

Small molecule inhibitors against enteric bacterial glycosyltransferases

by

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B.S., Nanjing Agricultural University, China, 2016

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AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Department of Diagnostic Medicine/Pathobiology
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Manhattan, Kansas, USA

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Abstract

Enteric bacterial pathogens are the major sources of foodborne diseases. Infection caused by these pathogens leads to gastroenteritis thus of great threat to public health. Pathogenic bacteria evolved to develop various secretion systems to deliver virulence proteins termed ‘effectors’. These virulence factors promote the invasion of pathogens as well as evasion of host innate immune response. Type III secretion system (T3SS) is one of the most complex secretion systems used by Gram-negative bacteria *Escherichia coli*, *Salmonella enterica*, and *Citrobacter rodentium*. T3SS resembles a molecular syringe structure to inject secreted effector proteins directly from the bacterial cell into the host cell. Our goal is to look for anti-bacterial virulence therapies by targeting important T3SS effectors.

In our first project, we targeted T3SS effector proteins NleB and SseK (NleB ortholog), which are glycosyltransferases that glycosylate protein substrates on arginine residues. This modification is unusual because it occurs on the guanidinium groups of arginines, which are poor nucleophiles, and is distinct from the activity of the mammalian O-linked N-acetylglucosaminyltransferase (OGT). As the deletion of gene *nleB* significantly attenuates *C. rodentium* virulence, we aimed to find small molecular inhibitors which specifically bind to NleB. Therefore, we performed high-throughput screening (HTS) assays to identify NleB/SseK inhibitors. We identified two compounds (100066N and 102644N) that significantly inhibited NleB1, SseK1, and SseK2 glycosylation activities. They were also able to inhibit NleB1 glycosylation of the tumor necrosis factor receptor type 1-associated DEATH domain (TRADD) protein in mammalian cells. After pre-incubation with the two compounds in mouse monocyte/macrophage-like cells (RAW264.7), number of *Salmonella enterica* strain ATCC 14028 which replicated inside RAW264.7 cells greatly reduced 24 h post infection. Results

confirmed that 100066N and 102644N can inhibit NleB/SseK activity both *in vitro* and *in vivo*. Both compounds can work directly in mammalian cell culture to counter bacterial virulence. Furthermore, 100066N and 102644N show high potential in new drug development because they have advantages of not interfering with bacterial growth curve when added to the LB culture, no significant toxicity to mammalian cells, and do not cross-react with human OGT.

However, we encountered difficulties when trying to apply 100066N and 102644N on mice infection models. The two compounds were not easy to synthesize, and their derivatives did not show significant inhibitory effect on bacterial glycosyltransferases. Therefore, in the second project, we screened more NleB/SseK inhibitors by enlarging our libraries to perform HTS assays. These libraries include Analyticon natural products library, ChemDiv 3D Biodiversity, ChemDiv Peptidomimetics, FDA proved drugs, Selleck Bioactives and MayBridge mini library. We identified YM155 (sepantronium bromide) which can inhibit EHEC NleB1, *Salmonella* SseK1, SseK2, and *Citrobacter* NleB activities. Like 100066N and 102644N, YM155 also does not interfere with enteric bacterial growth curve when added to the LB culture, has no significant toxicity to mammalian cells nor cross-react with human OGT. Above all, YM155 has the advantage of being more soluble and amenable to chemical derivatization.

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Chapter 1 - Literature Review

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Human enteric pathogens

Bacterial pathogens must circumvent both the host immune system and competition from commensal bacteria to establish an infection. Innate immunity is a primary stage of defense required for elimination of the invading pathogens (Medzhitov & Janeway 1997). In addition to physical and chemical barriers, the innate immune system also consists of surface proteins classified as pattern recognition receptors (PRRs) that sense components of pathogens known as pathogen-associated molecular patterns (PAMPs) (Suresh & Mosser 2013). These receptor ligand associations lead to the induction of the NF- κ B signaling pathway that ultimately culminates in the production of pro-inflammatory cytokines (Liu et al. 2017).

In turn, bacterial pathogens have evolved various strategies to target and inhibit NF- κ B signaling pathway to promote their colonization (Le Nègre 2012).

Bacteria have evolved multiple secretion systems to deliver arsenals of virulence proteins termed ‘effectors’ to counteract host innate immunity. Human enteric pathogens enterohemorrhagic *Escherichia coli* (EHEC), enteropathogenic *E. coli* (EPEC), *Citrobacter rodentium*, and *Salmonella enterica* mainly use type three secretion system (T3SS) to inject effector proteins directly into the target cell cytoplasm. Type three secretion system is a syringe-like secretion apparatus that injects effectors that bind host proteins. These interactions

ultimately subvert the generation of an inflammatory response to the infecting pathogen (Daniell et al. 2001).

EPEC and EHEC

Enteric bacterial pathogens are important threats to human health and are major sources of foodborne disease. According to Central for Disease Control and prevention, more than 48 million food borne illness causing 128,000 hospitalization and 3,000 deaths annually in the United States. *Escherichia coli* (*E. coli*) is a common group of bacteria colonizing the intestinal tract of human and warm-blood animals. Pathogenic *E. Coli* infections are part of the main sources of foodborne disease contributing to severe gastroenteritis. Among them, Enterohemorrhagic *E. coli* (EHEC) are especially serious bacterial pathogens because they cause a type of renal failure (hemolytic uremic syndrome; HUS) for which therapies are limited (Tzipori et al. 2004). A related attaching/effacing (A/E) pathogen, enteropathogenic *E. coli* (EPEC), is an important cause of infantile diarrhea.

Escherichia coli was first discovered by Theodor Escherich in 1885, isolated from infant fecal samples with his own anaerobic culture methods and Hans Christian Gram's new staining technique (Escherich 1989). The bacteria from this family share common characteristics as rod like, none-spore-forming facultative anaerobes. They can be categorized into two groups: commensal *E.coli* (non-pathogenic) and pathogenic *E. coli* (Croxen et al. 2013). Commensal *E.coli* is a component of host common flora and it is beneficial to the maintenance of intestinal homeostasis. On the other hand, pathogenic *E. coli* leads to various food borne diseases threatening human health.

Pathogenic *E. coli* can cause intestinal diseases such as diarrhea or dysentery. Some strains are also related with extra-intestinal infections, including urinary tract infections and meningitis

(Kaper et al. 2004). Virulence traits of pathogenic *E. coli* are considered to derive from gaining or losing of genes in the bacteria genome. Pathogenic *E. coli* is able to gain new virulence genes through conjugation, transposition, transformation and transduction to help survive and multiply in the environment and host body (Kaper et al. 2004).

The traditional way to classify pathogenic *Escherichia coli* is through serotyping. Different strain types are differentiated through their O (somatic), H (flagellar), and K (capsular) surface antigen profiles (Fratamico et al. 2016).

Another way to classify pathogenic *E. coli* is through their pathotypes. Based on different mechanism and virulence factors, pathogenic *E. coli* strains are divided into Extra-intestinal Pathogenic *E. coli* (ExPEC) and seven pathotypes of intestinal pathogenic *E. coli*. (Kaper et al. 2004). The seven pathotypes of intestinal pathogenic *E. coli* are enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), Shiga toxin-producing *E. coli* (STEC) (e.g. enterohemorrhagic *E. coli*(EHEC), enteropathogenic *E. coli* (EPEC), and enteroaggregative *E. coli* (EAEC), diffuse-adherent *E. coli* (DAEC), and adherent invasive *E. coli* (AIEC).

EPEC is an attaching/effacing (A/E) pathogen that causes infantile diarrhea, primarily in developing countries (Clarke et al. 2002). EPEC adherence is mediated by an interaction between the bacterial Tir and intimin proteins to create intimate contact with host intestinal cells (Kenny et al. 1997). Polymerized actin accumulates under the A/E lesion, forming a pedestal-like structure (Campellone & Leong 2003). The EPEC chromosome harbors the locus of enterocyte effacement (LEE), a pathogenicity island that encodes a T3SS and multiple effectors (McDaniel et al. 1995). LEE encoded effectors are generally designated by the term ‘Esp’ to denote EPEC secreted proteins, while effectors encoded outside of the LEE are generally designated as ‘Nles’,

to denote non-LEE encoded effectors. However, for many effectors the nomenclature remains ambiguous, as some effectors designated as ‘Esp’s are encoded outside of the LEE.

EHEC is a human pathogen that causes hemorrhagic colitis and hemolytic uremic syndrome (HUS) (Newell & La Ragione 2018). EHEC also contains the Esps and Nles, but also encodes Shiga-like toxins (EHEC O157:H7) that are responsible for causing more serious disease. HUS can affect patients from young to old while EPEC remains a pathogen of infants less than 2 years old (Robins-Browne & Hartland 2002).

EHEC and EPEC share many virulence determinants and features. They both are A/E pathogens that colonize the intestinal mucosa through the formation of A/E lesions on enterocyte surface (Wong et al. 2011). LEE is essential in this process because it encodes the structure components of T3SS. Bacteria inject translocated intimin receptor (Tir) into enterocytes through T3SS and binds to outer membrane protein intimin. The intimin-Tir interaction leads to actin polymerization and promotes intestinal colonization via formation of A/E lesion. On the other hand, the Nles function as enzymes targeting NF- κ B and MAPK signaling pathway, inhibiting host inflammatory response (Baruch et al. 2011; Gao et al. 2013).

Salmonella enterica

Salmonella enterica is a zoonotic pathogen of substantial concern to global human and animal health. *Salmonella enterica* remains one of most frequently reported causes of foodborne illness (Yombi et al. 2015). *Salmonella enterica* is a rod-shaped, Gram-negative facultative intracellular pathogen. The genus *Salmonella* was named after Daniel Salmon, an American veterinary pathologist. Together with Theobald Smith, his research assistant, they isolated *Hog-cholera bacillus* (now known as *S. enterica* serovar *Choleraesuis*) from pigs with swine fever in

1884. Some serovars have a broad host range (*S. Typhimurium* and *S. Enteritidis*), others are host-adapted (*S. Typhi* and *S. Paratyphi A* and *C*). Ingestion of contaminated foods, especially poultry, is the primary source of infection, although contaminated water and direct contact with animals are also risk factors. *S. enterica* invades the intestinal epithelium in the ileum and colon, either to cause a neutrophilic gastroenteritis or disseminate to systemic sites and cause sepsis. It thrives in the intracellular niche, allowing intrinsic antimicrobial resistance and chronic colonization in rare cases (Knodler et al. 2019). *Salmonella enterica* encodes multiple T3SSs. T3SS1, encoded by the *Salmonella* pathogenicity island (SPI)-1, has many functions, including the invasion of epithelial cells and the induction of an inflammatory response at the intestinal surface (Marcus et al. 2000). T3SS2 is encoded by SPI-2 and is a key virulence determinant for systemic disease in mice (Ochman et al. 1996). T3SS2 is induced intracellularly (Hensel, 2000)

A Schematic of the EHEC O157:H7 EDL933 genome. Black rectangles, O-islands; red dots, prophage elements. **B** Schematic of the *S. Typhimurium* genome. Red arrows, *Salmonella* pathogenicity islands (SPIs); Grey and yellow rectangles, prophage elements.

C. rodentium

C. rodentium is a natural pathogen of mice that was discovered during a collapse of mouse colonies due to transmissible murine colonic hyperplasia (TMCH) (Luperchio et al. 2000).

TMCH can further induce diarrhea in *C. rodentium* infected mice. As both EHEC and EPEC are human pathogens which have little virulence on mice (Mundy et al. 2005), the genomic and mechanistic similarities between *C. rodentium* and EPEC have made *C. rodentium* a model system for studying A/E pathogens *in vivo* (Silberger et al. 2017). Thus, *C. rodentium* infected mice model has long been used to understand the molecular basis of EPEC and EHEC infection on human (Collins et al. 2014).

Gram-negative bacterial secretion systems

Bacterial secretion systems are protein complexes present on the cell membranes of bacteria for secretion of substances. For pathogenic bacteria, secretion of these virulence substances helps them to invade host cells and survive under host defense mechanisms. Scientists have discovered diverse systems that enable bacteria to transport virulence proteins from the cytosol into host cell or host environment across one or two membranes. They can be classified into different types based on their specific structure, composition, and activity.

For example, protein directly transported from the cytoplasm of bacteria to host cell membrane (one-step activity); protein first transported out of the inner cell membrane into the periplasm then went through the outer cell membrane into host cell (two-step activity). In generally, four types of secretion systems have been found in gram-positive bacteria (cell envelopes contain one membrane, monoderm). Nine secretion systems have been found in Gram-negative bacteria (cell envelopes contain two membranes, diderm), including Type I, II, III, IV, V and VI secretion system (T1SS, T2SS, T3SS, T4SS, T5SS, and T6SS) (Desvaux et al. 2009; Green et al. 2016).

Type I secretion system

T1SS is also known as ATP-binding cassette (ABC) dependent transporter. It has a simple structure applied by many gram-negative bacteria to one-step transport substrates across their inner and outer bacteria membrane with the help of periplasmic membrane-fusion protein (MFP) (Koronakis et al. 1991). T1SS was first discovered by identifying two membrane proteins of *E. coli*, HlyB and HlyD, which are important for transporting cytotoxin hemolysin A (HlyA) (Nicaud et al. 1985).

T1SS activates when a signal sequence HlyA binds HlyB on the cell membrane. This signal sequence is an ATP-binding cassette transporter. The HlyAB complex activates HlyD which uncoils and moves to the outer cell membrane. The terminal signal is recognized by TolC in the inner membrane. The HlyA is secreted out of the outer membrane through a tunnel-like protein channel (Koronakis et al. 2000).

T1SS transports various molecules including ions, carbohydrates, and proteins. The secreted molecules vary in size from the small *Escherichia coli* peptide colicin V, which is 10 kDa (Gilson et al. 1987), to the *Pseudomonas fluorescens* cell adhesion protein LapA, which is 520 kDa (Boyd et al. 2014). Among the most well-known molecules are repeats in toxin (RTX) protein family and lipase enzymes, such as HlyA.

Type II secretion system

T2SS is a large membrane bound multiprotein complex used by many Gram-negative bacteria to translocate proteins from bacterial cytoplasm across bacterial outer membrane into extracellular space. The T2SS pathway was first found in *Klebsiella oxytoca* to secrete pullulanase, a starch-hydrolyzing lipoprotein (d'Enfert et al. 1987). Some of the human pathogens hold one or more T2SS, including *Vibrio cholerae*, EHEC and EPEC, *Pseudomonas aeruginosa*, *Klebsiella*, *Legionella pneumophila*, *Yersinia enterocolitica*, and *Aeromonas hydrophila* (Korotkov et al. 2012). Unlike T1SS, T2SS uses a two-step process to secrete proteins outside the cell. The first step requires the help of Sec and Tat secretory pathways to transport substances across the inner membrane to periplasm (Natale et al. 2008). The second step involves a complex structure similar to T4SS pili to drive proteins from periplasm across bacterial outer membrane (Peabody et al. 2003). T2SS contains approximately 40-70 proteins of 12-15 different types, named general

secretory proteins (GSPs) as part of the main terminal branch of the general secretion pathway (Sandkvist 2001).

T2SS structure contains four main components: the pseudopilus, the secretion ATPase, the inner membrane complex and the outer membrane complex. The pseudopilus is a periplasmic fibrous structure made up of multiple copies of five pseudopilins (pillin like proteins), known as GspG, GspH, GspI, GspJ and GspK (Korotkov et al. 2012). The maturation of pseudopilins involves signal peptide GspO cleavage of the pre-pseudopilins N-terminal amino acids residues when pre-pseudopilins is driven across bacteria inner membrane into periplasm by the Sec pathway (Filloux 2004). The inner membrane complex is made up of four different proteins GspC, GspF, GspL, and GspM. Assembly of these proteins also require the Seq pathway (Filloux 2004). GspF and GspL are important to the binding of secretion ATPase GspE, while GspC interacts with outer membrane secretin GspD to open up the gate and release of proteins outside cell (Johnson et al. 2006). Briefly, GspE catalyzes the hydrolysis of ATP and releases energy to form pseudopilus, driving the exoproteins transported to periplasm by Sec or Tat pathway across the outer membrane β -barrels channels to outside of the cell.

