising immune-responsive vaccine adjuvants.

Caspases are proteases that control inflammation and cell death. Much is still unknown about the role of caspases as innate immune receptors, particularly lipopolysaccharide-sensing caspase-4. We have performed two small molecule screens to identify caspase-4 inhibitors for use as both therapeutics and signaling probes. From this, we have discovered a new role for non-steroidal anti-inflammatory drugs as multi-caspase inhibitors, which may shape patient applications of these essential drugs. We have also identified novel caspase-4 specific small molecules for sepsis treatment.

Antimicrobial peptides are small, amphipathic peptides able to both disrupt bacterial membranes and influence inflammation. We have designed a series of peptidomimetics able to mimic the properties of host-defense peptides. These peptidomimetics are active against a wide array of Gram-positive and Gram-negative pathogens, and reduce the inflammatory response. In an era of prevalent antibiotic resistance, peptidomimetics offer a path for the design of new antibiotics.
Dedication

To my loving husband, friends, and family,
without whom this thesis would have been completed years earlier.
Acknowledgements

First, I would like to thank Professor Hubert Yin and my labmates for their daily support, motivation, and knowledge.

Next, I would like to acknowledge my colleagues and mentors at the University of Colorado. Too many people to mention have provided me with friendship and scientific help over the past five years. I am particularly thankful to Theresa Nahreini, who offered a friendly ear and made all of my experiments possible. I would like to thank my thesis committee: Thomas Cech, Corrie Detweiler, Johannes Rudolph, and Dylan Taatjes for their insight and encouragement. It really takes a village to raise a Ph.D. biochemist.

Last but not least, I would like to thank my friends and family who kept me fed, sane, and happy during this arduous process. My family ensured I had the confidence to dream big, and the fortitude to see my dreams through. Finally, a special thanks to my husband, Brad, who has unconditionally loved me and encouraged my goals.
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Chapter 1

Chemical Control of the Innate Immune System

Portions of this chapter have been published as:


For both of these articles, C. Smith and X. Wang collaborated on the content, writing, editing, and figure preparation.

1.1 The Innate Immune System

The innate immune system serves as our first line of defense against an incessant siege of infectious microorganisms. This defense includes factors that target infectious microorganisms, through either direct killing or by making the host environment inhospitable. It also includes germline-encoded receptors that are able to recognize broad threats such as bacterial membrane components or viral RNAs. Upon sensing a hazard, the innate immune system induces inflammation to stave off infection. However, this protection is a double-edged sword. While an insufficient inflammatory response will permit infection to spread unchecked, over-inflammation can harm the host cells. As such, it is important to maintain carefully controlled levels of inflammation to ensure a healthy immune response. Chemical biology offers an enticing approach for regulating inflammation by controlling individual components of the innate immune system, such as Toll-
like receptors, caspases, and antimicrobial peptides. In the next section I will provide a brief introduction to these three different components of the innate immune system. I will also explore the design and characterization of chemical probes that regulate innate immune signaling. These compounds may have applications as next-generation therapeutics or signaling probes to improve our understanding of these crucial cellular responses.

1.1.1 Toll-Like Receptors (TLRs)

Toll-like receptors (TLRs) are evolutionarily conserved type I transmembrane proteins that detect pathogen associated molecular patterns (PAMPs) and danger associated molecular patterns (DAMPs) [203]. The ability to sense these molecular patterns make TLRs key regulators of both innate and adaptive immune responses. TLRs are comprised of an extracellular domain containing leucine-rich repeats (LxxLxLxxN, LRRs), a transmembrane domain, and an intracellular Toll/interleukin-1 receptor (TIR) domain. All TLR ectodomains share a common horseshoe structure, but different amino acid composition allows for the recognition of a diverse set of PAMPs and DAMPs (Table 1.1). TLRs are expressed on both the plasma (TLR 1/2, 3, 4, 5, 2/6) and endosomal membranes (TLR 3, 7, 8, 9). This variety in localization allows TLRs to protect the host against threats present in the extracellular environment as well as those internalized. Upon ligand recognition, TLR homo- or heterodimers trigger signaling and activate the intracellular TIR domain. TIR domain recruitment of MyD88 and other adaptor proteins activates signal transduction cascades, which culminate in the production of pro-inflammatory cytokines and chemokines (Figure 1.1). Native ligands have been identified for TLR1-9 as well as TLR13. TLRs are named Toll-like receptors due to their structural and functional similarities to the protein encoded by the toll gene in Drosophila [9].

1.1.1.1 Applications of TLR-targeting Small Molecules

Human and animal genetic studies have shown that the dysregulation of innate immune TLR signaling contributes to the development and progression of various diseases including sepsis,
autoimmune disease, and neuropathic pain (Table 1.2), a topic that has been extensively reviewed in the literature [25, 143]. Due to their significant biomedical relevance, TLRs have emerged as important drug targets [38]. Currently, there is great interest in the development of TLR small molecule modulators (Table 1.3) for interrogating TLR signaling and treating diseases caused by TLR signaling malfunctions. The development of TLR-targeting small molecules is explored in Chapter 3 and Chapter 6.

Table 1.1: Pathogen associated molecule patterns (PAMPs) and danger associated molecule patterns (DAMPs) recognized by human and murine TLRs. [226]

<table>
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<tr>
<th>TLR</th>
<th>Localization</th>
<th>PAMPs/DAMPs</th>
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<tbody>
<tr>
<td>TLR1/TLR2</td>
<td>Plasma membrane</td>
<td>Triacylated lipoprotein, peptidoglycan, saturated fatty acids</td>
</tr>
<tr>
<td>TLR2</td>
<td>Plasma membrane</td>
<td>Peptidoglycan, polysaccharide krestin, -defensin 3, phospholipomannan,</td>
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<td></td>
<td></td>
<td>serum amyloid A, haemagglutinin, biglycan, perins, lipoarabinomannan,</td>
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<tr>
<td></td>
<td></td>
<td>Gp96, HMGB1, glucuronoxylomannan, anti-phospholipid antibodies,</td>
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<td></td>
<td></td>
<td>surfactant proteins A and D, eosinophil-derived neurotoxin</td>
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<tr>
<td>TLR3</td>
<td>Endosome membrane</td>
<td>dsRNA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>LPS, heme, saturated fatty acids, VSV glycoprotein G, RSV fusion protein,</td>
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<td></td>
<td></td>
<td>resistin, biglycan, lactoferrin, MMTV envelope protein, mannan,</td>
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<td>glucuronoxylomannan, Gp96, nickel, S100, glycosylinositolphospholipids,</td>
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<td>sulfate fragment, HMGB1, neutrophil elastase, wheat amylase trypsin</td>
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<td>Plasma membrane</td>
<td>OxLDL, amyloid- fibril</td>
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<td>TLR5</td>
<td>Plasma membrane</td>
<td>flagellin</td>
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<td>TLR2/TLR6</td>
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<td>Diacylated lipopeptides, lipoteichoic acid, zymosan, saturated fatty acids</td>
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<td>TLR7</td>
<td>Endosome membrane</td>
<td>ssRNA, Anti-phospholipids antibodies, RNA associated auto-antigens</td>
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<td>TLR13b</td>
<td>Endosome membrane</td>
<td>23s rRNA</td>
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aMice lack TLR10.

bTLRs 11-13 are represented in humans by a pseudogene and are not functional.
Figure 1.1: TLR signaling pathway. TLRs expressed on both the plasma and endosomal membranes recognize a PAMP or DAMP and induce an inflammatory signaling cascade. Through TIR domain interactions with either TRIF or the Myddosome, signaling culminates in the recruitment of transcription factors (IRF3, NF-κB, AP-1) and the release of interferons and cytokines.
Table 1.2: TLR family proteins as targets in drug development. [226]

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<thead>
<tr>
<th>Disease</th>
<th>TLR involved</th>
<th>Therapeutic approach</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sepsis</td>
<td>TLR2/4/9</td>
<td>Antagonist</td>
</tr>
<tr>
<td>Asthma</td>
<td>TLR7/8/9</td>
<td>Agonist</td>
</tr>
<tr>
<td>Acute/chronic inflammation</td>
<td>TLR2/4</td>
<td>Antagonist</td>
</tr>
<tr>
<td>Chronic obstructive pulmonary disease</td>
<td>TLR2/4</td>
<td>Antagonist</td>
</tr>
<tr>
<td>Cardiovascular disease</td>
<td>TLR2/4</td>
<td>Antagonist</td>
</tr>
<tr>
<td>Neuropathic pain, chronic pain</td>
<td>TLR4</td>
<td>Antagonist</td>
</tr>
<tr>
<td>Vaccine adjuvants</td>
<td>TLR3-5, 7-9</td>
<td>Agonist</td>
</tr>
<tr>
<td>Autoimmune diseases (including systemic lupus erythematosus, rheumatoid arthritis, systemic sclerosis, Sjogren's syndrome)</td>
<td>TLR1-9</td>
<td>Antagonist</td>
</tr>
<tr>
<td>Cancer (including colon cancer, gastric cancer, breast cancer, melanoma, hepatocellular carcinoma, lung cancer, glioma, prostate cancer, ovarian cancer, cervical squamous cell carcinomas, chronic lymphocytic leukemia)</td>
<td>TLR1-9</td>
<td>Agonist</td>
</tr>
</tbody>
</table>

Table 1.3: Small molecule TLR modulators. While TLR-targeting small molecules are an ever expanding field, this table includes examples of recent TLR agonists and antagonists. [226]

<table>
<thead>
<tr>
<th>TLR</th>
<th>Small molecule agonist</th>
<th>Small molecule antagonist</th>
</tr>
</thead>
<tbody>
<tr>
<td>TLR1/TLR2</td>
<td>Pam3CSK4, Poly (I:C), Poly (A:U), IPH-3102, Rintatolimod</td>
<td>synthetic phospholipids</td>
</tr>
<tr>
<td>TLR3</td>
<td>Lipid A mimetic (D1), Eritoran, E5531, xanthohumol, JTT-705, auramofin, sulforaphane, cinnamaldehyde, taxanes, 6-shogao, isoleiquiritigenin, OSL07, glycyrrhizin, isoleiquiritigenin, caffeic acid phenethyl ester, β-lactamase fragments, (+)-Naloxone.(+)-Naltrexone, TAK-242, NCI126224, Paclitaxel, Heme, Curcumin, Chitohexaose</td>
<td>CU-CPT4</td>
</tr>
<tr>
<td>TLR4</td>
<td>LPS, Lipid A, CRX-547, CRX-527, E6020, morphine-3-glucuronide, saturated fatty acids, monophosphoryl lipid A (MPLA), morphine</td>
<td>Lipid A mimetic (D1), Eritoran, E5531, xanthohumol, JTT-705, auramofin, sulforaphane, cinnamaldehyde, taxanes, 6-shogao, isoleiquiritigenin, OSL07, glycyrrhizin, isoleiquiritigenin, caffeic acid phenethyl ester, β-lactamase fragments, (+)-Naloxone.(+)-Naltrexone, TAK-242, NCI126224, Paclitaxel, Heme, Curcumin, Chitohexaose</td>
</tr>
<tr>
<td>TLR5</td>
<td>Flagellin-derived peptides</td>
<td>None</td>
</tr>
<tr>
<td>TLR2/TLR6</td>
<td>Pam3CSK4, FSL1, MALP-2</td>
<td>None</td>
</tr>
<tr>
<td>TLR7</td>
<td>Isatoribine, Loxoribine, Imiquimod, gardiquimod, AZD8848, IMO-8400, ANA773, IMO-3100, SM360320, 82A</td>
<td>2’-O-methyl-modified RNAs, IRS-954 (DV-1079)</td>
</tr>
<tr>
<td>TLR8</td>
<td>VTX-1463, VTX-2337, IMO-8400, 2,3-Diamino-furo[2,3-c]pyridine</td>
<td>VTX-763</td>
</tr>
<tr>
<td>TLR9</td>
<td>IMO-8400, IMO-3100, SAR-21609, AZD1419, SD-101, IMO-2055, IMO-2125, QAX-935, AVE0675, DIMS0150, MGN-1703, MGN-1706, ISS1018, Agatolimod</td>
<td>IRS-954 (DV-1079)</td>
</tr>
<tr>
<td>TLR13</td>
<td>ACGGAAAGACCCC</td>
<td>None</td>
</tr>
</tbody>
</table>
1.1.2 Cysteine Aspartic Proteases (Caspases)

Caspases are cysteine-aspartic acid proteases that play an essential role in inflammation, cell death, and disease. This polyspecific protease family recognizes a tetrapeptide motif and cleaves substrates following an aspartic acid (Table 1.4). Caspases are expressed as inactive zymogens consisting of a large subunit, a small subunit, and an N-terminal prodomain. Prodomains affect caspase recruitment by forming interactions with recruitment scaffolds. Upon activation, caspases form an active heterotetramer comprised of two large and two small subunits.

While largely functioning downstream of PAMP or DAMP recognition, caspases are invaluable in regulating inflammation and cell death. There are eleven human caspases, which can be subdivided by substrate motif or function. Initiator caspases (caspase-2, -8, -9, -10) are recruited to multi-protein complexes such as inflammasomes and apoptosomes. These signaling hubs form in response to receptor recognition of PAMPs or DAMPs, and culminate in caspase activation. This family influences whether cell death occurs through the extrinsic (receptor-mediated) or intrinsic (damage-mediated) pathway. Executioner caspases (caspase-3, -6, -7, -14) are proteolytically cleaved by initiator caspases to propagate the signaling cascade. The aptly named inflammatory caspases (caspase-1, -4, -5) are involved in pyroptosis, a form of inflammatory cell death culminating in cytokine release. Recently, a role for caspases-4 and -5 (human) and -11 (mice) as innate immune receptors has emerged (Figure 2.6). The unique properties of these novel inflammatory caspases will be discussed in greater detail below.

1.1.2.1 Caspase-4/5/11 is a Novel Innate Immune Receptor

LPS is a major structural element of the outer membrane of Gram-negative bacteria that triggers an innate immune response through TLR4. It has recently come to light that in addition to its canonical TLR4-mediated responses, intracellular LPS activates pro-inflammatory caspase-11 and induces an immune response independent of TLR4 [68, 103]. This non-canonical response allows caspase-11 to serve as an innate immune sensor for cytosolic bacteria such as *Burkholderia*
Table 1.4: Caspase substrates and functions. Caspases are cytosolic proteases that recognize tetrapeptides strictly terminating in aspartic acid. Caspases can be subdivided based on their substrate recognition motif and their biological function. [40, 140, 212, 222]

<table>
<thead>
<tr>
<th>Caspase</th>
<th>Optimal Substrate</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caspase-1</td>
<td>WEHD</td>
<td>Inflammation</td>
</tr>
<tr>
<td>Caspase-2</td>
<td>DEHD</td>
<td>Stress-induced apoptosis</td>
</tr>
<tr>
<td>Caspase-3</td>
<td>DEVD</td>
<td>Apoptosis and Differentiation</td>
</tr>
<tr>
<td>Caspase-4</td>
<td>(W/L)EHD</td>
<td>Inflammation</td>
</tr>
<tr>
<td>Caspase-5</td>
<td>(W/L)EHD</td>
<td>Inflammation</td>
</tr>
<tr>
<td>Caspase-6</td>
<td>VEHD</td>
<td>Apoptosis and Differentiation</td>
</tr>
<tr>
<td>Caspase-7</td>
<td>DEVD</td>
<td>Apoptosis and Differentiation</td>
</tr>
<tr>
<td>Caspase-8</td>
<td>LETD</td>
<td>Extrinsic apoptosis and differen-</td>
</tr>
<tr>
<td>Caspase-9</td>
<td>LEHD</td>
<td>Intrinsic apoptosis and differen-</td>
</tr>
<tr>
<td>Caspase-10</td>
<td>LEXD</td>
<td>Extrinsic apoptosis and differen-</td>
</tr>
<tr>
<td>Caspase-11 (murine)</td>
<td>(W/L)EHD</td>
<td>Inflammation</td>
</tr>
<tr>
<td>Caspase-14</td>
<td>WEHD</td>
<td>Cornification</td>
</tr>
</tbody>
</table>

*thailandensis*. Hypotheses regarding the mechanism by which LPS induces caspase-11 activation were based on our understanding of the activation of other members of the caspase family, particularly caspase-1. Briefly, an inflammasome scaffold recruits and activates caspase-1 through conserved caspase activation recruitment domain (CARD) interactions between the adaptor protein apoptosis-associated speck-like protein containing a CARD (ASC) and caspase-1 [28]. Based on this existing paradigm, the mysterious non-canonical intracellular LPS receptor was suspected to contain a CARD domain. In a recent report in Nature, Shi *et al.* successfully identified the intracellular receptor of LPS [191].

Focusing on CARD-containing proteins, Shi *et al.* screened 18 proteins for caspase-11 activation; however, none was able to induce pyroptosis. Instead, the authors observed that during caspase-4/5/11 preparation, auto-homooligomerization occurred among recombinant caspases purified from bacterial preparations, but not among caspases purified from insect cell preparations. Thus, it was hypothesized that caspase-4/5/11 may be oligomerized by a bacterial component, such as LPS. Transfection of LPS into human monocytes resulted in a pyroptotic response, which was also observed in other cell types (HeLa and HL-60). Pyroptosis required caspase-4, but was independent of caspase-1 and the adaptor protein ASC, suggesting an activation mechanism distinct to previous
models of caspase activation, as it does not require canonical inflammasome components. Direct binding of LPS to caspase was shown using surface plasmon resonance (SPR), thus establishing caspase-4/5/11 as intracellular LPS receptors. This was further confirmed by mutagenesis studies, which revealed that several lysine residues in the CARD domain mediate LPS binding, conjecturing a critical charge interaction. These results not only expose LPS as the activator of caspase-4/5/11, but also widen LPS sensing to non-immune cells, with implications to our understanding of the pathology of sepsis.

1.1.2.2 A Unifying Hypothesis for Caspase Activation Through Aggregation

Most intriguingly, LPS binding-induced oligomerization of caspases is a prerequisite for activation. These results are consistent with the importance of polymeric assembly in other inflammasomes [28, 131]. Thus, self-assembly and aggregation may be a unifying mechanism for caspase activation and signaling propagation. When adaptor proteins or caspase-activating molecules (e.g., LPS) aggregate, caspases recruited to the complex are placed in close proximity. Under these conditions, when a single caspase molecule becomes activated, a self-propagating signaling cascade may result from the induced proximity, allowing robust signal amplification and rapid signal transduction (Figure 2.6). This mechanism would explain ASC polymerization-induced caspase-1 activation, small molecule aggregate-induced caspase-3 activation, death-inducing signaling complex (DISC) induced caspase-8 activation, and apoptosome-induced caspase-9 activation [28, 91, 166, 229]. For example, recent studies by the Wells group showed that a small molecule agent, 1541, forms fibrils which bind caspase-3 and allow activation [243].

It is conceivable that LPS can form a similar structure for caspase-4/5/11 localization and oligomerization. LPS is an amphipathic molecule that can self-aggregate into supramolecular complexes with an apparent critical micelle concentration (CMC) of 10-20 mg/mL [178]. In previously reported intracellular LPS shock models, cells are treated with high doses (1-2 mg/mL) of LPS [68, 103]. Nonetheless, the intracellular environment is highly crowded, which means local LPS concentrations might reach the CMC and facilitate association with caspase-4/5/11. Mutagene-
Figure 1.2: A schematic overview of LPS-induced caspase-4/5 activation. LPS (red) is released from Gram-negative bacteria in outer membrane vesicles. These vesicles are endocytosed, delivering LPS to the cytosol. LPS binds to CARD and activates caspase-4/5 (green). After activation, caspase initiates signaling through substrate cleavage. Gasdermin (orange) cleavage releases the N-terminal fragment, which associates with the plasma membrane and forms pores. This disrupts the membrane and kills the cell. Caspase-4/5 may also activate the canonical inflammasome (blue), culminating in caspase-1 (purple) activation. Caspase-1 cleaves cytokines (yellow) to their mature form, inducing inflammation.
sis studies demonstrated the essential role of the positive residues in the CARD domain for LPS binding. Caspase-4/5/11 therefore may bind to the negatively charged polar heads of LPS micelle aggregates via these positively charged residues. In turn, this may result in assembly of caspase-4/5/11 and proximity-induced auto-activation. Indeed, disruption of LPS aggregation by the detergents Triton-100 and Tween-20 prevents caspase-4/5/11 activation, consistent with an aggregation hypothesis. A recent paper by Vanaja et al. has uncovered that LPS enters the cytosol through endocytosis of bacterial outer membrane vesicles (OMVs) \[217\]. The importance of vesicles to cellular uptake and caspase activation further supports this hypothesis.

1.1.2.3 Applications of Caspase-targeting Small Molecules

LPS binds directly to caspase-4/5/11, presenting a novel activation mechanism for pyroptosis and sepsis. TLR4 activation has a previously established role in septic shock, which is caused by a rampant inflammatory response to LPS during infection. However, advanced drug candidates targeting TLR4 (e.g. TAK242, Eritoran) have met with little success in late stage clinical trials as anti-septics, despite their high potency and specificity \[101, 104, 152, 172\]. It was speculated that these drugs failed because TLR4 inhibition alone is not sufficient. The presence of high doses of LPS during septic shock, some of which may be aberrantly located in the cytoplasm during infection, may also require caspase inhibition as part of a therapeutic approach. Further, the identification of caspase-4 as a functional human homolog of capsase-11 expands murine sepsis models to human applications, and caspase-4 expression in non-macrophage cells enhances the scope of septic inflammation. Applications of caspase inhibitors in septic shock will be further discussed in Chapter 7.

1.1.3 Antimicrobial Peptides (AMPs)

Antibiotic resistance is a major global health threat, and the development of new antibiotics is critical. Antimicrobial peptides (AMPs) are small endogenous peptides that provide an innate defense against a broad spectrum of bacterial pathogens (Figure 1.3). These largely cationic
(+2-9) and amphipathic (≤50% hydrophobic) peptides disrupt negatively charged bacterial membranes through hydrophobic insertion, combating infection [24]. Membrane permeabilization occurs through either a pore-forming or carpet mechanism [23]. The particular type and organization of residues is inconsequential, and AMPs can assume α-helical, β-sheet, or unordered secondary structures. The lack of structural specificity is intriguing, as a wide variety of peptides can serve as antibiotics without the need for strict binding motifs.

In addition to a direct bactericidal mechanism, AMPs can affect inflammation, leading to their synonymous designation as host defense peptides (HDPs). Immune modulation occurs through the production of cytokines and chemokines, or suppression of pro-inflammatory factors. For example, LL-37 is a human cathelicidin that has been shown to improve sepsis survival in mice by inhibiting NF-κB [141]. The precise anti-inflammatory mechanism is unclear, and no TLR specificity is observed [3]. Emerging roles for AMPs include gene expression, leukocyte recruitment, cell differentiation, and wound healing [110].

1.1.3.1 Applications of Antimicrobial Peptides and Peptidomimetics

In an era of antibiotic resistance, AMPs provide a valuable template for the design of next-generation antibiotics. Evolved resistance to AMPs is rare, due to the diversity of peptide sequences and the lack of a receptor-mediated interaction. Resistance occurs through alteration of net surface charges, reduced membrane fluidity, or evolved degradation pathways [23]. Interestingly, these are the same factors that protect mammalian membranes from AMPs. Despite remote occurrences of antibiotic resistance, AMPs are widely studied for their antibiotic potential. However, endogenous AMPs are not effective therapeutics, as they are expressed in low concentrations, are easily degraded by proteases in the body, and are difficult to synthesize.

Recent therapeutic efforts have focused on the design and characterization of peptidomimetics. These peptides have an atypical peptide backbone or side chains, and may include other motifs, such as hydrocarbon chains. Peptidomimetics are designed to mimic the primary characteristics of natural host defense peptides, such as charge and amphiphilicity, without the problems of re-
Figure 1.3: AMPs have antibacterial and immunomodulatory capabilities. AMPs are small amphipathic peptides that participate in host defense. Biological activities include disruption of the bacterial membrane, and modulation of the host immune system.
duced potency due to protease degradation or side effects such as toxicity. As peptidomimetics are chemically tunable (length, ring size, hydrophobicity, lipidation, cationic charge) and replicate the cellular behaviors of AMPs, these are valuable probes for structure-analysis and signaling studies. The design and characterization of peptidomimetics will be further discussed in Chapters 4 and 5.

1.2 Chemical Biology Approaches to Probing Innate Immunity

There are several common strategies used to design chemical probes capable of modulating the innate immune system. Approaches include rational design based on known ligands, substrates, or binding interactions. If rational design is not possible, high-throughput experimentation may be conducted using a variety of computational, in vitro, or cellular approaches. These strategies are discussed below, along with examples demonstrating their efficacy.

1.2.1 Rational Design of Chemical Probes

Rational design may generate targeted probes without the need for labor-intensive screening. As crystal structures become more prevalent with both apo and ligand-bound forms it becomes possible to make informed decisions for small molecule design. Particularly when engineering specificity between highly-related protein families, such as TLRs or caspases, rational design may fine tune protein-drug interactions. Rational design can also use nature’s design as a template. Known ligands and substrates can serve as inspiration for the synthesis of novel chemical probes.

1.2.1.1 Structure-based Rational Design

**TLR rational design** The X-ray crystal structure of TLR4 complexed with the adaptor protein myeloid differentiation factor 2 (MD-2) reveals critical molecular recognition sites at the TLR4/MD-2 interface. Kuroki and coworkers have developed a synthetic peptide that corresponds to the Glu(24)-Lys(47) binding site on TLR4. This peptide effectively suppresses the association of MD-2 and TLR4, attenuating LPS-induced NF-κB activation and IL-8 secretion [144]. Likewise, Yin and coworkers designed a truncated 17-mer peptide (MD-2-I) that maintains TLR4-binding...
affinity of the full-length MD-2 [193]. This rationally designed peptide disrupts the TLR4/MD-2 interactions and blocks the TLR4-mediated inflammatory response both in vitro and in vivo [126].

TLR7 and TLR8 are two closely related proteins that both recognize ssRNA ligands. Recently, the crystal structures for TLR7 and TLR8 bound to their endogenous ligand, as well as to imidazoquinoline small molecules have become available. With this information, it has become possible to design specific small molecules that take advantage of the subtle differences in the ligand binding pockets. This approach is explored further in Chapter 6.

Caspase rational design While HTS has identified the majority of caspase-targeting small molecules, rational design is useful for engineering specificity. Caspases all recognize a tetrapeptide motif terminating in aspartic acid, and have highly conserved active site interactions. With this in mind, the P4 position is the largest determinant of specificity. Sunesis Pharmaceuticals explored a pyridine scaffold with an array of P4 substituents to identify specific caspase-3 inhibitors [6, 35]. Caspase specificity is further explored in Chapters 2 and 7.

AMP rational design AMPs are typically defined as short (12-50 amino acids), cationic (+2-9), and amphipathic (up to 50% hydrophobic) [24]. Further, while many AMPs form α-helical or β-sheet secondary structures, unordered peptides may also have antimicrobial properties. With this in mind, rational design of AMPs and peptidomimetics conforming to these guidelines have been synthesized. This topic will be further explored in Chapters 4 and 5.

1.2.1.2 Ligand and Substrate Derivatives

Toll-like Receptor Ligand Mimetics TLRs recognize evolutionarily conserved PAMPs and DAMPs. Native ligands for all human TLRs except for TLR10 have been identified (Table 1.1), which have provided excellent templates for the development of small molecule modulators. In addition to functioning as targeted therapeutics, these compounds facilitate probing the TLR signaling pathway for further signaling information. Native TLR ligands may be expensive or unstable, limiting day-to-day applications. Small molecules or mimetics circumvent these issues, yet it is important to remember that while these compounds are essential tools, they may not
completely emulate the behaviors of endogenous ligands. In the next paragraphs, I will describe some commonly used and promising TLR mimetics.

**TLR3** recognizes double stranded RNA (dsRNA), providing an important defense against viral infections. Polyinosinic-polycytidylic acid (poly(I:C)) and polyadenylic-polyuridylic acid (poly(A:U)) are synthetic analogues of dsRNA. Due to their specific TLR3 activity, these molecules are widely used as TLR3 agonists in research [74]. Other dsRNA mimetics such as IPH-3102 have been suggested for cancer treatment, since TLR3 is over-expressed in a large subset of cancer patients [17].

Lipopolysaccharides (LPS) are a group of natural ligands for TLR4 present on the outer cell membrane of Gram-negative bacteria. The generic structure of LPS consists of an outer core, an inner core, and a lipid A motif. Lipid A activates TLR4 signaling by binding to the TLR4 accessory protein myeloid differentiation-2 (MD-2). Structural differences in lipid A can affect immune responses, such as changes in the number and length of acyl chains and the functional groups at the bioisosteric position of the 1-phosphate. These types of modifications can alter the activities of lipid A mimetics from canonical agonism to antagonism [201]. For example, novel lipid A mimetics such as IAXO-101 inhibit TLR4 by preventing LPS binding to accessory proteins (e.g. CD14, MD2) [158].

Eritoran (E5564) is a lipid A analogue developed as an investigational drug for the treatment of sepsis [15]. Eritoran prevents septic over-inflammation by competing with LPS for binding to MD-2. However, a Phase III clinical trial of Eritoran in patients with sepsis failed due to the lack of efficacy. Nonetheless, the high-resolution structure of TLR4/MD-2/Eritoran has laid the crucial groundwork for future development of TLR4 inhibitors [104].

Monophosphoryl lipid A (MPLA) is a low-toxicity derivative of LPS with beneficial immune-stimulatory activity [159]. MPLA is an adaptor-inducing, interferon-β (TRIF)-biased TLR4 agonist which lowers toxicity due to a decrease in pro-inflammatory factors [136]. As such, the US Food and Drug Administration (FDA) has approved MPLA for use as a human vaccine adjuvant. Vaccine adjuvants combine antigens with immune activators in order to increase the adaptive immune
response. In fact, TLR agonists generally make excellent vaccine adjuvants because they activate both the innate and adaptive immune responses as well as escalate B and T cell immunity.

TLR2 recognizes lipoteichoic acid and diacylated lipopeptides from Gram-positive bacteria and yeast, in concert with TLR6 as a heterodimer. Pam$_2$CSK$_4$ and MALP-2 are synthetic diacylated lipopeptides and potent TLR2/TLR6 agonists. For Pam$_2$CSK$_4$ agonism, the Cys-Ser dipeptide unit and at least one C16 acyl group with an appropriately oriented ester carbonyl group are essential [4, 234]. While not required for agonist activity, four lysines are included in the structure of Pam$_2$CSK$_4$ to increase solubility.

**Caspase Substrate Mimetics** Caspases recognize a tetrapeptide substrate ending in aspartic acid. Caspases can be subdivided based on their tetrapeptide motif, though one substrate may be recognized by multiple enzymes. These peptide substrates have been extensively studied as both tools and therapeutics. The most prevalent pan-caspase inhibitor N-Benzylxycarbonyl-Val-Ala-Asp(O-Me) fluoromethyl ketone (z-VAD-FMK) capitalizes on promiscuity to irreversibly bind the active site. This inhibitor mimics the substrate, but the fluoromethyl-ketone group prevents proteolysis. A recent review by Poreba et al. further explores the plethora of other peptide inhibitors containing both canonical and non-canonical amino acids [167]. Substrate mimics with fluorescent or luminescent conjugates have also been developed as enzymatic assays [62, 147].

1.2.2 High Throughput Screening for Identifying Chemical Probes

High-throughput screens (HTS) are an excellent method for rapidly identifying chemical probes, however, these approaches are not without challenges. Despite this, in silico, target-based, and phenotypic screening methods have been successfully used to identify small molecules. Here we briefly discuss the applications and limitations of these methods. It should be noted that each method for high-throughput small molecule discovery has its advantages and disadvantages. Combinations of different approaches (e.g. phenotypic screening and structure-based rational design) may provide more desirable outcomes. The small molecules targeting the innate immune system not only provide the opportunity to identify promising drug candidates, but also enable novel biology
to be unveiled.

1.2.2.1 *In silico* screening

As computational capabilities increase, *in silico* docking has become en vogue for small molecule design. With available structural information for the target protein, a small molecule can be docked and ranked according to predicted affinities. This approach may also garner information about binding interactions and orientations without the need to re-cry stallize. While initial successes have been promising, *in silico* screening is still in its infancy. Docking software is unrefined, and unable to predict interactions with consistent accuracy. Further, ligand binding may introduce difficult-to-predict conformational changes, and the extent of that change can be unique to each ligand [207].

**TLR in silico screening** In the last decade, significant progress has been made in TLR structural analysis [196]. The structures of TLR/ligand complexes including TLR1/TLR2/Pam$_3$CSK$_4$, TLR3/dsRNA, TLR4/MD-2/LPS, TLR5/flagellin, TLR2/TLR6/Pam$_2$CSK$_4$, and TLR7 and TLR8 with ssRNA have been solved [94, 207, 240]. These structures allow for the development of TLR small molecule modulators using *in silico* screening as an economical alternative.

MD-2 is an accessory protein of TLR4, with an indispensable role in LPS activation of TLR signaling [203]. Among all TLRs, TLR4 is the only one that has been confirmed to form an active heterodimer with MD-2, although there is still speculation about whether TLR2 also binds to MD-2 [48]. Therefore, targeting the TLR4/MD-2 interaction is a promising strategy for the development of TLR4-specific modulators. Yin and coworkers have developed a novel *in silico* screening methodology incorporating Molecular Mechanics (MM)/implicit solvent methods to evaluate binding free energies. This new method has been applied to the identification of disruptors of the TLR4/MD-2 complex [92]. As a brief outline of this methodology, fast molecular docking is used to generate binding orientations and subsequent molecular dynamics (MD) simulation is used to rank the ligand according to their binding affinities as implemented in QUANTUM. The selected hits were then counter-screened against approximately 500 representative human proteins
to eliminate the non-specific inhibitors. Using this method, T5342126 has been identified as a highly specific TLR4/MD-2 interaction disruptor. Follow-up studies showed that T5342126 suppresses LPS-induced activation of the serine-threonine kinase, Akt-1, and pro-inflammatory factors induced by *E. coli* [29]. Furthermore, T5342126 potentiates morphine-induced analgesia in vivo [225].

In search of TLR3 small-molecule antagonists, Yin and coworkers have performed *in silico* screening with the Enamine database (approximately 1.2 million compounds) against the extracellular domain of TLR3 [30]. Analysis of the database using the *Glide* program identified nine initial hits. The majority of these identified hits shared a D-amino acid scaffold. This motif was suggested to represent a novel pharmacophore for targeting the RNA-binding site of TLR3. Further SAR studies lead to CU-CPT4a as a potent TLR3-specific antagonist. CU-CPT4a prevents dsRNA binding to TLR3, thereby blocking dsRNA-induced inflammation in murine macrophage RAW 264.7 cells.

**Caspase *in silico* screening** Caspase family structural biology has been well explored, with available structures for caspases 1-9, with the exception of caspase-4 and -5. With information available for both the apo and ligand bound states, as well as with a plethora of known inhibitors, there is a solid starting point for *in silico* design.

Caspase-3 isatin sulfonamide analogues have been identified through computational docking of 59 inhibitors to the X-ray structure [224]. These results were then analyzed by 3D-QSAR, a computational structure-activity relationship metric. In addition to confirming experimental results exploring isatin sulfonamide binding, this approach indicated that hydrophobicity and hydrogen bonding are the key factors for caspase-3/7 specificity.

*In silico* design from a 3000 compound library also identified a caspase-8 specific activator that targets the homodimer interface [26]. Interestingly, this molecule was only functional in the presence of TNF-related apoptosis-inducing ligand (TRAIL). This compound may sensitize cancer cells that are resistant to TRAIL, inducing necessary apoptosis.

**AMP *in silico* screening** Computational design of AMPs has seen a recent flood of interest. While AMPs are crucial to innate immunity, natural peptides make poor therapeutics
due to high production costs and degradation. *In silico* screening has been pursued to circumvent these issues and allow for identification of lead compounds without the need for lengthy synthesis.

AMPs operate by using cationic and hydrophobic residues to associate with and then disrupt bacterial membranes. As such, there is no target protein for docking analysis. Instead, *in silico* analyses of protein or genome sequences are correlated with known AMP properties [7]. This platform may be used to identify novel antibiotics, or more targeted peptide therapeutics such as anti-hypertensive or anti-parasitic peptides [107, 139].

1.2.2.2 Target-based Screening

Target based screening is one of the two main experimental approaches used to identify therapeutics. In this method, the direct effect of a compound is observed on a desirable target such as a TLR. This approach is useful for reducing the incidence of false positives, but in vitro analysis cannot account for biological complications such as cytotoxicity and cell permeability.

**TLR target-based screening** In order to identify peptides directly targeting TLR4, Yang and coworkers used a yeast two-hybrid system to screen for TLR4 antagonists. The screen of a random 16-mer randomized peptide library was conducted using full-length TLR4 as bait [235]. Peptide no. 9 has been found to suppress LPS-induced NF-κB activation, Iκ-Bα phosphorylation, and the release of inflammatory factors IL-1, IL-6, and TNF-α. Further analysis showed that peptide no. 9 functions through specifically binding to the TLR4 extracellular domain.

Peptides that reproduce the interactions between TLR4 and LPS are also potential TLR4 modulators. To identify peptidomimetics that target the TLR4/LPS interface, Tiwari and coworkers have screened a 7-mer phage-display peptide library [190]. Experimental data showed that the LPS mimics are capable of inducing inflammatory cytokine secretion. Additionally, these mimics can act as TLR4 agonists and functional adjuvants for vaccine development.

MyD88 mediates all TLR signaling pathways except for TLR3 [11]. Therefore, the TLR/MyD88 interaction is a general target for regulating TLR signaling. Tobias and coworkers have constructed a β-lactamase (Bla) fragment complementation system for identifying small molecules that dis-
rupt the TLR4/MyD88 interaction \[116\]. When TLR signaling is activated, the two \(\beta\)-lactamase fragments complement each other by virtue of spontaneous TLR4/MyD88 binding via their TIR domains. Inhibition of MyD88/TLR4 binding leads to the disruption of enzyme complementation and a loss of lactamase activity. After screening approximately 16,000 compounds using this reporter system, 45 hits were identified. Five MyD88/TLR4 interaction disruptors were selected after eliminating compounds that directly inhibit \(\beta\)-lactamase. These inhibitors specifically suppress LPS-induced pro-inflammatory factors, while showing no apparent cytotoxicity.

**Caspase target-based screening**  Due to the relative ease of purification and enzymatic characterization, target-based screening is popular for caspases \[167\]. Numerous caspase small molecule inhibitors have been identified, often with core scaffolds including heterocyclic structures such as quinoxalinones, pyridines, pyrimidinones, pyrimidinediones, and quinolones \[148\]. However, specificity is difficult to engineer in caspases due to the similarities in structure, particularly in the substrate binding site. For example, quinazoline derivatives broadly target caspases \[187\]. Target based screening of caspases will be further explored in Chapters 2 and 7.

**AMP target-based screening**  AMPs are generally not a good candidate for target-based screening. Since AMPs target the membrane through a non-specific interaction, there is not a clear target to assay.

### 1.2.2.3 Phenotypic Screening

Phenotypic screens are the second type of commonly used HTS method. In this approach, a cellular readout is used to signify a change in a disease or signaling pathway. This is advantageous, as the readout is representative of a biological system, and accounts for cytotoxicity and cell permeability. However, cellular machinery is convoluted, and often it is difficult to find the small molecule target.

**TLR phenotypic screening**  HEK 293 NF-\(\kappa\)B reporter cells offer a quick and specific system for TLR screening. All TLRs share NF-\(\kappa\)B as a common downstream signaling factor \[1\] \[203\]. NF-\(\kappa\)B activity can be measured in HEK-Blue human TLR cells, which are stably transfected
with human TLRs and a secreted embryonic alkaline phosphatase (SEAP) reporter gene. This approach has been successfully employed to identify morphine as a TLR4 agonist \[83, 225\]. This approach was also employed in Chapter 6 to identify specific TLR8-targeting small molecules. By using an array of HEK-Blue cells each expressing an independent TLR, this method can be utilized to assess specificity and confirm the cellular target.

Nitric oxide (NO) is an important pro-inflammatory factor in TLR signaling. Yin and coworkers have screened the National Cancer Institute (NCI) Diversity library (1364 compounds) using a phenotypic assay that monitors the NO production in macrophage cells. CU-CPT22 has been identified as a TLR1/TLR2-specific antagonist that blocks Pam3CSK4 binding to the TLR1/TLR2 complex \[31\]. In a similar fashion, NCI 126224 was identified as a potent TLR4 signaling inhibitor that suppresses LPS-induced NO over-production \[239\]. Here we have used nitric oxide production to identify saccharine-derived JAK/STAT1 inhibitors in Chapter 8.

Caspase phenotypic screening  Much like target-based screening, caspase catalysis has been successfully assayed in phenotypic screens. Active caspase will cleave its substrate, releasing a fluorescent or luminescent tag that can be detected in a plate format. Common reagents include FLICA or the Promega Caspase-Glo series \[8, 51, 62\]. These assays are useful for measuring caspase activity in cell culture, thus accounting for cell permeability and toxicity. Due to their role in cell death, caspase screens can also be performed with a viability assay. For example, cell death was monitored to successfully identify a TRAIL-sensitizing compound that functions through caspase-8 \[51\]. This approach may also be used to measure other pathways that culminate in caspase activation.

AMP phenotypic screening  To identify new AMPs as antibiotic candidates, one approach uses in situ synthesized peptide libraries affixed to a solid surface and screened against a target bacterium \[75, 76\]. Conversely, spotted peptide microarrays have large peptide libraries printed on a glass slide. For example, Diehnelt and coworkers performed an array screen using bacteria labeled with a viability dye and an amine-reactive dye to stain the outer membrane \[46\]. Peptides that bind and kill bacteria produce one readout, while viably bound bacteria fluoresce in
two colors.

Phage-display libraries have also been used to identify novel AMPs [81]. One complication of this method is that membrane mimetics are subpar, meaning whole bacterium screening is preferable. Further, it is difficult to elute the bound phage from the membrane using mild detergents. Still, this approach has been used to identify peptides targeting multi-drug resistant bacteria [19, 164, 197].

1.2.3 Discussion

Chemical biology is an exciting and diverse field that can be used to devise probes and therapeutics targeting the innate immune system. We have focused on three branches of innate immunity: TLRs, caspases, and AMPs. Through a variety of screening and rational design approaches, we have created small molecules and peptidomimetics for applications as adjuvants, sepsis treatments, antibiotics, and anti-inflammatory agents. This thesis will explore the design and characterization of chemical probes targeting the innate immune system for therapeutic applications.
Chapter 2

NSAIDs are Caspase Inhibitors

This section is currently undergoing revisions for publication as Christina Smith, Subada Soti, Torey Jones, Akihisa Nakagawa, Ding Xue, Hang Yin. NSAIDs are caspase inhibitors. Cell Chemical Biology, (under revision), 2017. This section may differ from the published manuscript.

*C. elegans* experiments were performed by A. Nakagawa and D. Xue. All other experiments were conceived, designed, performed, and analyzed by C. Smith with assistance from S. Soti and T. Jones. Individual contributions are outlined below using the CRediT taxonomy.


2.1 Introduction

Non-steroidal anti-inflammatory drugs (NSAIDs) are among the most prevalent pharmaceuticals in the world, with an estimated 30 billion doses taken annually in the United States alone [63, 151]. The anti-inflammatory properties of NSAIDs have been segued into analgesic and antipyretic treatments targeting conditions ranging from mild aches and pains to rheumatoid arthritis to cancer [171]. The best characterized anti-inflammatory properties of NSAIDs stem from inhibition of cyclooxygenase (COX) isoenzymes [61, 163, 189, 218]. COX inhibition reduces the production of prostaglandins, which are lipid autacoids involved in diverse cellular processes such
as angiogenesis, apoptosis, and cell migration \[171\]. However, much remains unclear about the existence and contributions of additional NSAID targets \[129\]. These targets may contribute to NSAID anti-inflammatory mechanisms, applications, and the occurrence of adverse drug reactions \[129\]. Reported NSAID functions include inhibition of NF-κB, inhibition of proteasome function, activation of intrinsic and extrinsic pathways of apoptosis, cell cycle arrest, and activation of stress kinases \[18, 20, 45, 56, 89, 105, 117, 162, 186, 230, 242\]. However, many of these effects are seen only at superphysiological concentrations, limiting their biological relevance \[59, 138\]. Here, we have identified cysteine-aspartic proteases (caspases) as novel targets for some NSAIDs such as ibuprofen, naproxen, and ketorolac at physiologic concentrations. During inflammation, these NSAIDs inhibit caspase catalytic activity, reducing cell death and the induction of inflammatory cytokines.

Caspases are a family of cysteine proteases that cleave peptide substrates after aspartic acid. Conserved from metazoans to humans, caspases play crucial roles in cell death, differentiation, and inflammation \[112\]. Initiator caspases (caspase-2, -8, -9, -10) contain large prodomains for recruitment into multi-protein complexes, such as inflammasomes and apoptosomes. Upon activation, initiator caspases will be proteolytically activated and subsequently activate executioner caspases (caspase-3, -6, -7), resulting in immunologically silent cell death through apoptosis \[111\]. The aptly named inflammatory caspases (caspase-1, -4, -5) generate biologically active cytokines such as IL-1β and IL-18, and induce pyroptotic cell death \[102, 191, 211\]. Caspases-4 and -5 are of particular interest, having been identified as innate immune receptors that directly detect bacterial lipopolysaccharide \[68, 103, 191\]. Inhibitors of caspase-4 may provide opportunities to treat inflammation and septic shock in response to bacterial infections \[194\]. Due to the important role of caspases in inflammation and disease, it is essential to understand the contributions of caspase inhibition in NSAID pharmacology.

While previous research has observed pro-apoptotic and chemoprotective NSAID behaviors, these studies were not conducted under inflammatory conditions, which are more relevant to patient physiology \[18, 20, 45, 117, 162, 242\]. As caspases are upregulated in response to inflammation, this approach masks the independent contributions of caspases and suggests that environment may affect
the NSAID anti-inflammatory mechanism. In the presence of an inflammatory stimulus, caspase activation propagates inflammation through the release of pro-inflammatory cytokines and danger-associated molecular patterns (DAMPs) from dying cells [41]. Consequently, caspase inhibition by NSAIDs will reduce inflammation. However, this behavior is a double-edged sword, as the reduction of cytokines and DAMPs is paired with the prevention of beneficial apoptosis. Armed with an expanded understanding of how caspase inhibition affects NSAID pharmacology, it may be possible to improve patient safety and account for the occurrence of negative side effects. As caspases are an attractive therapeutic target in their own right, it is also feasible that drug repurposing could expand applications of some NSAIDs. Caspases have known roles in cancer, cardiovascular disease, rheumatoid arthritis, inflammation, and neurodegenerative diseases [137].

Here we identify a novel role for NSAID-family drugs such as ibuprofen, naproxen, and ketorolac as competitive caspase inhibitors, which bind the S1 subsite and obscure the catalytic cysteine. At physiologically relevant concentrations, numerous NSAIDs impede caspase catalysis, reducing inflammation and cell death. Through study of a *C. elegans* model, we have determined that caspase inhibition is COX-independent, and represents a new NSAID anti-inflammatory target. We further used principal component analysis to identify the physical-chemical properties of NSAIDs that contribute to effective inhibition. As caspases play a pivotal role in inflammation and cell death, inhibition could contribute to the NSAID anti-inflammatory mechanism and inform patient safety.

2.2 Results

2.2.1 High-throughput Screening Identifies NSAIDs as Caspase-4 Inhibitors

Caspase-4 is an exciting therapeutic target, having been recently identified as an innate immune receptor for lipopolysaccharide. Unlike other caspases, which associate with inflammasomes or apoptosomes to propagate an inflammatory signal, caspase-4 is able to directly bind to lipopolysaccharide [191]. Caspase-4 inhibitors have promising anti-inflammatory applications in
septic shock treatment, however to date no caspase-4 small molecule inhibitors have been developed. Further, recent efforts in drug repurposing and computational analysis have identified a myriad of previously unknown targets for Food and Drug Administration (FDA)-approved pharmaceuticals. To this end, we aimed to use high-throughput screening to identify therapeutic inhibitors of caspase-4 enzymatic activity. We screened the 1,280 compound Prestwick Chemical Library, which is comprised of FDA-approved bioavailable drugs selected for chemical and pharmacological diversity. Screening at 33 µM yielded 27 compounds that reduced caspase-4 catalytic activity to less than 25%, a 2% hit rate. Due to the nature of the Promega Caspase-Glo coupled-enzyme screen, these hits included known luciferase inhibitors, such as resveratrol and adenosine 5'-monophosphate (AMP). Interestingly, NSAIDs comprised half of the hits, and eight of the top ten most potent inhibitors. Hits were not limited to one NSAID class (e.g. propionic acid class), and are structurally diverse. This was intriguing, as while caspases and cyclooxygenase share interrelated signaling, the enzymes themselves are largely dissimilar. Further, earlier studies aiming to observe NSAID off-target effects would not detect contributions from caspases, which are upregulated only in an inflammatory environment.

Despite the prevalence of NSAIDs as caspase-4 inhibitors, not all NSAIDs tested reduced enzymatic activity. To ascertain the chemical properties that contribute to caspase inhibitory activity, we performed a principal component analysis to compare NSAIDs in terms of physical-chemical properties such as size, aromaticity, polarity, and flexibility. From this evaluation, several trends emerged. The primary determinant was observed to be compound size (correlation coefficient 0.72). While aromaticity was not a factor, extremes in polarity or hydrophobicity were not tolerated, and increased flexibility discouraged binding (correlation coefficient 0.39). For example, exceptionally polar compounds such as oxicam-class NSAIDs or lipophilic compounds such as coxib-class NSAIDs are unable to inhibit caspase-4. These factors are consistent with known caspase active site inhibitors. The NSAID size restriction mirrors existing caspase inhibitors that often contain small core scaffolds. It may be that larger NSAIDs are incapable of fitting into the S1 pocket, and are unable to compensate by forming sufficient contacts.
Table 2.1: Total hits from caspase-4 screening with the Prestwick Chemical Library. Compounds identified by HTS that reduced caspase-4 catalytic activity to less than 25% were considered to be hits. Information on the therapeutic categories for each compound was obtained from drugs.com. Values shown represent the average and standard deviation of two independent replicates. Due to the nature of the Promega Caspase-Glo 9 coupled-enzyme system, luciferase inhibitors will also appear as false positives. Known luciferase inhibitors are denoted with an asterisk, including resveratrol and adenosine 5'-monophosphate (AMP).

<table>
<thead>
<tr>
<th>Name</th>
<th>CAS Number</th>
<th>Therapeutic Category</th>
<th>Caspase-4 Activity</th>
</tr>
</thead>
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<tr>
<td>Fenbufen</td>
<td>36330-85-5</td>
<td>NSAID</td>
<td>3.7 ± 2.4%</td>
</tr>
<tr>
<td>Terazosin</td>
<td>63590-64-7</td>
<td>Alpha-adrenergic blocker</td>
<td>3.2 ± 0.9%</td>
</tr>
<tr>
<td>Ketorolac Tromethamine</td>
<td>74103-07-4</td>
<td>NSAID</td>
<td>4.1 ± 0.3%</td>
</tr>
<tr>
<td>Indoprofen</td>
<td>31842-01-0</td>
<td>NSAID</td>
<td>4.2 ± 0.7%</td>
</tr>
<tr>
<td>Tiaprofenic Acid</td>
<td>33005-95-7</td>
<td>NSAID</td>
<td>4.3 ± 0.1%</td>
</tr>
<tr>
<td>Ketoprofen</td>
<td>22071-15-4</td>
<td>NSAID</td>
<td>6.5 ± 1.3%</td>
</tr>
<tr>
<td>Flurbiprofen</td>
<td>5104-49-4</td>
<td>NSAID</td>
<td>5.8 ± 0.7%</td>
</tr>
<tr>
<td>Ebselen</td>
<td>60940-34-3</td>
<td>NSAID</td>
<td>5.9 ± 2.3%</td>
</tr>
<tr>
<td>Resveratrol*</td>
<td>501-36-0</td>
<td>Dietary supplement</td>
<td>7.5 ± 1.1%</td>
</tr>
<tr>
<td>Tolmetin</td>
<td>64490-92-2</td>
<td>NSAID</td>
<td>8.6 ± 1.5%</td>
</tr>
<tr>
<td>Luteolin</td>
<td>491-70-3</td>
<td>Flavonoid</td>
<td>9.7 ± 0.3%</td>
</tr>
<tr>
<td>Felbinac</td>
<td>5728-52-9</td>
<td>NSAID</td>
<td>8.1 ± 2.2%</td>
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<tr>
<td>Suprofen</td>
<td>40828-16-4</td>
<td>NSAID</td>
<td>8.7 ± 0.9%</td>
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<tr>
<td>Niclosamide</td>
<td>50-65-7</td>
<td>Anthelmintic</td>
<td>11.3 ± 4.4%</td>
</tr>
<tr>
<td>Tranilast</td>
<td>53902-12-8</td>
<td>H-antihistaminic</td>
<td>11.4 ± 1.0%</td>
</tr>
<tr>
<td>Carprofen</td>
<td>53716-49-7</td>
<td>NSAID</td>
<td>12.2 ± 2.4%</td>
</tr>
<tr>
<td>Diacerein</td>
<td>13739-02-1</td>
<td>Interleukin-1 receptor antagonist</td>
<td>9.4 ± 5.4%</td>
</tr>
<tr>
<td>Tiabendazole</td>
<td>148-79-8</td>
<td>Anthelmintic</td>
<td>13.5 ± 3.1%</td>
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<tr>
<td>Monobenzone</td>
<td>103-16-2</td>
<td>Demelanizing agent</td>
<td>15.1 ± 2.3%</td>
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<td>Prazosin</td>
<td>19237-84-4</td>
<td>Alpha-adrenergic blocker</td>
<td>13.3 ± 0.3%</td>
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<tr>
<td>Leflunomide</td>
<td>75706-12-6</td>
<td>Disease-modifying anti-rheumatic drug</td>
<td>16.3 ± 1.5%</td>
</tr>
<tr>
<td>Flunixin</td>
<td>42461-84-7</td>
<td>NSAID</td>
<td>17.9 ± 2.0%</td>
</tr>
<tr>
<td>Gemfibrozol</td>
<td>25812-30-0</td>
<td>Fibrate</td>
<td>23.8 ± 2.1%</td>
</tr>
<tr>
<td>Adenosine 5'-Monophosphate*</td>
<td>18422-05-4</td>
<td>Nucleotide</td>
<td>20.9 ± 1.1%</td>
</tr>
<tr>
<td>Flufenamic acid</td>
<td>530-78-9</td>
<td>NSAID</td>
<td>23.4 ± 0.3%</td>
</tr>
<tr>
<td>Phenazopyridine</td>
<td>136-40-3</td>
<td>Analgesic</td>
<td>23.0 ± 3.2%</td>
</tr>
<tr>
<td>Anethole Trithione</td>
<td>532-11-6</td>
<td>Sialagogue</td>
<td>24.5 ± 0.1%</td>
</tr>
</tbody>
</table>
with the rest of the active site (Fig 2.2A). Further, at the P1 position, there is a nearly inalienable preference for aspartic acid, and the S1 pocket is narrow and extremely electropositive [227]. Thus, extremely hydrophobic or charged NSAIDs would be unable to bind. Finally, many NSAIDs contain a carboxylic acid that could form hydrogen bonds with the S1 pocket, mimicking aspartic acid.

Nine NSAIDs (fenbufen, indoprofen, ketoprofen, ketorolac, felbinac, tiaprofenic, aspirin, ibuprofen, and naproxen) were selected to further examine the effect of NSAIDs on caspase catalytic activity. These compounds represent diverse NSAID classes, scaffolds, sizes, polarities, rigidities, and caspase-4 inhibitory potencies.

### 2.2.2 NSAIDs are Multi-caspase Inhibitors

The caspase enzyme family is subdivided by their function as inflammatory, initiator, or executioner caspases, or segregated by recognition of particular tetrapeptide substrates [55, 210]. Despite these functional differences, caspases are structurally analogous, posing a challenge for chemical targeting of individual caspases [167, 227]. To explore NSAID specificity, we assayed the catalytic activity of caspases-1, -3, -4, -5, and -9 (Figure 2.3). These caspases were selected to represent inflammatory, initiator, and executioner caspases with diverse tetrapeptide substrates. Inhibition was not specific to caspase-4, as NSAIDs reduced catalysis of multiple caspases (Table 2.2). Aspirin is a notable exception, with no inhibitory properties. However, aspirin has a unique acetylation mechanism that will not affect caspase catalysis [115].

Multi-caspase inhibition suggests that NSAIDs are binding to a conserved subsite such as S1. In general, NSAID inhibition was more potent for caspases-4, -5, and -9, with weaker activity against caspase-3 and -1. This may be due to differences in the substrate recognition pocket, as caspases-4, -5, and -9 all recognize the LEHD tetrapeptide, whereas caspase-1 recognizes WEHD and caspase-3 DEVD. It is feasible that interactions with the adjacent but less conserved S2 and S1′ subsites account for the small differences in activity observed [167, 204].

We next expanded specificity testing to encompass cathepsin B, a lysosomal cysteine protease
Figure 2.1: ChemGPS-NP analysis compares the physical-chemical properties of screened NSAIDs. The physical-chemical properties of NSAIDs were compared using a ChemGPS-NP principal component analysis. (A) The relationship between NSAID size and caspase-4 activity. Size increases in the positive direction of the x-axis. Generally, smaller NSAIDs were more potent caspase-4 inhibitors, with the exception of aspirin and terazosin. The correlation coefficient of this data set is 0.46, however with these two outliers removed the correlation coefficient is 0.72. This suggests a strong correlation between NSAID size and inhibitory activity. (B) The relationship between NSAID aromaticity and caspase-4 activity. Aromaticity increases in the positive direction of the x-axis. There is no apparent relationship between aromaticity and caspase-4 activity, with a correlation coefficient of 0.12. (C) The relationship between NSAID polarity and caspase-4 activity. Compounds are more lipophilic in the positive direction of the x-axis, and increasingly polar in the negative direction. The correlation coefficient is -.12, indicating no correlation. (D) The relationship between NSAID flexibility and caspase-4 activity. NSAID flexibility increases in the positive direction of the x-axis, while rigidity increases in the negative direction. There is a weak correlation between compound rigidity and caspase-4 inhibitory activity, with a correlation coefficient of 0.39.
Figure 2.2: A biophysical and computational model of caspase binding interactions. Caspase-3 (PDB 2DKO) is shown with a tetrapeptide substrate or NASIDs (green). The catalytic cysteine is pink, tryptophans are yellow, and other residues are colored to depict hydrophobicity (blue more polar, red more hydrophobic). The inset shows the surface of the binding pocket. (A) The crystal structure of caspase-3 with a DEVD peptide substrate. The nomenclature of caspase and substrate interactions are denoted, and the cleavage site is indicated in red. Peptide residues are named P4-P1’, from N-terminal to C-terminal. The corresponding caspase binding pocket is S4-S1’. These associations are noted in blue. (B) Titration with indoprofen or aspirin quenches caspase-3 tryptophan fluorescence. The apparent K_D for indoprofen (circles) is 29.63 ± 2.02 μM. The apparent K_D for aspirin (squares) is 1289.68 ± 9.54 μM. Data are representative of the average and standard deviation of at least two independent experiments, normalized with the titrated solvent control as 100%, and buffer titrated with compound as 0%. (C) A representation of the top predicted cluster of indoprofen bound to caspase-3. Predictions place indoprofen in the S1 pocket of the active site. (D) A representation of the top predicted cluster of aspirin bound to caspase-3. Predictions place aspirin away from the active site.
Figure 2.3: Dose-response inhibitory plots of NSAIDs with caspases-1, -3, -4, -5, -9. Five caspases were treated with 66 µM to 26.1 nM of the indicated NSAIDs to obtain a dose-response curve. Curves for each caspase are denoted with a different color: caspase-1 in black, caspase-3 in pink, caspase-4 in red, caspase-5 in green, and caspase-9 in blue. The associated IC$_{50}$ values are available in Table 2.2. Data are representative of the average and standard deviation of at least two independent replicates. Data are normalized with substrate as 100%.
Table 2.2: NSAID multi-caspase inhibition of caspases-1, -3, -4, -5, -9. IC$_{50}$ values were determined for each NSAID and caspase combination. NSAIDs were tested from 66 µM to 26.1 nM to obtain a dose-response curve. Aspirin shows no inhibition against any caspase, however all other tested NSAIDs demonstrate pan-caspase inhibition. NSAIDs with no inhibition in the tested range are denoted as no activity, while weak inhibitors have an IC$_{50}$ value >66 µM. The associated dose-response curves are available in 2.3.