T2SS is important for promoting bacterial virulence by secretion of degradative enzymes such as hemagglutinin-protease (HAP) and endochitinase (Sandkvist 2001; Connell et al. 1998), also various toxins such as cholera toxin (CT) of *V. cholerae* and *Escherichia coli* heat-labile enterotoxin (LT) (Holmes et al. 1975; Dorsey et al. 2006).

Type III secretion system

The term Type III secretion system was described in 1993 as ‘signal sequence-independent’ protein secretion pathway (Salmond 1993). T3SS can be classified into non-flagellar or pathogenic T3SS and flagellar T3SS. The former class promotes bacterial virulence by secreting effector proteins into host cells while the later class relates with bacterial motility (Erhardt et al. 2010). T3SS is famous for its needle-like structure to ‘inject’ virulence effectors into host cells to regulate host-bacteria interaction. For example, EPEC and EHEC inject their receptor protein Tir into host cells through T3SS (Frankel et al. 2001), where it inserts in the host cell membrane and binds to a bacterial outer membrane protein called intimin, initiating a cascade of signaling events, leading to actin polymerization and pedestal-like structure formation, resulting in intimate attachment on the microvilli, formation of A/E lesion (Batchelor et al. 2000). The T3SS is essential for understanding the pathology of various gram-negative pathogenic bacteria. The general structure of T3SS is highly conserved across species (Erhardt et al. 2010). It is composed of more than 20 different structural proteins, assembling a structure named injectisome (or T3SS apparatus). This needle complex can be categorized into four parts according to the functions and substructures: export apparatus, basal body, needle, and translocon (Tsai et al. 2015). To avoid confusion, here we use unified nomenclature ‘secretion and cellular translocation’ (Sct) to describe conserved proteins of the type III secretion apparatus (Hueck 1998).

Table 1 Injectisome components of T3SS

Unified nomenclature	Function	EPEC/EHEC	<i>Salmonella</i> SPI-1	<i>Salmonella</i> SPI-2
Basal body				
SctC	OM ring	EscC	InvG	SsaC
SctD	IM ring	EscD	PrgH	SsaD
SctJ	IM ring	EscJ	PrgK	SsaJ
Export Apparatus				
SctR	IM component	EscR	SpaP	SsaR
SctT	IM component	EscT	SpaR	SsaT
SctS	IM component	EscS	SpaQ	SsaS
SctU	Autoprotease	EscU	SpaS	SsaU
SctV	Export Gate	EscV	InvA	SsaV
ATPase complex				
SctQ	Cytoplasmic ring	SepQ	SpaO	SsaQ
SctN	APTase	EscN	InvC	SsaN
SctO	StalK	EscO	InvI	SsaO
SctL	Stator	EscL	OrgB	SsaK
SctW	Gate-keeper	SepL	InvE	SsaL
Needle complex				
SctF	Needle	EscF	PrgI	SsaG
SctA	Needle tip/ filament	EscA	SipD	SseB
SctI	Inner rod	EscI	PrgJ	SsaI
SctP	Needle-length regulator	EscP	InvJ	SsaP
Translocon				
SctE	Translocation pore	EspD	SipB	SseC
SctB	Translocation pore	EspB	SipC	SseD

Basal body

The basal body of T3SS is composed of three ring-like membrane structure which form a tunnel to connect bacteria inner and outer membrane (Radics et al. 2014). The outer membrane ring consists of a 12mer secretin protein SctC (Gauthier et al. 2003). SctC functions as a channel for substance secretion across the outer membrane while its N-terminal domain stretches deeply into the periplasm (Deng et al. 2004). The membrane and supramembrane (MS) ring have an inner and outer toroid. The inner toroid is a periplasm-facing lipoprotein SctJ; the outer toroid is formed by a single transmembrane domain (TM) protein SctD (Portaliou et al. 2016). SctJ and SctD interaction maintains the stabilization of MS ring while SctD and SctC periplasmic domain interactions maintain the stabilization of T3SS basal body structure (Schraidt et al. 2011)

Export apparatus and ATPase

The export apparatus is covered by the MS ring (Diepold et al. 2014). It consists of five different types of bacterial inner membrane proteins, SctR, SctS, SctT, SctU and SctV. The minor part of export apparatus SctR, SctS and SctT are embedded in the TM protein SctD. The major part of export apparatus SctU, SctV also have C-terminal domains forming a ring structure in the cytoplasm, covering proteins SctR, SctS and SctT (Abrusci et al. 2013). The T3SS export apparatus associates with the base of needle complex and is important for the recruitment of substrates to enter the syringe (Minamino et al. 2010).

The ATPase complex is surrounded by the six pods of protein SctQ (C-ring). Similar to F1F0-ATPase synthase, this complex is made up of ATPase SctN, its positive regulator SctO and negative regulator SctL (Romo-Castillo et al. 2014). ATP is synthesized from ADP and inorganic phosphate through a unique rotary mechanism where two subcomplexes move relative

to each other and stabilized by the 'stator stalk' (Weber 2007). The stator α -helical SctL functions as chaperone to prevent oligomerization of ATPase SctN (Biemans-Oldehinkel et al. 2011). The stalk protein SctO promotes oligomerization of ATPase SctN and stimulates its hydrolytic activity (Romo-Castillo et al. 2014).

Needle

Different bacteria have similar T3SS needle structure that varies in size. Needle length can be short as 23 nm for EPEC injectisome, or long as 80 nm for *Salmonella enterica* (Ogino et al. 2006; Cornelis, 2006). The length of needle is dependent on molecular ruler SctP (Monjaras Feria et al. 2012). The needle is composed of three parts, the inner rod protein SctI, the extracellular protein SctF and needle tip/ filament SctA. SctI sits inside the MS inner toroid protein SctJ and extends to the outer membrane. Secretion of the extracellular needle SctF initiates the signal to pump out effector proteins from cytoplasm, which requires SctI interaction with SctU (Sal-Man et al. 2012). SctA is the tip or filament at the end of the needle structure, which functions similarly in T3SS (Fujii et al. 2012; Sal-Man et al. 2012). Most secreted proteins should remain unfolded to pass the 2-3nm diameter hole of needle (Akeda et al. 2005).

Translocon

T3SS Translocon consists of two translocator proteins SctB and SctE which associate with translocation of effector proteins across the host cell membrane (Ide et al. 2001). SctB and SctE are together anchored on the needle tip/ filament SctA, delivered into host cell plasma membrane

to form the translocator pore. Bacterial effector proteins are secreted into host cell through the translocator pore (Mattei et al. 2011).

Apart from T3SS structure proteins which build up the injectisome, bacteria secrete T3SS effector proteins into the host cell and promote infection or suppress host immune response. These effector proteins are considered binding to chaperones in bacteria cytoplasm which protect their cognate substrates and guide them towards the needle complex (Chen et al. 2013).

Type IV secretion system

T4SS is related to bacterial conjugation system, thus different bacteria can exchange their DNAs. It can transport single proteins, as well as protein-protein and DNA-protein complexes through same or different gram-negative bacteria species. The structure of type IV secretion system is composed of 12 protein subunits, VirB1 - VirB11 and VirD4 (Wallden et al. 2010). The translocation channel scaffold, the ATPases, and the pilus together constitute Type IV secretion system. The translocation channel scaffold contains VirB6 - VirB10. The ATPases consist of VirB4, VirB11, and VirD4, which drive the substrate motion through the channel and provide the system with energy. The pilus is composed of VirB2 and VirB5, with VirB2 being the major component. After DNA is packaged in a conjugative system it is recruited by ATPase analogues to the VirD4 coupling protein, then translocated through the pilus.

Type V secretion system

T5SS, also known as autotransporter system, is capable of transporting surface-localized proteins to cross bacterial outer membrane autonomously without consuming ATP (Leo et al. 2012).

Type V secretion system uses Sec system for unfolded proteins to cross the inner membrane.

Proteins which use this pathway have the capability to form a beta-barrel with their C-terminus which inserts into the outer membrane, allowing the rest of the peptide to reach the outside of the cell (Remmert et al. 2010). Autotransporters are often cleaved, leaving the beta-barrel domain in the outer membrane and freeing the passenger domain. Neither ATP nor proton gradient is necessary for surface display of the transported passenger domains. Type V secretion system can be further classified into type Va to Vc autotransporters (Henderson et al. 2004; Remmert et al. 2010). Va is a classical autotransporter secretion which contains a autotransporter protein that its translocator domain assembles β -barrel out membrane with help of periplasmic chaperone, then the rest part of the protein is guided out of the cell and released. Vb is called two-partner secretion where one partner carries the β -barrel domain while the other partner serves as the secreted protein (Henderson et al. 2004); Vc is named chaperone-usher secretion or trimeric

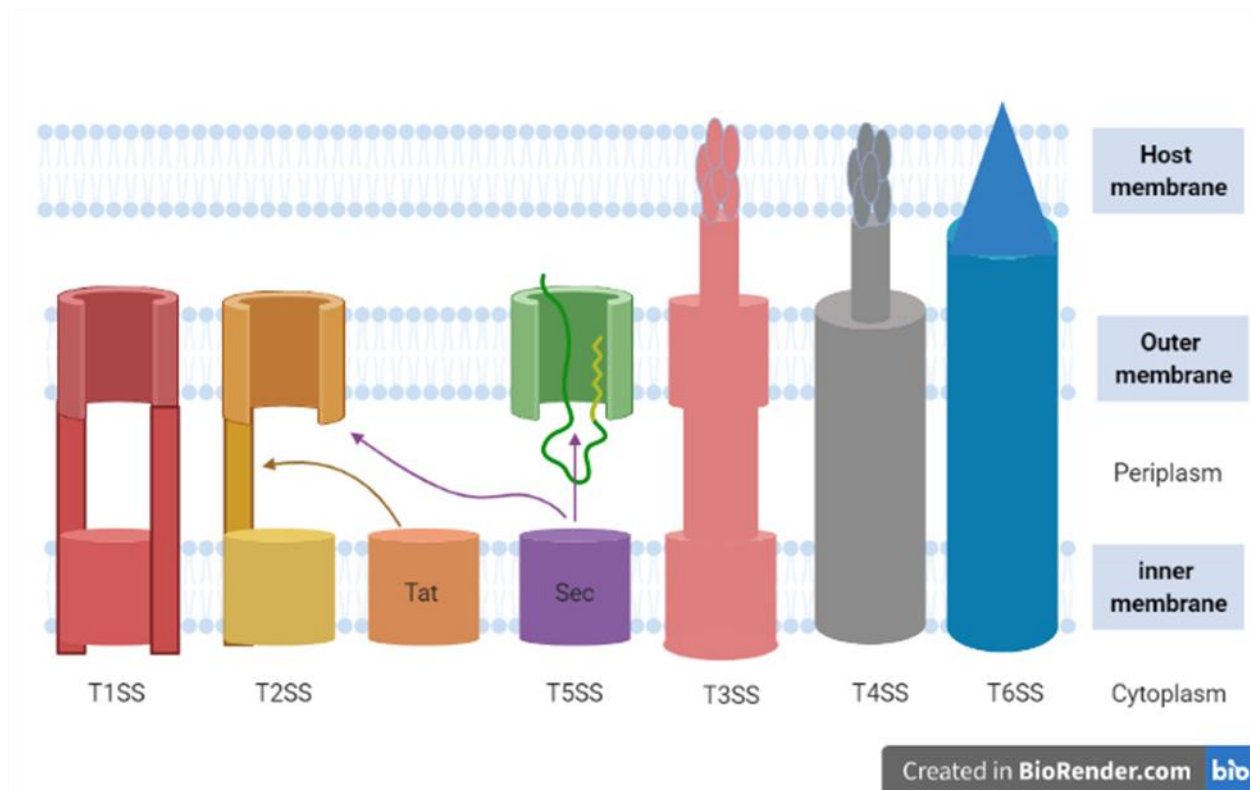
autotransporters in that the secretion protein utilizes both periplasmic chaperone and usher protein (formation of β -barrel) (Waksman et al. 2009).

Type VI secretion system

T6SS was first discovered in two bacterial pathogens, *Vibrio cholerae* and *Pseudomonas aeruginosa* (Pukatzki et al. 2006; Mougous et al. 2006). They found mutations in the Hcp and VrgG genes in *Vibrio cholerae* lead to decreased virulence and pathogenicity. Type VI secretion system is unique for not only delivering toxins into eukaryotic cells, but more often into prokaryotic cells in a contact dependent manner (Shneider et al. 2013).

T6SS consists of around 13 conserved proteins that assemble into three sub-complexes: a phage tail-like tubule, a phage baseplate-like structure, and cell-envelope spanning membrane complex. It is believed that the inverted phage tail like structure pumps out proteins outside of bacteria cells. Evidence have shown that T6SS not only injects effectors into eukaryotes cells to promote virulence, but more importantly this system was used to firing toxins into neighboring bacteria, serving as anti-competitor weapon (Smith et al. 2020).

Figure 2 Gram-negative bacteria secretion systems



Summary of Six Secretion Systems

Different from Gram-positive bacteria which have a thick peptidoglycan layer and no outer lipid membrane, Gram-negative bacteria have three layers: the inner membrane; the periplasmic space with a thin layer of peptidoglycan; and the outer membrane consists of lipopolysaccharides.

Gram-negative bacteria use different mechanism to transport protein through one, two or three phospholipid membranes.

Some proteins are secreted through a two-step mechanism. They first use Tat or Sec pathway to be transported from the inner membrane to periplasm, and then be transport to the outer membrane using a secondary method. T2SS and T5SS utilize a two-step process. As T5SS autotransporters have to be unfolded before crossing the outer membrane to be displayed, they only depend on Sec pathway to enter the periplasm.

Other proteins are secreted through one step, Sec or Tat-independent process by directly going through both bacterial membranes. T1SS, T3SS, T4SS, and T6SS use periplasm spanning channels and transport proteins form cytoplasm to outside outer membrane in a straightforward way. Among them, T3SS, T4SS, and T6SS can penetrate host cell membrane, directly secreting proteins to cytosol to manipulate invasion.

All six secretion systems described above forms a β -barrel channel in the outer membrane. One bacterium can use multiple secretion systems for different protein transport purposes. Besides the six main secretion systems, there are also T7SS, T8SS and T9SS which translocation mechanisms yet not fully understood. Secretion of proteins across lipid membranes is important for bacterial pathogens to invade host cells, promote virulence and suppress host immune response. Studying of bacterial secretion systems remains a vital task in understanding bacterial physiology and pathogenesis.

***E. coli* and *Citrobacter* Effectors**

NleA inhibits Nod-Like Receptor 3 (NLRP3)- mediated inflammasome activation via a direct association with NLRP3. NleA binding to NLRP3 prevents its deubiquitylation, which is required for inflammasome assembly and activation (Yen et al. 2015). As a consequence, interleukin 1 beta (IL-1 β) secretion decreases due to caspase 1 inhibition (Yen et al. 2015). NleA also binds the Sec24 subunit of the mammalian coatamer protein II complex (COPII) to inhibit vesicle trafficking (Kim et al. 2007).

NleB is a glycosyltransferase that glycosylates host proteins with N-acetylglucosamine (GlcNAc) on arginine residues (El Qaidi et al. 2017; Li et al. 2013; Pearson et al. 2013). Deleting *nleB* significantly attenuates *C. rodentium* virulence (Kelly et al. 2006). While a single *nleB* gene is found in *C. rodentium*, two *nleB* genes, *nleB1* and *nleB2*, are found in most EPEC and EHEC strains. EPEC NleB1 glycosylates the tumor necrosis factor receptor (TNFR) type 1-associated DEATH domain protein (TRADD) and the FAS-associated death domain-containing protein (FADD) (Li et al. 2013; Pearson et al. 2013), with preferential affinity for FADD (Scott et al. 2017). Glycosylation of TRADD inhibits its dimerization, thus preventing the proper assembly of the TNFR complex and the activation of the NF- κ B pathway (Li et al. 2013; Pearson et al. 2013). EHEC NleB1 and *C. rodentium* NleB also glycosylate glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (El Qaidi et al. 2017; Gao et al. 2013), which reduces GAPDH binding to TRAF2, subsequently limiting the extent of TRAF2 ubiquitination, leading to reduced NF- κ B activity (Gao et al. 2013).

NleC is a zinc metalloprotease that cleaves the NF- κ B p65 subunit, leading to NF- κ B pathway inhibition (Baruch et al. 2011; Giogha et al. 2015; Yen et al. 2010). NleC-mediated cleavage of p65 occurs between C38 and E39, generating a p65¹⁻³⁸ fragment that also competes with full-

length p65 for binding to the ribosomal protein RPS3, an additional NF- κ B subunit (Hodgson et al. 2015). This competition reduces the extent of RPS3 nuclear translocation, thus additionally inhibiting NF- κ B pathway activation (Hodgson et al. 2015).

NleD is also a zinc metalloprotease that cleaves and inactivates the mitogen-activated protein kinase signaling proteins c-Jun N-terminal kinase (JNK) and p38 (Baruch et al. 2011). JNK cleavage by NleD occurs before Y185 in the TPY (T183-P184-Y185) motif within the kinase activation loop of JNK, whereas p38 cleavage occurs at residue M213 (Creuzburg et al. 2017). As p38/JNK activation influences pro-inflammatory cytokine gene expression, their cleavage by NleD contributes downregulated inflammatory responses. In a random mutagenesis study of NleD, R203 was found to be critical for protease activity against p38 but not JNK (Creuzburg et al. 2017). In another study, mutation of E143 within the metalloprotease motif (H¹⁴²ELLH¹⁴⁶) abrogated JNK cleavage (Baruch et al. 2011). NleE was originally described to inhibit the nuclear translocation of the p65 NF- κ B subunit by preventing I κ B α phosphorylation (Newton et al. 2010). The C terminal IDSY(M/I)K motif was found to be critical for NleE-mediated inhibition of p65 translocation (Newton et al. 2010). It was later shown that NleE is an S-adenosylmethionine (SAM)-dependent methyltransferase that modifies a zinc-coordinating cysteine in the Npl4 zinc finger (NZF) domains in TAB2 and TAB3, which are two ubiquitin-chain sensory proteins involved in NF- κ B signaling (Zhang et al. 2012). Upon NleE mediated methylation of C673 and C692 in TAB2 and TAB3 respectively, the two proteins lose their ability to bind zinc and ubiquitin chains (Zhang et al. 2012). The NleE crystal structure displays a SAM pocket that particularly involves two critical residues, R107 and Y212 (Yao et al. 2014). The same study also identified ZRANB3 as a target of NleE. NleE mediated methylation of the

ZRANB3-NZF domain disrupted its ubiquitin chain binding without affecting ZRANB3 recruitment to sites of DNA damage (Yao et al. 2014).

NleF is important to EHEC colonization of germ-free piglets (Echtenkamp et al. 2008). NleF is a pro-inflammatory effector because it induces NF- κ B activation during the early stages of the infection (Pallett et al. 2014; Pollock et al. 2017). The C-terminal region of NleF is essential for its interaction with caspase 4, 8, and 9 (Blasche et al. 2013). NleF counteracts the host inflammatory response by preventing the heteromerization of caspase-4-p19 and caspase-4-p10, which is essential for caspase-4 activation and the eventual expression of IL-1 β (Blasche et al. 2013; Song et al. 2017). The transient pro-inflammatory activity of NleF is later overcome by the anti-inflammatory functions of other effectors such as NleE, NleH, and NleB. NleH1 and NleH2 are two paralog effectors (with 84 % homology) that contain a C-terminal kinase domain (Grishin et al. 2014). Both proteins bind RPS3, but only NleH1 inhibits RPS3 translocation to the nucleus (Gao et al. 2009). NleH1 interaction with RPS3 prevents its phosphorylation on S209 by IKK β (Wan et al. 2011). NleH1 phosphorylates the v-crk sarcoma virus CT10 oncogene-like protein (CRKL), which may play an important scaffolding role between NleH1 and IKK β (Pham et al. 2013). K159 in NleH1 and K169 in NleH2 are critical residues for the kinase activity of these effectors (Gao et al. 2009). EspJ was first described as a prophage-carried effector that influences the dynamics of pathogen clearance (Dahan et al. 2005) and inhibits opsonophagocytosis (Marches et al. 2008). More recently, its mode of action was clarified by the demonstration that this effector functions as an adenosine diphosphate (ADP) ribosyltransferase that inhibits the host Src kinase by amidation and ADP ribosylation of a Src glutamic acid residue (Young et al. 2014). Additional host kinases in the Src, Tec, and Syk NRTK families have also been described as EspJ substrates *in vivo* (Pollard et al. 2018).

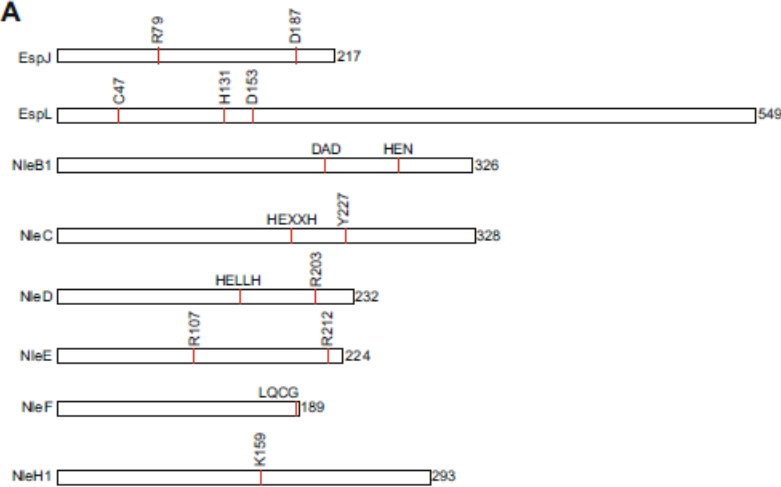
EspL is a cysteine protease that directly cleaves the receptor interacting protein (RIP) homotypic interaction motif (RHIM) domain containing proteins RIPK1, RIPK3, TRIF, and ZBP1/DAI, leading to their rapid inactivation, with subsequent inhibition of necroptosis and inflammatory signaling (Pearson et al. 2017). A conserved cysteine protease motif with catalytic residues C47, H131, and D153 was defined (Pearson et al. 2017). The proteolytic activity of EspL is important to the duration of *C. rodentium* intestinal colonization of mice, with $\Delta espL$ being cleared faster than wild-type *C. rodentium*, despite similar initial colonization magnitudes (Pearson et al. 2017).

Tir. In addition to its well-studied role in bacterial adhesion, Tir also inhibits NF- κ B pathway activation by interacting with TNFR-associated factor (TRAF) adaptor proteins leading to their proteasomal-independent degradation (Ruchaud- Sparagano et al. 2011) (Figure 3).

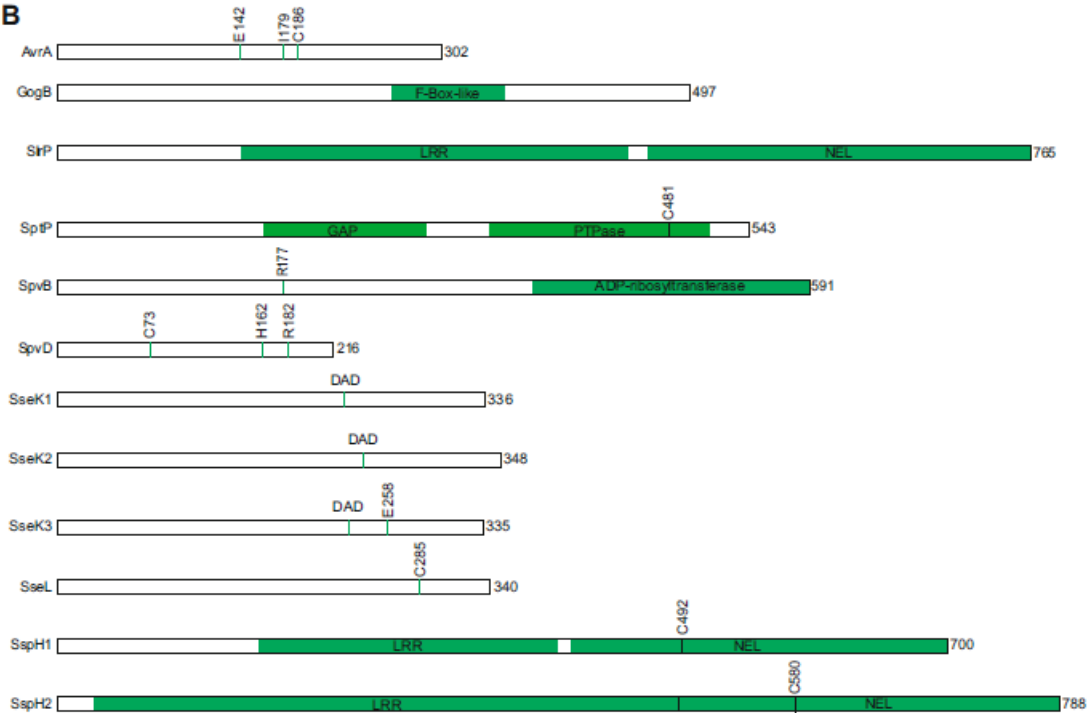
Figure 3 Effector amino acids and functional domains.

A

A



B



Schematic representation of T3SS effectors targeting host innate immunity. Critical amino acids and secondary structures are highlighted. A EHEC/EPEC/*C. rodentium* effectors. B *S. Typhimurium* effectors

***Salmonella* Effectors**

GogB is the first open reading frame (ORF) in the Gifsy-1 prophage in *Salmonella enterica* serovar Typhimurium (McClelland et al. 2001). GogB is a leucine-rich repeat protein that is secreted by both type three secretion systems encoded in *Salmonella* Pathogenicity Island-1 (SPI-1) and SPI-2 (Coombes et al. 2005). GogB is regulated by its transcriptional regulator SsrB through SPI-2 induction, and then localizes to the host cell cytoplasm (Xu and Hensel 2010). GogB alters the function of the host SKP, cullin, F-box containing complex (SCF) E3 type ubiquitin ligase by interacting with the human F-box only 22 (FBXO22) protein and S-phase kinase associated protein 1 (Skp1) (Pilar et al. 2012). GogB-deficient *Salmonella* fail to limit NF- κ B activation in RAW264.7 cells, indicating that GogB is an anti-inflammatory factor (Pilar et al. 2012).

PipA, GtgA, and GogA comprise a family of proteases that cleave both the RelA (p65) and RelB NF- κ B transcription factors, thus limiting host inflammatory responses (Sun et al. 2016).

Salmonella mutants in the pipA, gogA, or gtgA genes show increased NF- κ B pathway stimulation and virulence in C57/BL6 mice, as compared to wild-type *Salmonella* (Sun et al. 2016), indicating that this family of effectors inhibits host immune responses.

Salmonella encodes three members of the novel E3 Ligase (NEL) family, SlrP, SspH1, and SspH2. SlrP functions as an E3 ubiquitin ligase for mammalian thioredoxin (Bernal- Bayard and Ramos-Morales 2009), which may affect the redox state and activity of transcription factors.

SlrP also binds to the chaperone ERdj3 in the endoplasmic reticulum (Bernal-Bayard et al. 2010), possibly impacting the unfolded protein response and apoptosis. SspH1 and SspH2 have 39 % and 38 % identity to SlrP (Miao and Miller 2000). SspH1 inhibits NF- κ B signaling (Haraga

and Miller 2006). SspH1 interacts with and ubiquitinates human protein kinase N1 (PKN1), to activate its catalytic function (Keszei et al. 2014). Mutating SspH1 C492 abolishes its ability to ubiquitinate PKN1 (Keszei et al. 2014). However, the inhibition of NF- κ B signaling is not due to the ubiquitination and proteasome-dependent degradation of PKN1 (Keszei et al. 2014).

SspH2 increases Nod1-mediated IL-8 secretion by ubiquitinating Nod1 (Bhavsar et al. 2013). Mutating SspH2 C580 abolishes its ubiquitination activity (Quezada et al. 2009).

SpvB is encoded by the *spv* operon in the *Salmonella* virulence plasmid (Caldwell and Gulig 1991). SpvB ADP-ribosylates actin on R177 (Hochmann et al. 2006), causing both actin depolymerization and apoptosis (Guiney and Lesnick 2005). P-bodies are enzymes involved in mRNA turnover and post-transcriptional regulation. SpvB is also an important regulator of P-body disassembly (Eulalio et al. 2011).

SpvC is also encoded by the *spv* operon (Coynault et al. 1992) and is translocated by both T3SS-1 and T3SS-2 (Heiskanen et al. 1994). SpvC has a phosphothreonine lyase activity that targets ERK, p38, and JNK mitogenactivated protein kinases (MAPKs) (Mazurkiewicz et al. 2008). SpvC limits host inflammatory responses by reducing pro-inflammatory cytokine gene transcription (Haneda et al. 2012). Recently, kinetic studies have been performed on SpvC, revealing that this enzyme is extremely specific for MAPKs (Chambers et al. 2018) SpvD functions as a cysteine hydrolase with a papain-like fold and a catalytic triad consisting of C73, H162, and D182 (Grabe et al. 2016).

SpvD interacts with the exportin Xpo2m, which mediates nuclear-cytoplasmic protein recycling (Rolhion et al. 2016). The disruption of importin-alpha recycling from the nucleus results in p65 translocation deficiency and NF- κ B inhibition (Rolhion et al. 2016).

SteC is encoded within SPI-2 and has amino acid similarity to human kinase Raf-1, a protooncogene serine/threonine-protein kinase (Poh et al. 2008). An steC mutant showed no decrease for bacterial replication and virulence, but was defective for SPI-2 dependent F-Actin meshwork formation (Poh et al. 2008). SteC phosphorylates MEK and activates a signaling pathway involving MEK and ERK, MLCK, and Myosin IIB (Odendall et al. 2012). A steC mutant shows greater replication in HeLa cells, as compared to wild-type *Salmonella*, indicating that SteC may limit bacterial replication by regulating F-Actin meshwork formation (Odendall et al. 2012).