<table>
<thead>
<tr>
<th>NSAID</th>
<th>Caspase</th>
<th>IC$_{50}$ (µM)</th>
<th>R. Squared</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspirin</td>
<td>1</td>
<td>No activity</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>No activity</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>No activity</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>No activity</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>No activity</td>
<td>N/A</td>
</tr>
<tr>
<td>Felbinac</td>
<td>1</td>
<td>35.05 ± 2.50</td>
<td>0.984</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>13.98 ± 2.21</td>
<td>0.970</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>1.57 ± 0.11</td>
<td>0.988</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>2.60 ± 0.27</td>
<td>0.987</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>1.75 ± 0.17</td>
<td>0.989</td>
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<tr>
<td>Fenbufen</td>
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<td>0.996</td>
</tr>
<tr>
<td></td>
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<td>1.21 ± 0.20</td>
<td>0.972</td>
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<td>4</td>
<td>0.57 ± 0.04</td>
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<td></td>
<td>5</td>
<td>0.87 ± 0.09</td>
<td>0.982</td>
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<tr>
<td></td>
<td>9</td>
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<tr>
<td>Ibuprofen</td>
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<td>3</td>
<td>&gt;66</td>
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<td>8.84 ± 0.64</td>
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<td>Indoprofen</td>
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<td>9</td>
<td>0.30 ± 0.01</td>
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<td>Ketorolac</td>
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</tr>
<tr>
<td></td>
<td>9</td>
<td>0.85 ± 0.07</td>
<td>0.990</td>
</tr>
<tr>
<td>Ketoprofen</td>
<td>1</td>
<td>&gt;66</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>50.23 ± 10.15</td>
<td>0.856</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>7.55 ± 1.08</td>
<td>0.989</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>8.94 ± 1.48</td>
<td>0.972</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>8.79 ± 1.20</td>
<td>0.960</td>
</tr>
<tr>
<td>Naproxen</td>
<td>1</td>
<td>&gt;66</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>55.14 ± 7.33</td>
<td>0.798</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>14.69 ± 1.20</td>
<td>0.988</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>11.36 ± 0.87</td>
<td>0.977</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>8.24 ± 1.03</td>
<td>0.983</td>
</tr>
<tr>
<td>Tiaprofenic Acid</td>
<td>1</td>
<td>1.57 ± 0.05</td>
<td>0.999</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>0.81 ± 0.07</td>
<td>0.991</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>0.38 ± 0.01</td>
<td>0.993</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>0.47 ± 0.03</td>
<td>0.986</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>2.19 ± 0.18</td>
<td>0.997</td>
</tr>
</tbody>
</table>
No inhibition of cathepsin B was observed, demonstrating specificity for caspases over other cysteine proteases and ruling out the presence of redox cycling compounds (RCCs) (Figure S2.3A). RCCs generate hydrogen peroxide in the presence of strong reducing agents, and indirectly inhibit enzyme activity through oxidation of the catalytic cysteine [93]. We also accounted for luciferase inhibition, though NSAIDs have been widely tested in luciferase-based assays (Figure S2.3B) [105, 117, 132, 192]. We did not observe inhibition of luciferase or cathepsin B, suggesting that NSAID inhibition is specific to the caspase family. This finding links NSAIDs to a class of enzymes with known roles in cancer, cardiovascular disease, rheumatoid arthritis, and neurodegenerative diseases [137].

2.2.3 NSAIDs are Competitive Inhibitors

We next sought to further characterize the interaction between NSAIDs and various caspases. To assess binding, we observed caspase-3 tryptophan fluorescence. Caspase-3 has two active site tryptophan residues (Trp206 and Trp214) which provide information on the active site microenvironment [109]. We observed quenching of caspase-3 tryptophan fluorescence with indoprofen titration, giving an apparent $K_D$ of $30 \pm 2 \mu M$ (Figure 2.2B). Aspirin quenched with a substantially weaker apparent $K_D$ of $1290 \pm 10 \mu M$. The disparate binding affinities of these two NSAIDs corroborates our enzymatic data.

The hypothesized binding interaction was further explored with computational modeling and enzymatic competition assays. Computational docking over the entire caspase-3 surface places indoprofen and fenbufen in the caspase-3 S1 subsite, obscuring the substrate binding pocket at the catalytic cysteine (Figure 2.2C, Figure S2.1) [53, 65]. This localization would suggest NSAIDs act as competitive inhibitors. The S1 subsite is the most conserved, reflecting the strict requirement for aspartic acid at the P1 position. NSAIDs binding to this highly conserved site would not confer caspase specificity, in agreement with the multi-caspase activity observed. The carboxylic acid moiety of indoprofen and fenbufen is able to form hydrogen bonds (Arg64, Arg207, Gln161) mimicking the aspartic acid substrate. This hypothesis was experimentally validated with a substrate competition
assay. At increasing substrate concentrations, the observed IC$_{50}$ value increases, consistent with inhibitor and substrate competing for the same site ((Table S2.2)).

Conversely, despite the presence of a carboxylic acid, aspirin does not preferentially dock to the active site (Figure 2.2D). Taken together with the observed caspase inhibition, these results suggest that NSAIDs may be competitive caspase inhibitors recognizing the active site.

2.2.4 NSAIDs are Physiologically Relevant Caspase Inhibitors

We next studied inhibition of caspases by NSAIDs in a cellular context. We observed dose-dependent inhibition of both caspase-1 and -3 catalytic activity in HeLa and THP-1 cultured cells (Figure 2.4). Cell viability and IL-1$\beta$ production were also assessed as hallmarks of caspase catalysis. THP-1 cells treated with 100 µM NSAIDs demonstrated improved viability when challenged with nigericin and staurosporine, consistent with inhibition of caspase-mediated cell death (Figure 2.5). In agreement with a reduction in caspase-1 catalysis, we also observed a decrease in IL-1$\beta$ production in NSAID treated cells (Figure 2.6). Unlike previously identified NSAID targets observed only at high doses (mM), caspase inhibition occurs at micromolar concentrations in biological systems, this is consistent with physiological levels of NSAIDs (5-500 µM), and comparable to the IC$_{50}$ values for COX [59, 61, 100, 162]. To establish that the observed caspase response in cells was COX-independent, we knocked down COX-2 and observed no difference in caspase-3 catalysis (Figure S2.4).

2.2.5 Caspase Inhibition by NSAIDs is COX-independent

As inflammatory signaling has extensive cross-talk, it is difficult to parse out the individual contributions of caspase and COX inhibition by NSAIDs in a biological system. We further investigated NSAID treatments in the model organism C. elegans, in which the caspase homologs cell death proteins (CED) play a central role in apoptosis. C. elegans is an excellent model as apoptotic machinery is highly conserved, but lacks expression of either COX isoform [118, 236]. We performed cell death analysis using the ced-1(e1735) mutant that is defective in cell corpse engulf-
Figure 2.4: NSAIDs inhibit caspase activity in cell culture. (A) NSAIDs show dose-dependent caspase-3 inhibition in HeLa cells treated with 1 µM staurosporine. (B) THP-1 cells treated with 100 µM of selected NSAIDs in the presence of 1 µM nigericin (caspase-1) or staurosporine (caspase-3) show inhibited caspase catalytic activity. Data are representative of the average and standard deviation of three biological replicates, normalized with nigericin or staurosporine as 100% caspase activity, and z-VAD-FMK treated as 0%. The statistical significance was determined using a one-way ANOVA followed by Tukey’s test (*P≤0.05, ***P≤0.001 compared to 100% control).

Figure 2.5: NSAIDs rescue apoptotic THP-1 cells. THP-1 cells treated with (A) 25 µM nigericin or (B) 10 µM staurosporine undergo apoptosis. Treatment with 100 µM of selected NSAIDs improves viability. Data are shown as the average and standard deviation of three biological replicates normalized with nigericin at 0% viability, and untreated cells as 100% viability. The statistical significance was determined using a one-way ANOVA followed by Tukey’s test (*P≤0.05, ***P≤0.001 compared to 0% control).
Figure 2.6: NSAIDs reduce IL-1β production. THP-1 cells were treated with nigericin to activate caspase-1, producing the inflammatory cytokine IL-1β. Data are the average and standard deviation of three biological replicates, with nigericin treatment normalized to 100% IL-1β production, and untreated cells as 0%. The statistical significance was determined using a one-way ANOVA followed by Tukey’s test (*P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001 compared to 100% control).

Figure 2.7: (A) A representative image of a C. elegans embryo with persistent apoptotic cell corpses (indicated by arrows). (B) Cell death assays in C. elegans embryos treated with fenbufen or indoprofen. L4-stage ced-1(e1735) animals were exposed to 100 μM fenbufen or indoprofen in 0.5% DMSO on NGM agar plates, and their progenies were analyzed. Embryonic cell corpses were scored at comma, 1.5-fold, 2-fold, 2.5-fold, 3-fold, and 4-fold stages. The y-axis represents the mean and standard deviation of cell corpses scored (n = 15). The statistical significance was determined using a two-way ANOVA followed by Tukey’s test. (**P ≤ 0.001 compared to 0.5% DMSO control).
ment process, which sensitized the cell death assay \[39, 142\]. With 100 µM fenbufen or indoprofen treatment, we observed statistically significant decrease of cell death at all stages, whereas 0.5% DMSO did not cause any effect (Figure 2.7). This experiment presents an in vivo COX-independent model for NSAID inhibition of caspases. While both pathways will be simultaneously affected in patients, caspase inhibition is a previously unacknowledged avenue that may contribute to NSAID pharmacology.

2.3 Discussion

Here we report a novel anti-inflammatory mechanism for NSAIDs such as ibuprofen, naproxen, and ketorolac. We observe that these NSAIDs are competitive pan-caspase inhibitors, binding to the S1 subsite and obscuring the catalytic cysteine. Unlike previously identified non-COX NSAID targets, caspase inhibition is observed at physiological concentrations. The caspase inhibitory profile is comparable in potency to COX, with micromolar IC\(_{50}\) values \[61, 100, 162\]. We also observe that caspase inhibition is COX-independent, implicating a novel pathway for NSAID pharmacology in patients. At physiological concentrations, it is likely that both COX and caspase pathways are simultaneously modulated, each contributing to the anti-inflammatory mechanism. We propose that caspase contributions are relevant to the NSAID anti-inflammatory mechanism of action by reducing cell death and the generation of pro-inflammatory cytokines. However, despite the beneficial anti-inflammatory results of caspase inhibition, the possible detriments should not be discounted. Reducing healthy caspase signaling increases the incidence of viral and bacterial infection, and deregulates inflammation and cell proliferation \[67, 137\]. Interestingly, we observed a trend between the potency of caspase inhibition and drug recall and incidence of side effects. For example, fenbufen and indoprofen have been largely recalled from global markets due to liver and gastrointestinal toxicity \[52, 121\]. However, aspirin, ibuprofen, and naproxen remain prevalent, and demonstrate weaker caspase inhibition. Future efforts should look to further explore how caspase inhibition by these NSAIDs affects patients. Drug repurposing may expand NSAID applicability to caspase-focused pharmacological targets such as cancer, cardiovascular disease, rheumatoid
arthritis, and neurodegenerative diseases. An improved understanding of how caspases control the NSAID anti-inflammatory mechanism may lead to the design of more specific drugs, and improve patient safety.

### 2.4 Significance

While NSAIDs are commonly used drugs, the pharmacological contributions of non-canonical cellular targets remain unclear. Through high-throughput screening of FDA-approved drugs, we have identified a subset of NSAIDs capable of inhibiting caspases at physiological concentrations. In vitro characterization obtained IC\textsubscript{50} values with multiple caspases, representing wide applicability against diverse biological functions and substrates. Principal component analysis examined NSAID chemical space, and identified compound size as the discerning factor for inhibition. We explored active site binding interactions with tryptophan quenching, substrate competition, and computational modeling. Further, we examined the biological context of this work through cell culture and \textit{C. elegans} animal models. Here we observed a non-canonical NSAIDs mechanism that is COX-independent, establishing caspases as a novel pharmacological target.

While extensive effort has been invested into the study of NSAIDs, previous research linking NSAIDs to apoptosis was not conducted under inflammatory conditions. In these circumstances, the contribution of caspases would not be apparent, as these important enzymes are upregulated only under inflammatory conditions. Further, many studies are conducted at superphysiologic NSAID concentrations, and may not be relevant to patients. By measuring caspase signaling at physiologic concentrations under inflammatory conditions, we were able to observe a novel role for NSAIDs. Understanding how additional targets may contribute to NSAID pharmacology could clarify the anti-inflammatory mechanism, improve patient safety, and facilitate the design of next-generation therapeutics.
2.5 Materials and Methods

2.5.1 Protein Expression and Purification

For high-throughput screening, a caspase-4 C258A catalytically inactive mutant was expressed using a modified pET vector, which was generously gifted by Dr. Feng Shao [191]. To obtain the catalytically active mutant, New England Biolabs Q5 Site-Directed Mutagenesis Kit was used to revert the catalytically inactive caspase-4 to an active cysteine at site 258. Caspase-3 (pET23b-Casp3-His Addgene #11821) and -9 (pET23b-Casp9-His Addgene #11829) were gifts from Dr. Guy Salvesen [199, 241]. Proteins were expressed and purified using the protocol previously described by Denault and Salvesen [43]. Briefly, plasmids were expressed in E. coli BL21 pLysS cells and grown in 2x TY culture media at 18 C overnight after induction with 50 µM IPTG. Cells were then pelleted and sonicated to collect the lysate, and purified using Nickel NTA Agarose Beads (GoldBio). Fractions were analyzed by SDS-PAGE. For IC\textsubscript{50} determination, caspase-1, -4, and -5 were purchased from Enzo Life Sciences.

2.5.2 High-throughput Screening

High-throughput screening for inhibitors of human caspase-4 was performed using the 1280 compound Prestwick Chemical Library at the University of Colorado High-throughput Screening Core Facility. The screen was designed in a 384-well format (Greiner 781207), using 33 µM compound, 1 µM recombinant human caspase-4 in high citrate buffer (50 mM Tris-HCl, pH 7.5, 1 M sodium citrate, 10 mM DTT, 10% sucrose), and 20 µM Promega Caspase-Glo 9 Assay substrate [147, 174]. While the Caspase-Glo 9 assay is designed for use with caspase-9, the LEHD substrate is also recognized by caspase-4 [174, 204]. The assay Z’ factor is 0.66, recommending this method as suitable for high-throughput experimentation (Figure S2.2) [238]. Compounds were incubated with enzyme for at least fifteen minutes prior to substrate addition to allow equilibration. Plates were read with endpoint luminescent analysis 20 minutes and 60 minutes after substrate addition. Compounds were considered to be hits if caspase-4 activity was inhibited to less than 25% relative
to the solvent control. Using these criteria, we identified 21 hits, which are summarized in Table S2.1.

### 2.5.3 Chemicals

Fenbufen, indoprofen, ketoprofen, ketorolac, felbinac, naproxen, ibuprofen, and tiaprofenic were purchased from Sigma Aldrich. Aspirin was purchased from TCI America. Pan-caspase inhibitor z-VAD-FMK was obtained from InvivoGen. All compounds were dissolved in DMSO.

### 2.5.4 Caspase Catalytic Activity, IC\textsubscript{50} Determination, and Specificity

To assess the potency and specificity of each NSAID, IC\textsubscript{50} values were determined against caspases-1, -3, -4, -5, and -9. Experiments were performed in a 384-well format per the conditions noted here, which were optimized for each enzyme.

Table 2.3: Caspase specificity methodology. Caspase specificity experiments were carried out using the conditions noted below.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>[Enzyme] (nM)</th>
<th>[Substrate] (µM)</th>
<th>Buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caspase-1</td>
<td>2.5</td>
<td>WEHD, 6.5</td>
<td>Enzo Caspase</td>
</tr>
<tr>
<td>Caspase-3</td>
<td>200</td>
<td>DEVD, 3.3</td>
<td>Standard Caspase</td>
</tr>
<tr>
<td>Caspase-4</td>
<td>1</td>
<td>LEHD, 10</td>
<td>High Citrate</td>
</tr>
<tr>
<td>Caspase-5</td>
<td>20</td>
<td>LEHD, 10</td>
<td>High Citrate</td>
</tr>
<tr>
<td>Caspase-9</td>
<td>200</td>
<td>LEHD, 6.5</td>
<td>High Citrate</td>
</tr>
</tbody>
</table>

Enzo Caspase Buffer: 50 mM HEPES, pH 7.4, 100 mM NaCl, 0.5% Tween 20, 10 mM DTT, 10% glycerol. Standard Caspase Assay Buffer: 20 mM PIPES, pH 7.5, 100 mM NaCl, 1mM EDTA, 10 mM DTT, 10% sucrose. High Citrate Assay Buffer: 50 mM Tris-HCl, pH 7.5, 1 M sodium citrate, 10 mM DTT, 10% sucrose.

Substrate was present in at least 16-fold excess, and enzyme concentrations were kept well below IC\textsubscript{50}. Activity was measured as the change in luminescent signal for at least thirty minutes. Analysis was performed using the linear portion of the curve, however endpoint analysis yielded values within error. Percent inhibition was determined compared to the DMSO only control. Each assay included solvent and z-VAD-FMK controls, and assay acceptance criteria required at least
a ten-fold signal to noise ratio. Compounds were tested between 66 µM and 26.1 nM to obtain a dose-response curve. Higher concentrations were not tested since higher DMSO concentrations (> 1%) decrease luminescence. IC\textsubscript{50} values were calculated using OriginPro 2016 using a dose-response curve with variable Hill slope, where A1 and A2 represent the asymptotes, p is the Hill slope, and Logx0 is the center.

\[ y = A_1 + \frac{A_2 - A_1}{1 + 10^{(\log x_0 - x)/p}} \]

The asymptotes were fixed at 100% and 0% to represent the solvent and z-VAD-FMK controls respectively. Data shown is the average and standard deviation of at least two technical replicates repeated in two independent assays.

### 2.5.5 Cathepsin B Catalytic Activity

Recombinant human liver cathepsin B was obtained from Enzo Life Sciences. NSAID inhibition of cathepsin B was tested in a 384-well format (Greiner 781209) in buffer containing 352 mM KH\textsubscript{2}PO\textsubscript{4}, 48 mM Na\textsubscript{2}HPO\textsubscript{4}, 4 mM EDTA, 8 mM cysteine, and 0.1% Triton-X at pH 6.0 with 66 µM of compound [16]. Cathepsin B was assayed at 75 nM with 2.5 µM z-Arg-Arg-7-amido-4-methylcoumarin hydrochloride (Sigma-Aldrich). N-Acetyl-Leu-Leu-Methional from BioVision Incorporated was used at 1 µM as an inhibitory control. Data shown is the average and standard deviation of at least 3 technical replicates repeated in three independent assays (n=9). Data was normalized such that cathepsin B with solvent is 100%, and 0% as ALLM inhibitor.

### 2.5.6 Caspase Catalytic Activity in Cultured Cells

HeLa (human epithelial) cells cultured in DMEM with 10% FBS and 1% Penicillin/Streptomycin were plated at 10,000 cells/well in a white 96-well plate (Greiner 655083) and grown overnight. THP-1 (human peripheral blood monocyte) cells were grown in RPMI with 10% FBS, 1% Penicillin/Streptomycin, and 0.05 mM 2-mercaptoethanol. THP-1 cells were plated at 25,000 cells/well in a white 96-well plate and differentiated with 10 ng/mL phorbol myristate acetate (PMA) overnight. The day after plating the cell culture media was replaced. THP-1 cells were primed
for 4 hours with 1 µg/mL E. coli O111:B4 lipopolysaccharide (InvivoGen). HeLa cells were not primed. Cells were then treated with 1 µM staurosporine (Sigma-Aldrich) or nigericin (InvivoGen) and the indicated concentration of inhibitor [128, 134, 150, 157, 205]. After incubation, caspase-1 (2 hours incubation) or -3 (4 hours incubation) activity was assayed by Promega Caspase-Glo 1 Inflammasome Assay or Promega Caspase-Glo 3/7 Assay per manufacturer’s recommendations. Data was normalized with 10 µg/mL z-VAD-FMK as 0% caspase activity, and 1 µM staurosporine or nigericin as 100% activation. Data shown is the average and standard deviation of at least three biological replicates. Statistical significance was determined using a one-way ANOVA test followed by Tukey’s test to compare compound treated with nigericin or staurosporine only.

2.5.7 Cell Viability Determination

THP-1 or HeLa cells were cultured and plated as above in a 96-well black plate (Greiner 655086). Apoptosis was induced using 10 µM staurosporine or 25 µM nigericin, and viability was measured using the Promega CellTiter-Fluor Viability Assay per manufacturer’s recommendations. Staurosporine data was collected after two hours, and nigericin after three. Promega CellTiter-Glo Luminescent Cell Viability Assay was used to assess compound toxicity, and also to observe luciferase inhibition. Data was normalized using untreated cells as 100% viability and staurosporine or nigericin cells as 0%. For CellTiter-Glo, 0% is lysed cells. Data shown is the average and standard deviation of at least three biological replicates. Statistical significance was determined using a one-way ANOVA test followed by Tukey’s test to compare compound treated with nigericin or staurosporine only.

2.5.8 Enzyme-linked Immunosorbent Assay (ELISA)

THP-1 cells were plated in a 96-well plate (Costar 3596) and treated as described above. IL-1β cytokine production was determined using the BD Biosciences Human IL-1β ELISA Set II per manufacturer’s instructions. Data was normalized with 100% as nigericin treated, and untreated cells as 0%. Data shown is the average and standard deviation of at least three biological replicates.
Statistical significance was determined using a one-way ANOVA test followed by Tukey’s test to compare compound treated with nigericin only.

2.5.9 siRNA Knockdown and Western Blot

HeLa cells were plated at 300,000 cells/well in a 6-well tissue-culture treated plate (Costar 3516) and grown in supplemented DMEM (1% Penicillin/Streptomycin, 10% FBS). Cells were transfected using 1.2 µL Lipofectamine RNAiMAX (ThermoFisher) and 25 nM of each siRNA per well. COX2 siRNA was obtained from Dharmacon (ON-TARGETplus Human PTGS2 SMARTpool 5743). Silencer Select CASP3 (4290824) and GAPDH (4390849) siRNAs were obtained from ThermoFisher. For each experiment, a mock control was performed without siRNA. Cells were maintained for 48 hours, then transferred into a 96-well plate at 10,000 cells/well and grown overnight. The following day, 72 hours post-transfection, cells were assayed for caspase-3 activity as described above, or collected for protein expression analysis by Western blot. For Western blot, cell pellets were collected, lysed in RIPA buffer (ThermoScientific) and assayed for total protein content using the Pierce BCA Protein Assay Kit (ThermoScientific) per manufacturer’s instructions. For caspase-3 and GAPDH detection, 20 µg of total protein was loaded per lane. Due to the lower expression levels of COX2, 50 µg of total protein was loaded per lane. COX2 (#4842), GAPDH (#2118), and caspase-3 (#9662) primary antibodies were obtained from Cell Signaling Technologies. Peroxidase-conjugated Affinity Pure Goat Anti-rabbit IgG secondary antibody was obtained from Jackson ImmunoResearch Laboratories Inc. (111-035-003). Knockdown was quantified using Fiji. Data shown is the average and standard deviation of at least two biological replicates. Data was normalized such with mock staurosporine treated as 100%, and 0% as z-VAD-FMK treated.

2.5.10 Tryptophan Fluorescence

Tryptophan fluorescence was detected on a HORIBA Jobin Yvon Fluorolog using FluorEssence software. Fluorescence of 250 nM caspase-3 was measured with excitation at 295 nm and emission from 300 nm to 450 nm. Indoprofen or aspirin was titrated into the sample and the
change in fluorescence at 342 nm was quantified. Samples were incubated for five minutes after titration in order to ensure equilibrium was reached. Data was normalized as caspase-3 titrated with DMSO as 100% and buffer titrated with compound as 0%. This accounts for quenching by DMSO, and for the intrinsic fluorescence of the NSAID. Data shown is the average and standard deviation of at least two independent trials.

2.5.11 COX-independent Apoptosis in *C. elegans*

The phenotypic analysis in animals was conducted using a *C. elegans* mutant, *ced-1(e1735)*, that is defective in cell corpse clearance process. 100 µL of 100 µM fenbufen or indoprofen with 0.5% DMSO were added to the nematode growth media (NGM) plates seeded with bacteria (OP-50), and incubated for three minutes at room temperature for absorption. One hundred L4 animals were placed on the plates and incubated for one day at 20 C to allow them lay embryos. The live embryos were then subjected to cell corpse counting as described previously [156]. Statistical significance was determined using a one-way ANOVA test followed by Tukey’s test to compare compound treated with the 0.5% DMSO control.

2.5.12 Computational Docking Analysis

Docking of NSAIDs with caspase-3 was performed using SwissDock [65, 64]. Compounds (indoprofen: ZINC 391, aspirin: ZINC 53, fenbufen: ZINC 1427) were docked against caspase-3 (PDB 2DKO) [53]. Drug structures were obtained from ZINC, provided by the Irwin and Shoichet Laboratories in the Department of Pharmaceutical Chemistry at the University of California, San Francisco (UCSF) [86, 87, 200]. Clusters were scored using their FullFitness, and the top hit was visualized. Molecular graphics and analyses were performed with the UCSF Chimera package. Chimera is developed by the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco [160].
2.5.13 Chemical Space Analysis

ChemGPS-NP was used to explore the chemical space of screening hits [113]. ChemGPS-NP was developed and made available by the Backlund group at Uppsala University. Using principal component analysis, compounds were compared based on size, aromaticity, polarity, and hydrophobicity.

2.6 Acknowledgements

The authors would like to thank Dr. J. Isaac Godfroy, Dr. Johannes Rudolph, Dr. Corella Detweiler, and Adam Csakai for discussions and scientific insight. Thank you to Martha O’Brien at Promega for her technical expertise. Finally, thank you to Dr. Rosaura Padilla-Salinas, Dr. J. Isaac Godfroy, and Adam Csakai for critical review of the manuscript. The authors declare that they have no competing financial interests. This work was funded by NIH R01 GM101279 (to H. Y.) and R01 GM059083 (to D. X.).

2.7 Supplementary Information
Supplementary Figure 2.1: Computational modeling of fenbufen binding to caspase-3. Caspase-3 (PDB 2DKO) was docked with NSAIDs to predict the binding mode. The catalytic cysteine is pink, tryptophans are yellow, and other residues are colored to depict hydrophobicity (blue more polar, red more hydrophobic). The inset shows the surface of the binding pocket. A representation of the top predicted binding cluster of fenbufen is indicated.

Supplementary Figure 2.2: Validation of a caspase-4 high-throughput screening (HTS) assay. Human caspase-4 (1 μM) was validated for HTS using the Promega Caspase-Glo 9 system (20 μM) in a 384-well format. By comparing the assay signal to noise ratio and variability, a Z’ factor of 0.66 was calculated, indicating a suitable HTS method. Human caspase-4 shows a substantial increase in luminescence when compared to the buffer only control, corresponding to catalytic activity. A DMSO (3% v/v) control was used to account for compound solvents, and does show a decrease in luminescent output. The pan-caspase inhibitor z-VAD-FMK (10 μg/mL) and catalytically inactive caspase-4 do not show activity. Data shown is the average and standard deviation as determined by endpoint luminescence 20 minutes after substrate addition.
Supplementary Figure 2.3: NSAIDs do not inhibit the cysteine protease cathepsin B or luciferase. (A) NSAIDs do not inhibit the enzymatic activity of the cysteine protease cathepsin B, indicating specificity for the caspase family. Data are representative of the average and standard deviation of three independent replicates. Data was normalized such that cathepsin B with solvent is 100%, and 0% as ALLM inhibitor. (B) The Promega CellTiter-Glo viability assay utilizes an identical luciferase enzyme to that found in the Promega Caspase-Glo assay. 100 µM NSAID treatment in HeLa cells does not show compound toxicity or inhibit luciferase. Aspirin appears to be pro-apoptotic, in agreement with previous studies. Data are representative of the average and standard deviation of three biological replicates. Data was normalized with untreated cells at 100%, and lysed cells as 0%.
Supplementary Figure 2.4: Caspase enzymatic analysis is unaffected by COX expression. (A) Cyclooxygenase-2 (COX2), caspase-3 (CASP3), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were knocked down in HeLa cells by siRNA. Western blot confirmed a knockdown of gene expression 48 and 72 hours post-transfection. (B) Caspase-3 activity was then measured using the Promega Caspase-Glo system. CASP3 knockdown showed a substantial decrease in activity relative to the untreated mock control, while the cells with either GAPDH or COX2 knockdown did not. Data are is representative of the average and standard deviation of two biological replicates. Data were is normalized such with mock staurosporine treated as 100%, and 0% as z-VAD-FMK treated.
Supplementary Table 2.1: Caspase-4 inhibition by NSAIDs present in the Prestwick Chemical Library. This list may not be inclusive of all NSAIDs present in the library. Values shown represent the average and standard deviation of two independent replicates.

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<tr>
<th>Name</th>
<th>CAS Number</th>
<th>Caspase-4 Activity</th>
</tr>
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<tbody>
<tr>
<td>Fenbufen</td>
<td>36330-85-5</td>
<td>3.7±2.4%</td>
</tr>
<tr>
<td>Ketorolac Tromethamine</td>
<td>74103-07-4</td>
<td>4.1±0.3%</td>
</tr>
<tr>
<td>Indoprofen</td>
<td>33065-95-7</td>
<td>4.3±0.1%</td>
</tr>
<tr>
<td>Ketoprofen</td>
<td>22071-15-4</td>
<td>6.5±1.3%</td>
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<tr>
<td>Flurbiprofen</td>
<td>5104-49-4</td>
<td>5.8±0.7%</td>
</tr>
<tr>
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<td>60940-34-3</td>
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<td>S-(+)-Ibuprofen</td>
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<td>25.0±1.7%</td>
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<tr>
<td>Niflumic Acid</td>
<td>4394-00-7</td>
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<tr>
<td>(R) Naproxen sodium salt</td>
<td>55837-30-4</td>
<td>33.8±0.1%</td>
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<tr>
<td>Pranoprofen</td>
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<td>31.0±4.1%</td>
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<tr>
<td>Naproxen</td>
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<td>35.2±3.6%</td>
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<tr>
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<td>Sulindac</td>
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<tr>
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<tr>
<td>Piroxicam</td>
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Supplementary Table 2.2: Competition of fenbufen with substrate. Increasing substrate concentrations increase the IC50 value of fenbufen to caspase-4. This suggests fenbufen acts as a competitive inhibitor, and may be competed out by the presence of additional substrate. The data shown are representative of three independent replicates.

<table>
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<th>IC50 (nM)</th>
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<tr>
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<td>3240 ± 200</td>
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<tr>
<td>99.9</td>
<td>4120 ± 300</td>
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Chapter 3

Saccharin Derivatives as Inhibitors of Interferon-Mediated Inflammation

This chapter has been published as:


Chemical synthesis was designed and executed by A. Csakai with support from S. Coulup. High-throughput screening was performed by A. Martinko with support from C. Smith. Further biological experiments were designed and performed by C. Smith with support from E. Davis. Some content has been removed from manuscript to more concisely highlight the contributions of C. Smith, particularly with regard to compound synthesis and characterization. Individual contributions are outlined below using the CRediT taxonomy.


3.1 Abstract

A series of novel, saccharin-based antagonists have been identified for the interferon signaling pathway. Through in vitro high-throughput screening with the Colorado Center for Drug Discovery (C2D2) Pilot Library, we identified hit compound 1, which was the basis for extensive structure-
activity relationship studies. Our efforts produced a lead anti-inflammatory compound, tert-butyl N-(furan-2-ylmethyl)-N-4-[(1,1,3-trioxo-2,3-dihydro-1λ6,2-benzothiazol-2-yl)methyl]benzoylcarbamate CU-CPD103 (103), as a potent inhibitor using an established nitric oxide (NO) signaling assay. With further studies of its inhibitory mechanisms, we demonstrated that 103 carries out this inhibition through the JAK/STAT1 pathway, providing a drug-like small molecule inflammation suppressant for possible therapeutic uses.

3.2 Introduction

Interferons (IFN) are a linchpin of inflammatory signaling, assisting in host defense against pathogens, antigen presentation, and immunomodulation. There are two main classes of interferons: type I (IFN-α/β) and type II (IFN-γ) \[184\]. Interferons bind to their respective transmembrane receptors, inducing dimerization and regulation of inflammatory gene expression through the JAK/STAT signaling pathway \[185\]. Janus kinases (JAKs) are tyrosine kinases that interact with interferon receptors, resulting in recruitment and phosphorylation of signal transduction and activator of transcription (STAT) proteins \[181\]. The JAK/STAT association in turn promotes transcription of pro-inflammatory genes including inducible nitric oxide synthase (iNOS) \[106\].

Interferons coordinate the inflammation response in concert with other innate immune pathways, particularly Toll-like receptor (TLR) signaling. TLRs are pattern recognition receptors that respond to infectious markers and induce a pro-inflammatory response \[202, 25\]. These two pathways synergistically interact in macrophages to elicit an immune response towards infective threats. Macrophage priming with IFN-γ improves the inflammatory response to TLR ligands, such as lipopolysaccharide (LPS) for TLR4. In turn, TLRs upregulate type I interferons, and NF-κB assists in transcription of multiple interferon-inducible genes \[185\]. Of particular interest, the iNOS promoter has binding sites for both STAT1 and NF-κB \[130\]. Transcription of iNOS may be activated by multiple inflammatory factors, including LPS, type I, and type II interferons \[58\]. STAT1 activation by IFN-β has an autocrine/paracrine mechanism preceding iNOS activation, and serves as a necessary transcription factor for synthesis \[58\]. It has been demonstrated that knockdown of
the interferon-α/β receptor 1 (IFNAR1) mitigates iNOS expression, even in the presence of LPS [216]. IFN-γ activates STAT1 through interaction with its cognate receptor IFNGR, upregulating iNOS [5]. Thus, activation of the TLR and interferon interrelated pathways can be regulated at their convergence in JAK/STAT signaling. Nonetheless, it has been a significant challenge to regulate individual pathways with high specificity and selectivity.

Generally, inflammatory signaling is beneficial, and may protect the host against infection. However, in autoimmune pathologies such as systemic lupus erythematosus and multiple sclerosis, an overabundance of interferon signaling can have deleterious effects [177, 155, 13]. Indeed, it has been noted that among centenarian women, polymorphisms that result in decreased IFN-γ may contribute to longevity [125]. Additionally, a small molecule modulator of IFN signaling may provide a useful tool in the study and treatment of autoimmune diseases. Here, we report a small molecule that is able to inhibit the interferon-induced JAK/STAT1 signaling pathway, without compromising the effectiveness of other components of the innate immune system, such as TLRs.

### 3.3 Results and Discussion

#### 3.3.1 C2D2 Compound Library Screening

We started our search for anti-inflammatory agents by looking for inhibitors of LPS-induced TLR4 activation. The Colorado Center for Drug Discovery (C2D2) compound library.(Anon n.d.)

The C2D2 pilot library consists of 2,200 drug-like compounds that represent a variety of diverse and commercially available scaffolds. The initial screen was performed using LPS-activated RAW 264.7 cells in a 96-well plate format to monitor nitric oxide (NO) production. This assay uses a Sandmeyer reaction to convert 2,3-diaminonapthalene to fluorescent napthalenetriazole in the presence of NO. As NO is produced in the TLR inflammatory response, this readout provides information on the extent of TLR signaling. Initial screening yielded 31 hits, representing 5 scaffolds (Figure 3.1). We selected the scaffold with an isothiazolone 1,1-dioxide core for further development as it produced the most numerous and potent hits. Compounds 1 and 2 (Figure 3.1), represent two of the more
potent hits.

Figure 3.1: C2D2 hit molecules. Two representative hit molecules selected from the screening of the Colorado Center for Drug Discovery (C2D2) Pilot Library.

3.3.2 Synthesis and Structure Activity Relationship (SAR)

The synthesis of our hit molecule, 1 (Figure 3.2), began with the oxidative dimerization of commercially available 3 to give dithiodipropionic acid 4 [122]. Conversion to the dithiodipropionyl chloride was completed with thionyl chloride, and subsequent treatment with anhydrous ammonia gave dithiodipropionamide 5 as a mixture of diastereomers [122]. As previously described by Lewis and co-workers, oxidative cyclization was performed with sulfuryl chloride to give an inseparable mixture of 6 and 7 [122]. This mixture of isothiazolinones was then deprotonated with sodium hydride, and alkylated with benzyl chloride 9. The alkylation yields two synthetically useful intermediates 12 and 13, which are easily separable by column chromatography. Chloro-intermediate 12 was oxidized with meta-chloroperoxybenzoic acid (m-CPBA) to give the isothiazolone 1,1-dioxide core, 14.

Chloro-intermediate 14 was coupled to 3,4-dimethylphenylboronic acid, using previously reported conditions to give the parent library hit, 1 [37]. Identical conditions were used to synthesize a related β-naphthalene analog, 15. These conditions failed, however, when 4-pyridinylboronic acid was used. Making use of intermediate 13 with Heck conditions and 4-bromopyridine•HCl success-
Figure 3.2: Isothiazolinone synthesis. i. I₂, KI, NaOH, H₂O, (4 = 99%), ii. 1) SOCl₂ 2) NH₃, CH₂Cl₂ (5 = 66%), iii. SO₂Cl₂, EtOAc (6% 7 = 51%), iv. piperidine or furfurylamine, DIPEA, CH₂Cl₂ (9 = 95%, 10 = 99%), v. Boc₂O, DMAP, THF (11 = 81%), vi. 1) NaH, DMF 2) 9 (12 = 70%, 13 = 67%), vii. m-CPBA, CH₂Cl₂ (14 = 49%), viii. ArB(OH)₂, Pd(dppf)Cl₂•CH₂Cl₂, K₂CO₃, 1,4-dioxane (1 = 47%, 15 = 32%), ix. 4-bromopyridine•HCl, Pd(OAc)₂, KOAc, DMA (16 = 54%), x. m-CPBA, CH₂Cl₂ (17 = 84%)
fully gave 16. The coupling product 16 was fully oxidized at both sulfur and the pyridyl nitrogen to give 17. The synthesis of compounds 15 and 17 provide analogs that exhibit both higher and lower log P values, respectively, as compared to 1. However, 15 only showed a small improvement in potency, and 17 showed greatly reduced potency as compared to 1 (Figure 3.3).

The 2-benzyl-4-methyl-5-phenylisothiazol-3-one 1,1-dioxide core of 1, 15, and 17 was sensitive to a variety of mild reaction conditions. We commonly observed complex mixtures in efforts to synthesize other analogs. The few successfully synthesized analogs exhibited only modest activity, so we thought it best to make more drastic structural changes. In an effort to simplify synthesis and increase stability, we took inspiration from saccharin 18 (Figure 3.4).

Saccharin inspired analogs, 19 and CU-CPD103 (103), were easily synthesized from commercially available saccharin and previously synthesized benzylchlorides 9 and 11. 103 is easily deprotected with TFA to give 20. A significant improvement in activity was observed with intermediate 103, so we sought related analogs 26-43 (Figure 3.5). The lithium aluminum hydride reduction of saccharin 18, previously described by Porter and coworkers, provided 2,3-dihydro-1,1-dioxo-1,2-benzisothiazole 21 [168]. Alkylation of this sultam with 9 and 11 provided analogs 26 and 27. Commercially available 1-isooindolinone 22, phthalimide potassium salt 23, 1,2-benzisothiazol-3(2H)-one 24, and 3-hydroxybenzisoxazole 25, were alkylated with 9 and 11 to give analogs 29, 30, 32, 33, 35, 36, 38, and 39 (Figure 3.5). Treatment of analogs 35 and 36 with m-CPBA at 0 °C gave analogs 38 and 39 respectively, as racemic mixtures. All Boc protected analogs were treated with trifluoroacetic acid in methylene chloride to give deprotected analogs 28, 31, 34, 37, 40 and 43.

The removal of the saccharin series 3-position carbonyl gave us the sultam series 26, 27, and 28 (Figure 3.6). As we had seen with the parent saccharin series, the Boc protected amide 27 was the most potent of the series. 27, however, was unable to match the potency 103. For this reason, we believe the 3-position carbonyl to be somewhat important to either binding or cell permeability. Replacement of the saccharin series 1-position SO₂ moiety with a methylene unit gave us the isoindolinone series 29, 30, and 31. Unlike others, the analog bearing a Boc protected amide 30 shows the lowest potency. All isoindolinone analogs, however, are poor inhibitors. This led us
Figure 3.3: SAR summary and toxicity of 4-Aryl-3-methylisothiazolone 1,1-dioxide analogs. Summary of IC_{50} values and toxicity for structure activity relationship studies. IC_{50} values were obtained using RAW 264.7 cells treated with 20 ng/mL LPS and varying concentrations of compound. A cell viability assay was used to determine cytotoxicity at each tested concentration. LC_{50} is the concentration at which cytotoxicity results in 50% cell viability. The purity of tested compounds was evaluated via $^1$H NMR (> 95% sample purity).

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<tr>
<td>100 μM</td>
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Figure 3.4: Saccharin derived analogs. xi. 1) NaH, DMF 2) 9 (19 = 65%) or 11 (103 = 62%), xii. TFA, CH_2Cl_2 (20 = 87%)
Figure 3.5: Fused ring analogs. xiv. 1) NaH, DMF 2) 9 (26 = 84%) or 11 (27 = 81%), xv. TFA, CH₂Cl₂ (28 = 92%), xvi. 1) NaH, DMF 2) 9 (29 = 57%) or 11 (30 = 10%), xvii. TFA, CH₂Cl₂ (31 = 71%), xviii. 18-crown-6, DMF, 9 (32 = 84%) or 11 (33 = 81%), xix. TFA, CH₂Cl₂ (34 = 81%), xx. 1) NaH, DMF 2) 9 (35 = 48%) or 11 (36 = 27%), xxi. TFA, CH₂Cl₂ (37 = 97%), xii. m-CPBA, CH₂Cl₂, 35 (38 = 85%) or 36 (39 = 79%), xiii. TFA, CH₂Cl₂ (40 = 92%), xxiv. 1) NaH, DMF 2) 9 (41 = 55%) or 11 (42 = 14%), xxv. TFA, CH₂Cl₂ (43 = 87%)
to believe that the 1-position SO$_2$ moiety makes a significant interaction, perhaps as a hydrogen bond acceptor. As previously discussed, the 3-position carbonyl of 103 appears less significant, so the carbonyl of the isoindolinone 30 may be making a weaker hydrogen bond interaction as a poor bioisostere of the SO$_2$ moiety. Otherwise, the isoindolinone series would likely have even lower potency.

To further investigate the role of the SO$_2$ moiety, the 3-position carbonyl of 103 was maintained, and the SO$_2$ moiety replaced with an additional carbonyl, as shown with the phthalimide analogs 32, 33, and 34. The activity of 103 is nearly equal with 33. As with the parent saccharin analogs, 32 and 34 show little or no activity. This suggests that a carbonyl is a suitable bioisostere to the SO$_2$ moiety, so long as the 3-position carbonyl is intact, unlike 30.

Furthermore, a loss of one, or both, oxygen atoms from the SO$_2$ moiety of 103 results in a complete loss of activity, as seen with 36 and 39. As seen before, the deprotected species 37 and 40 show poor activity. As expected, the piperidine amide analog, 38 shows a further decrease in potency. However, 35 inexplicably shows comparable potency as compared to 103, and is a great improvement over the related analog 26. When the unoxidized sulfur of 36 is replaced with a smaller oxygen atom 42, the activity is once again comparable to that of 103. Again, the deprotected analog 43 shows significantly lower activity, though 41 shows some modest activity.

We further investigated the role of the SO$_2$ moiety by synthesizing the fully reduced and partially reduced variants, 35-40. If the SO$_2$ moiety does play the role of hydrogen bond acceptor, then these reduced analogs would be less efficacious. As expected, we observed either a loss in activity, or no change in activity from analogs 36-40. The fully reduced analog 35, however, inexplicably shows activity comparable to that of 103. We also synthesized 41-43 as smaller, more basic bioisosteres of 35-37. This modification resulted with a drop in activity for 41 as compared to 35, and a great improvement with 42 as compared to 36. In fact, the potency of 42 is comparable to that of 103, 27, and 33. This suggests that an oxygen hydrogen bond acceptor is important to activity. The steric constrains of this position seem flexible, given two of the more active analogs in this series, 103 and 42, are the largest and smallest of the series, respectively. The less active of
**Figure 3.6: SAR summary and toxicity of saccharin inspired analogs.** Summary of IC$_{50}$ values and toxicity for structure activity relationship studies. IC$_{50}$ values were obtained using RAW 264.7 cells treated with 20 ng/mL LPS and varying concentrations of compound. A cell viability assay was used to determine cytotoxicity at each tested concentration. LC$_{50}$ is the concentration at which cytotoxicity results in 50% cell viability. The purity of tested compounds was evaluated via $^1$H NMR (>95% sample purity).
this series are of varying size, but are consistently less basic than the more active analogs.

From this series of molecules, there are five analogs that have IC$_{50}$ values of less than or roughly equal to 10 µM. Four of these five analogs have the same Boc-protected furfurylamide (103, 27, 33, and 42). All four of these analogs lose significant activity when deprotected. This implies that there is an important interaction(s) being made with the Boc group, and/or that an amide N-H causes a deleterious interaction. To better understand this observation, analogs 50, 52, 54, 55, 56, 57, and 58 were synthesized from the corresponding carboxylic acid 45 (Figure [3.7](#221)).

Interestingly, intermediate ester 44 showed modest activity (Figure [3.6](#)), while intermediate carboxylic acid 45 shows no activity. This may imply that the previously successful analogs bearing Boc groups might be benefiting from hydrophobic interactions with the tertiary butyl moiety. Boc protected amides 52 and 56, however, have shown very poor activity. Initial efforts to synthesize 56 produced the double-Boc protected amide 50, which also has poor activity, suggesting that the furan substituent was necessary. We also investigated a very minor change by synthesizing the extended linker analog 54. Surprisingly, a significant loss in activity was observed.

To test if the presence of an acidic amide N-H could be causing a negative effect on activity, we synthesized 55. However, we observed significantly lower potency, perhaps suggesting that the rotational constraints of a tertiary amide are not conducive to activity. Given this observation, we speculated that perhaps a carbonyl component was still required for activity. However, the N-methylcarbamate and N-acyl analogs, 57 and 58, were significantly less potent than even 20. We concluded that the furfurylamide component was essential, and that Boc protection was the optimal substituent. Based on these results, 103 was selected as our lead compound.

### 3.3.3 Anti-Inflammatory Mechanism Studies of 103

Our initial screen identified compounds that could inhibit the LPS-induced inflammatory response. To determine if 103 specifically targets TLRs, NO signaling was assessed with three different TLR ligands. LPS (TLR4), Poly I:C (TLR3), and Pam$_2$CSK$_4$ (TLR2/6) were chosen
Figure 3.7: Amide modifications. xxvi. 1) NaH, DMF 2) 4-bromomethylbenzoic acid tert-butyl ester (44 = 62%), xxvii. TFA, CH₂Cl₂ (45 = 98%), xxviii. SOCl₂ (>99%), xxiv. Procedure A: 46 in CH₂Cl₂, then ammonia in THF (49 = 92%), or methylamine in THF (51 = 79%), or 2-furan-2-y1-ethylamine and DIPEA (53 = 85%), or N-methylfurfurylamine and DIPEA (55 = 86%). Procedure B: 1) tert-butylcarbamate, LiHMDS, THF 2) 46 in CH₂Cl₂. Procedure C: 1) NaH in DMF, then 47 or 48 2) 46 in CH₂Cl₂ (57 = 41%, 58 = 17%), xxv. Boc₂O, DMAP, CH₂Cl₂ (50 = 73%), xxvi. Boc₂O, DMAP, CH₂Cl₂ (52 = 81%), xxvii. Boc₂O, DMAP, CH₂Cl₂ (54 = 33%)
Figure 3.8: SAR summary and toxicity of saccharin derived analogs. Summary of IC\textsubscript{50} values and toxicity for structure activity relationship studies. IC\textsubscript{50} values were obtained using RAW 264.7 cells treated with 20 ng/mL LPS and varying concentrations of compound. A cell viability assay was used to determine cytotoxicity at each tested concentration. LC\textsubscript{50} is the concentration at which cytotoxicity results in 50% cell viability. The purity of tested compounds was evaluated via \textsuperscript{1}H NMR (>95% sample purity).

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<td>-NHCH\textsubscript{3}</td>
<td>58</td>
<td>42.6 ± 5.19 μM  &gt; 100 μM</td>
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to encompass the most variety in signaling, including differences in TLR localization and adaptor proteins (Figure 3.3). As Figure 3.9 shows, 103 inhibits NO signaling regardless of ligand treatment. This suggests that 103 does not bind specifically to an individual TLR, but rather inhibits a common downstream factor of these TLRs. The dose-response curves for all three ligands show comparable inhibition, with minor deviations due to TLR expression levels and the effectiveness of the ligand to induce inflammation. Primary macrophage cells demonstrated the same behavior as RAW 264.7 cells, with 103 inhibiting LPS signaling with an IC\textsubscript{50} value of 9.61 ± 1.45 µM (Figure 3.2). It is important to note that 103 shows no cytotoxicity at concentrations up to 100 µM (Figure 3.4).

To further confirm that iNOS was being down regulated by treatment with 103, quantitative real time polymerase chain reaction (RT-PCR) and western blot experiments were performed. RT-PCR data was obtained using RAW 264.7 cells treated with LPS and varying concentrations of 103. Figure 3.10 demonstrates that treatment with 103 decreases iNOS mRNA in a dose-dependent fashion. Western blots were performed with a pan NOS antibody, and again iNOS is seen to decrease in a dose-dependent fashion (Figure 3.11), indicating that 103 suppresses iNOS at mRNA, protein, and cell signaling levels. While LPS was used as the inflammation-inducing ligand in the NO production assay to maintain consistency, its effects are indirect. Therefore, a secondary assay that monitors IFN-γ induced mRNA changes was carried out to confirm the validity of the NO assay as the primary readout for understanding SAR and compound optimization (Figure 3.7). These results demonstrated the same trend of inhibition for 103, 36, and 49. Compound 103 is able to inhibit greater than 70% of IFN-γ induced mRNA, whereas 36 and 49 show a weaker potency, with maximum inhibition of 50%. Importantly, these results corroborate the IC\textsubscript{50} values determined in the previously described NO production assay.

Regardless of their ligand or localization, all TLRs activate NF-κB (Figure 3.3). In order to test the inhibitory effects of 103 on NF-κB activity, a secreted embryonic alkaline phosphatase (SEAP) assay was performed in HEK 293T cells. When tested at concentrations up to 100 µM, the compound did not down regulate NF-κB activation through TLR3 or TLR4 (Figure 3.5). To determine if any modulation occurs through other NF-κB pathways, TNF-α was used to activate
Figure 3.9: 103 inhibits NO signaling mediated by different TLRs with comparable IC₅₀s. RAW 264.7 cells were treated with LPS (TLR4 ligand), Poly I:C (TLR3 ligand), or Pam2CSK4 (TLR2/6 ligand). The IC₅₀ values are 2.61 ± 0.40 µM, 10.9 ± 0.74 µM, and 1.69 ± 0.43 µM, respectively. Treatment with 103 decreased NO production with all TLR ligands in a dose-dependent fashion. These results demonstrate that 103 does not specifically inhibit a particular TLR, but rather, a common downstream effector. Data was normalized [(raw data-untreated cells)/(TLR agonist + solvent control-untreated cells)] such that TLR agonist + solvent is 100% activation, and untreated cells are 0% activation. Data points shown are the average of nine replicates, with error bars represented as the standard deviation.
Figure 3.10: 103 treatment decreases iNOS mRNA in a dose-dependent fashion. RAW 264.7 cells were incubated with 20 ng/mL LPS and varying concentrations of 103 for 20 hours. Data is shown with ligand-induced cells normalized to a fold change of 1. Treatment with 103 decreases iNOS mRNA up to 67%. Data shown is the average quantification of three biological replicates, each in technical duplicate, with error bars represented as the standard deviation. ** p ≤ 0.01, **** p ≤ 0.0001.
Figure 3.11: A) 103 treatment reduces iNOS protein expression in a dose-dependent fashion. The iNOS protein is induced by LPS treatment, and decreases with compound treatment, suggesting that compound reduces the inflammation that results in iNOS expression. The image shown is a representative image, with brightness and contrast adjusted for clarity. B) Quantification of iNOS western blot. Data was normalized to GAPDH as a loading control. Data shown is the average quantification of three biological replicates, with error bars represented as the standard deviation. ***p ≤ 0.001.
NF-κB signaling. As seen in the Figure 3.4, NF-κB signaling through tumor necrosis factor receptor (TNFR) is also unaffected. This data suggests that 103 does not directly modulate the TLR signaling pathway at any point, as NF-κB is essential to all TLR signaling. We next sought to confirm this result through observation of NF-κB-induced cytokines, particularly TNF-α. A commercially available enzyme-linked immunosorbent assay (ELISA) was used to measure TNF-α in RAW 264.7 cells. Figure 3.6 in the supplementary information shows that there was no change in TNF-α cytokine levels with compound treatment. These results confirm in two cell types that there is no modulation of NF-κB by 103, regardless of ligand or signaling pathway. However, previous results demonstrated that TLR-induced NO activation is inhibited by 103. The iNOS promoter has binding sites for both NF-κB and STAT1. Since NF-κB activation is not being affected with 103 treatment, the inhibition of iNOS was therefore likely to occur within the JAK/STAT1 pathway.

As no direct antagonism was observed through TLRs and NF-κB, additional tests were carried out to identify the potential anti-inflammatory mechanism of 103. The interferon I (IFN-α/β) and interferon II (IFN-γ) pathways cause upregulation of iNOS, which results in production of NO. As such, we speculated that observed NO inhibition might occur through inhibition of the JAK/STAT signaling pathway. To test this hypothesis, IFN-γ was used as a ligand to activate iNOS in RAW 264.7 cells. Inhibition of NO occurred in a dose-dependent fashion with treatment of 103. The IC\(_{50}\) value with IFN-γ is 7.88 ± 1.25 µM, which corroborates the IC\(_{50}\) value of LPS (Figure 3.12). This indicated that the JAK/STAT1 pathway is involved in the inhibitory function of 103. Additionally, as TLR activation results in production of type I interferons, synonymous inhibition with IFN-γ proposes a shared target between these two pathways. Thus, it is likely that the molecular target of 103 lies in the interferon-induced STAT1 pathway. Additionally, a commercial JAK/STAT RT-PCR array was used to determine if JAK/STAT pathway signaling is by and large modulated with 103 treatment. A summary of the results is available in Table 3.1. The modified genes, including NOS2, Cebpβ, and Gtp1 suggest that 103 modulates STAT1 signaling \[109, 176, 19\]. Additional validation is required to target the specific site of action for 103. Taken together, our results provide consistent evidence that 103 functions through the interferon-induced JAK/STAT1 pathway.
Figure 3.12: 103 inhibits IFN-γ signaling in a dose-dependent fashion. RAW 264.7 cells were treated with 5 ng/mL IFN-γ to activate JAK/STAT1 signaling. Treatment with 103 decreased NO production in a dose-dependent fashion. These results suggest that 103 inhibits NO through the JAK/STAT1 pathway. Data was normalized \((\text{raw data-untreated cells})/(\text{TLR agonist + solvent control-untreated cells})\) such that TLR ligand + solvent is 100% activation, and untreated cells are 0% activation. Data points shown are the average of nine replicates, with error bars represented as the standard deviation.
signaling pathway, suppressing iNOS.

3.4 Conclusion

In summary, we report the identification of a group of novel IFN inhibitors based on a saccharine core. Extensive SAR studies have shown a narrow tolerance for change at the lone amido position. A Boc protected furfurylamide has proven to be the most consistently successful substituent. Our results demonstrate that 103 is a potent inhibitor of iNOS on both an mRNA and protein expression level. However, this occurs without NF-κB modulation, indicating a TLR-independent mechanism. Further biochemical studies imply potential inhibition in the STAT1 pathway, as this is shared between type I and II interferons, and associated genes were observed to change via PCR array. 103 may provide therapeutic insight for inflammatory diseases such as systemic lupus erythematosus and multiple sclerosis.

3.5 Materials and Methods

3.5.1 General Chemistry Methods and Synthesis

For the sake of brevity, these methods have been omitted from this chapter. See the full manuscript for a complete description.

3.5.2 Nitric Oxide Structure Activity Relationship Studies

Raw 264.7 cells were plated on day one at 375,000 cells/mL in a tissue culture treated 96-well plate. Cells were plated in supplemented RPMI medium (10% fetal bovine serum, 1% L-glutamine, 1% Penicillin/Streptomycin) and incubated at 37 C. On day two, supplemented media was removed from the cells, and unsupplemented RPMI was added. Cells were treated with 20 ng/mL LPS (Invivogen) and varying concentrations of the appropriate compound. Samples were incubated with compound for 18-24 hours at 37 C. On day three, 90 µL of media was transferred to a black 96-well plate (ThermoScientific). 10 µL of 0.05 mg/mL 2,3-diaminonaphthalene (Sigma)
in 0.62 M HCl was added to the media. The plate was covered in aluminum foil and shaken at room temperature for 15 min. A Beckman Coulter DTX 880 Multimode Detector was used to quantify results. Samples were excited at 360 nm and emission was measured at 430 nm. Data was normalized as (well raw data-untreated cells)/(ligand + solvent control-untreated cells) such that ligand + solvent is 100% activation, and untreated cells are 0% activation. Experiment was conducted with a minimum of three biological replicates, in triplicate. IC_{50} values were obtained by using OriginPro 9 to calculate a dose response curve.

An assessment of nitric oxide production was also conducted using primary rat macrophage cells donated by Linda Watkins’ lab at the University of Colorado at Boulder. The experiment was performed as described above, with 500 ng/mL LPS treatment.

### 3.5.3 TLR Specificity

As discussed above, Raw 264.7 cells were used in a nitric oxide signaling assay. Cells were treated with one of three ligands: 20 ng/mL LPS, 2.5 µg/mL Poly I:C, or 200 ng/mL Pam2CSK4. Data was normalized as (well raw data-untreated cells)/(ligand + solvent control-untreated cells) such that ligand + solvent is 100% activation, and untreated cells are 0% activation. Experiment was conducted with a minimum of three biological replicates, in triplicate.

### 3.5.4 Secreted Embryonic Alkaline Phosphatase (SEAP) Reporter Assay

TLR3 or TLR4 transfected HEK Blue cells were designed in house. These cells have a secreted embryonic alkaline phosphatase (SEAP) reporter gene with has been stably transfected along with the appropriate TLR and accessory proteins. 1E5 HEK Blue cells were plated in a 96-well plate in supplemented DMEM (10% fetal bovine serum, 1% Penicillin/streptomycin, 1% L-glutamine) on day one. Cells are incubated overnight at 37 C. On day 2, media was replaced with unsupplemented DMEM and appropriate concentrations of compound. Signaling was activated with either 20 ng/mL LPS, 2.5 µg/mL Poly I:C, or 50 ng/mL TNF-α. Cells were again incubated overnight at 37 C. After 18-24 hours of incubation, 20 µL of media was removed and placed in
a new 96-well plate. 180 µL of Quanti-Blue (Invivogen) was added to the media, and the plate incubated at 37 C until color change was observed (30 min-2 hours). Plates were then quantified on a Beckman-Coulter DTX 880 Multimode Detector by measuring absorbance at 620 nm. Data was normalized as (well raw data-untreated cells)/(ligand + solvent control-untreated cells) such that ligand + solvent is 100% activation, and untreated cells are 0% activation. Experiment was conducted with a minimum of three biological replicates, in triplicate.

3.5.5 Enzyme-linked Immunosorbent Assay (ELISA)

ELISA was performed using a kit from BD Biosciences to measure TNF-α expression levels. Raw 246.7 cells were treated as described in the nitric oxide assay. 75 µL of supernatant from nitric oxide plates was taken and used for ELISA. Assay was performed per manufacturer’s specifications. Experiment was conducted with a minimum of three biological replicates, in triplicate.