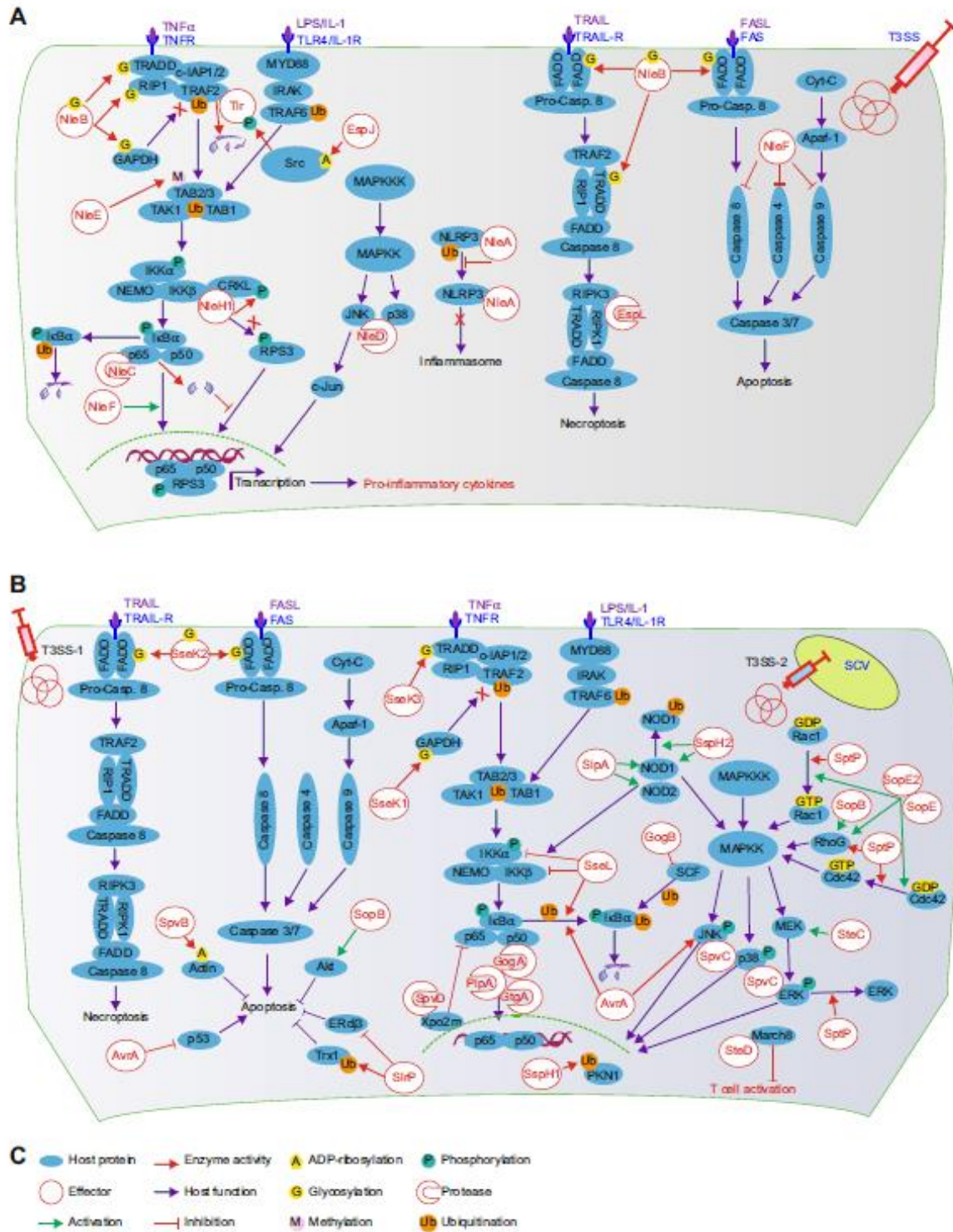
SteD stimulates MARCH8, a member of the MARCH family E3 integral membrane ubiquitin ligases (Bayer-Santos et al. 2016). SteD causes ubiquitination and depletion of surface-localized mature MHC class II (mMHCII), thus helping to reduce T cell activation (Bayer-Santos et al. 2016).

Salmonella Secreted Effector L (SseL) is encoded within SPI-2 and is transported into the cytosol from the SCV via the T3SS-2. SseL functions as a deubiquitinase (DUB) to induce a delayed cytotoxic effect in *Salmonella*-infected macrophages (Rytönen et al. 2007). NF- κ B signaling in macrophages is significantly activated after infection with an sseL mutant, as compared to its activation by wild-type *Salmonella* (Le Nègre et al. 2008). Expression of SseL in mammalian cells impairs I κ B α ubiquitination and degradation, and mutating C285 abolished this effect (Le Nègre et al. 2008).

AvrA is encoded on SPI-1 and is a member of the YopJ/Avr protein family (Hardt and Galan 1997). AvrA is a multifunctional effector that influences eukaryotic cell pathways by regulating the ubiquitination (Sun et al. 2004) and acetylation (Wu et al. 2010) of target proteins to modulate proliferation (Liu et al. 2010), inflammation (Du and Galan 2009), and apoptosis

(Collier-Hyams et al. 2002). AvrA acetylates p53 (Wu et al. 2010) and also inhibits JNK signaling by acetylating MKK4 (Jones et al. 2008) and MKK7 (Du and Galan 2009). AvrA also has a deubiquitinase activity that targets β -catenin, resulting in stabilization of β -catenin and increasing intestinal epithelial cell proliferation (Wu et al. 2010). Mutating E142, I179, and C186 reduces AvrA acetyltransferase activity (Wu et al. 2010); C186 also appears to be critical for AvrA deubiquitinase activity (Ye et al. 2007). It should be noted that the activity of AvrA has largely been reconciled to function as a acetyltransferase, similar to its homolog, the *Yersinia* outer protein J (YopJ) and many early observations regarding a potential AvrA protease activity may have been artifacts (Ma and Ma 2016) (Figure 4).

Figure 4 Host inflammatory pathways and their targeting by T3SS effectors



Upon TNF α stimulation, TRADD forms a complex with TRAF2, leading to RIP1 and TRAF polyubiquitination followed by recruitment of TAK1. TAK1 activation results in activation of the IKK complex and phosphorylation of I κ B α , which is then ubiquitinated and degraded, releasing NF- κ B for nuclear translocation and pro-inflammatory gene transcription. Stimulation of TLR4 or IL-1R by LPS or IL-1 β initiates a signaling cascade that leads to TAK1 activation. Stimulation with FASL results in FADD oligomerization and activation of caspase 8, leading to caspase 3/7 maturation and apoptosis. Stimulation with TRAIL initiates necroptosis through a pathway involving the formation of a complex among RIP1, RIP3, TRADD, FADD, and activated protein kinases (MAPKs) pathway activation begins with the binding of Ras/Rho family proteins to a mitogen activated protein kinase kinase kinase (MAPKKK), which in turn leads to the phosphorylation and activation of a mitogen activated protein kinase kinase (MAPKK), followed by the phosphorylation of the mitogen activated protein kinases (MAPKs) ERK, p38, and JNK. NOD1 and NOD2 recruit receptor-interacting serine/threonine-protein kinase 2 (RIPK2), which further activates MAPKs and the IKK complex. (a) Strategies used by EHEC/EPEC/ *C. rodentium* to counteract inflammation. (b) Strategies used by *Salmonella enterica* Seroovar Typhimurium to counteract inflammation. (c) Shape and arrow annotations describing bacteria and host protein interactions.

Salmonella encodes up to three NleB orthologs named SseK1, SseK2, and SseK3 (Brown et al. 2011). The NleB and SseK effectors contain a conserved DXD motif, which is required for enzymatic function of glycosyltransferases of the GT-A family (Gao et al. 2013). SseK1 and SseK2 inhibit TNF-induced NF- κ B activation and cell death during infection in macrophages (Gunster et al. 2017). SseK3 binds, but does not glycosylate TRIM32, an E3 ubiquitin ligase involved in TNF and interferon signaling (Yang et al. 2015). SseK1 and SseK3 inhibit TNF-mediated NF- κ B pathway activation. SseK1 glycosylates GAPDH, while SseK2 glycosylates FADD (El Qaidi et al. 2017). SseK3 glycosylates TRADD and has an overall fold that is similar to other glycosyltransferase type-A (GT-A) family members (Esposito et al. 2018). Mutating E258 impairs the ability of SseK3 to glycosylate TRADD (Esposito et al. 2018).

Salmonella protein tyrosine phosphatase (SptP) shares significant homology in its C-terminal domain to the catalytic domains of eukaryotic tyrosine phosphatases (PTPases) and the *Yersinia* protein tyrosine phosphatases YopH (Kaniga et al. 1996). SptP inhibits ERK activation by interfering with Raf1 activation, a MAPKKK (Murli et al. 2001) Inhibition is dependent on

SptP-phosphatase activity, as a SptP C481S mutation no longer inhibits ERK activation (Lin et al. 2003). SptP phosphatase activity is also important for inhibiting TNF- α induction during *Salmonella* infection of macrophages (Lin et al. 2003).

Salmonella outer protein B (SopB, also known as SigD) has sequence homology to mammalian inositol polyphosphate 4-phosphatases and recombinant SopB functions as an inositol phosphate phosphatase (Norris et al. 1998). It is encoded within SPI-5 but delivered into cells via T3SS SPI-1 (Knodler et al. 2002). SopE is 69% identical to SopE2 (Bakshi et al. 2000). SopB, SopE, and SopE2, cooperate in a functionally redundant manner to activate Rho-family GTPases during *Salmonella* infection (Patel and Galan 2006), resulting in activation of MAPK and NF- κ B signaling pathways (Bruno et al. 2009). SopB functions as a phosphoinositide phosphatase to activate the RhoG exchange factor, (Patel and Galan 2006), while SopE and SopE2 act as exchange factors for Cdc42, Rac1, and RhoG (Stender et al. 2000).

Salmonella invasion protein A (SipA) is encoded within SPI-1 (Kaniga et al. 1995) and is transported into mammalian cells via the T3SS- 1 (Zhou et al. 1999a). SipA binds F-actin, contributing to *Salmonella* internalization and mediates the downstream activation of Rho GTPases (Zhou et al. 1999b). SipA induces NF- κ B activation and intestinal inflammation by activating the NOD1/NOD2 signaling pathway (Kestera et al. 2011). SipA also induces IL-8 expression by inducing Jun and p38 MAPK phosphorylation (Figueiredo et al. 2009).

Summary

Here we have described many of the *E. coli* and *Salmonella* T3SS effectors that target host innate immunity. Many of these effectors apply their inhibitory effect in a cumulative or synergistic manner by blocking multiple steps within a signaling pathway. Effector translocation

cell is not random but rather is temporally controlled by the pathogen (Lara-Tejero et al. 2011), allowing the pathogen to adjust the host inflammatory response not only to evade inflammation but also to alter the relative abundance of commensal flora. Microbial genomes provide information about how co-evolution has shaped pathogen targeting host immunity. Characterizing anti-inflammatory microbial proteins offers opportunities both to identify natural modulators of inflammation and to engineer these proteins for use as effective immune-therapeutics (Ruter and Hardwidge 2014). T3SS effectors represent a major pool of pathogen-derived immunomodulatory molecules that may have future utility in treating autoimmune disorders associated with perturbations in NF- κ B signaling (Sun et al. 2013). Indeed, *Shigella flexneri* and *Yersinia pestis* effectors have already been exploited to selectively inhibit MAPK pathways and to tune T-cell responses (Wei et al. 2012).

Chapter 2 - High-Throughput Screening for Bacterial Glycosyltransferase Inhibitors

I contributed to Figure 6D, 7B, 7D and 7E in Chapter 2. A modified version of this chapter is published in Front Cell Infect Microbiol (El Qaidi et al. 2018).

El Qaidi S, Zhu C, McDonald P, Roy A, Maity PK, Rane D, Perera C, & Hardwidge PR. (2018). High-Throughput Screening for Bacterial Glycosyltransferase Inhibitors. Front Cell Infect Microbiol. 2018 Dec 18;8:435. doi: 10.3389/fcimb.2018.00435.

Introduction

Many Gram-negative bacterial pathogens interact with mammalian cells by using a specialized type III secretion system (T3SS) to inject proteins directly into infected host cells (Notti and Stebbins, 2016; Deng et al. 2017). A subset of these injected protein “effectors” are enzymes that modify the structure and function of human proteins by catalyzing the addition of unusual post-translational modifications (Ribet and Cossart 2010; Salomon and Orth 2013). Some T3SS effectors play important roles in bacterial virulence and may be “druggable” targets for anti-virulence compounds that could be used to replace or augment traditional antibiotic regimens (Kimura et al. 2011; Warren et al. 2014; Gu et al. 2015; McShan and De Guzman 2015). The NleB1/NleB2 (enteropathogenic and enterohemorrhagic *E. coli*; EPEC and EHEC) and SseK1/SseK2/SseK3 (*Salmonella enterica*) T3SS effectors are glycosyltransferases that modify protein substrates on arginine residues (Li et al. 2013; Pearson et al. 2013). This modification is unusual because it occurs on the guanidinium groups of arginines, which are poor nucleophiles. This modification is biologically important because the glycosylation of arginines on protein substrates disrupts the normal functioning of the innate immune system (Li et al. 2013; Pearson et al. 2013; El Qaidi et al. 2017). Several death domain-containing proteins have been described

as substrates of some of the NleB/SseK orthologs, including the Fas-associated protein with death domain (FADD), tumor necrosis factor receptor type 1-associated DEATH domain protein (TRADD), and the receptor-interacting serine/threonine-protein kinase 1 (RIPK1) (Li et al. 2013). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Gao et al. 2013) and hypoxia-inducible factor 1- alpha (HIF1a) (Xu et al. 2018) are additional substrates. Recent structural characterization of a subset of the NleB/SseK orthologs revealed that these proteins are retaining glycosyltransferases consisting of three functional domains, namely a helix-loop-helix (HLH) domain, a lid domain, and a catalytic domain with a HEN motif (His-Glu-Asn) that is required for catalytic activity (Esposito et al. 2018; Park et al. 2018).

NleB1 is a signature of EHEC strains that have been observed to cause widespread foodborne outbreaks that develop into cases of hemolytic uremic syndrome (HUS) (Wickham et al. 2006). HUS, which is caused by the activity of a Shiga-like toxin, leads to destruction of the endothelial cells of the kidney and is often fatal (Loos et al. 2018). Antibiotic therapy against EHEC is typically contraindicated, because antibiotics may induce increased Shiga-like toxin expression, exacerbating patient symptoms (Wong et al. 2000). This motivated our work, which sought to identify small molecule inhibitors of NleB1 as a potential alternative therapeutic strategy to antibiotics. Such compounds may also be of utility as mechanistic probes for understanding the mechanism of the NleB/SseK effector family.

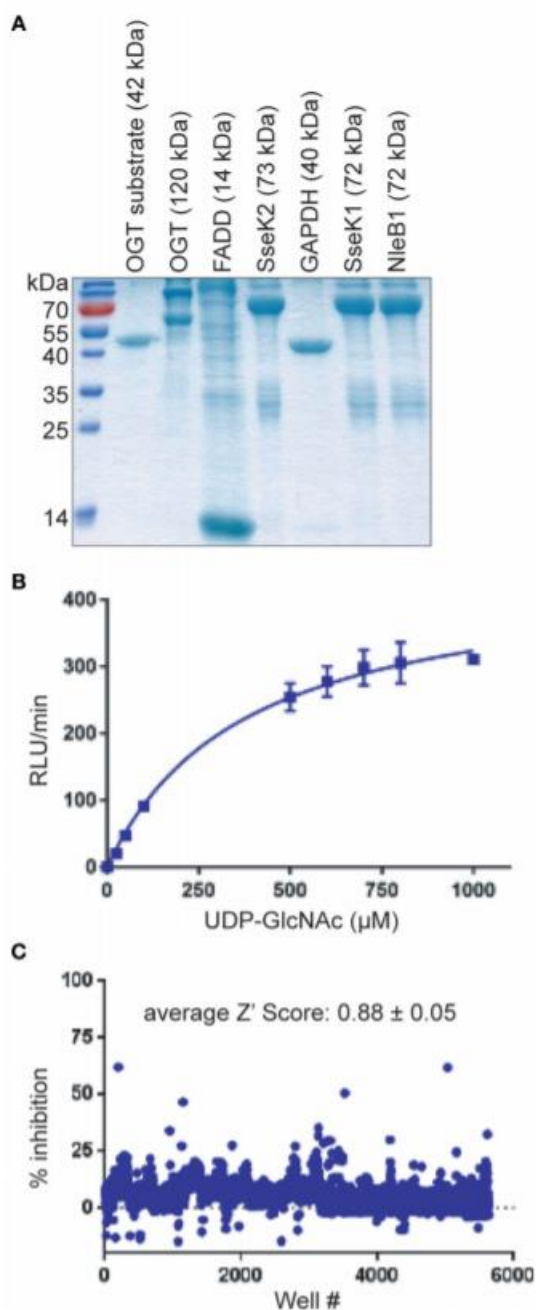
Results

NleB1 Inhibitor Screening

We developed and optimized a high-throughput screening (HTS) assay for NleB1 inhibitors. Purified, recombinant forms of wildtype *E. coli* O157:H7 NleB1 (Figure 5A) and, as a negative control, an inactive form of NleB1 (NleB1-AAA) in which the aspartic acid residues required for Mn²⁺ stabilization were mutated to alanines, were produced (Gao et al. 2013). We then optimized the conditions in which these enzymes could be studied in a solution-based assay that produces a luminescent signal when UDP-GlcNAc is hydrolyzed by NleB1 to liberate UDP (Figure 1B). Optimal NleB1 activity was observed at 0–25 mM NaCl and 10 mM MgCl₂, with >50 % loss of activity at 500 mM NaCl and at 50 mM MgCl₂. NleB1 activity increased linearly up to 2 h and no loss of activity was observed up to 4 % DMSO. The kinetic parameters of NleB1 (150 nM at 30 °C) were calculated as follows: V_{max}: 2,975.3 ± 125 RLU/min/μg protein; K_m: 379 ± 43 μM; K_{cat} (s⁻¹): 50, K_{cat}/K_m (s⁻¹, M⁻¹): 130,703.4. As expected, the NleB1-AAA mutant enzyme had no detectable activity (data not shown). We then screened the University of Kansas Center of Excellence in Chemical Methodologies and Library Development (KU CMLD, <https://cmlld.ku.edu/outreach>) library of 5,160 compounds. This library utilizes new principles of scaffold design, incorporates multiple scaffold cores into a single library, and contains compounds that are likely to have pharmacological activity based on sound drug design principles. The structures and bioactivity data of these compounds are described in PubChem (<https://www.ncbi.nlm.nih.gov/pcsubstance/?term=%22ku+outreach+library%2C+the+university+of+kansas%22%5Bsourcename%5D>).

An average Z' Score of 0.88 ± 0.05 was obtained across all 15 plate assays in the pilot screen for NleB1 inhibitors (Figure 5C). Compounds were tested in a concentration dose-response (10–160 μ M) assay. Of all the compounds that inhibited the primary assay at single concentration, 80 % of the hits inhibited NleB1 in a dose-responsive manner. Compounds (n = 52) that inhibited NleB1 to >3 standard deviations $\pm$ plate median were scored as hits in the assay (hit rate of 1 %; Supplemental Table 1). The two most potent compounds (100066N and 102644N) were resynthesized as fresh powders for further study (Figure 6A). 100066N is a flavone analog that has been previously synthesized (Ahn et al. 2007). 102644N is a substituted isoxazole whose synthesis has also been described (Waldo et al. 2008). Compound 104108N, which was not identified as a hit in our HTS assay, was used as a negative control in some subsequent experiments.

Figure 5 HTS assays

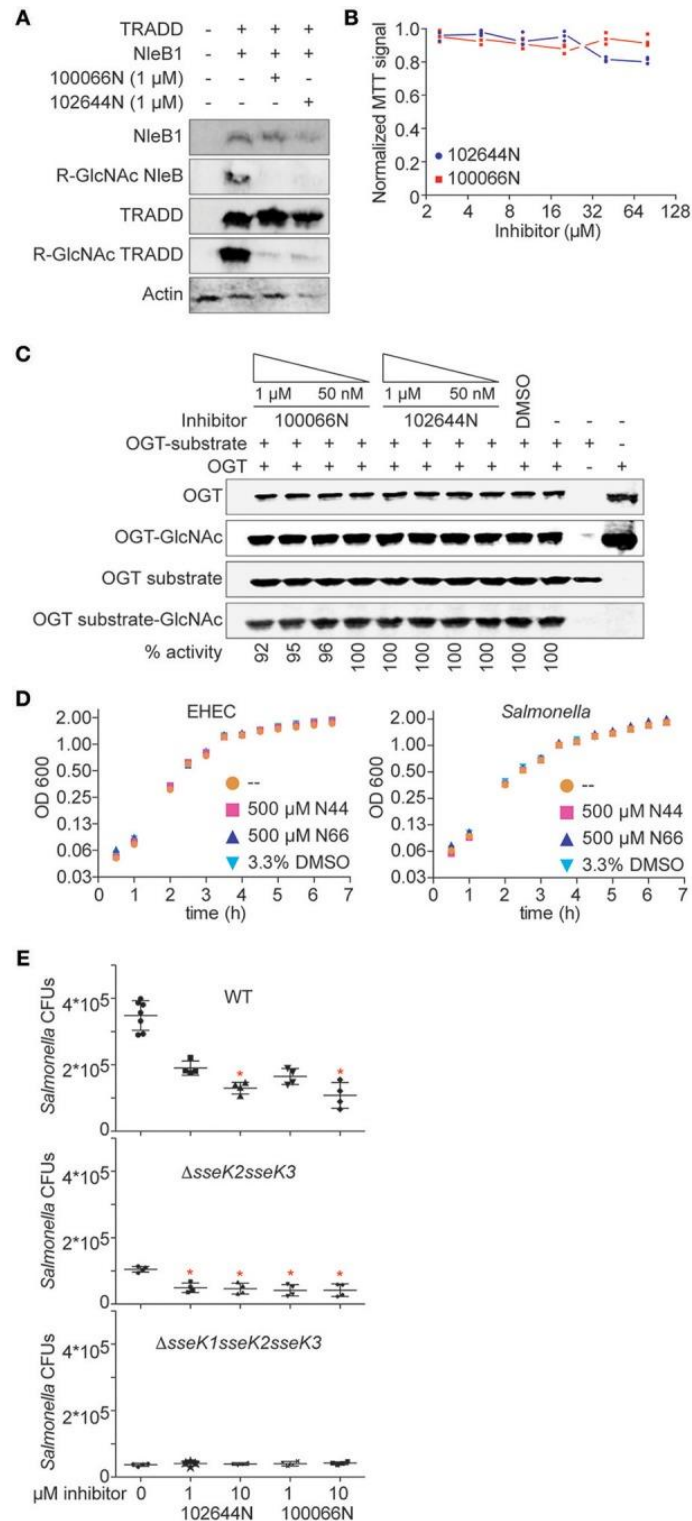


(A) SDS-PAGE analysis of the recombinant proteins used in this study. (B) UDP liberation (RLU/min) is plotted as a function of substrate (UDP-GlcNAc) concentration in the presence of NleB1. (C) Summary of HTS assay data. The % inhibition of UDP liberation is plotted as a function of the well number of each compound represented in the CMLD library of 5,160 compounds. Compounds ($n = 52$) that inhibited the assay to >3 standard deviations $\pm$ the plate median were identified as positive hits.

***In vitro* Glycosylation Assays**

We performed secondary screens to evaluate the ability of these compounds to inhibit NleB1 and SseK1 glycosylation of the human GAPDH protein substrate (Gao et al. 2013, 2016; El Qaidi et al. 2017). Both 100066N and 102644N were active against both NleB1 and SseK1 (Figures 6B, C). We corroborated these data by quantifying UDP liberation in a UDP-Glo assay as a function of inhibitor concentration. Both 100066N and 102644N inhibited NleB1 activity in a concentration dependent manner, whereas compound 104108N, the negative control, did not inhibit NleB1 (Figure 6D). SseK2 glycosylates the human FADD protein (ElQaidi et al. 2017). We also tested the inhibitory effect of 100066N and 102644N on SseK2 and found that both these compounds inhibited FADD glycosylation by SseK2 in a concentration-dependent manner (Figure 6E).

Figure 6 *In vitro* glycosylation assays



(A) 100066N, 102644N, and 104108N structures. (B) Western blot analysis of the inhibition of NleB1 and SseK1 glycosylation of GAPDH by 100066N and 102644N. (C) Quantification of panel B, $n = 3$. (D) UDP-Glo assays were

performed using 250 nM NleB1, in 125 mM Tris pH 7.4, 25 mM MnCl₂, 2.5 mM DTT, and 100 μM UDP-GlcNAc in the presence of inhibitor concentrations ranging from 1 nM to 500 μM. (E) Western blot analysis of the inhibition of SseK2 glycosylation of FADD by 100066N and 102644N.

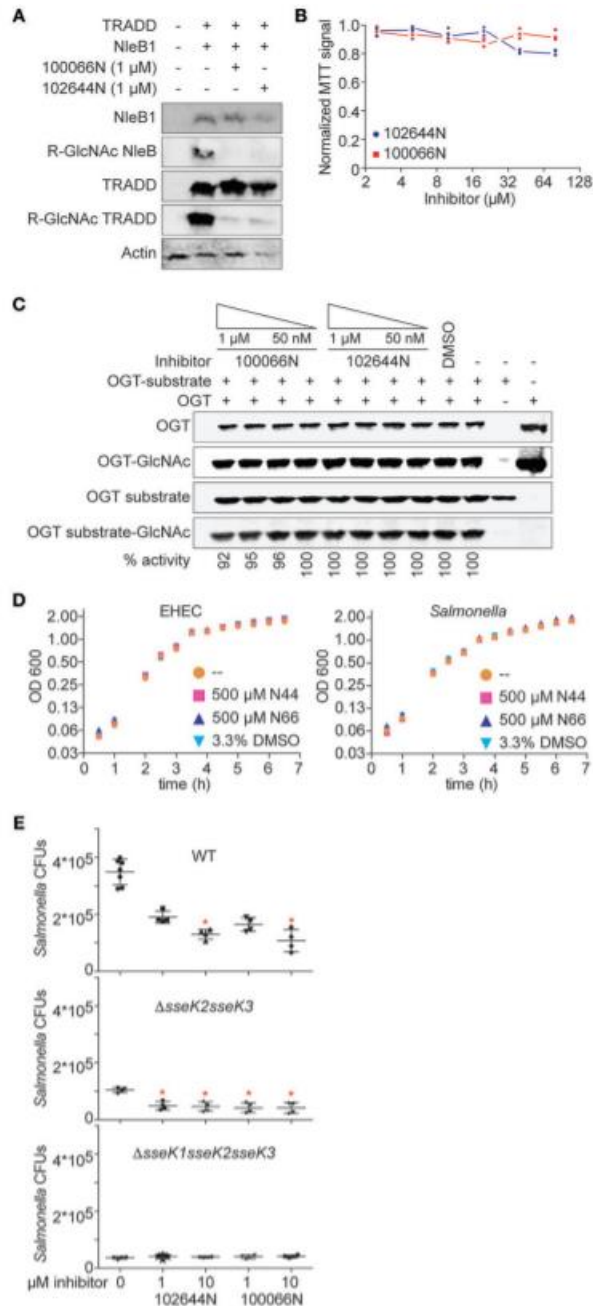
Cell Culture Assays

NleB1 glycosylates human TRADD on R235, thereby blocking death domain interactions and disrupting tumor necrosis factor signaling (Li et al. 2013). We next assessed whether 100066N and/or 102644N would be effective in inhibiting NleB1 activity in mammalian cells. We co-transfected HEK293 cells with NleB1 and TRADD expression plasmids in the presence or absence of these compounds and then performed immunoblotting assays to measure TRADD glycosylation. We observed that both compounds, when provided at 1 μM concentration to HEK293T cells, were effective in reducing the extent of TRADD glycosylation by NleB1 (Figure 7A). Neither inhibitor was significantly toxic to mammalian cells, as inferred from performing 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays as a function of inhibitor concentration (Figure 7B). The human O-linked N-acetylglucosaminyltransferase (OGT) is an essential serine/threonine N-acetylglucosamine (O-GlcNAc) transferase that maintains protein glycosylation homeostasis (Zhao et al. 2018). Deletion of OGT is lethal and deficiencies in OGT activity are associated with neurodegenerative diseases and gut inflammation (Zheng et al. 2016; Zhao et al. 2018). To consider the future use of 100066N and 102644N as potential therapeutics, we next tested whether they also inhibit OGT. Neither 100066N nor 102644N inhibited the activity of a recombinant form of human OGT (Figure 7C). These data suggest that the two NleB1 inhibitors we initially characterized are specific to the pathogen enzymes that glycosylate arginine residues, rather than cross-react with the mammalian OGT enzyme that glycosylates Ser/Thr residues (Aquino-Gil et al. 2017).

Neither 100066N nor 102644N directly impacted the growth rates of *E. coli* O157:H7 and *Salmonella enterica* when supplied at 500 μ M concentrations in bacterial cultures (Figure 7D). These data suggest that the inhibitors specifically target the NleB/SseK virulence factors, rather than act as general bacteriostatic or bactericidal agents.

Finally, we quantified the impact of these inhibitors on *Salmonella* replication in RAW264.7 cells. When 102644N and 100066N (1 or 10 μ M) were added to RAW264.7 cells prior to their infection with WT *Salmonella*, we observed that both inhibitors (at 10 μ M) significantly reduced the number of WT *Salmonella* 24 h after infection (Figure 7E, top). We performed similar experiments with *Salmonella* Δ sseK2sseK3 (expressing only SseK1) and observed that both inhibitors at both 1 and 10 μ M further reduced the replication of *Salmonella* Δ sseK2sseK3 (Figure 3E, middle). We also assessed the impact of 102644N and 100066N on *Salmonella* Δ sseK1sseK2sseK3 and found there was no further impact of these inhibitors on the already relatively-low abundance of this triple-mutant strain (Figure 7E, bottom).

Figure 7 Cellular assays



(A) Western blot analysis of the inhibition of NleB1 glycosylation of TRADD by 100066N and 102644N in HEK293T cells. (B) MTT assays. Quantification of normalized MTT signal intensities as a function of 100066N and 102644N concentrations added to HEK293T cells for 24 h. (C) OGT assays. OGT was incubated with a recombinant OGT-peptide substrate in the presence of 100066N and 102644N and then subjected to immunoblotting using an anti-O-GlcNAc monoclonal antibody. (D) Bacterial growth assays. EHEC (left) and *Salmonella* (right) were cultured in the presence of the indicated compounds and growth was monitored as a function of time. (E) 100066N and 102644N reduce *Salmonella* replication in macrophages. Inhibitors (1 or 10 μ M) were added to RAW 264.7

cells for 1 h prior to infection with 10^6 CFUs of the indicated *Salmonella* strains. Colony counts were enumerated 24 h later. Asterisks indicate a significant difference ($p < 0.05$) from samples lacking inhibitors, as determined using one-way analysis of variance (ANOVA) with Dunnett's multiple comparisons tests.

Discussion

The T3SS and its effectors are of critical importance to the virulence of many Gram-negative pathogens (Gaytan et al. 2016). Bacterial virulence is significantly decreased in T3SS deficient strains, whereas bacterial replication is not typically affected (Nordfelth et al. 2005). Thus, chemicals targeting the T3SS or its effectors are appealing, as they are less likely to be subject to evolutionary pressures toward resistance (Gu et al. 2015; McShan and De Guzman 2015). We screened for and identified two small molecules, 100066N and 102644N, that are effective in inhibiting the activities of NleB1, SseK1, and SseK2. We used a relatively small compound library for these initial studies, and it is likely that future HTS assays using larger compound libraries may reveal more effective compounds. It is notable that although our primary screen was designed to target only the EHEC NleB1 protein, both 100066N and 102644N showed activity against not only NleB1, but also SseK1 and SseK2. It was promising to note that neither 100066N nor 102644N were toxic to mammalian cells, nor did they directly affect *E. coli* or *Salmonella* growth. Given the significant difference in the NleB/SseK mechanism of action as compared to OGT, it was reassuring but perhaps not surprising to observe that neither inhibitor affected the O-linked glycosylation of an OGT substrate. We also noted that neither 100066N nor 102644N further impacted the colonization of the *Salmonella* $\Delta sseK1sseK2sseK3$ triple mutant, suggesting few significant off-target effects of these compounds. The extent to which the SseK enzymes are redundant and/or are important to survival in mouse macrophages is still unclear and some of our data are in contrast to previous studies. Initially it was concluded that effects of SseK1 and SseK2 are not evident in infections of RAW 264.7 cells in a comparison of

single- and double-mutants with wild-type serovar Typhimurium SL1344 (Kujat Choy et al. 2004). Later it was realized that this strain also expresses SseK3 and these experiments were reassessed (Brown et al. 2011). While no phenotype was observed in RAW264.7 cells, it was shown that a Δ sseK1sseK2 double mutant was attenuated in competitive index infections of mice (Brown et al. 2011). Our experiments instead used *Salmonella enterica* ATCC 14028 and thus our observation that both Δ sseK1sseK2 and Δ sseK1sseK2sseK3 were attenuated in macrophages in comparison to the wild-type strain may be due to strain-dependent differences. We also failed to observe significant macrophage death in our studies. Although a structure is available in the Protein Data Bank (PDB) for SseK3 [6EYR; (Esposito et al. 2018)], the lack of confirmed substrates for this effector limited our ability to assess the efficacy of 100066N and 102644N against SseK3. We previously characterized the structures of NleB2 (5H5Y), SseK1 (5H60), and SseK2 (5H61, 5H62, 5H63) (Park et al. 2018). We are currently attempting to generate co-crystal structures between SseK1 and both 100066N and 102644N to understand how these compounds inhibit these glycosyltransferases and to identify the residues they bind in the enzymes. We speculate that 100066N and 102644N could affect UDP-GlcNAc binding and/or hydrolysis, activation of the acceptor arginine, or affect the flexibility of the HLH domain which we have attributed to being important to the recognition of the acceptor protein substrates containing death domains (Park et al. 2018). Future approaches to study the efficacy of these inhibitors will also include derivatization of both 100066N and 102644N. Logical medicinal chemistry strategies include the exploration of substitution effects on the furan and phenyl moieties at the 3 and 5 positions of 102644N as well as bio isosteric replacement of the sulfonamide linker and determining the effect of substituents on phenyl ring for 100066N.