3.5.6 Polymerase Chain Reaction and Arrays

2e7 Raw 264.7 cells were plated in a 6 cm dish on day one in supplemented RPMI. On day two, cells were treated with 20 ng/mL LPS and varying concentrations of 103, diluted in unsupplemented RPMI. Cells were allowed to incubate at 37C for 20 hours. After 20 hours, cells were scraped and resuspended in PBS. RNA was extracted using the miRNeasy Mini Kit from QIAGen (No. 1038703). Reverse transcription was performed using the Qiagen RT First Strand Kit per manufacturers specifications. Reverse transcription was performed using a BioRad T1000 or Techne TC 3000G thermalcycler. qPCR was performed using soAdvanced SYBR Green Supermix from BioRad. RT qPCR NOS2 primers were obtained from QIAGEN. GAPDH primers were obtained from SABioscience. ΔCq calculated as (Sample primer average Cq-Control primer average Cq). ΔΔCq calculated as (Sample ΔCq-Control ΔCq). For NOS2 PCR, GAPDH was used as a reference gene. Individual PCR experiments were conducted with a minimum of three biological replicates, in triplicate. Values for statistical significance were calculated using Dunnett’s Multiple Comparisons Test, where all samples are compared to the control.
An additional experiment was carried out as above with 5 ng/mL IFN-γ as the ligand. Cells were incubated with IFN-γ and varying concentrations of 103, 36, or 49 for 16 hours. These compounds were chosen to represent varied SAR and IC₅₀ values. The results corroborated the PCR results with LPS activation, demonstrating a validated biological system with both ligands. The results are summarized below in Figure 3.7. Values for statistical significance were calculated using Dunnett’s Multiple Comparisons Test to show dose-dependence relative to the 1 µM treatment.

For PCR arrays, data was normalized to the average of four housekeeping genes provided as internal controls. Fold change calculated as \((2^{-\Delta\Delta C_q})\), such that the LPS control is normalized to 1. Array results were considered to be significantly different with compound treatment if mRNA expression was >2 fold or <0.5 fold. GAPDH, B2M, and HPRT1 serve as normalization controls for the array, and do not show a significant change in expression. Two independent biological replicates were performed, and the fold change averaged. The results which were duplicated, and which show the requisite fold changes are listed below in Table S3.1.

### 3.5.7 Western Blot

1E6 Raw 264.7 cells were plated in a 6-well plate in supplemented RPMI (10% fetal bovine serum, 1% L-glutamine, 1% Penicillin/Streptomycin) and incubated overnight at 37 C. On day two, media was replaced with unsupplemented RPMI. Cells were treated with 20 ng/mL LPS and appropriate concentrations of 103 and incubated overnight at 37 C. On day 3, cells were harvested with a cell lifter in PBS and lysed with SDS Buffer. Samples were heated at 95 C for 5 minutes. A 10% SDS-PAGE gel was run at 180 V. Bands were transferred to 0.2 µm pore size nitrocellulose (Invitrogen) at 100 mAmps for 1 hour. Nitrocellulose was blocked for 1 hour in 5% milk in Tris-buffered saline with Tween-20 (TBST). Primary pan NOS antibody (Cell Signaling Technology) was added overnight at 4 C (1:2000 dilution). Goat anti-rabbit IgG HRP conjugated secondary antibody (Invitrogen) was added and incubated at room temperature for two hours (1:2000 dilution). Nitrocellulose was developed using SuperSignal West Pico Chemiluminescent substrate (ThermoScientific) for less than thirty seconds. Images were obtained using a GE LAS 4000 from
the University of Colorado Biochemistry Instrument Core. Values for statistical significance were calculated using Tukey’s Multiple Comparisons Test.

3.6 Acknowledgements

We thank the National Institutes of Health (grants GM101279 and GM103843) for financial support. We thank Noah Kastelowitz for critical reading of the manuscript and assistance with statistical calculations.
3.7 Supplementary Information

Supplementary Figure 3.1: C2D2 hit scaffolds. The hit scaffolds (I-V), found from screening the Pilot Library provided by the Colorado Center for Drug Discovery (C2D2).
Supplementary Figure 3.2: 103 inhibits NO signaling in primary macrophages. The IC$_{50}$ value is 9.61 ± 1.45 µM. Data was normalized [(raw data-untreated cells)/(TLR agonist + solvent control-untreated cells)] such that TLR agonist + solvent is 100% activation, and untreated cells are 0% activation. Data points shown are the average of three replicates, with error bars represented as the standard deviation.
Supplementary Figure 3.3: TLR signaling pathway schematic. This figure was reprinted from Buchanan et al. with permission from the author [25]. TLRs homodimerize or heterodimerize in response to ligand binding, activating a signaling cascade that culminates in NF-B activation and cytokine production.
Supplementary Figure 3.4: 103 is non-toxic at concentrations up to 100 µM. Raw 264.7 cells were prepared as described above for the nitric oxide assay. After supernatant was removed for nitric oxide testing, cells were treated with a 1:10 dilution of cell proliferation reagent WST1 (Roche). Cells were incubated at 37 C until a color change was observed (30 min-2 hours). Data was quantified on a Beckman-Coulter DTX 880 Multimode Detector using absorbance at 450 nm. Data was normalized as (raw data-100% DMSO)/(untreated cells-100% DMSO) such that untreated cells are 100% survival, and 100% DMSO is 0% survival. Experiment was conducted with three biological replicates, in triplicate. Error bars represent the standard deviation of nine replicates.
Supplementary Figure 3.5: 103 does not affect the NF-κB pathway. HEK 293T cells that have been stably transfected with a SEAP reporter gene were treated with LPS, Poly IC, or TNF-α. No dose dependent change was observed. This suggests that 103 does not modulate NF-κB transcription. Data was normalized as [(raw data-untreated cells)/(ligand + solvent control-untreated cells)] such that ligand + solvent is 100% activation, and untreated cells are 0% activation. Error bars represent the standard deviation of nine replicates.
Supplementary Figure 3.6: 103 does not decrease TNF-α production. Data was normalized as \([\text{raw data-untreated cells)}/(\text{ligand + solvent control-untreated cells})]\) such that ligand + solvent is 100% activation, and untreated cells are 0% activation. Error bars represent standard deviation of nine replicates.
Supplementary Figure 3.7: 103 inhibits IFN-γ induced iNOS. RAW 264.7 cells were incubated with 5 ng/mL IFN-γ and varying concentrations of 103, 36, or 49 for 16 hours. Data is shown with ligand-induced cells normalized to a fold change of 1. Treatment with 103 decreases iNOS mRNA up to 75%, whereas weaker antagonists 36 and 49 show maximum inhibition of 50%. Data shown is the average quantification of three biological replicates, each in technical duplicate, with error bars represented as the standard deviation. * p ≤ 0.05, *** p ≤ 0.001. These results are consistent with the IC_{50} values determined using the primary NO production assay (2.61 ± 0.40 µM for 103, 68.0 ± 30.5 µM for 36, and 50.6 ± 10.3 µM for 49), confirming the validity of the SAR studies.
Supplementary Table 3.1: JAK/STAT PCR array. The genes listed had an mRNA change of >2 fold or <0.5 fold when compared to LPS control array. The listed genes are consistent with interferon modulation in the JAK/STAT pathway.

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Chapter 4

The Development of Antimicrobial α-AApeptides that Supress Pro-Inflammatory Immune Responses

This chapter has been published as:


The manuscript was written, prepared, and revised jointly by C. Smith, S. Padhee, and J. Cai. The concept of AApeptides is the primary intellectual work of the Cai research group at the University of South Florida. Synthesis and design of AApeptides was performed by S. Padhee and the Cai research group. Cellular assays (Figures 4.3, 4.4, 4.5, S4.5, S4.6, S4.7) were conceived, designed, performed, and analyzed by C. Smith with assistance from N. Manoj.

4.1 Introduction

Resistance to conventional antibiotics is a significant threat to global public health [70]. As an alternative approach, host-defense peptides (HDPs) have emerged as promising antibiotic agents. HDPs are present in virtually all life forms, acting as the first line of defense against microbial infection [70, 135]. The current paradigm is that HDPs initially adopt globally amphipathic structures, which in turn disrupt negatively charged bacterial membranes [70, 135]. Intriguingly, in addition to direct bacterial killing, some HDPs are reported to be able to modulate the innate immune response
by antagonizing Toll-like receptors (TLRs) [141]. Immunomodulatory HDPs suppress production of harmful pro-inflammatory cytokines, thereby preventing complications from infection [70]. For instance, the human cathelicidin LL-37 can inhibit the induction of tumor necrosis factor-α (TNF-α) by Gram-positive and Gram-negative bacterial infections [22, 188].

Despite their promising potential, HDPs face obstacles for further development due to their susceptibility to protease degradation [70]. There is significant interest in the development of unnatural mimics of HDPs, including peptoids [149, 34, 33, 82, 95, 96], β-peptides [99, 97, 98], and other classes of oligomers and polymers [32, 36, 60, 77, 79, 80, 124, 108, 145, 146, 198, 208, 232]. Antimicrobial peptidomimetics function similarly to HDPs in their ability to assume globally amphipathic structures, embracing segregated hydrophobic and cationic patches that facilitate bacterial membrane disruption. We have previously reported a group of unnatural HDP mimics, α-AApeptides (N-acylated-N-aminoethyl peptides), as effective antimicrobial agents against various bacterial strains. Further lipidation has been shown to improve the antimicrobial activities of α-AApeptides and reduce their antibiotic resistance. Herein, we report the development of modified α-AApeptides through simultaneous lipidation and cyclization. These novel cyclic lipo-α-AApeptides demonstrate significantly improved potency and broad-spectrum activity against a range of clinically relevant Gram-positive and Gram-negative bacteria. Furthermore, the lead cyclic lipo-α-AApeptide is the first of its kind shown to mimic some HDPs by antagonizing TLR4-induced nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and nitric oxide (NO) signaling responses, as well as inhibiting production of pro-inflammatory cytokines such as TNF-α [22, 188]. The linear analogue of the lead cyclic lipo-α-AApeptide shows weaker antimicrobial and inferior anti-inflammatory activity, indicating that both lipidation and cyclization are critical for the dual functionality. As such, cyclic lipo-α-AApeptides could emerge as a new class of antibiotic agents with both direct bacterial toxicity and immunomodulation ability.
4.2 Results and Discussion

4.2.1 Design and Synthesis of α-AApeptides

Our current design is based on the finding that cyclization decreases sequence flexibility and stabilizes amphipathic structures, leading to antimicrobial agents with improved potency \[198, 145, 146\]. Clinically approved cyclic lipopeptides daptomycin and polymyxin are used to treat infections caused by Gram-positive and Gram-negative bacteria, respectively \[228, 237\]. However, the development of antimicrobial peptidomimetics with lipo-cyclic motifs is rare. We have previously shown that antimicrobial α-AApeptides can be designed in a straightforward manner by joining α-AA peptide building blocks together. These building blocks assume amphipathic conformations upon interaction with bacterial membranes. Thus, we hypothesize that cyclic α-AApeptides containing amphipathic α-AApeptide building blocks and a membrane-directing lipid tail will lead to improved antimicrobials.

Previous studies have shown that both the number of amphipathic building blocks and the length of the lipid tails affect antimicrobial activity. This is because the specific interactions between bacterial membranes and peptide sequences are largely governed by overall hydrophobicity and cationic charges. In addition, the length of the lipid tails is believed to be important for penetrating bacterial membranes. Thus, a series of cyclic α-AApeptides containing different numbers of amphipathic α-AApeptide building blocks and different lengths of lipid tails were prepared following the synthetic route shown in Figure 4.1.

We designed amphipathic α-AApeptide building blocks that possess a cationic Lys side chain and hydrophobic lipid tails of varying lengths. Thus, different lipid tails can be conveniently introduced to cyclic α-AApeptides on the solid phase. C6-C16 alkyl tails are most commonly observed in lipopeptide antibiotics \[70\]. Therefore, we chose C6, C12, and C16 lipid tails to study their impact on the antimicrobial activity of cyclic lipo-α-AApeptides. The synthesis was accomplished by first attaching an α-AApeptide building block to the solid support, followed by the addition of an allyl carboxylate ester-containing AApeptide building block, which enables cyclization directly
Figure 4.1: Solid Phase Synthesis of Cyclic Lipo-α-AApeptides
on the solid phase. After the α-AApeptide oligomers were assembled, the allyl protecting group was removed by using Pd(PPh3)4. Following removal of the Fmoc group, the sequence was cyclized in the presence of PyBop. Such an approach circumvents the need to cyclize the sequences in solution after cleavage from the solid support, greatly simplifying the purification process.

### 4.2.2 Antimicrobial and Hemolytic Activities of α-AApeptides

We then tested these sequences against several clinically relevant, multidrug-resistant bacteria, including both Gram-positive and Gram-negative strains (Table 4.1). Both the number of cationic charges and the extent of hydrophobicity have been shown to be important for potent antimicrobial activity. Cyclic lipo-α-AApeptide 1, which contains three amphiphilic building blocks in the ring, as well as a C6 lipid tail, did not show any antimicrobial activity under the tested conditions. However, with the same ring structure, replacement of the C6 lipid tail in 1 with a C12 lipid tail yielded 2, which showed good activity against Gram-positive bacteria. Further increase of the lipid tail length to C16 led to 3, which demonstrated excellent potency against both Gram-positive and Gram-negative bacteria. The relationship between tail length and potency could suggest that short lipid tails cannot penetrate bacterial membranes. This is especially relevant for Gram-negative bacteria, which have both outer and inner membranes. A similar correlation between lipid tail length and potency was also observed for 5 and 6. Additionally, it seems that increasing ring size does not significantly affect antimicrobial activity, as seen with 3, 4, and 6. In fact, 3 has a smaller ring size and has the most potent antimicrobial activity against Gram-positive bacteria. Our results indicate that we might have reached an optimal ring size. This is also consistent with the findings that many natural cyclic peptide antimicrobial agents also contain six to eight amino acid residues in their ring structures [228, 237]. When compared to the previously developed linear α-AApeptide NB-119-2 and the HDP analogue pexiganan, this new class of cyclic lipo-α-AApeptides is more potent.

The capability of cyclic lipo-α-AApeptides to mimic HDPs and disrupt bacterial membranes was assessed by fluorescence microscopy. MRSA ATCC 33591 was stained with the membrane-
Table 4.1: The antimicrobial and hemolytic activities of cyclic lipo-α-AApeptides. The microbial organisms used are *K. pneumoniae* (ATCC 13383), and *P. aeruginosa* (ATCC 27853), Methicillin-resistant *S. epidermidis* (RP62A), Vancomycin-resistant *E. faecalis* (ATCC 700802), Methicillin-resistant *S. aureus* (ATCC 33591). The minimum inhibitory concentration (MIC) is the lowest concentration that completely inhibits growth after 20 h. HC<sub>50</sub> is the concentration causing 50% hemolysis. NB-119-2 and Pexiganan are listed for comparison.

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permeable dye 4,6-diamidino-2-phenylindole (DAPI) and the non-membrane-permeable dye propidium iodide (PI) in the presence or absence of cyclic lipo-α-AApeptide 3 (Figure 4.2). Upon treatment with 3, both DAPI and PI were able to permeate MRSA membranes, indicating perturbed integrity. The aggregation of bacteria after treatment might be due to the loss of membrane potential, consistent with the observation of membrane disruption [153, 232].

4.2.2.1 Anti-inflammatory Properties of α-AApeptides

In addition to potent antimicrobial activity, an interesting behavior of HDPs is that they might repress inflammation, providing a new approach for the treatment of bacterium-induced inflammatory disorders [22, 188]. Our recent data have shown that certain HDP-mimicking γ-AApeptides also have the similar functions. Therefore, as structural and functional mimics of HDPs, cyclic lipo-α-AApeptides are proposed to harness the innate immune system in a similar fashion. As such, we investigated the ability of 3 to modulate immune responses through TLR4 signaling. TLRs are evolutionarily conserved type I transmembrane proteins responsible for the inflammatory response to invasive pathogens. The native ligand for TLR4 is lipopolysaccharide (LPS), a component of Gram-negative bacterial cell walls. Through TLR4, LPS activates the NF-κB signaling pathway and downstream production of cytokines such as TNF-α. Currently, there is great interest in developing molecules to modulate TLR signaling as therapeutic candidates for immunity malfunctions. As an elegant example, Tew and co-workers demonstrated that antimicrobial agents might exhibit immunomodulatory activities [209, 195].

To test the ability of cyclic lipo-α-AApeptides to modulate TLR4 signaling, we first tested the inhibitory effects of the lead compound, 3, in a nitric oxide (NO) production assay (Figure 4.3A) [30]. NO produced in the downstream inflammatory signaling cascade for all TLRs plays an important role in immunological processes such as the generation of free radical bacterial toxins and regulation of phagocytosis [133]. RAW 264.7 murine monocyte macrophage cells were treated with varying concentrations of 3 in the presence of 20 ng/mL LPS. As shown in Figure 4.3A, 3 is capable of inhibiting TLR4-induced NO production in a dose-dependent fashion, with an IC₅₀
Figure 4.2: Fluorescence micrographs of MRSA treated with 5 µg/mL cyclic lipo–AApeptide 3 for 2 h. A1) control, no treatment, DAPI-stained; A2) control, no treatment, PI-stained; A3) control, no treatment, merged view. B1) treatment, DAPI-stained; B2) treatment with 3, PI stained; B3) treatment with 3, merged view.
value of 3.06±0.21 µM. Importantly, 3 showed negligible cytotoxicity up to 100 µM (Figure 4.3B), confirming that its TLR4 suppression is not due to cytotoxicity [170, 219].

Figure 4.3: Anti-inflammatory and cytotoxic properties of 3. A) Reduced TLR4-induced NO production in the presence of 3. RAW 264.7 cells were treated with 20 ng/mL LPS and varying concentrations of 3. Data was normalized to 20 ng/mL LPS as 100% activation and untreated cells as 0% activation. Our results indicate that 3 reduces NO production in a dose-dependent fashion. B) Compound 3 is nontoxic up to 100 µM, as demonstrated by a crystal violet cell viability assay. Data is normalized with untreated cells as 100% survival and 100% DMSO as 0% survival.

To further confirm the effect of 3 on TLR signaling, a previously established secreted embryonic alkaline phosphatase (SEAP) assay was used to assess NF-κB activation in TLR4-overexpressing human embryonic kidney (HEK293) cells [85]. NF-κB transcription is directly correlated to TLR4 activation, as signaling results in NF-κB nuclear translocation [244]. Compound 3 inhibited NF-κB with an IC_{50} value of 2.27±0.08 µM (Figure 4.4A). Furthermore, production of the proinflammatory cytokine TNF-α was also inhibited, with a comparable IC_{50} value (Figure 4.4B), confirming the inflammation-suppressing effects of 3. As TNF-α dysregulation importantly contributes to medical conditions such as systemic lupus erythematosus and multiple cancers, cyclic lipo-α-AApeptides could provide a group of novel candidates for the treatment of these diseases [127, 175].

We also investigated if cyclization is critical for anti-inflammatory activity by comparing 3 with its linear analogue, 3-linear. Interestingly, the ability of 3-linear to decrease NO production was comparable to that of 3 (Figure 4.5B), whereas its inhibition of NF-κB and TNF-α is significantly
Figure 4.4: NF-κB and TNF-α inhibition by 3. A) NF-κB activation is inhibited in HEK293 cells by 3. Cells were plated in 96-well plates and treated with 20 ng/mL LPS and various concentrations of 3. Data were normalized to 20 ng/mL LPS as 100% activation and untreated cells as 0% activation. B) Inhibition of TLR4-induced TNF-α production by 3. A mouse TNF (Mono/Mono) enzyme-linked immunosorbent assay (ELISA) in RAW 264.7 cells demonstrated that TNF-α production decreased upon treatment of 3. Data were normalized to 20 ng/mL LPS as 100% activation and untreated cells as 0% activation.
less potent (Figures 4.6 and 4.7). These results suggest that although linear and cyclic AApeptides do not behave identically, both possess anti-inflammatory activity. As such, we conclude that cyclization is not required to suppress inflammation. However, 3-linear shows much decreased antimicrobial activity when compared to 3 (Figure 4.5A), suggesting that cyclization is more critical for antimicrobial activity than anti-inflammatory activity. It is known that nonpeptidic antimicrobial agents suppress TLR signaling by binding specific TLR ligands (e.g., LPS) [195, 209]. By contrast, cyclic lipo-α-AApeptides appear to function through a different mechanism (Figure 4.5). In our experiments, both pretreatment and cotreatment with 3 resulted in complete inhibition of NO production at 10 µM. Our data suggest that cyclic lipo-α-AApeptides reduce inflammatory responses by using a novel mechanism, while simultaneously fighting bacterial infection.

4.3 Conclusions

In summary, we report a new class of antimicrobial and anti-inflammatory peptidomimetics, cyclic lipo-α-AApeptides, which mimic the structures and functions of HDPs. These cyclic lipo-α-AApeptides can potently arrest the growth of multidrugresistant Gram-negative and Gram-positive bacteria. Furthermore, they can modulate immune responses by strongly antagonizing TLR4 signaling and suppressing LPS-induced production of proinflammatory cytokines. Here we report not only the design and synthesis of an unprecedented cyclic lipo version of α-AApeptides but also the first example of α-AApeptides that display both potent antimicrobial and anti-inflammatory activity. Cyclic lipo-α-AApeptides could serve as a proof-of-concept for immunomodulatory antimicrobial peptidomimetics that do not bind to an extracellular ligand but instead directly interact with components of the TLR signaling cascade. As the mechanistic nature of HDPs remains an outstanding problem in the field, these cyclic lipo-α-AApeptides might help us to understand an alternative mechanism for how HDPs function.
Figure 4.5: 3 does not interact with LPS to inhibit nitric oxide production. Raw 264.7 cells were incubated with 10 M 3 for 30 minutes. Pre-treatment cells were washed with media to remove 3 from the media, and 20 ng/mL LPS added. Co-treatment cells were treated with 20 ng/mL LPS, leaving 3 in the media. Both pre-treatment and co-treatment samples show complete inhibition of nitric oxide production, suggesting that 3 does not bind to LPS. Data is normalized to 20 ng/mL LPS as 100% activation, and untreated cells as 0% activation.
4.4 Materials and Methods

4.4.1 Solid-phase Synthesis, Purification and Characterization of Cyclic Lipo-α-AApeptides

A standard Fmoc chemistry protocol for solid-phase synthesis was used to synthesize lipidated cyclic α-AApeptides on a Burell Wrist-Action shaker on Rink amide resin using peptide vessels (Figure 4.3). Each coupling cycle consisted of Fmoc deprotection with 20% piperidine/DMF, 6 h coupling with 2 equiv of building blocks in the presence of 4 equiv of diisopropylcarbodiimide (DIC) and 3,4-dihydro-3-hydroxy-4-oxo-1,2,3-benzotriazine (DlhtOH) in DMF. Firstly, the lipidated building block was attached to the solid phase support, followed by coupling with the building blocks using standard Fmoc chemistry. After the desired sequence was assembled, the allyl group was removed by usage of Pd(PPh3)4 (0.2 equiv) and PhSiH3 (10 equiv) in CH2Cl2 for 2 h (repeated twice). The Fmoc group was then removed, and intramolecular cyclization was achieved by PyBop/HOBt/DIEA/DMF. Lastly, the resin was transferred into a 4 mL vial, and the cyclic lipo-α-AApeptide was cleaved from the solid support by TFA/CH2Cl2/triisopropylsilane (50:48:2, v/v) over 5 h. The solvent was evaporated, and the residues were analyzed and purified by a Waters HPLC system on analytical (1 mL/min) and preparative (20 mL/min) modules, respectively. The purities of cyclic lipo-α-AApeptides (>95%) were determined by analytical HPLC.

4.4.2 Antimicrobial Assay for MIC Determination

The bacterial strains used to test the efficacy of cyclic lipo-α-AApeptides included multidrug-resistant Staphylococcus epidermidis (RP62A), vancomycin resistant Enterococcus faecalis (ATCC 700802), methicillin-resistant Staphylococcus aureus (ATCC 33591), Klebsiella pneumoniae (ATCC 13383), and multidrug-resistant Pseudomonas aeruginosa (ATCC 27853). The antimicrobial activities of the cyclic lipo-α-AApeptides developed were determined by the serial dilution method. Bacterial cells were grown overnight at 37°C in 5 mL medium, after which a bacterial suspension of approximately 106 CFU/mL in Luria broth or trypticase soy was prepared, ensuring that the
bacterial cells were in the mid-logarithmic phase. Aliquots of the bacterial suspension (50 µL) were added to 50 µL of medium containing the cyclic lipo-α-AApeptides, for a total volume of 100 µL in each well. The cyclic lipo-α-AApeptides were dissolved in PBS buffer in twofold serial dilutions. The concentration range used for peptides was 25 to 0.5 µg/mL. The 96-well plates were incubated at 37°C for about 20 h, and the optical density (OD) was measured at a wavelength of 600 nm. The lowest concentration at which complete inhibition of bacterial growth is observed is defined as the minimum inhibitory concentration (MIC). The experiments were repeated three times, each time in duplicate.

4.4.3 Hemolysis Assay

Freshly drawn human red blood cells (hRBCs) were used for the assay. The blood sample was washed with PBS buffer several times and centrifuged at 700g for 10 min until a clear supernatant was observed. The hRBCs were resuspended in 1PBS to get a 5% (v/v) suspension, which was used to perform the assay. Twofold serial dilutions of cyclic lipo-α-AApeptides were prepared in PBS buffer. Concentrations ranging from 250 µg/mL to 1.56 µg/mL were tested by adding the cyclic lipo-α-AApeptides solutions to sterile 96-well plates to make up to a total volume of 50 µL in each well. Then, 50 µL of 5% (v/v) hRBC solution was added to achieve a total volume of 100 µL in each well. The 0% hemolysis point and 100% hemolysis point were determined in 1PBS and 0.2% Triton-X-100, respectively. The 96-well plate was incubated at 37°C for 1 h and centrifuged at 3500 rpm (Rotor: Sorvall Heraeus 6444) for 10 min. The supernatant (30 µL) was then diluted with 1PBS (100 µL), and hemoglobin was detected by measuring the OD360 with a Biotek Synergy HT microtiter plate reader. Percent hemolysis = (Abs sample - Abs PBS) / (Abs Triton - Abs PBS) * 100. Peptide concentrations corresponding to 50% hemolysis were determined from the dose-response curves. The experiments were repeated three times, each time in duplicate.
4.4.4  Fluorescence Microscopy

A double staining method with DAPI (4,6-diamidino-2-phenylindole dihydrochloride, Sigma, >98%) and PI (propidium iodide, Sigma) as fluorophores was used to visualize and differentiate between viable and dead S. aureus (ATCC 33591) cells. DAPI, being a double-stranded DNA binding dye, stains all bacterial cells irrespective of their viability. Ethidium derivatives such as propidium iodide (PI) are capable of passing through only damaged cell membranes and intercalating with the nucleic acids of injured and dead cells to form a bright red fluorescent complex. Bacterial cells were grown until they reached mid-logarithmic phase, and then cells (2103) were incubated with the cyclic lipo-α-AApeptide 3 at 5 µg/mL for 4 h. The cells were then pelleted by centrifugation at 3000g for 15 min. The supernatant was decanted, and the cells were washed with 1PBS several times and incubated with PI (5 µg/mL) in the dark for 15 min at 0C. Excessive PI was removed by washing the cells several times with 1PBS. Lastly, the cells were incubated with DAPI (10 µg/mL in water) for 15 min in the dark at 0C. Then, excessive DAPI solution was removed, and the cells were washed with 1PBS. Controls were treated following the exactly same procedure but in the absence of 3. The bacteria were examined by using a Zeiss Axio Imager Z1 optical microscope (100).

4.4.5  Fluorescent Detection of Nitric Oxide

RAW 264.7 (mouse leukemic monocyte macrophage cell line) cells were grown in RPMI 1640 medium with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin, and 1% l-glutamine. On day one, cells were plated in a 96-well plate at 75000 cells per well in complete RPMI 1640 medium. Cells were grown overnight at 37C and 5% CO2 in a humidified incubator. On day 2, the medium was removed, and cells were placed in unsupplemented RPMI 1640 medium. LPS (20 ng/mL) and the appropriate concentration of AApeptides were added to a final volume of 200 µL. All stock solutions of AApeptides were prepared in PBS as 20 mmstocks and then diluted to the desired concentration with PBS. There were PBS controls in each experiment. Plates were then incubated for 24 h after treatment. Following incubation, 100 µL of medium was removed from each well
and added to a flat black 96-well microfluor plate (Thermo Scientific). 2,3-Diaminonamthalene (10 µL, 0.05 mg/mL) in HCl (0.62M) was added to the medium, and the mixture was incubated for 20 min in the dark. The reaction was quenched with NaOH (3.0M, 5 µL), and the plate was read on a DTX880 plate reader (Beckman Coulter, CA, USA). Data were collected with excitation at 360 nm and emission at 430 nm. Data were normalized with the ligand-only control as 100% activation and the untreated cells as 0% activation. Fold inhibition=\((\text{sample 430 nm untreated cells 430 nm})/(\text{ligand control 430 nm untreated cells 430 nm})\). The IC\(_{50}\) values were calculated graphically by using OriginPro v8.6 software.