Experiment Procedures

Cloning and Cell Lines

The plasmids used in this study are listed in Table 1. Human embryonic kidney 293 cells (HEK 293) and Abelson murine leukemia virus induced, macrophage-like cells from BALB/c mice (RAW264.7) were purchased from ATCC. Both cell lines were grown in DMEM (Thermo Scientific), supplemented with 10% FBS (Atlanta Biologicals) and 100µg/ml penicillin/streptomycin (Sigma) in 5% CO₂. *nleB1* (EHEC EDL933), *sseK1*, and *sseK2* (*Salmonella enterica* ATCC 14028) were cloned into pET42a. A human OGT substrate peptide (KKKYPPGGSTPVSSANMM) was fused to the N-terminus of GAPDH and expressed in pET28a. Human FADD was expressed in pET15a. Plasmids were transformed into *E. coli* BL21 (DE3). Protein overexpression was performed for 4 h at 37°C upon induction with 0.5 mM IPTG. His-tagged proteins were purified using nickel agarose beads (Qiagen) as described previously (El Qaidi et al. 2017). *Salmonella* mutants were constructed using lambda red recombination (Datsenko and Wanner 2000).

Table 2 Plasmids used in this study

Description	Source
GST-EHEC-NleB1	El Qaidi et al. 2017
GST-EHEC-NleB1(AAA)	Gao et al. 2013
GST-SseK1	El Qaidi et al. 2017
GST-SseK2	El Qaidi et al. 2017
HA-EHEC-NleB1	Gao et al. 2013
His-FADD	El Qaidi et al. 2017

His-GAPDH	Gao et al. 2013
His-OGT	This study
His-OGT substrate (KKKYPPGGSTPVSSANMM)-GAPDH	This study
TRADD-FLAG	This study

Protein Purification

E. coli BL21 (DE3) strains harboring protein expression plasmids were grown at 37 °C in LB containing 50 µg/ml kanamycin until an OD600 of 0.4, at which time 0.5 mM IPTG was added for 4 h further growth. Cells were harvested using centrifugation and the pellet was resuspended in 1/40 culture volume of lysis buffer (50 mM sodium phosphate NaH₂PO₄ pH 8.0, 0.5 mg/ml lysozyme). The suspension was incubated on ice for 30min with occasional shaking and then an equal volume of 50 mM sodium phosphate pH 8.0, 2 M NaCl, 8 mM imidazole, 20 % glycerol, 2 % Triton X-100, was added for an additional 30 min of incubation. Cell lysates were sonicated, centrifuged, and then the resultant supernatant was added to 2 ml Ni-NTA resin (Qiagen) for 1 h of rotation at 4°C. The mixture was loaded on a Poly-Prep Chromatography Column (Bio-Rad) and washed with 10ml of 50mM sodium phosphate pH 8.0, 600 mM NaCl, 60mM imidazole, 10 % glycerol. Proteins were eluted in 2ml 50mM sodium phosphate pH 8.0, 600 mM NaCl, 250 mM imidazole, 10 % glycerol and dialyzed into the same buffer lacking imidazole overnight at 4°C. We note that some of the proteins appeared to show cleavage of the epitope tag after purification and other proteins formed higher molecular weight aggregates when resolved using SDS-PAGE.

HTS Assay Optimization

The glycosyltransferase activity of NleB1 was tested using the luminescence based UDP-Glo™ Glycosyltransferase Assay (Promega) according to the manufacturer's instructions. The optimized assay was performed by incubating purified NleB1 (150 nM) with UDP-GlcNAc (300 μM) for 2 h at 30 °C, followed by the addition of UDP detection reagent to convert UDP to ATP, which is then utilized by luciferase to generate light in proportion to the UDP concentration. The luminescence was read using a BioTek Synergy Neo (BioTek Inc.). The assay was optimized for buffer components (MgCl₂, NaCl), linearity of the assay, the concentration ranges of NleB1 and UDP-GlcNAc, optimal temperature, and the K_m of NleB1 for UDP-GlcNAc.

Pilot Library Screening

The optimized assay was used to screen a KU Legacy collection of 5,160 diversity compounds synthesized by The Chemical Methodologies and Library Development (CMLD) Center. Assays were performed in buffer containing 50 mM Tris pH 7.5, 100 mM NaCl, 1 mM DTT, 10 mM MgCl₂ and 0.5 % DMSO. NleB1 (150 nM) was pre-incubated for 30 min in 384 well-microplates, containing CMLD compounds (20 μM) transferred acoustically using ECHO555 (Labcyte Inc). Luminescence was read 2 h after the addition of the UDP detection reagent. Each screening plate contained 16 wells of minimum signal (no enzyme, substrate only in 0.5 % DMSO, for average and standard deviation calculations, SD min) and 16 wells of maximum signal (enzyme + 0.5% DMSO + Substrate, for average and standard deviation calculations, max). The distribution of controls across each plate was used to calculate Z' scores, using the equation $z' = (1 - 3SD_{min} + 3SD_{max} \text{ Average max} - \text{Average min})$. The percent inhibition was

normalized to the controls and compounds that inhibited the assay to >3 standard deviations plus the plate median were identified as hits.

Glycosylation Assays

In vitro glycosylation assays were performed as described previously (El Qaidi et al. 2017).

Briefly, 200 nM of enzyme (NleB1, SseK1, or SseK2) was incubated with 1 μ M of GAPDH or FADD in the presence or absence of the studied inhibitors in buffer containing 50 mM Tris-HCl pH 7.4, 1 mM UDP-GlcNAc, 10 mM MnCl₂, and 1mM DTT. After 2 h incubation at RT, samples were subjected to Western blotting using an anti-RGlcNAc monoclonal antibody (Abcam). Assays with OGT were performed in buffer containing 25 mM Tris-HCl pH 7.4, 1 mM UDP-GlcNAc, 12.5 mM MgCl₂, and 1mM DTT. After 4 h of incubation at RT, samples were subjected to Western blotting using an anti-O-GlcNAc monoclonal antibody (Millipore). Signal intensities were quantified using Li-COR Image Studio software and inhibition was calculated by quantifying the relative reduction in substrate glycosylation after normalization to the effect of DMSO.

UDP-Glo Assays

UDP-Glo luminescence assays were performed in 96 well-microplates, containing 250 nM NleB1 in 125 mM Tris pH 7.4, 25 mM MnCl₂, 2.5 mM DTT, and 100 μ M UDP-GlcNAc in the presence of inhibitor concentrations ranging from 1 nM to 500 μ M. Reactions were incubated for 2 h at 30 °C and then developed and quantified using a FLUOstar microplate reader (BMG Labtech).

Transfections

HEK293T cells were co-transfected with NleB1-HA and TRADD-FLAG plasmids in the presence or the absence of inhibitors. At 24 h post-transfection, cells were washed with PBS, suspended in SDS sample buffer, boiled for 10min, and immunoblotted with an anti-R-GlcNAc monoclonal antibody (Abcam).

MTT Assays

MTT assays were performed as specified by Millipore using HEK 293 cells in the presence of 2-fold serial dilutions of 100066N or 102644N. Formazan absorbance was measured using an Epoch Microplate Spectrophotometer (BioTek).

Macrophage Infections

Macrophage infection assays were performed to measure the intracellular invasion of bacteria in the presence of 100066N or 102644N. RAW264.7 cells were seeded at 1×10^5 cells/well in 24-well plates and inhibitors were added at 1 or 10 μ M final concentrations 1 h before infection. Bacterial cultures were grown overnight and 10^6 CFUs were added to each well for 30 min. Cells were then incubated in medium containing 100 μ g/mL gentamicin for 1 h, and then 10 μ g/mL gentamicin for an additional 23 h. Bacteria were released from RAW264.7 cells using 1 % saponin, diluted in PBS, and plated for colony counts.

Statistical Analyses

Data were analyzed statistically using one-way analysis of variance (ANOVA) with Dunnett's multiple comparisons tests. P-values < 0.05 were considered significant.

Chapter 3 - YM155 Inhibits NleB and SseK Arginine

Glycosyltransferase Activity

I contributed to all figures except for Figure 8 and Figure 9 in Chapter 3. A modified version of this chapter is published in Pathogens (Zhu et al. 2021).

Zhu C, El Qaidi S, McDonald P, Roy A, & Hardwidge PR. (2021). YM155 Inhibits NleB and SseK Arginine Glycosyltransferase Activity. Pathogens. 2021. doi: 0.3390/pathogens10020253

Introduction

Enteric bacterial pathogens are important threats to human health and are major sources of foodborne disease. Enterohemorrhagic *E. coli* (EHEC) are especially important bacterial pathogens because they cause a type of renal failure (hemolytic uremic syndrome; HUS) for which therapies are limited (Tzipori et al. 2004). A related attaching/effacing (A/E) pathogen, enteropathogenic *E. coli* (EPEC), is an important cause of infantile diarrhea. Recent EHEC outbreaks (e.g. O104:H4) have involved a combination of virulence traits characteristic of EHEC and of enteroaggregative *E. coli* (EAEC; Kampmeier et al. 2018). *Salmonella* is also a leading cause of foodborne illnesses (Martinez-Becerra et al. 2018). Understanding how these bacteria evade the mammalian immune system and develop small molecules to subvert these bacterial strategies is an important area of investigation. We and others have characterized a conserved family of type III secretion system (T3SS) effector proteins that inhibits innate immune responses to infection (Esposito et al. 2018; Gao et al. 2013; Li et al. 2013; Park et al. 2018; Pearson et al. 2013). These proteins (named NleB1 in EHEC and EPEC, NleB in *Citrobacter rodentium*, and SseK in *Salmonella enterica*) are glycosyltransferases that glycosylate host protein substrates with β -D-N-acetylglucosamine (GlcNAc) on arginine residues. These substrates include receptor-interacting serine/threonine-protein kinase 1 (RIPK1), tumor necrosis

factor receptor (TNFR) type 1-associated DEATH domain protein (TRADD), and the Fas-associated protein with death domain (FADD). Some NleB/SseK orthologs also glycosylate glyceraldehyde 3-phosphate dehydrogenase (GAPDH) to reduce GAPDH binding to TRAF2 and inhibit NF- κ B signaling (Gao et al. 2013). Arginine glycosylation occurs on the guanidinium groups of arginines, which are poor nucleophiles. This modification is biologically important because the glycosylation of arginines on host protein substrates leads to target protein inactivation and disrupts the innate immune response. Mammals do not add N-GlcNAc residues to arginine, while this modification is important to *E. coli* and *Salmonella* virulence. Thus, inhibitors that prevent the formation of this unusual post-translational modification represent a potentially novel mechanism by which to combat infections. We previously developed and optimized a high-throughput screening (HTS) assay for NleB1 inhibitors. This assay produces a luminescent signal when UDP-GlcNAc is hydrolyzed by NleB1 to liberate UDP (El Qaidi et al. 2018). We showed from an initial screen of 5160 compounds from the University of Kansas Center of Excellence in Chemical Methodologies and Library Development library that two compounds, 100066N and 102644N, both inhibited NleB1, SseK1, and SseK2 activities (El Qaidi et al. 2018). The addition of these compounds to cultured mammalian cells inhibited NleB1 glycosylation of TRADD. These compounds were also capable of inhibiting *Salmonella* replication in mouse macrophage-like cells. Neither inhibitor was significantly toxic to mammalian cells, nor showed *in vitro* cross-reactivity with the mammalian O-linked N-acetylglucosaminyltransferase (OGT). The utility of 100066N and 102644N was limited because of the lack of their commercial availability and poor solubility. We were therefore motivated to screen a larger library of compounds to identify more diverse scaffolds that may be amenable to future chemical optimization. Here, we report the results of a 42,498 compounds screening

assay, as well as the preliminary characterization of the activity of sepantronium bromide (YM155) against NleB/SseK.

Results

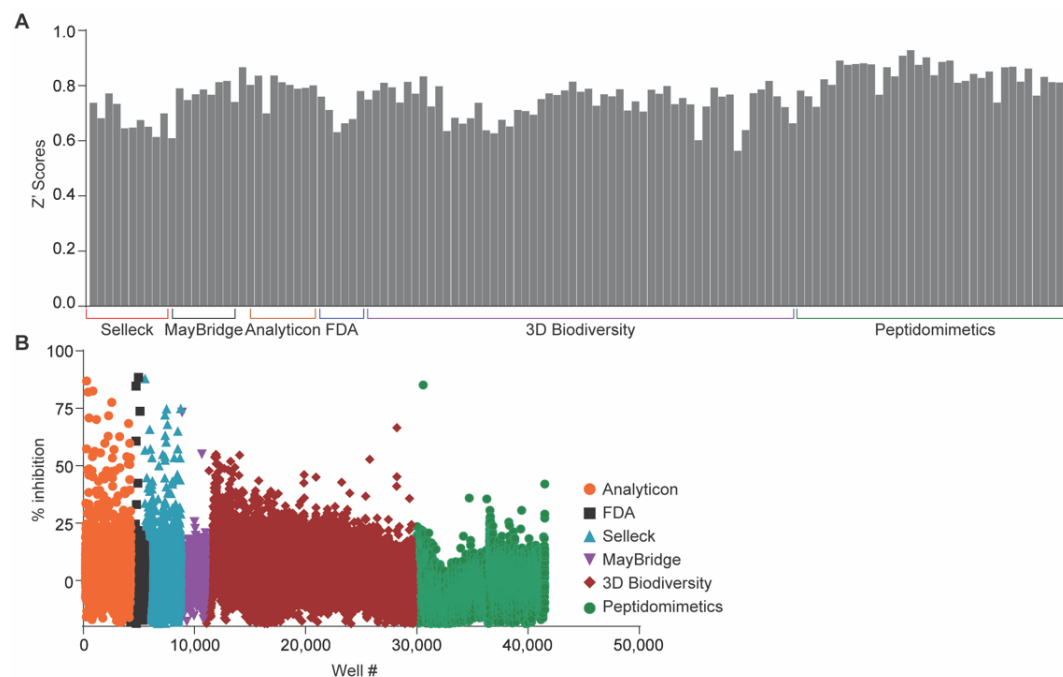
NleB1 Inhibitor Screening

We used a previously described high-throughput screening (HTS) assay for NleB1 inhibitors that produces a luminescent signal when UDP-GlcNAc is hydrolyzed by NleB1 to liberate UDP (El Qaidi et al. 2018). The assay was performed by incubating purified NleB1 (150 nM) with UDP-GlcNAc (300 μ M) for 2 h at 30 °C, followed by conversion of UDP to ATP, which is then utilized by luciferase to generate light in proportion to the UDP concentration.

We screened 42,498 compounds (20 μ M) derived from the University of Kansas Infectious Diseases Assay Development (KU-IDAD) laboratory from diversity sets representing a diverse scaffold collection including: Analyticon Natural products library (n = 4,197), ChemDiv 3D Biodiversity (n = 19,528), ChemDiv Peptidomimetics (n = 12,139), FDA (n = 1,280), Selleck Bioactives (n = 3,354), and MayBridge mini (n = 2,000) for their ability to inhibit NleB1 activity.

Overall, we obtained a 0.47 % hit rate (200 compounds) with an average z' -score of 0.77 +/- 0.08 (Figure 8A). Among the 200 compounds that significantly inhibited NleB1 activity, the hits were distributed among the libraries as follows : Analyticon Natural products library (n = 76), ChemDiv 3D Biodiversity (n = 59), ChemDiv Peptidomimetics (n = 8), FDA (n = 25), Selleck Bioactives (n = 28), and MayBridge mini (n = 4) (Figure 8B).

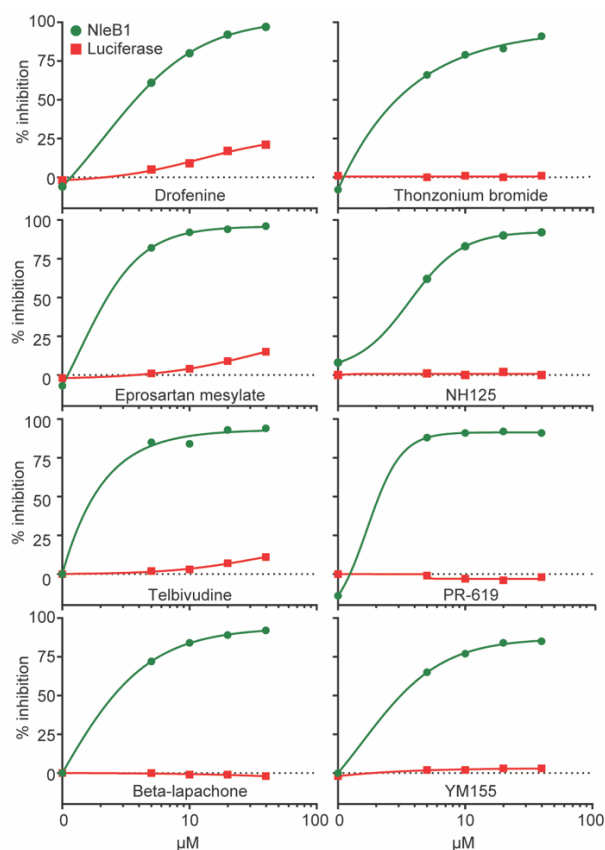
Figure 8 Primary screening results



(A) Distribution of Z' scores across plates in NleB1 inhibition assays. An average z'-score of 0.77 +/- 0.08 was obtained, reflecting good separation between the distributions of the positive and negative controls among all assay plates. (B) HTS results. The percentage of inhibition was calculated by computing luminescence signals from UDP liberated from NleB activity. Inhibition was plotted as a function of each compound per well number for all 42,498 targets in the combined libraries.

We validated 8 of the primary hits by performing additional reporter assays and determined that each of the tested compounds inhibited NleB activity without directly impacting the luciferase enzyme used in the primary assay (Figure 9).

Figure 9 Reporter assay reconfirmation.



NleB inhibition and luciferase inhibition assay results of eight primary targets from the FDA and Bioactives libraries with $IC_{50}s < 5 \mu M$. Luciferase assays were conducted as described for HTS assays. NleB activity inhibition (green) and luciferase activity inhibition (red), both are plotted as a function of individual compound concentration (0-40 μM).

Thonzonium Bromide is a monocationic surface-active agent with surfactant and detergent properties used as an additive in ear and nasal drops (Chafetz et al. 1986). Telbivudine is an antiviral drug used to treat hepatitis B infection (Han 2005). Eprosartan mesylate is an angiotensin II receptor antagonist used to treat high blood pressure (Teitelbaum et al. 2004). NH125 is a selective inhibitor of eukaryotic elongation factor 2 kinase with anti-bacterial properties (Abouelhassan et al. 2019). Drofenine hydrochloride is a competitive inhibitor of butyrylcholinesterase (Bodur et al. 2001). Beta-Lapachone is a naturally occurring topoisomerase I inhibitor that induces apoptosis by inhibiting cell cycle progression (Li et al. 1993). PR-619 is a

broad-range deubiquitylase inhibitor that induces ER stress (Kuan-lin et al. 2019). One of the new compounds we identified from this screen is sepantronium bromide (YM155; Figure 10A). YM155 is an inexpensive, commercially-available compound that has been previously characterized as a suppressor of survivin, a member of the inhibitor of apoptosis (IAP) gene family (Nakahara et al. 2007). YM155 suppresses proliferation in a broad range of human cancer cell lines (Nakahara et al. 2011). We investigated the activity of YM155 against NleB/SseK further.

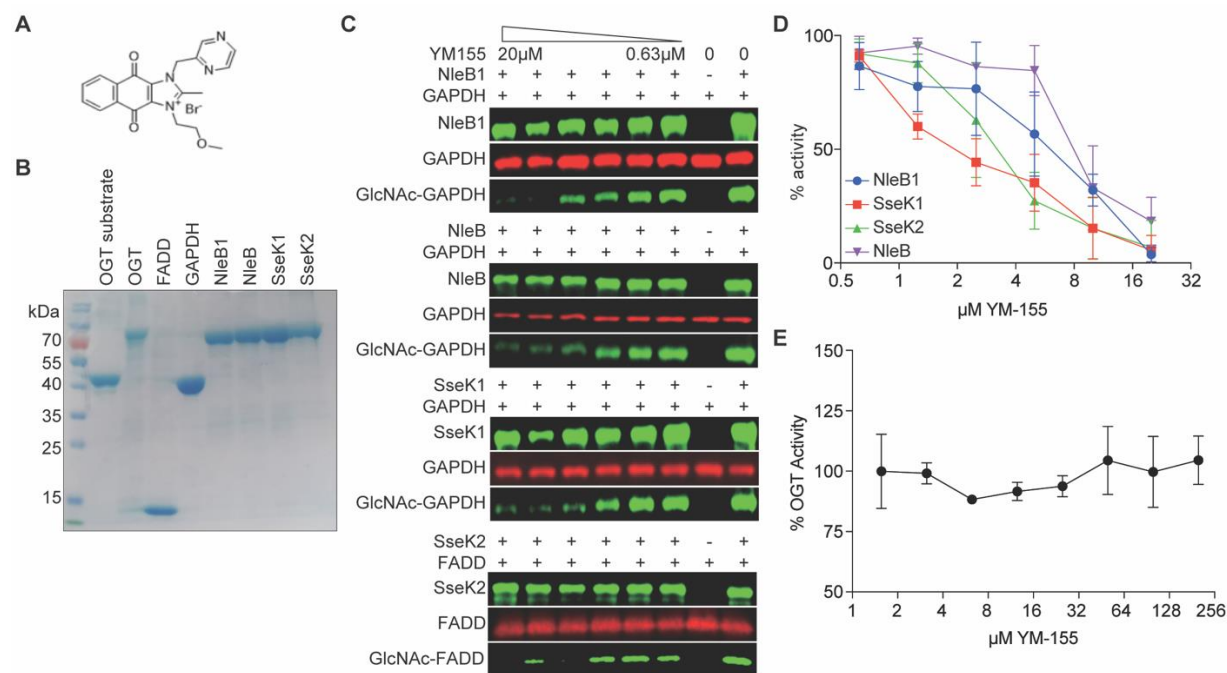
Glycosylation Assays

We tested the ability of YM155 to inhibit substrate glycosylation by the NleB/SseK enzymes. All recombinant enzymes (rNleB1, rNleB, rSseK1, and rSseK2) and substrates (rGAPDH and rFADD) were first purified using affinity chromatography (Figure 10B). YM155 inhibited EHEC NleB1, NleB, and SseK1 glycosylation of GAPDH in a concentration dependent manner (Fig. 3C). YM155 also inhibited SseK2 glycosylation of the human FADD protein (Figure 10C).

Western blot intensities were quantified as shown in Figure 10D.

We next tested whether YM155 also inhibited the human OGT enzyme, an essential serine/threonine N-acetylglucosamine (O-GlcNAc) transferase that maintains protein glycosylation homeostasis (Zhao et al. 2018). To do this, we performed a bioluminescence-based UDP-Glo glycosyltransferase assay to quantify OGT activity in presence of YM155. YM155 had no impact on OGT activity (Figure 10E), suggesting it is specific to the NleB/SseK Arg glycosyltransferases.

Figure 10 *In vitro* glycosylation assays



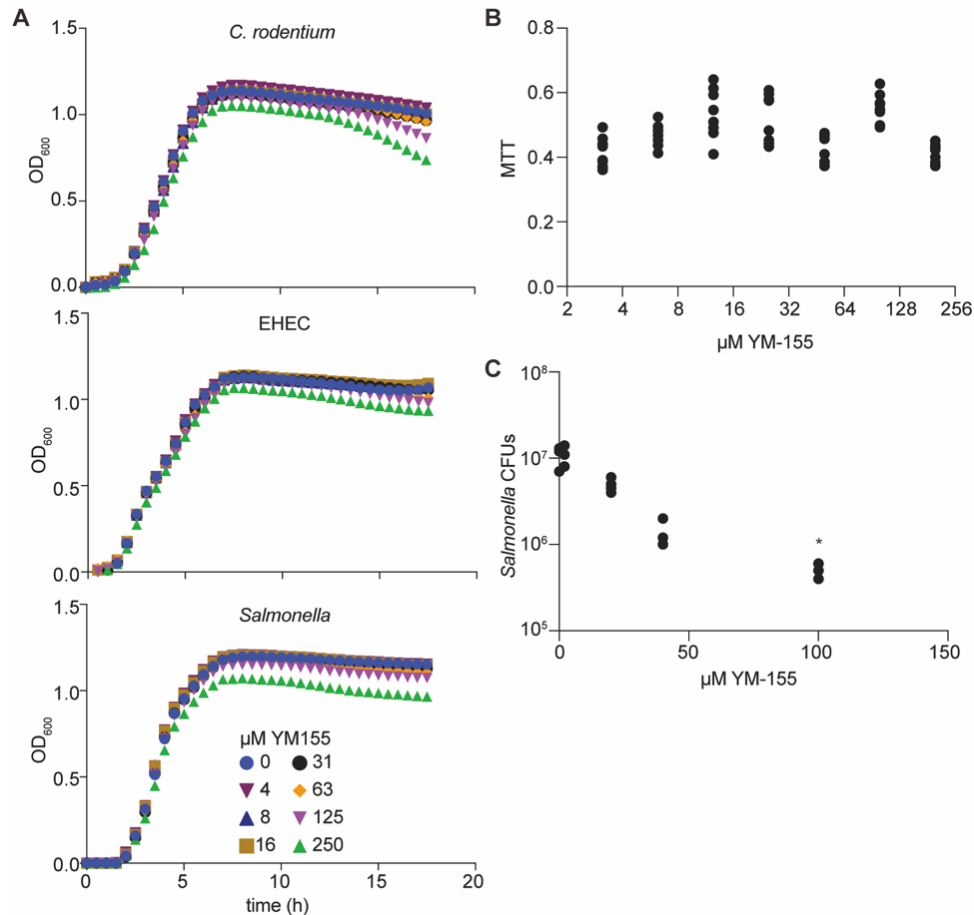
(A) YM155 structure. (B) SDS-PAGE analysis of recombinant proteins. (C) Western blot results for YM155 inhibition of NleB1, NleB, and SseK1 glycosylation of GAPDH and SseK2 glycosylation of FADD. (D) Quantification of western blot signal intensities, $n = 3$. (E) UDP Glo Assay. OGT activity as a function of [YM155], $n = 3$.

Growth Assays

YM155 did not impact the growth rates of *C. rodentium*, EHEC, or *Salmonella enterica* when supplied at concentrations of up to 125 μM in bacterial cultures (Figure 11A). These data suggest that YM155 does not act as a general bacteriostatic or bactericidal agent. YM155 was not significantly toxic to mammalian cells, as inferred from 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay data (Figure 11B). These data suggested the potential use of YM155 as an anti-virulence small molecule. To determine whether YM155 might function *in vivo* to reduce pathogen burdens, we quantified the impact of YM155 on *Salmonella* survival in cell culture. When YM155 was added to RAW264.7 cells prior to their infection with

Salmonella, we observed that the number of *Salmonella* 24 h after infection was significantly reduced (Figure 11C).

Figure 11 Cellular assays



(A) Bacterial growth assays. *C. Rodentium*, EHEC, and *S. enterica* were cultured in LB media in the presence of YM155; bacterial growth was monitored as a function of time. (B) MTT assays. YM155 was added to RAW264.7 cells for 24 h and cell viability was assayed by monitoring MTT signal intensity. (C) *Salmonella* infection assays. RAW264.7 cells were seeded at 1×10^5 cells/well in 24-well plates and YM155 was added 1 h before infection with 10^6 CFUs of *Salmonella* for 30 min. Cells were incubated in medium containing 100 μg/mL gentamicin for 1 h, and then 10 μg/mL gentamicin for an additional 23 h. Bacteria were released from RAW264.7 cells using 1 % saponin, diluted in PBS, and plated for colony counts.

Discussion

We previously identified two small molecules, 100066N and 102644N, that inhibited the activities of NleB1, SseK1, and SseK2 (El Qiadi et al. 2018). In this study, we screened a larger library of 42,498 compounds and identified 200 other molecules that may have activity against NleB/SseK (Figure 8). We validated the activity of 8 of these compounds in secondary screening assays (Figure 9). However, most of these compounds had unfavorable properties that precluded their further study. PR-619 exhibits cytotoxicity (Seiberlich et al. 2012) and Beta-Lapachone causes DNA damage (Vanni et al. 1998). NH125 exhibited direct antimicrobial activity, whereas thonzonium bromide, telbivudine, eprosartan mesylate, and drofenine hydrochloride did not significantly reduce NleB1 activity when studied further in Western blotting assays (*data not shown*). We therefore focused on YM155.

YM155 was discovered as a small molecule inhibitor that suppresses survivin, a member of the inhibitor of apoptosis (IAP) family (Nakahara et al. 2007). Survivin functions to inhibit caspase activation (Ambrosini et al. 1997). YM155 suppresses the proliferation of a broad range of human cancer cell lines (Nakahara et al. 2011). YM155 also inhibits topoisomerase function and prevents double DNA strand repair (Hong et al. 2017).

Here we determined that YM155 also functions as an NleB/SseK glycosyltransferase inhibitor. IC₅₀s for the NleB/SseK orthologs were estimated at ~2 μ M from luciferase reporter (Figure 9) and Western blotting assays (Figure 10C-D). YM155 had no discernible toxicity to RAW264.7 cells, nor did it affect bacterial growth rates at concentrations less than 250 μ M. Furthermore, YM155 had no apparent cross-reactivity with the mammalian OGT enzyme (Figure 10E). YM155 reduced the survival of *Salmonella* in RAW264.7 cells as a function of YM155 concentration (Figure 11C), suggesting its potential utility as an anti-virulence compound.

Although YM155 appears to be less active than 100066N and 102644N (El Qiadi et al. 2018), it has the advantage of being more soluble and amenable to chemical derivatization.

While our primary goal is to discover, characterize, and use NleB/SseK inhibitors as anti-virulence therapeutics, it has not escaped our attention that an additional benefit of such inhibitors may be by synergizing with existing antibiotic regimens. Thus, future applications of our work would be to perform minimum inhibitory and minimal bactericidal concentration testing and quantify to what extent the NleB/SseK inhibitors may improve antibiotic efficacy. The future repurposing of previously characterized small molecules to prevent or treat bacterial could promote a medicinal chemistry campaign on NleB/SseK inhibitor development, as well as the development of other small molecules that inhibit effectors catalyzing unusual post-translational modifications, which adds impact to potential future studies.