### 4.4.6 Secreted Embryonic Alkaline Phosphatase (SEAP) Reporter of NF-κB Transcription

HEK293 cells were grown in Dulbecco’s Modified Eagle’s Medium (DMEM) with 10% FBS, 1% penicillin/streptomycin, and 1% l-glutamine. HEK293 cells were stably transfected with human TLR4, as well as the required accessory proteins MD-2 and CD14. Additionally, the cells possess an optimized alkaline phosphatase reporter gene under the control of a NF-κB-inducible promoter\(^4\). On day one, cells were plated in a 96-well plate at 40000 cells per well in complete DMEM medium. Cells were grown overnight at 37°C and 5% CO\(_2\) in a humidified incubator. On day 2, medium was removed, and cells were placed in Optimem+0.5% FBS medium. LPS (20 ng/mL) and the appropriate concentration of AApeptides were added to a final volume of 200 µL. All stock solutions of AApeptides were prepared as 20 mm stocks in PBS and then diluted to the desired concentration with PBS. There were PBS controls in each experiment. Plates were then incubated for 24 h after treatment. Following incubation, the medium was assayed by using the Phospha-Light SEAP Reporter Gene Assay System (Applied Biosystems) per the manufacturer’s instructions. The plate was read on a DTX880 plate reader (Beckman Coulter). Data were collected with luminescence at 430 nm. Data were normalized with the ligand-only control as 100% activation and the untreated cells as 0% activation. Fold inhibition=\((\text{sample 620 nm untreated cells 620 nm})/(\text{ligand control 620 nm untreated cells 620 nm})\). The IC\(_{50}\) values were calculated graphically by using OriginPro
4.4.7 Enzyme-linked Immunosorbent Assay (ELISA) Detection of TNF-α

RAW 264.7 cells were grown in RPMI 1640 medium with 10% FBS, 1% penicillin/streptomycin, and 1% L-glutamine. On day 1, cells were plated in a 96-well plate at 75000 cells per well in complete RPMI 1640 medium. Cells were grown overnight at 37°C and 5% CO2 in a humidified incubator. On day 2, the medium was removed, and cells were placed in unsupplemented RPMI 1640 medium. LPS (20 ng/mL) and the appropriate concentration of AApeptides were added to a final volume of 200 µL. We prepared all stock solutions of AApeptides as 20 mm stocks in PBS and then diluted to the desired concentration with PBS. There were PBS controls in each experiment. Plates were then incubated for 24 h after treatment. Following incubation, samples were assayed for TNF-α per the method outlined in the BD Biosciences Mouse TNF (Mono/Mono) ELISA Set (BD Biosciences, CA, USA). The plate was read on a DTX880 plate reader (Beckman Coulter). Data were collected with absorbance at 450 nm. Data were normalized with the ligand-only control as 100% activation and the untreated cells as 0% activation. Fold inhibition=[(sample 450 nm untreated cells 450 nm)/(ligand control 450 nm untreated cells 450 nm)]. The IC_{50} values were calculated graphically by using OriginPro v8.6 software.

4.5 Crystal Violet Toxicity Assay

Cells that were treated with compound for NO experimentation were also tested for compound toxicity by using crystal violet stain. The medium was decanted, and the cells were fixed for 20 min in paraformaldehyde (4%). After fixing, the formaldehyde was removed, and cells were incubated for one hour with crystal violet stain (0.05%). After incubation, cells were rinsed with deionized water to remove excess stain and reconstituted in 100% MeOH for 10 min. The plate was read on a DTX880 plate reader (Beckman Coulter). Data were collected with absorbance at 535 nm. Data were normalized with the untreated cells control as 100% survival and 100% DMSO as 0% survival. Fold inhibition=[(sample 535 nm 100% DMSO 535 nm)/(untreated cells 535 nm 100% DMSO 535 nm)].
4.6 Acknowledgements

We thank the University of South Florida for financial start-up support for J.C. and the National Institutes of Health for grants GM101279 and GM103843 to H.Y.

4.7 Supplementary Information

4.7.1 General Experimental Information

α-Amino acid esters and Knorr resin (0.66 mmol/g, 200-400 mesh) were provided by ChemImpex International, Inc. All other reagents and solvents were purchased from either Sigma-Aldrich or Fisher Scientific. The α-AApeptide building blocks were synthesized following previously reported procedure. NMR spectra of the -AApeptide building blocks were obtained on an Agilent DD800 instrument. Cyclic lipo-α-AApeptides were prepared on a Rink amide resin in peptide synthesis vessels on a Burrell Wrist-Action shaker. The cyclic lipo-α-AApeptides were analyzed and purified on an analytical and a preparative Waters HPLC system, respectively, and then dried on a Labconco lyophilizer. Molecular weights of cyclic lipo-α-AApeptides were identified on a Bruker AutoFlex MALDI-TOF mass spectrometer.

4.7.2 Synthesis and Characterization of α-AApeptide Building Blocks

The α-AApeptide building blocks were synthesized following the previously reported procedure (Figure 4.1). The structures of building blocks used for the solid phase synthesis are shown below (Figure 4.2). Compounds m1 and m2 were reported previously.

Compound m3. Yield 70% in two steps. 1H NMR (CDCl3, 800 MHz) δ (two rotamers) 7.73 (2H, t, J = 8), 7.56 (2H, d, J = 8), 7.37 (2H, t, J = 8), 7.29 (2H, t, J = 8), 5.98-5.87 (1H, m), 4.60-4.25 (4H, m), 3.79 (3H, m), 3.09 (2H, m), 2.40-2.00 (4H, m), 1.42 (9H, s), 1.14 (10H, d), 0.9-0.8 (3H, m). 13C NMR (CDCl3, 200 MHz) δ 175.0, 174.3, 157.2, 156.6, 143.8, 141.2 127.7, 127.1, 125.1,
Compound m4. Yield 52% in two steps. 1H NMR (CDCl₃, 800 MHz) δ (two rotamers)
7.72 (2H, t, J = 8), 7.55 (2H, d, J = 8), 7.36 (2H, t, J = 8), 7.27 (2H, t, J = 8), 6.01 (1H, m),
4.70-4.05 (4H, m), 3.70-3.00 (5H, m), 2.40-2.00 (4H, m), 1.80-1.00 (31H, m), 0.95 (3H, t, J = 8).
13C NMR (CDCl₃, 200 MHz) δ 174.0, 172.1, 156.6, 156.1, 143.8, 141.2, 127.7, 127.1, 125.1, 119.9,
79.2, 67.1, 63.1, 48.4, 47.1, 41.0, 39.6, 34.2, 33.3, 31.9, 30.0, 29.6, 29.3, 29.1, 29.4, 25.2, 23.8, 22.7,

Compound m5. Yield 60% in two steps. 1H NMR (CDCl₃, 800 MHz) δ (two rotamers) δ =
7.72 (2H, t, J = 8), 7.55 (2H, d, J = 8), 7.36 (2H, t, J = 8), 7.27 (2H, t, J = 8), 5.95 (1H, m),
4.80-3.80 (5H, m), 3.70-3.20 (5H, m), 3.06 (2H, m), 2.31 (2H, m), 1.80-1.50 (2H, m), 1.41 (9H, s),
1.30-1.10 (28H, m), 0.86 (3H, t, J = 8). 13C NMR (CDCl₃, 200 MHz) δ 175.2, 174.5, 156.7, 156.1,
148.0, 141.2, 127.7, 127.1, 125.1, 119.9, 79.1, 67.1, 61.1, 48.4, 47.1, 40.2, 39.6, 33.8, 33.4, 31.9,
30.6, 29.7, 29.4, 29.3, 29.1, 28.4, 25.2, 23.8, 22.7, 14.1. HR-ESI: [M+H]+cacl: 750.5052 , found:
750.5036.

4.7.3 Supplementary Figures
Supplementary Figure 4.1: Synthesis of α-AAppeptide building blocks. DhbtOH = 3-4-Dihydro-3-hydroxy-4-oxo-1-2-3-benzotriazine.

Supplementary Figure 4.1: Synthesis of α-AAppeptide building blocks. DhbtOH = 3-4-Dihydro-3-hydroxy-4-oxo-1-2-3-benzotriazine.
Supplementary Figure 4.2: Building blocks used for the solid phase synthesis.
Supplementary Figure 4.3: Solid phase synthesis of cyclic lipo-α-AA peptides.
Supplementary Figure 4.4: The structures of synthesized cyclic lipo-α-AApeptides.
Supplementary Figure 4.5: Characterization of 3-linear. A) the structural of 3-Linear (non-cyclic analog of 3) and its antimicrobial and hemolytic activity. B) TLR4-induced nitric oxide production in the presence of 3 or 3-Linear. RAW 264.7 were treated with 20 ng/mL LPS and varying concentrations of 3 or 3-Linear. Data is normalized to 20 ng/mL LPS as 100% activation, and untreated cells as 0% activation. Our results indicate that 3 and 3-Linear reduce nitric oxide production in a comparable fashion.
Supplementary Figure 4.6: NF-κB activation is inhibited in HEK 293 cells by 3 and 3-Linear. Cells were plated in 96-well plate and treated with 20 ng/mL LPS and various concentrations of 3 and 3-Linear. Data is normalized to 20 ng/mL LPS as 100% activation, and untreated cells as 0% activation.
Supplementary Figure 4.7: Inhibition of TLR4-induced TNF-α production by 3 and 3-Linear. Mouse TNF (Mono/Mono) enzyme-linked immunosorbent assay (ELISA) in RAW 264.7 cells demonstrates that TNF-α production is decreased with treatment of 3 and 3-Linear. Data is normalized to 20 ng/mL LPS as 100% activation, and untreated cells as 0% activation.
Chapter 5

Lipidated Cyclic $\gamma$-AApeptides Display Both Antimicrobial and Anti-Inflammatory Activity

This chapter has been published as:


The manuscript was written, revised, and prepared jointly by C. Smith, Y. Li, and J. Cai. The concept of AApeptides is the primary intellectual work of the Cai research group at the University of South Florida. Synthesis and design of AApeptides was performed by Y. Li and the Cai research group. Cellular assays (Figures 5.3, 5.4, 5.5, S5.4) were conceived, designed, performed, and analyzed by C. Smith with assistance from N. Manoj and J. Cardiello.

5.1 Abstract

Antimicrobial peptides (AMPs) are host-defense agents capable of both bacterial membrane disruption and immunomodulation. However, the development of natural AMPs as potential therapeutics is hampered by their moderate activity and susceptibility to protease degradation. Herein we report lipidated cyclic -AApeptides that have potent antibacterial activity against clinically relevant Gram-positive and Gram-negative bacteria, many of which are resistant to conventional antibiotics. We show that lipidated cyclic -AApeptides mimic the bactericidal mechanism of AMPs
by disrupting bacterial membranes. Interestingly, they also harness the immune response and inhibit lipopolysaccharide (LPS) activated Toll-Like Receptor 4 (TLR4) signaling, suggesting that lipidated cyclic -AApeptides have dual roles as novel antimicrobial and anti-inflammatory agents.

5.2 Introduction

Rapidly emerging resistance of antibiotics is a major public health concern [120]. As such, natural antibiotics including antimicrobial peptides have captured researchers’ attention. Antimicrobial peptides (AMPs) are short cationic amphiphilic peptides present in almost every living organism [70]. Due to their broad-spectrum antimicrobial activity, AMPs are a promising new class of drug candidates. AMPs are effective due to their ability to form a globally amphipathic structure with segregated hydrophobic and cationic regions, capable of disrupting bacterial membranes [70, 135]. Such disruption is rooted in the physical properties of bacterial membranes, making the development of resistance difficult [32]. Additionally, AMPs are known as host defense peptides (HDPs) for their role in modulating host innate immunity and diminishing septic responses after bacterial infection [188]. The immunomodulatory activities of AMPs are speculated to be a crucial contributor to host defense, since the direct antimicrobial activity of AMPs is often weak under physiological conditions [70, 21]. One of the most important immune responses against bacterial infection is lipopolysaccharide (LPS)-activated Toll-like receptor 4 (TLR4) signaling. LPS is a characteristic component of Gram-negative bacterial cell walls that activates the immune response through TLR4. As such, AMPs may have dual roles as both antibiotic and anti-inflammation agents in the treatment of pathogen invasions. As host inflammatory responses to bacterial infection can lead to deadly septic shock, the current treatment for severe infections involves the administration of both anti-bacterial and anti-inflammatory drugs simultaneously. Therefore, the dual functional roles of AMPs have potential as new generation of anti-infective therapeutics.

Nonetheless, AMPs are susceptible to proteolytic cleavage, hampering their development into novel therapeutics [209]. Peptidomimetics as an alternative approach may circumvent these problems. This is because peptidomimetics, with modified peptide backbones, are more stable
against protease degradation. Examples of successful antimicrobial peptidomimetics include β-peptides [99, 98, 97], peptoids [34, 82], and others [32, 36, 79, 80, 145, 146, 153, 195, 198, 209, 228, 237]. Similar to AMPs, these peptidomimetics are designed to form globally amphipathic structures upon interaction with lipid bilayers, disrupting bacterial membranes. Although many peptidomimetics have broad-spectrum antibacterial activity, the reports of immunomodulatory responses are very rare [195, 209]. Tew et al. identified that AMP mimics antagonize lipoteichoic acid (LTA)-activated Toll-like receptor 2 (TLR2) signaling [195]. Here we present a new class of immunomodulatory antimicrobial peptidomimetics, which inhibit TLR signaling without ligand interaction.

We recently developed a new class of peptidomimetics termed γ-AApeptides based on a chiral peptide nucleic (PNA) backbone. Due to the versatility of γ-AApeptides, we were able to synthesize potent antimicrobials with broad-spectrum activities against both Gram-positive and Gram-negative bacteria. The design of antimicrobial γ-AApeptides is straightforward, which is achieved through joining of amphipathic γ-AApeptide building blocks. Herein, we report the design and synthesis of a new generation: lipidated cyclic γ-AApeptides. We show that this class of compounds has potent and broad-spectrum antimicrobial activity against both Gram-positive and Gram-negative bacteria, including community-acquired multidrug-resistant pathogens. Interestingly, several lipidated cyclic γ-AApeptides inhibit Toll-Like Receptor 4 (TLR4) signaling and block production of the proinflammatory cytokine tumor necrosis factor-α (TNF-α), implying the potential for a new generation of antibiotic agents with dual functionality.

5.3 Results and Discussion

5.3.1 Synthesis and Antimicrobial Activity of γ-AApeptides

We have shown previously that antimicrobial AApeptides with potent and broad-spectrum activity can be designed and prepared easily by joining amphipathic AApeptide building blocks together [145]. This is because AApeptides formed by this approach are capable of adopting globally
amphipathic structures, which are crucial for the disruption of bacterial membranes. Moreover, cyclization or lipidation can increase their potency. Cyclization reduces structure motility and facilitates bacterial membrane disruption; while lipidation encourages antimicrobial agent interactions with membranes. We therefore speculated that γ-AApeptides that are both cyclized and lipidated would be more potent in killing bacterial pathogens. In fact, lipidated cyclic peptides polymyxin and daptomycin have been used as last-resort antibiotics to treat infections caused by Gram-negative and Gram-positive bacteria, respectively [228, 237]. However, the exploration of antimicrobial peptidomimetics with lipidated cyclic structural motifs is scarce. To test our hypothesis, a series of lipidated cyclic γ-AApeptides (Figure 5.1) containing different numbers of amphiphilic γ-AApeptide building blocks were designed and synthesized on the solid phase. Their antibacterial activity was then measured against a range of multi-drug resistant Gram negative and Gram-positive bacteria (Figure 5.1).

We initially designed and synthesized lipidated cyclic γ-AApeptides (HW-B-73, -77 and -78, 5.1) in which the lipid tail C16 is directly connected to the ring structure. Unfortunately, these sequences only possess weak activity against bacteria. We hypothesize that this is because the lipid tail on the ring structure has limited orientation, therefore cannot position itself for membrane insertion even after the amphipathic ring contacts the bacterial membranes. Indeed, a trend was apparent in other membrane-active lipidated cyclic peptide antibiotics, including daptomycin and polymyxin B, which contain at least two amino acid residues connecting both lipid tails and ring structures [228, 237]. To test our hypothesis, we moved the lipid tail outside the cyclic ring, leading to the sequence YL-1. As expected, YL-1 showed strong activity against Gram-positive bacteria, although it only contains three amphiphilic building blocks. To determine how ring size affects antibacterial activity, we then included more amphiphilic building blocks into the ring structure. However, an additional amphiphilic building block (YL-4) did not boost activity. A similar phenomenon has been observed in cyclic peptoids and other cyclic peptide antimicrobial agents, which normally contain 6-8 residues in their ring structures [32, 231, 215]. In fact, YL-4 has weaker antimicrobial activity than YL-1, which may due to the increased flexibility of the larger
Figure 5.1: The structures of lipidated cyclic γ-AAPeptides. Two γ-AAPeptides, HW-C-22-2 and HW-C-22-4, which is either linear or cyclic and do not contain lipid tails, were used for comparison. Compounds are named according to their notebook codes.
Table 5.1: The antimicrobial and hemolytic activities of lipidated cyclic γ-AApptides. The microbial organisms used are *E. coli* (ATCC 25922), *K. pneumoniae* (ATCC 13383), *P. aeruginosa* (ATCC 27853), Methicillin-resistant *S. epidermidis* (RP62A), Vancomycin-resistant *E. faecalis* (ATCC 700802), and Methicillin-resistant *S. aureus* (ATCC 33591). The minimum inhibitory concentration (MIC) is the lowest concentration that completely inhibits growth after 24 h. HC50 is the concentration causing 50% hemolysis. Pexiganan and previously reported cyclic γ-AApptide HW-B-13 are included for comparison. YL-36, the compound with the most potent and broad-spectrum activity, is shaded in grey.

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<th>Hemolysis (µg/mL) (µM)</th>
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</table>
ring. This trend is also seen in HW-B-73, HW-B-77, and HW-B-78.

We have previously observed that the exact amphiphilic structures can affect the antimicrobial activity of a sequence \[153\]. The reversal of cationic and hydrophobic groups results in antimicrobial agents with different potency. It appears that while the overall amphipathicity dominates antimicrobial activity, the precise distribution of functional groups in molecules will affect the strength of peptidomimetic interaction with bacterial membranes. As such, YL-12, containing one reversed amphiphilic building block in relation to YL-1, was prepared. This sequence has improved antimicrobial activity against both Gram-negative and Gram-positive bacteria. The reversal of additional building blocks leads to the most potent lipidated cyclic \(\gamma\)-AApeptides YL-34 and YL-36. Particularly, YL-36, with a small amphipathic ring and a C16 lipid tail, shows very potent activity against all tested drug-resist Gram-positive and Gram-negative strains. It not only has much improved antimicrobial activity over the AMP Pexiganan, but is also superior when compared with the previously reported cyclic \(\gamma\)-AApeptide HW-B-13 of much larger ring size, especially against Gram-negative pathogens.

We hypothesize that lipidated cyclic \(\gamma\)-AApeptides are able to kill bacteria through membrane disruption analogous to AMPs, as they possess globally cationic amphipathic structures and broad-spectrum activity against both Gram-positive and Gram-negative bacteria. As such, a fluorescence microscopy experiment was carried out to study the ability of the most potent lead YL-36 to affect the membranes of \textit{S. aureus} (Gram-positive) and \textit{E. coli} (Gram-negative). Briefly, both bacteria were stained with the membrane permeable dye 4,6-diamidino-2-phenylindole (DAPI) and the non-permeable dye propidium iodide (PI) in the absence or presence of YL-36 (Figure 5.2). YL-36 treatment resulted in PI becoming visible using fluorescence microscopy, suggesting bacterial membranes of both \textit{S. aureus} and \textit{E. coli} were damaged. Aggregation of \textit{S. aureus} after treatment with YL-36 is observed, which is generally believed to arise from the loss of membrane potential after the disruption of membranes.
Figure 5.2: Fluorescence micrographs of *S. aureus* (A) and *E. coli* (B) that are treated (or no treatment) with 5 µg/mL lipidated cyclic γ-Apeptide YL-36 for 2 h. A1) control, no treatment, DAPI stained; A2) control, no treatment, PI stained; B1) YL-36 treatment, DAPI stained; B2) YL-36 treatment, PI stained.
5.3.1.1 Anti-inflammatory Properties of γ-AApeptides

In addition to bacterial membrane disruption, some AMPs are speculated to modulate the immune system through TLR signaling [141]. Since lipidated cyclic γ-AApeptides were designed to mimic AMPs, we anticipated that they may be also capable of harnessing immune responses. As such, YL-1, YL-12, YL-29, YL-34, and YL-36 were also assessed for the ability to modulate the LPS-induced TLR4 signaling response by measuring nitric oxide production (Figure 5.3A) [30]. Nitric oxide is produced as a downstream inflammatory factor in all TLR signaling, and is part of the global inflammatory response. Figure 5.3A shows that lipidated cyclic γ-AApeptides are a potent class of anti-TLR4 signaling agents, capable of reducing nitric oxide production. To further investigate the anti-inflammatory capabilities of lipidated cyclic γ-AApeptides, we chose the most potent antimicrobial agent YL-36 for further investigation. The IC$_{50}$ of YL-36 was obtained by treating RAW 264.7 cells with varying concentrations of YL-36 in concert with 20 ng/mL LPS. The resulting inflammatory response was monitored through measurement of nitric oxide production.

YL-36 shows an inhibitory effect of the nitric oxide signaling with an IC$_{50}$ value of 1.98 $\mu$M (Figure 5.3B). HW-C-22-2 and HW-C-22-4 demonstrate that lipidation increases the effectiveness of γ-AApeptides as TLR signaling antagonists, as neither construct is active under the tested concentrations (Figure 5.3). Nitric oxide is produced by TLR activation, and correlates to the level of inflammatory response. Additionally, YL-36 shows little cytotoxicity up to 100 $\mu$M (Figure 5.4), suggesting the inhibition of nitric oxide is not due to the toxicity of YL-36. As such, we further explored the ability of YL-36 to inhibit downstream NF-κ activation and the production of pro-inflammatory cytokines. Inhibition of the NF-κ activation (Figure 5.4A) was determined by a previously developed secreted embryonic alkaline phosphatase (SEAP) assay [193]. Furthermore, the pro-inflammatory cytokine tumor necrosis factor- (TNF-α) is also strongly inhibited (IC$_{50}$ = 2.05 $\mu$M) (Figure 5.4B) [239]. Both NF-κ and TNF-α are produced due to TLR activation. Their inhibition by YL-36 shows that TLR signaling is being inhibited in a dose dependent fashion. Neither HW-C-22-2 nor HW-C-22-4 shows inhibitory activities, further demonstrating anti-inflammatory
Figure 5.3: Inhibition of nitric oxide production by lipidated cyclic γ-AApeptides. A) Nitric oxide production in the presence of the lipidated cyclic γ-AApeptides YL-1, YL-12, YL-29, and YL-36. Two γ-AApeptides lacking alkyl tails, either linear (HW-C-22-2) or cyclic (HW-C-22-4), were included to demonstrate the importance of γ-AApeptide lipidation on their TLR activities. RAW 264.7 cells/well were treated with 20 ng/mL LPS and varying concentrations of lipidated cyclic γ-AApeptides in a 96-well plate. Data is normalized to 20 ng/mL LPS as 100% activation, and untreated cells as 0% activation. B) Dose-dependent inhibition of nitric oxide production by YL-36. This experiment was performed as described in 3a, and a dose response curve was obtained for calculation of an IC$_{50}$ value.
activity depends on the lipidation of γ-AApeptides.

Tew et al. has previously elegantly identified antimicrobial agents that reduce TLR-induced inflammatory response [195, 209]. Interestingly, our lipidated cyclic γ-AApeptides appear to have a different mechanism of inhibition, not interacting with the TLR ligands. If γ-AApeptides interacted with LPS, pre-treatment with YL-36 prior to LPS addition would abolish its anti-TLR4 activities. Our data though demonstrates that both pre-treatment and co-treatment with γ-AApeptides and LPS results in complete antagonism of nitric oxide production (Figure 5.5), indicating that γ-AApeptides do not bind to LPS directly.

### 5.4 Conclusions

As a new class of antimicrobial peptidomimetics that mimic AMPs, lipidated cyclic γ-AApeptides exhibit potent and broad-spectrum activity against a range of multi-drug resistant Gram-negative and Gram-positive bacteria. Maybe even more importantly, lipidated cyclic γ-AApeptides also imitate AMPs in their immunomodulatory capabilities. These γ-AApeptides have been shown to antagonize the LPS activated NF-κ signaling response, and to potently suppress release of harmful pro-inflammatory cytokines including tumor necrosis factor-α (TNF-α). Additionally, lipidated cyclic γ-AApeptides do not bind to TLR ligands, suggesting a novel mechanism for immunomodulation. These peptidomimetics provide an exciting new approach to treat bacterial infections by exerting dual-functional roles: they are a new generation of antibiotic agents that kill both multi-drug resistant Gram-positive and Gram-negative bacteria directly, as well as anti-inflammatory agents through harnessing immune responses. Furthermore, as deregulation of TNF-α is also related to diseases such as cancer and lupus erythematosus, lipidated cyclic γ-AApeptides may also provide other therapeutic applications in the future [127, 175].
Figure 5.4: Inhibition of NF-κB and TNF-α by lipidated cyclic γ-AApeptides. A) NF-κ activation in HEK 293 cells in the presence of various γ-AApeptides. HEK 293 cells were stably transfected with TLR4 and its accessory proteins, as well as a secreted embryonic alkaline phosphatase (SEAP) reporter gene. Cells were plated in 96-well plate with 40,000 cells/well and treated with 20 ng/mL LPS and YL-36. Data is normalized to 20 ng/mL LPS as 100% activation, and untreated cells as 0% activation. Increasing concentrations of YL-36 decrease transcription of SEAP in a dose-dependent fashion. HW-C-22-2 and HW-C-22-4 were included for comparison. B) YL-36 reduces production of TNF-α. TNF-α production was measured with a monoclonal mouse ELISA. Data is normalized to 20 ng/mL LPS as 100% activation, and untreated cells as 0% activation. Increasing concentrations of YL-36 result in decreased production of TNF-α. HW-C-22-2 and HW-C-22-4 were included for comparison.
Figure 5.5: YL-36 inhibits TLR4 activation independent of LPS binding. Raw 264.7 cells were incubated for 30 minutes with 10 µM YL-36. After incubation, pre-treatment cells were washed with medium and activated with 20 ng/mL LPS. Co-treatment cells were not washed, and were treated with 20 ng/mL LPS while YL-36 remained in the medium. Antagonism for both cell treatments is 100%, suggesting that YL-36 does not bind to the ligand as a mode of inhibition. Data is normalized to 20 ng/mL LPS as 100% activation, and untreated cells as 0% activation.
5.5 Materials and Methods

5.5.1 General Experimental Methods

Rink amide MBHA resins (200-400 mesh, 0.7 mmol/g) were purchased from Chem-Impex Int’l Inc. Other chemicals were ordered from either Sigma-Aldrich or Fisher Scientific, and used without further purification. 1H NMR spectra of the building blocks were obtained on an Agilent DD800 instrument. The solid phase syntheses of the lipidated cyclic γ-AApeptides were carried out in a peptide reaction vessel on a Burrell Wrist-Action shaker, were analyzed and purified using an analytical and preparative Waters HPLC system, respectively. The final products were dried in a Labcono lyophilizer. Molar masses were identified using a Bruker AutoFlex MALDI-TOF mass spectrometer.

5.5.2 Solid Phase Synthesis of Lipidated Cyclic γ-AA Peptides

The syntheses of lipidated cyclic γ-AApeptides were carried out on the solid phase as reported previously [145, 232]. For each coupling cycle, 20% Piperidine in DMF was used to remove the Fmoc protecting group, followed by the coupling of 1.5 equiv of building blocks with 4 equiv of HOBT (1-hydroxybenzotriazole monohydrate)/DIC (diisopropylcarbodiimide) in DMF for 6 h. The allyl group was removed by 0.2 equiv of Pd(PPh3)4 in the presence of 10 equiv of PhSiH3/CH2Cl2 (2 h for each, repeated twice). The exposed carboxyl group reacted with the N-terminus of the sequence to complete the cyclization using PyBOP/HOBOT/DIPEA/DMF. The lipidated γ-peptides was cleaved from solid support in 50:48:2 TFA/CH2Cl2/TIS (triisopropylsilane) for 2 h. The solvent was evaporated and the sequences were analyzed and purified using a Waters HPLC system monitored at 215 nm. The desired fractions were collected and lyophilized, and their molecular weights were confirmed by the Bruker AutoFlex MALDITOF mass spectrometer.
5.5.3 Antimicrobial Assays

The lipidated cyclic γ-AApeptides were tested for their antimicrobial activity against various microbial organisms including *E. coli* (ATCC 25922), *K. pneumonia* (ATCC 13383), multi-drug resistant *P. aeruginosa* (ATCC 27853), Methicillin-resistant *S. epidermidis* (MRSE, RP62A), Vancomycin-resistant *E. faecalis* (ATCC 700802), Methicillin-resistant *S. aureus* (ATCC 33592). The highest concentration of the tested AA-peptides was 25 µg/mL. The bacteria in 5 mL of medium were grown at 37 C overnight and then diluted to make a suspension of approximate 1 \(10^6\) CFU/mL. Aliquots of 50 QL of bacterial suspension were mixed with 50 QL of medium containing different concentrations of lipidated cyclic γ-AA-peptides. The plate was incubated at 37 C overnight with cell growth monitored by a Biotek Synergy HT microtiter plate reader under the 600 nm wavelength. MIC was determined when the lowest concentration of the compounds inhibit the cell growth completely in 24 h. The results were repeated at least three times with duplicates for each time.

5.5.4 Hemolysis Assay

Freshly drawn, K2 EDTA treated human red blood cells (hRBCs) were washed with PBS buffer twice and centrifuged at 1000g for 10 min. After the clear supernatant was removed, the cell pellets were resuspended in PBS and mixed with serial diluted lipo-cyclic γ-AApeptides in a 96-well plate. The plate was incubated at 37 C for 1 h and centrifuged at 3500 rpm for 10 min. The supernatant was separated and diluted in PBS, and the absorbance was detected at 360 nm using a Biotek Synergy TH plate reader. % hemolysis = (Abssample AbsPBS)/(AbsTriton AbsPBS) 100%. 0% hemolysis (negative control) was determined by mixing blood with PBS and 100% hemolysis (positive control) was determined by mixing blood with Triton X-100 (final concentration 0.1%). The results were repeated at least three times with duplicates for each time.
5.5.5 Fluorescence Microscopy

DAPI (4, 6-Diamidino-2-phenylindole dihydrochloride, Sigma, >98%) and PI (Propidium iodide, Sigma) were used to stain the bacteria cells of *E. coli* or *S. aureus*. DAPI is a DNA binding dye staining all bacterial cells regardless of their viabilities, and PI is an ethidium derivative which only can pass through damaged bacterial membranes and intercalates with their nucleic acids. Briefly, bacteria in mid-logarithmic phase were incubated with lipidated cyclic γ-AA peptides (2 MIC) for 2 h, and then were centrifuged at 3000g for 15 min. The bacteria cell pellets were separated then incubated with PI, followed by washing and incubation with DAPI (each dye incubation was performed at 0°C for 15 minutes in dark). Controls were bacteria culture without peptides following the same procedure described above. The stained bacteria cells were observed under Zeiss Axio Imager Z1 optical microscope using the 100X oil-immersion objective.

5.5.6 Fluorescent Detection of Nitric Oxide

Raw 264.7 (Mouse leukaemic monocyte macrophage cell line) cells were grown in RPMI 1640 medium containing 1% L-glutamine, 1% Penicillin/streptomycin and 10% fetal bovine serum (FBS). Cells were plated in a 96-well plate at 75,000 cells/well in complete RPMI 1640 medium, and allowed to grow overnight at 37°C and 5% CO2 in a humidified incubator. The media was removed, and cells were placed in unsupplemented RPMI 1640 medium. 20 ng/mL LPS and the appropriate concentration of lipidated cyclic γ-AA peptides (20 mM stock solutions in PBS) were added to a final volume of 200 µL. PBS controls were included in each experiment. Plates were then incubated for 24 h, and then 100 µL of media was transferred to a flat black 96-well microfluor plate (Thermo Scientific, MA, USA). Following that, 10 µL of 0.05 mg/mL 2,3-diaminonamthalene in 0.62 M HCl was added to the media and incubated for 20 minutes in the dark. The reaction was quenched with 5 µL of 3.0 M NaOH, and the plate was read on a Beckman Coulter DTX880 plate reader (Beckman Coulter, CA, USA). Data was collected with excitation at 360 nm and emission at 430 nm. Data was normalized with the ligand only control as 100% activation, and the untreated cells
as 0% activation. Fold inhibition = [(Sample 430 nm Untreated cells 430 nm)/(Ligand Control 430 nm Untreated cells 430 nm)]. The EC50 values were calculated graphically using OriginPro v8.6 software.

5.5.7 Secreted Embryonic Alkaline Phosphatase (SEAP) Reporter of NF-κ Transcription

HEK293 (Human Embryonic Kidney 293) cells were grown in Dulbecco’s Modified Eagle Medium (DMEM) in the presence of 1% Penicillin/streptomycin, 10% fetal bovine serum (FBS), and 1% L-glutamine. HEK293 cells are stably transfected with human TLR4, as well as the required accessory proteins MD-2 and CD14. Moreover, the cells also possess an optimized alkaline phosphatase reporter gene under the control of a NF-κ inducible promoter. Cells were first plated in a 96-well plate at 40,000 cells/well in complete DMEM medium and allow to grow overnight at 37°C and 5% CO2 in a humidified incubator. Then the media was removed, and cells were placed in Optimem + 0.5% FBS medium. 20 ng/mL LPS and the appropriate concentration of lipidated cyclic γ-AApeptides were added to a final volume of 200 µL. PBS buffer was included as control. Plates were then incubated for 24 h, and then medium was assayed per the instructions of the Phospha-Light SEAP Reporter Gene Assay System (Applied Biosystems, NY, USA). The plate was read on a Beckman Coulter DTX880 plate reader (Beckman Coulter, CA, USA). Data was collected with luminescence at 430 nm. Data was normalized with the ligand only control as 100% activation, and the untreated cells as 0% activation. Fold inhibition = [(Sample 430 nm Untreated cells 430 nm)/(Ligand Control 430 nm Untreated cells 430 nm)]. The EC50 values were calculated graphically using OriginPro v8.6 software.

5.5.8 Enzyme-Linked Immunosorbent Assay (ELISA) Detection of TNF-α

Raw 264.7 (Mouse leukaemic monocyte macrophage cell line) cells were grown in RPMI 1640 medium with 10% fetal bovine serum (FBS), 1% Penicillin/streptomycin, and 1% L-glutamine. Cells were plated in a 96-well plate at 75,000 cells/well in complete RPMI 1640 medium and allowed
to grow overnight at 37C and 5% CO2 in a humidified incubator. Then media was removed, and cells were placed in unsupplemented RPMI 1640 medium. 20 ng/mL LPS and the appropriate concentration of lipidated cyclic γ-AApeptides (20 mM stock solutions) were added to a final volume of 200 µL. PBS was included as the control. Plates were then incubated for 24 hours, and then samples were assayed for TNF-α per the method outlined in the BD Biosciences Mouse TNF (Mono/Mono) ELISA Set (BD Biosciences, CA, USA). The plate was read on a Beckman Coulter DTX880 plate reader (Beckman Coulter, CA, USA). Data was collected with absorbance at 450 nm. Data was normalized with the ligand only control as 100% activation, and the untreated cells as 0% activation. Fold inhibition = \[\frac{(\text{Sample 450 nm Untreated cells 450 nm})}{(\text{Ligand Control 450 nm Untreated cells 450 nm})}\]. The EC50 values were calculated graphically using OriginPro v8.6 software.

5.5.9 Crystal Violet Toxicity Assay

Cells which were treated with compound for nitric oxide experimentation were also tested for compound toxicity using crystal violet stain. Cells were fixed for 20 minutes in 4% paraformaldehyde after the media was removed. After fixing, formaldehyde was removed and cells were incubated for 1h with 0.05% crystal violet stain. After incubation, cells were rinsed with deionized water and reconstituted in 100% methanol for 10 minutes. The plate was read on a Beckman Coulter DTX880 plate reader (Beckman Coulter, CA, USA). Data was collected with absorbance at 535 nm. Data was normalized with the untreated cells control as 100% survival, and the blank wells as 0% survival. Fold inhibition = \[\frac{(\text{Sample 535 nm Blank 535 nm})}{(\text{Untreated cells 535 nm Blank 535 nm})}\].

5.6 Acknowledgements

This work was supported by the USF start-up fund (J.C.) and National Institutes of Health (Grants GM101279 and GM103843) (H.Y.).
5.7 Supplementary Information

5.7.1 Synthesis and Characterization of the Lipidated Cyclic γ-AApeptides Building Blocks

The building blocks (Figure 5.1) used for the preparation of lipidated γ-AApeptides were synthesized following previous reported protocols [145, 153, 232]. Compounds 2, 3 and 4 have been reported, and the 1H and 13C NMR spectra of structures 1, 5 and 6 are obtained and shown below.

**Compound 1.** Yield 60%. 1H NMR (CDCl3, 800 MHz) (two rotamers) 7.71 (d, J = 8 Hz, 2H), 7.55 (d, J = 8 Hz, 2H), 7.35 (t, J = 8 Hz, 2H), 7.27 (t, J = 8 Hz, 2H), 4.70-4.26 (m, 3H), 4.14-4.02 (m, 2H), 3.85-3.33 (m, 3H), 3.05 -2.99 (m, 2H), 2.33-2.17 (m, 2H), 1.52-1.40 (m, 15H), 1.24-1.15 (m, 24H) ppm. 13C NMR (CDCl3, 200 MHz) (two rotamers) 175.3, 156.9, 156.2, 143.9, 143.8, 141.2 127.6, 127.0, 125.2, 119.9, 79.2, 66.9, 66.7, 50.5, 50.2, 47.2, 47.1, 40.2, 33.2, 32.5, 31.9, 29.7, 29.6, 29.4, 28.4, 25.1, 22.7, 14.2, 14.1 ppm. HR-ESI: [M+H]+ calc: 750.5052, found: 750.5069.

**Compound 5.** Yield 60%. 1H NMR (CDCl3, 800 MHz) (two rotamers) 7.74 (t, J = 8 Hz, 2H), 7.52-7.45 (m, 2H), 7.38 (t, J = 8 Hz, 2H), 7.29-7.15 (m, 7H), 4.33 (t, J = 8 Hz, 1H), 4.22-4.12 (m, 2H), 4.06-3.99 (m, 3H), 3.75-3.24 (m, 2H), 2.97-2.76 (m, 2H), 2.21-2.17 (m, 2H), 1.54-1.52 (m, 2H), 1.24-1.16 (m, 24H), 0.88 (t, J = 8 Hz, 3H) ppm. 13C NMR (CDCl3, 200 MHz) (two rotamers) 175.7, 175.6, 172.6, 171.9, 156.8, 155.9, 143.8, 143.7, 143.5, 141.2, 141.1, 137.1, 136.9, 131.2, 128.6, 127.0, 125.3, 125.2, 125.0, 124.9, 120.0, 119.9, 52.6, 52.3, 51.8, 50.4, 49.0, 46.9, 38.7, 38.0, 33.1, 32.6, 31.9, 29.7, 29.6, 29.4, 25.0, 22.7, 14.2 ppm. HR-ESI: [M+H]+ calc: 669.4262, found: 669.4269.

**Compound 6.** Yield 60%. 1H NMR (CDCl3, 800 MHz) (two rotamers) 8.65 (s, 2H), 7.75 (d, J = 8 Hz, 2H), 7.57 (d, J = 8 Hz, 2H), 7.38 (t, J = 8 Hz, 2H), 7.30 (t, J = 8Hz, 2H), 5.89-5.81 (s, 1H), 5.29-5.25 (m, 1H), 5.21-5.18 (m, 1H), 4.52 (d, J = 8 Hz, 2H), 4.35-4.12 (m, 3H), 3.76 (s, 2H), 3.64-3.39 (m, 1H), 3.28-2.97 (m, 4H), 2.73-2.44 (m, 4H), 1.57-1.42 (m, 15H) ppm. 13C NMR (CDCl3, 200 MHz) (two rotamers) 173.7, 173.2, 172.9, 172.8, 172.7, 159.9, 159.6, 158.6, 157.0, 156.7, 156.5, 143.8, 143.7, 143.5, 143.4, 141.2, 131.9, 131.8, 127.8, 127.1, 127.0, 125.2, 125.0, 124.9, 120.0, 119.9, 118.6, 118.5, 118.1, 67.6, 67.3, 65.5, 52.0, 51.4, 51.1, 50.6, 50.2, 47.0, 40.7, 39.9, 32.1,

5.7.2 Solid Phase Synthesis, Purification, and Characterization of Lipidated Cyclic γ-AA Peptides

The syntheses of our lipidated cyclic γ-AA peptides are based on building blocks strategy as reported previously [153, 232]. For each coupling cycle, 20% Piperidine in DMF is used to remove the Fmoc protecting group, followed by the coupling of 1.5 equiv of building blocks with 4 equiv of HOBT (1-hydroxybenzotriazole monohydrate)/DIC (diisopropycarbodiimide) in DMF for 6 hours. The cyclization is done on the resins with the aid of the building block 2. Before the cyclization, the allyl group is removed by 0.2 equiv of Pd(PPh3)4 in the presence of 10 equiv of PhSiH3/CH2Cl2 (2 hours for each, repeated twice). The exposed carboxyl group reacts with the N-terminus of the sequence to complete the cyclization using PyBOP/HOBT/DIPEA/DMF. After that, the cleavage of the peptides from solid support is achieved in 50:48:2 TFA/CH2Cl2/TIS (triisopropylsilane) for 2 hours. The solvent is evaporated and the sequences are analyzed and purified using a Waters HPLC system. The traces are detected at 215 nm. The lyophilized fractions (Figure 5.2) were collected and their molecular weights were shown below (Figure 5.3) on the Bruker AutoFlex MALDI-TOF mass spectrometer using -cyano-4-hydroxy-cinnamic acid.

5.7.3 Supplementary Figures
Supplementary Figure 5.1: Lipidated cyclic γ-AApeptide building blocks
Supplementary Figure 5.2: The synthesis and structures of lipidated cyclic γ-AApeptides. A) synthesis of HW-B-73, HW-B-77, and HW-B-78; B) synthesis of YL-1, YL-4, YL-12, YL-29, YL-34 and YL-36.
Supplementary Figure 5.3: MALDI analysis of lipidated cyclic $\gamma$-AA peptides.

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Supplementary Figure 5.4: YL-36 is not toxic to cultured cells. YL-36 is non-toxic with treatments up to 100 $\mu$M as demonstrated by a Crystal Violet cell viability assay. Data is normalized with untreated cells as 100% survival, and blank wells as 0% survival.
Chapter 6

Rational Design of TLR8-Specific Tetra-Substituted Imidazole Adjuvants

This chapter is a sample of a manuscript in preparation as:

Adam Csakai* Christina Smith*, Hiromi Tanji, Torey Jones, Subada Soti, Lindsey Broadwell, Toshiyuki Shimizu, and Hang Yin. Rational design of TLR8-specific tetra-substituted imidazole adjuvants. Manuscript in preparation, 2017. *These authors contributed equally to this work.

Rational design and synthesis of small molecules was performed by A. Csakai. C. Smith designed and performed all biological characterization including devising the two-site model. T. Jones, S. Soti, and L. Broadwell performed and analyzed a subset of biological experiments. H. Tanji and T. Shimizu are attempting to crystalize TLR8 in the presence of D-107. Individual contributions to date are outlined below using the CRediT taxonomy.


6.1 Introduction

Toll-like receptors (TLRs) are a crucial component of the innate immune system, providing the first line of defense against danger molecules from pathogens and damaged host cells [203]. After sensing a threat, TLRs induce inflammatory signaling resulting in the upregulation of NF-κB and cytokines. TLR dysregulation disrupts inflammatory homeostasis, contributing to a myriad of
health concerns. TLR under-activation causes in an insufficient inflammatory response, permitting infection to spread unchecked [71]. Over-activation occurs in autoimmune diseases, and results in an overabundance of inflammatory factors damaging the host [90]. To maintain a healthy inflammatory response, small molecules can be used to tune TLR signaling. However, much remains unclear about how specificity is regulated between closely-related TLRs, as well as how to specifically attenuate these pathways.

TLR7 and TLR8 are two closely-related endosomally expressed sensors of single-stranded ribonucleic acids (ssRNA) [44, 73]. Pathogen-related ligands for these TLRs include ssRNAs from influenza A and human immunodeficiency virus type 1, as well as bacterial ssRNAs [54, 73, 223]. However, activation may also occur in response to endogenous ssRNAs released during cell death, or from short interfering RNAs (siRNAs) [90]. As evidenced by their similar recognition patterns, TLR7 and TLR8 are highly analogous, and engineering chemical specificity has presented a substantial challenge. However, an increase in structural information has made rational design of TLR8 specific small molecules feasible [206, 207].

The solved crystal structures of TLR7 and TLR8 have provided essential information on ligand recognition and binding motifs. These TLRs possess two nucleotide binding sites that each recognize an ssRNA degradation product (Figure 6.1) [207, 240]. Site 1 is located at the dimer interface, and is sufficient to induce signaling in the presence of a suitably potent ligand. This site binds uridine (U) or guanosine (G) from degraded ssRNAs, as well as imidazoquinoline small molecules such as R848. While the location of site 1 is conserved between both TLRs, the particular residues and their binding partners vary (Figure 6.2). Further, though the recognition motif is conserved, the volume and electrostatic potential of site 1 varies between TLR7 and TLR8, and may contribute to specificity. Site 2 does not induce dimerization, but instead affects ligand affinity at site 1. Site 2 recognizes uridine-purine (UG or UA) or poly-uridine nucleotides. In TLR7, site 2 is located more closely to the dimer interface than site 1.

TLR agonists are promising vaccine adjuvants, as TLR activation can control the induction, extent, and duration of an adaptive immune response through the production of cytokines [220].
Figure 6.1: Two site ligand binding model for TLR7 (gray) and TLR8 (tan). These TLRs have two distinct nucleotide binding sites. Site 1 induces dimerization and binds to small molecule ligands, or uridine (red) and guanosine (purple) nucleotides. The location of site 2 differs between TLR7 (blue) and TLR8 (orange).

**Site 1: Induces Dimerization**
- TLR7: R848, guanosine (ssRNA)
- TLR8: R848, uridine (ssRNA)

**Site 2: Modulates Site 1 Ligand Affinity**
- TLR7: Poly-Uridine (ssRNA)
- TLR8: Uridine-Guanosine (ssRNA)

Figure 6.2: TLR7 and TLR8 Site 1 Interactions. The location of site 1 is highly conserved, however the residues vary. Residues near the ligands are denoted in blue, hydrophobic interactions in green, hydrogen bonding in orange, \( \pi \) interactions in pink, and salt bridges in yellow. The interactions are further subdivided into protein interactions with R848, ssRNA ligand, or protein-protein interactions at the binding interface.

<table>
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- Nearby Residues (Crystal)
- Hydrophobic Interactions
- Hydrogen Bonding
- \( \pi \) or NH-\( \pi \)
- Salt bridge

Description of residues and interactions is provided in the figure captions.
For example, monophosphoryl lipid A activates TLR4, and is one of only three FDA-approved adjuvants [226]. Despite the lack of formal approval, it has been observed that many vaccines unwittingly contain TLR agonists which act as adjuvants, including the Influvac and yellow fever vaccines [169, 183]. TLR8 is a particularly enticing target due to its expression on dendritic cells (DCs) and a proven capacity to activate the Th1 cell-mediated immune response (Figure 6.3) [220]. Unlike other TLRs, TLR8 can induce a robust inflammatory response in newborns, who are particularly vulnerable to infection [27, 47, 119, 161]. However, existing imidazoquinoline TLR8 agonists are ineffective adjuvants [220]. While imidazoquinolines successfully initiate the adaptive immune response, this is coupled with high cytokine concentrations that cause flu-like symptoms in humans. Imidazoquinolines also rapidly spread away from the injection site, and cause detrimental systemic inflammation. Improved TLR adjuvants must selectively induce an immune response only when and where it is needed. While several highly potent TLR7/8 adjuvants are under production, efforts have focused on solubility and biodistribution rather than specific activation [233]. A TLR agonist that is activated only in the presence of an antigen such as ssRNA would be an exciting advancement.

Here we report the discovery of a TLR8-specific small molecule (D-107) that induces inflammation only in the presence of inflammatory ssRNAs. Further, through structure-activity relationship studies, we explore chemical specificity between the closely related TLR7 and TLR8, paving the way for the design of other targeted therapeutics. This immune-specific agonist may have applications as an improved vaccine adjuvant with site-specific activation.

6.2 Results and Discussion

6.2.1 Rational Design of TLR8-Specific Chemical Probes

The crystal structures of TLR7 and TLR8 in the presence of varied ssRNA and imidazoquinoline ligands was used as a basis for small molecule rational design [206, 207, 240]. The potent imidazoquinoline ligand R848 is able to activate both TLR7 and TLR8 by binding to site 1, though
Figure 6.3: TLR8 agonists as vaccine adjuvants. TLR8 agonists are promising vaccine adjuvants due to their ability to activate both the innate and adaptive immune response. Following TLR8 activation, NF-κB transcription induces the production of pro-inflammatory cytokines. These cytokines regulate T cell differentiation and subsequent immune cell recruitment, B cell formation, and antibody production.
the residues involved vary slightly (Figure 6.2). The quinolone moiety of R848 mimics the nucleotide base, though additional residues participate in hydrophobic interactions. Interestingly, R848 is sufficient to induce TLR dimerization through site 1 binding when nucleotide ligands are not.

While no structural information is available, the closely-related imidazoquinoline R837 is specific to TLR7. Structure-activity comparisons between R848 and R837 reveal several trends (Figure 6.4). The presence of a 2-position ether chain appears to be important for TLR8 activity (Figure 6.4A, orange). Further, R848 and R837 each possesses a 1-position bulky substituent (Figure 6.4A, green), though R837 lacks an alcohol moiety. Based on the R848-bound TLR8 crystal structure, essential hydrophobic, hydrogen bonding, and π-stacking interactions can be predicted (Figure 6.4B). Based on this rationale, we generated a series of R848-derived small molecules to explore the chemical space contributing to TLR8 specificity and activity. We maintained the ether chain on the 2-position of the imidazole core previously seen to be important for TLR8 activity, and interchanged the 1-position isobutyl group and tertiary alcohol moiety from R848 and R837. As the quinoline portion of the imidazoquinoline scaffold has not been explored previously, we de-fused the pyridine ring portion to assess its role in specificity. These modifications aim to affect π-stacking, hydrogen-bonding, and hydrophobic interactions by the quinoline moiety. Further, changes in molecule size and electrostatic potentials may take advantage of the site 1 differences between TLR7 and TLR8. A summary of the compounds synthesized and characterized is available in Figure 6.5.

6.2.2 Biological Characterization of TLR8 Activity and Specificity

To assess the validity of the rational design process, we assayed our compounds for TLR7 and TLR8 activity in HEK 293 cells. TLR activation was monitored through an NF-κB secreted embryonic alkaline phosphatase (SEAP) reporter gene. Compounds were tested at 100 µM with and without R848 to assess both antagonistic and agonistic capabilities, and screened for toxicity (Figure 6.6). The majority of compounds did not demonstrate any activity for either TLR. This
Figure 6.4: Structural comparisons of R848 and R837. (A) R848 and R837 are imidazoquinoline agonists that display disparate TLR specificity. R848 binds both TLR7 and TLR8, while R837 is specific for TLR7. These compounds differ by an alcohol on the 1-position substituent, and the presence of an ether chain at the 2-position. (B) R848 interactions with TLR8. Two TLR8 monomers from the crystal structure are denoted in blue and green with R848 shown in red. Hydrogen bonding, hydrophobic, and π stacking interactions predicted to be critical for binding are shown.
Figure 6.5: Structure-activity relationship studies of imidazoquinoline-derived compounds. To assess TLR8 specificity and activity, a series of R848-derived compounds were synthesized. The R848 quinoline ring was de-fused, and attached to a variety of substituents to assess the affects of π-stacking, hydrogen-bonding, hydrophobicity, flexibility, and electrostatic interactions on specificity and activity.
is interesting, as the loss of agonistic activity from R848 indicates there is limited chemical space available for modification. For example, compound G-36 is closely related to R848, differing only in the cleavage of the pyridine ring to an amindino group. Yet this simple change completely abolishes agonism. This may indicate the importance of rigidity or π-stacking interactions in conferring activity. Further support for the importance of π-stacking comes from G-75, which loses all activity with removal of the benzene ring. Compounds D-58, D-74, D-97, D-107, and D-125 demonstrated inhibition of R848 that could not be attributed to toxicity and were considered further.

Structural analysis of inhibitory compounds revealed trends in potency and specificity (Figure 6.7). Compounds D-107, D-58, and D-125 demonstrate specific inhibition of R848-induced TLR8 signaling. The strength of inhibition is correlated with the addition of larger halogens such as iodine, though whether potency stems from decreased electronegativity or increased halogen size remains unclear. Further, the conversion of the tertiary alcohol to an isobutyl group revokes TLR8 specificity, consistent with R848 and R837 activity. As such, D-74 and D-97 inhibit multiple TLRs, demonstrating activity without specificity. While we anticipate D-98 follows the same trend, toxicity makes it difficult to draw conclusions. As D-107 was the most potent compound, it was selected for further characterization. Specificity analysis against a variety of TLR-expressing cells demonstrated that D-107 selectively inhibits R848 activation of TLR8, with an IC₅₀ value of 12.8 ± 1.1 μM (Figure 6.8).
Figure 6.6: Cellular activity of tetra-substituted imidazoles. R848-derived compounds were assayed for TLR7 and TLR8 inhibitory activity in the presence (A) and absence (B) of R848. No compounds displayed agonistic activity when added to otherwise untreated cells. Compounds D-58, D-74, D-97, D-107, and D-125 demonstrated inhibition of R848 that could not be attributed to toxicity. TLR activity is normalized with R848 treatment at 100%, and untreated cells as 0%. Toxicity is normalized with untreated cells as 100% viability, and 25% DMSO treated as 0%. Data are representative of at least three biological replicates.
Figure 6.7: TLR8 specificity and potency. Structure-activity relationship studies discovered a family of inhibitors (D-107, D-58, D-125, and G-52) that specifically inhibit R848 activation of TLR8. Potency correlates to halogen size, and inversely with electronegativity. Changing the tertiary alcohol to an isobutyl group abolished TLR8 specificity, consistent with observations from R848 and R837.
Figure 6.8: Potency and specificity of D-107. (A) D-107 inhibits R848-induced TLR8 signaling with an IC$_{50}$ value of 12.8 ± 1.1 µM. (B) D-107 specifically inhibits TLR8, and does not show activity against TLR3, TLR4, TLR5, TLR7, or TLR9.
6.2.3 The Biological Relevance of D-107

Next, we aimed to explore the effect of D-107 on biologically relevant ssRNA ligands. We used ssRNA40 and ORN06, 20-mer ligands that degrade to the relevant U and UG products. Unlike R848, these ligands affect both binding sites and emulate physiologic detection of virus or siRNA nucleotides. In the presence of these ssRNA ligands, D-107 synergistically induces TLR8 signaling greater than that of the ssRNA alone (Figure 6.9A). When compared to ssRNA ligand alone, ssRNA40 activity is increased 5-fold, and ORN06 2-fold. Interestingly, D-107 inhibits R848 signaling, and does not affect signaling alone. This indicates that D-107 increases NF-κB transcription only with simultaneous ssRNA treatment. We also explored combinatorial treatments of R848 with ssRNA ligands, as R848 served as the basis for rational design (Figure 6.9B). Like D-107, R848 was able to synergistically activate TLR8 signaling in the presence of ssRNAs. Signaling was increased 2-fold over R848 alone with ssRNA40, and 1.6-fold in the presence of ORN06. This likely occurs when UG from degraded ssRNA binds to site 2, increasing the affinity for R848 at site 1. The combination of ORN06 and ssRNA40 is not statistically different from either independent ligand, as each generates the same degradation products. This data provides a proof of concept for signaling synergy between the two sites of TLR8, corroborating previous structural observations. Based on these results, we have devised a two-site hypothesis to relate the effects of combinatorial treatment with D-107.

6.2.4 A Two Site Model for D-107 Activity

Due to the diverse biological responses D-107 has to varied TLR8 agonists, we aimed to identify the mechanism of action. There are two models which could account for D-107 antagonizing R848 while synergistically agonizing ssRNA ligands.

6.2.4.1 Site 1 Binding Model

The first model poses that D-107 binds to site 1 of TLR8, consistent with R848 binding that served as the foundation for rational design (Figure 6.10). In this instance, D-107 would act...
Figure 6.9: D-107 agonizes TLR8 signaling in the presence of ssRNAs. (A) In the presence of the ssRNA TLR8 ligands ssRNA40 and ORN06, D-107 synergistically agonizes NF-κB transcription. Data are normalized for each independent ligand as 100%, and untreated cells as 0%. Data are representative of the average and standard deviation of at least three biological replicates. (B) In the presence of ssRNA TLR8 ligands, R848 synergistically agonizes NF-κB transcription. Data are normalized with R848 as 100% activity, and untreated cells as 0%. Data are representative of the average and standard deviation of at least three biological replicates. *p ≤ 0.05
as a competitive inhibitor for R848, preventing TLR8 activation. Conversely, in the presence of endogenous ligand, ssRNA binding to site 2 would induce a conformational change that allowed D-107 to stabilize dimerization and induce signaling. As D-107 is a derivative of R848, this model is rational, as it presumes a similar binding interface. This is further supported by computational docking, which places D-107 in the site 1 pocket. However, competitive inhibition of R848 seems challenging, as R848 binds tightly to TLR8 with a $K_D$ of 0.2 $\mu$M [207]. The differences in pocket volume and electrostatics at site 1 could also explain how D-107 preferentially targets TLR8.

### 6.2.4.2 Site 2 Binding Model

The second model hypothesizes that D-107 binds to TLR8 site 2, and induces a conformational change that disparately affects R848 and uridine ligands bound to site 1 (Figure 6.11). This model is feasible since both R848 and ssRNA uridine bind site 1, though with varied interactions and orientations (Figure 6.2). While D-107 was rationally designed for site 1, the similarities between the U and UG binding sites may have shifted the binding location. Docking analysis of D-107 with the TLR8 crystal structure did not predict site 2 binding. As site 2 varies significantly between TLR7 and TLR8, this model could explain how D-107 specifically targets TLR8.

### 6.3 Conclusions and Future Directions

Here we report a first-in-class TLR8-specific small molecule. We aimed to design small molecules that could target TLR8 without affecting the closely related TLR7. The R848 bound TLR8 crystal structure was used as a basis for the rational design of imidazoquinoline-derived small molecules. After assessing the cellular activity of this compound family, we identified a series of molecules that demonstrated TLR8-specificity. Potency was linked to the size and electronegativity of the 4-position halogen, while specificity was dependent on the tertiary alcohol at the 1-position. From this analysis, D-107 emerged as the most promising TLR8-specific small molecule. Interestingly, D-107 demonstrates a ligand-dependent response on TLR8 signaling, inhibiting R848 activity while synergistically agonizing ssRNA signaling. As an ssRNA-dependent agonist, D-107 could have
Figure 6.10: Site 1 model of D-107 binding. (A) A schematic representation of the site 1 binding model. D-107 binds to site 1, competing out R848 and inhibiting TLR8 signaling. UG binding to site 2 induces a conformational change that improves site 1 affinity for D-107 or R848, increasing TLR8 activation. (B) Docking analysis of R848 and D-107 with the TLR8 crystal structure. Modeling predicts that R848 (green) and D-107 (blue) bind to site 1 (red). This interaction concurs with the structurally determined R848 binding site (yellow).
Figure 6.11: Site 2 model of D-107 binding. A schematic representation of the site 1 binding model. D-107 binds to site 2, preventing ssRNA UG binding and inducing a conformational change that affects site 1. Due to the differences in U and R848 binding, D-107 disparately affects the signaling of each. This causes synergistic activation of ssRNAs, and inhibition of R848 signaling.
applications as a vaccine adjuvant, or could be used to bolster the immune system in immunocompromised individuals. Further, D-107 can be used to explore how the two nucleotide binding sites of TLR8 affect each other and downstream inflammation.

Future work should continue to explore the mechanism of action for D-107. Binding analysis with isothermal titration calorimetry can determine the $K_D$ of D-107, allowing for comparison with existing ligands. Further cellular assays may tease out two-site interactions through transfection of U or UG in the presence of D-107. Synergistic agonism may be explored further using PCR or enzyme-linked immunosorbent assays (ELISA) to fully characterize the inflammatory response. Finally, a crystal structure of D-107 both alone and in complex with ssRNA ligands will clarify conformational changes imposed by binding. Structural analysis is currently underway through a collaboration with the Shimizu research group at the University of Tokyo.

6.4 Materials and Methods

6.4.1 Structural Basis for Rational Design

Rational design of specific small molecules considered the crystal structures of TLR7 and TLR8. PDB 3W3G, 3W3L, 4R07, 4R08, 3W3K, 3W3J, 5GMF, 5GMG, 5GMH [206, 207, 240].

6.4.2 Chemical Synthesis

All small molecule synthesis was designed, performed, and validated by A. Csakai. For the sake of brevity and to focus on the contributions of C. Smith, synthetic information is not included.

6.4.3 Cell Culture

HEK 293 cells transfected with the appropriate TLR and an NF-κB SEAP reporter were used to assess compound potency and specificity. HEK TLR cells were either purchased from Invivogen or prepared by Shuting Zhang. Cells were grown in DMEM media with 10% FBS and 1% Penicillin/Streptomycin. To select for TLR and reporter expression, the media was supplemented
with 10 µg/mL blasticidin and 100 µg/mL zeocin. 100,000 cells/well were plated in a tissue-culture treated 96-well plate (Costar 3596) in unsupplemented DMEM. Cells were then treated with 1 µg/mL R848, 5 µg/mL ssRNA40/LyoVec (InvivoGen), and/or 5 µg/mL ORN06/LyoVec (InvivoGen) and the indicated concentrations of compound. Cells were incubated overnight and assayed for NF-κB signaling using a SEAP assay as described below. Data shown is the average and standard deviation of at least three biological replicates. Statistical significance was determined using a one-way ANOVA test followed by Tukey’s test.

6.4.4 Secreted Embryonic Alkaline Phosphatase (SEAP)

To assess NF-κB activity via a SEAP reporter gene, cell media was assayed with QuantiBlue (Invivogen) per manufacturer’s recommendations. The SEAP reporter is constructed as a IFN-β promoter fused to five NF-κB and AP-1 binding sites. SEAP was quantified by measuring absorbance at 620 nm. Data was normalized with ligand + DMSO as 100% activity and untreated cells as 0% activity. Each data point represents the average and standard deviation of at least three biological replicates.

6.4.5 WST1 Toxicity Assay

Cellular toxicity was assessed using the Roche Cell Proliferation Reagent WST-1 per manufacturer’s recommendation. Briefly, WST-1 was diluted 1:10 into cell-containing media and assayed with absorbance at 450 nm. Data was normalized with untreated cells as 100% viability, and 25% DMSO treated samples as 0% viability. Each data point represents the average and standard deviation of at least three biological replicates.

6.4.6 Computational Docking

Docking of R848 and D-107 with TLR8 was performed using SwissDock [65, 64]. Docking was performed with both the full length TLR8 structures (3W3G, 3W3L, 4R07, 4R08) and truncated 3W3L (residues 292-639) which encompassed the dimer inclusive of both binding sites. Compounds
were generated and assigned 3D coordinates in ChemAxon MarvinSketch. Files were then converted to .mol2 using OpenBabel and processed in SwissDock.

6.4.7 Modeling

Molecular graphics and analyses were performed with the UCSF Chimera package. Chimera is developed by the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco [160].
Chapter 7

A Combinatorial Approach to Supressing Sepsis

This work is unpublished, and continues to be pursued by the Yin research group and collaborators.

Screening was designed by C. Smith, and optimized and performed by C. Smith, S. Soti, and T. Jones. Structural analysis and compound grouping was performed by A. Csakai and C. Smith. Re-synthesis of E-97 was performed by A. Csakai. Subsequent enzyme and cell experiments were designed by C. Smith and performed by C. Smith, S. Soti, and T. Jones. Individual contributions are outlined below using the CRediT taxonomy.

Conceptualization, Supervision, Project Administration: C.S. & H.Y.; Methodology, Formal Analysis, Writing-Original Draft: C. S.; Data Curation, Writing-Review & Editing, Visualization: C.S., A.C.; Validation, Investigation: C.S., S.S., T.J.; Resources, Funding Acquisition: H.Y.

7.1 Introduction

Septic shock is a serious condition that affects 750,000 Americans each year, and is the tenth leading cause of death in the United States [10, 12, 69, 179]. After overcoming the 80% mortality rate, survivors remain susceptible to organ damage, cognitive impairment, and other permanent physical disabilities. Despite this, little is known about the causes of septic shock, and existing treatments only target the symptoms. Toll-like receptor 4 (TLR4) has long been implicated in sepsis pathology, as high levels of lipopolysaccharide (LPS) are present in septic patients, and TLR4 expression has been correlated to sepsis in both mice and humans [68, 165]. However, potent
and specific TLR4 inhibitors such as TAK242 and Eritoran failed in clinical trials due to lack of efficacy \cite{101, 104, 152, 172}. This failure suggests that TLR4 inhibitors may not be sufficient to prevent sepsis, and another immune receptor may be involved in pathogenesis.

Recently, a new mechanism for TLR-independent LPS signaling was identified \cite{2, 68, 102}. Caspase-4 and -5 in humans and -11 in mice have been shown to be intracellular LPS innate immune receptors. These caspases detect cytosolic LPS as it binds directly to the caspase activation and recruitment domain (CARD) \cite{191}. While this is the first evidence of caspases serving as innate immune receptors, redundant immune sensors are common. For example, flagellin is a ligand for both TLR5 and the NLRC4 inflammasome. Based on this new non-canonical activation, we hypothesize that sepsis may be caused by simultaneous activation of TLR4 and the non-canonical inflammasome.

### 7.2 Results and Discussion

#### 7.2.1 Design of a Cell Viability Assay to Detect Caspase-4/5 Activation with Intracellular LPS Challenge

While promising during early studies, potent and specific TLR4 small molecule inhibitors such as TAK242 and Eritoran did not improve the survival of septic patients in clinical trials \cite{101, 104, 152, 172}. With the identification of caspase-4/5 as novel intracellular LPS receptors, it has become apparent that this cytosolic inflammatory machinery may contribute to sepsis \cite{68}. Thus, we aimed to determine if combined TLR4 and caspase-4/5 inhibition improves cell viability after LPS challenge (Figure 7.1). However, it was necessary to first establish a functional cell assay to quantify cell death after intracellular LPS challenge \cite{191}. As caspase-4 is expressed only in the cytosol, cells were electroporated to introduce LPS to the intracellular environment. This approach is not ideal, as the membrane is compromised during electroporation and the cell undergoes significant stress. However, this was necessary, as purified LPS is not taken up into the cytosol from the extracellular environment even at superphysiologic concentrations (Figure 7.2).
a patient, outer membrane vesicles from bacteria are endocytosed and released into the cytosol, but this is difficult to recapitulate in culture without lengthy purification steps [217]. As such, 250,000 cells were electroporated with 1 µg/mL *E. coli* LPS, and then incubated for four hours to induce caspase-4/5 activation. Cell viability was measured using the Roche WST-1 cytotoxicity assay.

Figure 7.1: A combinatorial approach to sepsis treatment. TLR4-specific therapeutics such as Tak242 failed during clinical trials. Instead, caspase inhibition may also be required. Caspase inhibition with the pan-caspase inhibitor z-VAD-FMK may sufficiently reduce cell death with intracellular LPS challenge, or simultaneous caspase and TLR inhibition may be required.

To validate a cytosolic LPS viability assay, we considered the contributions of immune priming, cell type variability, and caspase ligand specificity. Caspases are generally poorly expressed and require priming with an inflammatory stimulus to upregulate inflammasome expression. This is supported by the mouse studies of Hagar *et al.*, which demonstrated that blocking priming prevented caspase-11 activation and sepsis in mice [68]. However, we did not observe a requirement for priming in cultured immune cells such as BV-2 (murine microglia), U937 (human monocyte), and RAW 264.7 (murine macrophage) (Figure 7.3). It may be that with direct introduction of LPS to the cytosol by electroporation, the basal levels of caspase-4/5 expression are sufficient to induce pyroptosis. Interestingly, the structural requirements for LPS induction of pyroptosis appear to be
Figure 7.2: Superphysiological concentrations of LPS are not freely taken up into the cytosol. LPS added to the cell media at superphysiologic concentrations (up to 1 mg/mL) are not sufficient to induce pyroptosis. LPS is not freely taken up into RAW 264.7 or BV-2 cells to induce caspase activation and subsequent cell death. Viability was determined with untreated cells as 100% and 50% DMSO treated cells as 0%.
tightly regulated. To validate our assay, we electroporated in a variety of TLR ligands to confirm caspase-4/5 specificity for LPS previously reported \[191\]. Indeed, we saw no cell death with the TLR2 ligands Pam2CSK4, Pam3CSK4, and Lipomannan, the TLR3 ligand Poly (I:C), or a control plasmid (FGFR3-YFP) (Figure 7.4A). Even more exclusively, LPS from two different organisms displayed disparate caspase-4/5 activation. LPS from \textit{R. sphaeroides} (LPS-RS) differs from \textit{E. coli} LPS only in acylation state, yet does not induce pyroptosis (Figure 7.4B). While the structural requirements for LPS activation of caspase-4/5/11 remain unexplored, sensing appears to be sensitive to minor alterations. While this topic is not discussed further in this chapter, we have hypothesized that the differences in LPS activation may be linked to a capacity to form higher order structures, such as micelles \[194\]. Many inflammasome mechanisms are linked to production of a large protein scaffold for caspase activation and recruitment. Perhaps LPS micelles or aggregates serve a similar function.

Figure 7.3: Immune cells do not require priming to induce pyroptosis upon intracellular LPS challenge. Priming the cells does not change the extent of cell death upon intracellular LPS challenge. This may occur if the endogenous levels of caspase-4/5/11 are sufficient to induce pyroptosis. Data are normalized with untreated cells as 100% viability and 50% DMSO treated cells as 0% viability, and represent the average and standard deviation of at least biological triplicate.
Figure 7.4: LPS induces pyroptosis. A) RAW 264.7 cells were challenged with a variety of TLR ligands (Pam$_2$CSK$_4$, Pam$_3$CSK$_4$, Lipomannan, Poly (I:C)) and no pyroptosis was observed. Electroporation with a FGFR3-YFP plasmid similarly did not affect viability. Data are normalized with untreated cells as 100% viability and 50% DMSO treated cells as 0% viability, and represent the average and standard deviation of at least biological triplicate.B) LPS sensitivity appears to be species-dependent, with *E. coli* LPS inducing pyroptosis where *R. sphaeroides* (LPS-RS) does not.
7.2.2 Combinatorial Treatment to Improve Cell Viability with Intracellular LPS Challenge

In order to assess the efficacy of a combinatorial inhibition on cell viability, immune cells were electroporated with 1 µg/mL LPS in the presence of the TLR4-specific inhibitor TAK242 and/or the pan-caspase inhibitor z-VAD-FMK. No caspase-4/5 specific small molecules exist, so pan-caspase inhibition was used for a proof of concept study.

We observed that cells challenged with intracellular LPS demonstrated significantly improved viability in the presence of z-VAD-FMK, but not TAK242 (Figure 7.5). Combinatorial treatment did not show any improvement over z-VAD-FMK alone. We speculate that in a more complex biological system, combinatorial treatment may still be required. TAK242 may inhibit cell priming through TLR4, reducing the expression of caspase-4/5, and thus the likelihood of pyroptosis. However, in instances such as septic shock where high levels of endotoxin are detected, caspase inhibition may be required as a second line of defense. This would reduce cell death and the production of cytokines, forestalling systemic inflammation. It is also important to note that z-VAD-FMK is a pan-caspase inhibitor, and inhibition of all caspasers simultaneously may mask the contributions of caspase-4/5. In order to thoroughly pursue this hypothesis, it became necessary to develop a caspase-4 or caspase-4/5 specific inhibitor to tease out the contributions of these individual caspases. No specific caspase-4/5 inhibitors have been published to date.

7.2.3 High-Throughput Screening for Caspase-4 Inhibitors

[The methods in this section also appear in Chapter 2. The Prestwick Chemical Library was simultaneously screened with the SoftFocus Protease/Kinase Library.] High-throughput screening for inhibitors of human caspase-4 was performed using the 2100 compound SoftFocus Protease/Kinase Library at the University of Colorado High-throughput Screening Core Facility. The screen was designed in a 384-well format (Greiner 781207), using 33 µM compound, 1 µM recombinant human caspase-4 in high citrate buffer (50 mM Tris-HCl, pH 7.5, 1 M sodium citrate, 10
Figure 7.5: z-VAD-FMK inhibits pyroptosis. Immune cells electroporated with 1 µg/mL LPS undergo nearly complete cell death. TLR4 inhibition by TAK242 does not improve viability, but pan-caspase with z-VAD-FMK inhibition significantly improves cell survival. Combinatorial treatment is not superior to z-VAD-FMK treatment alone. Electroporation does induce cell stress, and results in a reduction of cell viability regardless of treatment. Data are normalized with untreated cells as 100%, and 50% DMSO treated cells 0% viability. *p ≤ 0.05 compared to LPS treated.
mM DTT, 10% sucrose), and 20 µM Promega Caspase-Glo 9 Assay substrate [147, 174]. While the Caspase-Glo 9 assay is designed for use with caspase-9, the LEHD substrate is also recognized by caspase-4 [204]. The assay Z’ factor is 0.66, recommending this method as suitable for high-throughput experimentation [238]. Compounds were incubated with enzyme for at least fifteen minutes prior to substrate addition to allow equilibration. Plates were read with endpoint luminescent analysis 20 minutes and 60 minutes after substrate addition. Compounds were considered to be hits if caspase-4 activity was inhibited to less than 25% relative to the solvent control. Using these criteria, we identified 48 hits, which are summarized in Figure 7.1. However, there was an issue identifying the source plates at the screening facility. Plates 1, 2, and 9 are not available, and so the facility is uncertain as to the identity of those compounds and these twelve hits could not be pursued further.

7.2.4 Biofocus Protease/Kinase Library Hit Validation and Specificity

The 33 hits with known structures were subdivided into six core scaffolds, as indicated in Figure 7.6. All protease/kinase library hits are six-membered and five-membered fused heterocycles containing two or more nitrogen or oxygen heteroatoms. The conserved structural motif between hits is promising, suggesting a structure-activity relationship. We selected a subset of hits and related molecules to undergo validation and to assess caspase-specificity. These compounds were cherry picked from both the 384-well screening plates and the 96-well source plates, however the limited volumes available restricted the extent of secondary screening. The two oxygen-containing hits were not cherry picked due to their disparate core. We re-validated the selected compounds for activity against caspase-4, caspase-9, and caspase-3. These caspases were selected to assess structural diversity and substrate specificity in order to select hits for re-synthesis and more extensive biological characterization. The following results represent the average and standard deviation of at least two independent experiments.

Scaffold 1 is comprised of five hits with activity ranging from 11-14%, representing 4% of scaffold representation in the library (Figure 7.7). These compounds have an imidazo[4,5-c]pyridine...
Table 7.1: Biofocus protease/kinase library screening hits. The Biofocus Protease/Kinase Library was assayed at 33 \( \mu \text{M} \) with recombinant human caspase-4. Compounds were determined to be hits if caspase-4 activity was reduced to \( \leq 25\% \) compared to the solvent control. Due to an issue at the screening facility, plates 1, 2, and 9 contain unknown compounds and could not be characterized further. Compounds indicated as no relationship did not fall into a general scaffold category.

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<td>6.8±2.2%</td>
<td>Structure Unknown</td>
</tr>
<tr>
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<td>3</td>
</tr>
<tr>
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<td>7.4±2.6%</td>
<td>4</td>
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<td>9-A2</td>
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<td>1-G8</td>
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<tr>
<td>6-L17</td>
<td>23-F9</td>
<td>8.4±2.3%</td>
<td>4</td>
</tr>
<tr>
<td>6-J15</td>
<td>23-E8</td>
<td>9.0±3.0%</td>
<td>4</td>
</tr>
<tr>
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<td>1-C10</td>
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</tr>
<tr>
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<td>25-E2</td>
<td>10.9±2.9%</td>
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<td>20-C3</td>
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<td>1</td>
</tr>
<tr>
<td>4-O5</td>
<td>13-H3</td>
<td>11.2±2.3%</td>
<td>3</td>
</tr>
<tr>
<td>1-M22</td>
<td>2-G11</td>
<td>12.4±6.3%</td>
<td>Structure Unknown</td>
</tr>
<tr>
<td>1-C19</td>
<td>1-B10</td>
<td>12.6±5.7%</td>
<td>Structure Unknown</td>
</tr>
<tr>
<td>4-G7</td>
<td>13-D4</td>
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<td>3</td>
</tr>
<tr>
<td>6-J7</td>
<td>23-E4</td>
<td>13.0±1.9%</td>
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</tr>
<tr>
<td>5-D6</td>
<td>20-B3</td>
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<td>1</td>
</tr>
<tr>
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<td>20-D3</td>
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</tr>
<tr>
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<td>19.9±1.4%</td>
<td>Structure Unknown</td>
</tr>
<tr>
<td>4-G19</td>
<td>13-D10</td>
<td>20.0±1.6%</td>
<td>2</td>
</tr>
<tr>
<td>6-N15</td>
<td>23-G8</td>
<td>20.4±4.0%</td>
<td>4</td>
</tr>
<tr>
<td>5-O11</td>
<td>17-H6</td>
<td>20.9±4.6%</td>
<td>5</td>
</tr>
<tr>
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<td>23-H5</td>
<td>21.1±5.2%</td>
<td>4</td>
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<td>23-F8</td>
<td>21.4±7.3%</td>
<td>4</td>
</tr>
<tr>
<td>3-P21</td>
<td>11-H11</td>
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<td>Oxygen-containing</td>
</tr>
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<td>12-D11</td>
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<td>23.6±7.5%</td>
<td>2</td>
</tr>
<tr>
<td>6-016</td>
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<td>3-N20</td>
<td>12-G10</td>
<td>24.0±3.8%</td>
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</tr>
<tr>
<td>1-K19</td>
<td>1-F10</td>
<td>24.6±10.1%</td>
<td>Structure Unknown</td>
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</tbody>
</table>
Figure 7.6: Summary of protease/kinase hit scaffolds. Caspase-4 inhibitors identified in high-throughput screening were subdivided into six groups based on their core scaffold. All scaffolds were fused heterocycles with nitrogen heteroatoms. Each scaffold contained an array of substituents and potencies.
core with a variety of aromatic rings (red), methyl or hydrogen substituents (green), and hydrogen or m-Tolyl groups (blue). Compound 20-C3 did not validate as a potent caspase-4 inhibitor, with 71% caspase-4 activity compared to 11% during screening. Interestingly, 20-C3 did display potent inhibition of caspase-3, though not caspase-4 or -9. This compound may be examined further as a caspase-3 inhibitor. The remaining four compounds were consistent with the screen, but did not demonstrate caspase-4 specificity.

Scaffold 2 consists of 2 hits with a imidazo[1,2-a]pyrazine core, and 1 related molecule with an imidazo[1,2-a]pyridine core (Figure 7.8). Activity for this scaffold ranges from from 19-24%, and represents 50% and 1% of hits possible with the respective cores. These compounds contain an electron rich phenol ring (red), and a meta-substituted phenyl ring (green). Compound 13-D10 and did not re-validate as a potent hit, but may be explored for caspase-3 activity. 13-C10 provided inconclusive results for caspase-4, but did not inhibit the other two caspases. 18-G9 demonstrates at least a 4-fold preference in activity for caspase-4, and should be evaluated further.

Scaffold 3 consists of 9 hits with activity ranging from 7-24%, representing 12% of the library compounds containing this core (Figure 7.9). This scaffold contains a pyrrolo[3,2-c]pyridine core with an electron rich phenol ring (red), a methyl or hydrogen substituent (green), and a phenyl or heterocyclic substituent (blue). These compounds do not display caspase-4 specificity, though the results are largely consistent with the initial screening.

Scaffold 4 consists of 9 hits with activity ranging from 7-23%, which is 14% of scaffold representation in the library (Figure 7.10). This scaffold contains a [1,2,4]triazolo[1,5-a]pyridine core with sulfonamides and amide substituents (red), as well as phenyl rings, furan, or n-propyl groups (blue). Caspase-4 enzymatic activity was assessed, and normalized with substrate as 100% activity, and z-VAD-FMK as 0% activity. As a secondary screen with limited volumes, each sample was tested twice. While compound 23-E4 demonstrated weak caspase-4 specificity, the overall scaffold was non-specific.

Scaffold 5 consists of 3 hits with activity ranging from 14-21% and a pyrazolo[1,5-a]pyrimidine core, with hits representing 20% of this scaffold in the library (Figure 7.11). This scaffold contains a
Figure 7.7: Scaffold 1 structures and activity. Scaffold 1 represents five hits with an imidazo[4,5-c]pyridine core. Caspase-4 enzymatic activity was assessed, and normalized with substrate as 100% activity, and z-VAD-FMK as 0% activity. As a secondary screen with limited volumes, each sample was tested twice. This scaffold does not display caspase-4 specificity.
Figure 7.8: Scaffold 2 structures and activity. Scaffold 2 represents three hits with imidazo[1,2-a]pyrazine or imidazo[1,2-a]pyridine cores. Caspase-4 enzymatic activity was assessed, and normalized with substrate as 100% activity, and z-VAD-FMK as 0% activity. As a secondary screen with limited volumes, each sample was tested twice. Compound 18-G9 demonstrates caspase-4 specificity.
Figure 7.9: Scaffold 3 structures and activity. Scaffold 3 represents three hits with a pyrrolo[3,2-c]pyridine core. Caspase-4 enzymatic activity was assessed, and normalized with substrate as 100% activity, and z-VAD-FMK as 0% activity. As a secondary screen with limited volumes, each sample was tested twice. This scaffold does not display caspase-4 specificity.
Figure 7.10: Scaffold 4 structures and activity. Scaffold 4 represents nine hits with a [1,2,4]triazolo[1,5-a]pyridine core. Caspase-4 enzymatic activity was assessed, and normalized with substrate as 100% activity, and z-VAD-FMK as 0% activity. As a secondary screen with limited volumes, each sample was tested twice. With the exception of 23-E4, this scaffold does not display caspase-4 specificity.
propyl chain terminating in OCH₃ or imidazole (red), and phenyl rings (blue). Caspase-4 enzymatic activity was assessed, and normalized with substrate as 100% activity, and z-VAD-FMK as 0% activity. As a secondary screen with limited volumes, each sample was tested twice. This scaffold does not demonstrate caspase-4 specificity, but may be pursued for caspase-3 inhibitors.

Scaffold 6 consists of 3 compounds with activity ranging from 11-31% (Figure 7.12), representing 5% of the scaffold’s presence in the library. This scaffold has a [1,2,4]triazolo[4,1-a]pyridine core with phenylacetylene or styrene (red) substituents, and benzene or hydrogen groups (blue). 25-E2 demonstrates caspase-4 specificity, with activity at least five-fold increase over the other caspases. 22-H8 is weakly specific, with a three-fold activity difference. 25-D2 does not inhibit caspase-3, which may provide a starting point for structure-activity relationship studies. This scaffold is the most promising for future specificity studies.

7.2.5 Caspase-4 Specific Inhibitors

Based on this secondary screening, we identified 18-G9, 25-E2, and 22-H8 as the most promising caspase-4 specific inhibitors (Figure 7.13). All of these compounds share a similar core, with nitrogens at the 1- and 4-position. The nitrogen position appears to be critical, as other scaffolds with one to three nitrogens lack specificity. All compounds possess a benzene-derived ring linked by two carbons at the 6-position off the pyridine. The substituent linker is sp or sp² hybridized, providing linear geometry and placing all carbons in a conjugated system. At the 3-position of the five-membered ring, all compounds except 22-H8 have a benzene-derived substituent. 22-H8 is the least specific compound, suggesting this substituent is crucial. The aromatic ring may participate in hydrophobic or π-stacking interactions. Further, these structures are largely planar, which may contribute to binding. When compared to similar scaffolds in the library that did not show caspase-4 activity, the hit molecules are substantially more hydrophobic. While current specificity is limited to a several-fold difference in activity, additional structure-activity relationship studies may develop more potent inhibitors based on the trends identified here.

To further validate these compounds, it became necessary to re-synthesize them to confirm
Figure 7.11: Scaffold 5 structures and activity. Scaffold 5 represents three hits with a pyrazolo[1,5-a]pyrimidine core. Caspase-4 enzymatic activity was assessed, and normalized with substrate as 100% activity, and z-VAD-FMK as 0% activity. As a secondary screen with limited volumes, each sample was tested twice. This scaffold does not display caspase-4 specificity.
Figure 7.12: Scaffold 6 structures and activity. Scaffold 6 represents three hits with a [1,2,4]triazolo[4,1-a]pyridine core. Caspase-4 enzymatic activity was assessed, and normalized with substrate as 100% activity, and z-VAD-FMK as 0% activity. As a secondary screen with limited volumes, each sample was tested twice. 25-E2 and 22-H8 are caspase-4 specific, while 25-D2 is specific for caspase-4 and -9.
both the compound identity and to have sufficient material for more extensive biological characterization. 22-H8 was synthesized and assayed for caspase-4 and -3 specificity. Consistent with our previous results, there was only a modest preference for caspase-4 (IC\textsubscript{50} 4.1 µM) compared to caspase-3 (IC\textsubscript{50} 15 µM). Further, this compound was toxic in cell culture. While these limitations mean that 22-H8 should not be pursued further, 18-G9 and 25-E2 remain to be synthesized, and should be more promising.

### 7.3 Conclusions and Future Directions

Caspase-4 specific inhibitors have exciting potential in sepsis treatment. Either alone or through combinatorial treatments with TLR4 inhibitors, novel therapeutics will prevent LPS-sensing in both the intracellular and extracellular environments. Here we have validated an assay for detecting caspase-induced pyroptosis after intracellular LPS challenge in multiple immune cell lines. We have also demonstrated that treatment with the pan-caspase inhibitor z-VAD-FMK improves cell viability. While a contribution for TLR4 was not observed in this model, in a more complex biological system TLR4 inhibition may be beneficial by blocking both immune priming and upregulation of caspase expression. Further, z-VAD-FMK is an irreversible pan-caspase inhibitor, and may obfuscate the contributions of individual caspases and TLRs. As no specific caspase-4 or -4/5 inhibitors have been reported to date, we performed a high-throughput screen of the Soft-Focus Protease/Kinase Library and identified 48 caspase-4 inhibitors. Of these, three compounds demonstrated caspase-4 specificity when assayed with caspase-9 and -3. These compounds have a [1,2,4]triazolo[4,1-a]pyridine or imidazo[1,2-a]pyridine core with nitrogens at the 1- and 4-position and aromatic substituents at the 3- and 6-position. 22-H8 was resynthesized, but demonstrated only a weak preference for caspase-4. Future work should synthesize 18-G9 and 25-E2 to validate caspase-4 activity both \textit{in vitro} and in a biological system. Additional structure-activity relationships may develop a more potent and specific caspase-4 inhibitor. Cheminformatic analysis of related compounds that did not demonstrate activity may serve as a starting point for design. Biological characterization should assess inhibitor contributions to cell viability after intracellular LPS
Figure 7.13: Summary of specific caspase-4 inhibitors. Compounds 18-G9, 25-E2, and 22-H8 all demonstrate preferential inhibition of caspase-4.
challenge using the experimental protocols designed here. If promising, animal models or infection with outer membrane vesicles could provide relevance for septic shock.

7.4 Materials and Methods

7.4.1 Electroporation

To assess pyroptosis in response to intracellular LPS challenge, immune cells were plated at 250,000 cells/mL in a 24-well plate and treated with the appropriate compound. The following day, the cells were pelleted and resuspended in 25 µL of Neon R buffer, or unsupplemented RPMI media. This concentration step is required for electroporation with the Neon 10 µL tips. Ligand was then added to the cells, and each cell replicate was electroporated in duplicate (10 µL each) to account for electroporation introduction of variability and cell stress. Electroporation occurred at 1680 V, 20 ms, 1 pulse. Cells were then placed into 48-well plates with 250 µL of antibiotic-free media. Cells were treated with 1 µg E. coli or R. sphaeroides LPS, Pam2CSK4, Pam3CSK4, lipomannan, poly(I:C), or FGFR3-YFP. Compound treatment occurred with 1 µM TAK242 or 10 µg z-VAD-FMK, and was added on day one and/or two accordingly. After four hours, cells were assayed for viability.

7.4.2 Cell Viability

Four hours after electroporation, cell viability was determined using the Roche WST-1 cytotoxicity assay. WST-1 was diluted ten-fold into cell-containing media, and incubated at 37 C until a color change was observed. The plate was quantified by reading absorbance at 450 nm. Data was normalized with untreated cells at 100%, and media only as 0% viability.

7.4.3 Caspase Specificity

To assess the potency and specificity of cherry picked compounds, activity was determined against caspases-3, -4, and -9. Experiments were performed in a 384-well format with 33 µM compound per the conditions noted here (Table 7.2), which were optimized for each enzyme. Substrate was present in at least 16-fold excess, and enzyme concentrations were kept low. Activity was
Table 7.2: Caspase specificity methodology. Caspase specificity experiments were carried out using the conditions noted below.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>[Enzyme] (nM)</th>
<th>[Substrate] (µM)</th>
<th>Buffer</th>
</tr>
</thead>
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<td>WEHD, 6.5</td>
<td>Enzo Caspase</td>
</tr>
<tr>
<td>Caspase-3</td>
<td>200</td>
<td>DEVD, 3.3</td>
<td>Standard Caspase</td>
</tr>
<tr>
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<td>LEHD, 10</td>
<td>High Citrate</td>
</tr>
<tr>
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<td>20</td>
<td>LEHD, 10</td>
<td>High Citrate</td>
</tr>
<tr>
<td>Caspase-9</td>
<td>200</td>
<td>LEHD, 6.5</td>
<td>High Citrate</td>
</tr>
</tbody>
</table>

measured as the change in luminescent signal for at least thirty minutes. Analysis was performed using the linear portion of the curve, however endpoint analysis yielded values within error. Percent inhibition was determined compared to the DMSO only control. Each assay included solvent and z-VAD-FMK controls, and assay acceptance criteria required at least a ten-fold signal to noise ratio.

Standard Caspase Assay Buffer: 20 mM PIPES, pH 7.5, 100 mM NaCl, 1mM EDTA, 10 mM DTT, 10% sucrose. High Citrate Assay Buffer: 50 mM Tris-HCl, pH 7.5, 1 M sodium citrate, 10 mM DTT, 10% sucrose.
Bibliography

[1] Colorado Center for Drug Discovery, Department of Chemistry at Colorado State University.