Materials and Methods

Cell lines

Abelson-murine-leukemia-virus-induced, macrophage-like cells from BALB/c mice (RAW264.7) were purchased from ATCC and grown in Dulbecco's Modified Eagle Medium (DMEM), supplemented with 10 % FBS (Atlanta Biologicals) and 100 µg/ml penicillin/streptomycin (Sigma, St. Louis, MO, USA) in 5 % CO₂.

Library Screening

HTS assays were performed similarly to as described previously (El Qiadi et al. 2018) in 50 mM Tris-HCL buffer pH 7.5, 100 mM NaCl, 1 mM DTT, 10 mM MgCl₂ and 0.5 % DMSO. NleB1 (150 nM) was pre-incubated for 30 min in 384 well-microplates, containing compounds (20 µM) transferred acoustically using ECHO555 (Labcyte Inc). Luminescence was read 2 h after the

addition of the UDP detection reagent. The percent inhibition was normalized to the controls and compounds that inhibited the assay to >3 standard deviations plus the plate median were identified as hits.

Protein Purification

Protein purification was performed similarly to as described previously (El Qiadi et al. 2017). *E. coli* BL21 (DE3) strains harboring pET42a-, pET28a-, and pET15b-expression plasmids were grown at 37 °C in LB until an OD600 of 0.4, at which time 0.5 mM IPTG was added for 4 h. Cells were centrifuged, and the pellet was resuspended in lysis buffer (50 mM sodium phosphate buffer pH 8.0, 0.5 mg/ml lysozyme). The suspension was incubated on ice for 30 min with occasional shaking and then treated with 50 mM sodium phosphate buffer pH 8.0, 2 M NaCl, 8 mM imidazole, 20 % glycerol, 2 % Triton X-100, for 30 min. Cell lysates were sonicated, centrifuged, and then the resultant supernatant was added to 2 ml Ni-NTA resin (Qiagen) for 1 h of rotation at 4 °C. The mixture was loaded on a Poly-Prep Chromatography Column (Bio-Rad) and washed with 10 ml of 50 mM sodium phosphate buffer pH 8.0, 600 mM NaCl, 60 mM imidazole, 10 % glycerol. Proteins were eluted in 2 ml 50 mM sodium phosphate buffer pH 8.0, 600 mM NaCl, 250 mM imidazole, 10 % glycerol and dialyzed into the same buffer lacking imidazole.

Glycosylation Assays

In vitro glycosylation assays were performed as described previously (El Qiadi et al. 2017). Enzymes (200 nM of NleB1, NleB, SseK1, or SseK2) were incubated with 1 µM of GAPDH or FADD +/- serial dilutions of YM155 in 50 mM Tris-HCl buffer pH 7.4, 1 mM UDP-GlcNAc, 10

mM MnCl₂, and 1 mM DTT. After 2 h incubation at RT, samples were subjected to western blotting using anti-R-GlcNAc and anti-His tag monoclonal antibodies (Abcam, Cambridge, MA, USA). Signal intensities were quantified using LI-COR Image Studio software (LI-COR Biosciences, Lincoln, NY, USA), and inhibition was calculated by quantifying the relative reduction in substrate glycosylation.

OGT assays

UDP-Glo luminescence assays were performed to determine whether YM155 inhibits the mammalian OGT enzyme. Reactions were performed in 96 well-microplates following manufacturer's instructions with the UDP-Glo™ Glycosyltransferase Assay Kit (Promega). Reactions contained 200 nM OGT in 25 mM Tris-HCL buffer pH 7.5, 12.5 mM MgCl₂, 0.06 mg/ml BSA, 1 mM DTT, 50 μM OGT peptide substrate and 100 μM UDP-GlcNAc, and included YM155 from 1-200 μM. Reactions were incubated for 1 h at 22 °C, and luminescence signals were quantified using a FLUO star microplate reader (BMG Labtech, Cary, NC, USA).

MTT Assays

MTT assays were performed as specified by Millipore using RAW264.7 cells in the presence of 2-fold serial dilutions of YM155. Formazan absorbance was measured using an Epoch Microplate Spectrophotometer (BioTek, Winooski, VT, USA).

Bacterial Growth Assays

Bacterial cultures were grown overnight, diluted 1:200 in LB in the presence of 2-fold serial dilutions of YM155 (0–250 μ M), and then grown at 37 °C for 18 h. Bacterial growth was monitored by measuring the absorbance of the culture medium at OD₆₀₀.

Macrophage Infection assays

RAW264.7 cells were seeded at 1×10^5 cells/well in 24-well plates and YM155 was added 1 h before infection. Bacterial cultures were grown overnight and 10^6 CFUs were added to each well for 30 min. Cells were treated with 100 μ g/mL gentamicin for 1 h, and then 10 μ g/mL gentamicin for an additional 23 h. Bacteria were released from RAW264.7 cells using 1 % saponin (Sigma), diluted in PBS, and plated for colony counts.

Chapter 4 - Discussion. Strategies Used in New Enzyme Inhibitor

Discovery

An enzyme inhibitor is a molecule that binds to an enzyme and decreases enzymatic activity. It is in contrast of the term enzyme activator, which increases enzymatic activity. Enzyme inhibitors decrease enzyme activity through various mechanisms.

Inhibitor classification

Generally, inhibitors are divided into two categories: Reversible inhibitors and irreversible inhibitors.

Reversible inhibitors bind to enzymes with non-covalent interactions such as hydrogen bonds, hydrophobic interactions, and ionic bonds. In common, enzyme activity affected by reversible inhibitors can be recovered through removing the inhibitor. There are four classes of reversible enzyme inhibitors corresponding to their effect on the kinetic constants of the enzyme:

competitive inhibitor, uncompetitive inhibitor, non-competitive inhibitor, and mixed inhibitor. In competitive inhibition, the inhibitor usually binds to the same active site of enzyme where the substrate aims to bind. Enzymatic activity is decreased because inhibitor and substrate compete for the same active site resulting in less substrate bound to the enzyme. For uncompetitive inhibitor, the inhibitor binds to the substrate-enzyme complex instead of the enzyme itself. In non-competitive inhibition, the inhibitor binds to a different site of enzyme rather than where the substrate binds to, causing a structural change in enzyme where the enzyme-substrate-inhibitor complex cannot form product. A mixed inhibitor can bind to the enzyme at the same time as the enzyme's substrate. Differ from non-competitive inhibition, binding of a mixed inhibitor affects the binding of the substrate (Strelow et al. 2004). The kinetics of enzyme-catalyzed reactions can

be used to identify different types of inhibition, according to their effects on K_m (Michaelis constant) and V_{max} (maximum velocity) (Cleland et al. 1963).

The irreversible inhibitors usually covalently bind to an enzyme, and inhibition can not be reversed. Irreversible inhibitors change enzyme active site and display time-dependent inhibition (Srinivasan 2020).

Use of inhibitors

Enzyme inhibitors are widely used in agricultural and medical field. They either come from natural products or synthesized in labs and industries.

Natural inhibitors often act as poisons from animals and plants to prey for food or defend their predators. Most natural poisons are neurotoxins which can cause paralysis. For example, penicillin from *Penicillium* moulds act as β -lactam antibiotics to fight against a broad spectrum of bacteria (Wainwright, 1987); glycoalkaloids from plants are acetylcholinesterase (AChE) inhibitors which break down neurotransmitter acetylcholine into acetate and choline (Hostettmann, 2006); Botulinum toxin from *Clostridium botulinum* and related species (Montecucco, 2005) interferes with neural transmission by blocking the release of acetylcholine at neuromuscular junction, leading to muscle paralysis (Burgen et al. 1949). Therefore, botulinum toxin is commonly used in the medical field for muscle disorders treatment and cosmetic applications (Ramachandran and Yaksh, 2014).

Artificial synthesized inhibitors have developed broader and more specific use compared to natural inhibitors. They can also be AChE inhibitors used in agriculture as insecticides (malathion) and herbicides (glyphosate) (Colovic et al. 2013). Others are designed into drugs for

medical purpose, such as DNA replication inhibitors used in cancer chemotherapies and inhibitors which regulate the metabolic pathways.

Methodologies to discover new inhibitors

There are three ways to discover new inhibitors: Use of natural products, high-throughput screening (HTS), and rational design with the help of computer modeling (molecular docking). Natural toxins are often enzyme inhibitors secreted by plants or animals to help survive in nature. They can be used to hunt for food or defend against predators. Many drugs we use nowadays are natural compounds or their derivatives (Newman, 2000). Despite the complexity of natural products which made them difficult to isolate and analyze, the development of modern analytical and synthetic techniques has greatly broadened our understanding of the active composition and molecular structure of natural products. Therefore, natural products still have high potential for new inhibitors discovery as we can find new enzyme targets of existing products and synthesize derivatives according to their well-studied structures (Clardy and Walsh, 2004).

High-throughput screening (HTS) is based on the concept that testing a large library of compounds against one target enzyme, in hope of finding some of the compounds can significantly alternate target enzyme activity. This methodology was the only choice for new drug discovery in the past as we had limited knowledge to predict protein-protein interactions. Therefore, the quality of screening was based on which library we chose and how many compounds we screened. Scientists have gained rich experience during practice of HTS trials to overcome difficulties in finding suitable inhibitors. For example, using dimethyl sulfoxide (DMSO) to dissolve compounds which are poorly soluble in water; developing colorimetric methods for quantification of specific enzyme reactions to increase accuracy and decrease

complexity during new compounds search; Targeting smaller-size compounds profile and existing drug libraries for easier understanding the absorption, distribution, metabolism, excretion, and toxicity (ADMET) process of identified targets (Gribbon and Sewing, 2005). If choosing the right screening strategy and following the hit to lead process, HTS will still be the classical and practical method in new drug discovery.

Rational design and molecular docking became possible approach in the past decade with the fast development of structural biology. Crystallography technique improvements such as s high-throughput crystallization (Mueller et al. 2001) and high-performance synchrotron beamlines (Hendrickson, 2000) have revealed more structure information than ever before. Therefore, it becomes possible to conclude enzyme activity through their 3D structure and synthesize compounds of required functional domains. Rational design is based on the structure of a known enzyme especially its functional region to predict products which could be possible inhibitors. These candidates are then tested against the target enzyme to discover novel inhibitor which can reduce specific enzymatic activity. Obtaining the novel inhibitor and enzyme complex structure can further help the refinement of inhibitor, such as modifying the functional groups of inhibitor to increase affinity to the enzyme. The repetition of refinement process can theoretically lead to ideal inhibitor finding in the concept of rational design. Instead of HTS, initial screening for rational design used computational methods to predict the affinity between two molecules, known as ‘molecular docking’. This technique simulates the docking process by comparing two molecule’s shape complementarity and calculating interaction energies when the enzyme and a potential inhibitor are binding together. Molecular docking can go through virtual libraries that is larger than any physical library in relatively short time, thus making it powerful technique to support rational design of new drugs.

Identification of bacterial arginine glycosyltransferase inhibitors

EPEC, EHEC and *Salmonella* are food borne pathogens of high threat to public health. Annually increasing in their antibiotic drug resistance rise challenge for new drug development. These Gram-negative pathogens inject virulence proteins into host cell through type III secretion system. The secreted T3SS effectors help them to invade host cells and survive under host defense mechanisms, making them attractive targets for antimicrobial treatments.

Many small inhibitors against T3SS have been identified in the past decade. Animal studies have supported the concept that aiming at bacterial virulence factors, rather than killing the bacteria, can significantly decrease the chance of bacteria developing drug resistance. Therefore, finding small molecule inhibitors of T3SS is a promising way to develop antivirulence therapies. For example, N-Hydroxybenzimidazoles can inhibit LcrF transcription activator by targeting multiple adaptational response (MAR) proteins, in turn suppress the expression of cytotoxic Yop proteins in *Yersinia* (Garrity-Ryan et al. 2010); Compound 2-imino-5-arylidene thiazolidinone (TTS29) disrupts T3SS needle assembly in *S. typhimurium* without affecting bacterial growth, and also can function on *Y. spp.*, *P. aeruginosa* and *Francisella novicida* (Felise et al. 2008).

Our goal is to find small molecule inhibitors of enteric pathogens' T3SS effectors. We targeted effector NleB because the deletion of gene *nleB* significantly attenuates *C. rodentium* virulence. We identified three compounds (100066N, 102644N and YM155) through HTS that significantly inhibited NleB1, SseK1, and SseK2 glycosylation activities. Combination of 50 % 100066N and 50 % 102644N achieved enhance inhibition of NleB1 activity. NleB1 is a glycosyltransferase composed of a helix-loop-helix (HLH) domain, a lid domain, and a catalytic domain. The HLH

domain is important to the recognition of the acceptor protein substrates containing death domains. The conserved HEN motif (His-Glu-Asn) in the active site is important for enzyme catalysis and bacterial virulence (Park et al. 2018).

We hypothesize several mechanisms for identified inhibitors to work. They could affect UDP-GlcNAc binding and/or hydrolysis, activation of the acceptor arginine, the flexibility of the HLH domain, the folding of enzyme catalytic domain or the amino residues of DXD motif.

Obtaining the co-crystal structure of inhibitors 100066N and 102644N bind to enzymes NleB/SseK complex is our next goal to understand their mechanisms of inhibition. This step is critical for designing derivatives of these compounds (or find structure similar drugs) to improve inhibitory performance. New round of screening these derivatives, repetition of refinement process can theoretically lead to ideal inhibitor finding. We aim to find one compound that is highly efficient in reducing EHEC/ EPEC and *Salmonella* virulence, highly specific to bacterial glycosyltransferase with less toxicity or side effects, and commercially affordable which can be further developed as antibacterial drug.

Despite screening for more potential small molecule inhibitors and testing YM155 derivatives, we considered also look into molecules which can inhibit formation of T3SS apparatus. NleB1 is not the only gene controlling EHEC virulence, inhibitor of T3SS assembly can theoretically be more effective on reducing pathogen virulence. As T3SS structure is highly conserved among species, the discovery of new inhibitors is likely to block virulence of a wide array of animal and plant Gram-negative bacterial pathogens. However, as more evidence of bacterial secretion system effectors possess intra-bacterial activities appears (El Qiadi et al. 2020), we may not be able to significantly attenuates bacteria virulence without affecting their metabolism.

Chapter 5 - Conclusions

During evolution, enteric pathogens developed various strategies to invade, survive, and multiply in their hosts. One of the most important methods is to deliver virulence proteins to the host through multiple secretion systems. These virulence factors termed ‘effectors’ can counteract host immune systems and play critical roles in bacterial pathogenesis.

In Chapter 1, we discussed about the general background information of three common enteric pathogens *Escherichia coli*, *Salmonella enterica*, and *Citrobacter rodentium*. This part includes the classification, clinical symptoms, and major virulence traits of EHEC, EPEC, *Salmonella enterica* and *Citrobacter rodentium*. In the second part, we discussed six major secretion systems of gram-negative bacteria separately, including Type I, II, III, IV, V and VI secretion system. We described each of their composition, structure differences and compared their mechanisms of protein secretion. Our laboratory work focuses on T3SS of enteric attaching/effacing (A/E) pathogens. We reviewed detailed information for each individual T3SS effectors of EHEC, EPEC, *Salmonella enterica*, and *Citrobacter rodentium*. This information includes where the effector is encoded, what are their enzyme activities, and how they interact with the host. Site mutations data and animal studies of individual effectors are also included. To conclude, many T3SS effectors block multiple steps within a host signaling pathway (e.g. NF- κ B signaling pathway) to suppress innate immune response.

In Chapter 2, we specifically targeted T3SS effector NleB1, a signature of EHEC strains causing hemolytic uremic syndrome. As deletion of *nleB* or *nleB* Y219A, E253A point mutations significantly attenuates *C. rodentium* virulence (Kelly et al. 2006; Wong et al. 2016), we aim to identify small molecule inhibitors of NleB1 against EHEC infection. We developed and optimized a HTS assay to screen for NleB1 inhibitors through the KU CMLD library of 5,160

compounds. The inhibition of NleB1 glycosylation was performed in a solution-based assay that produces a luminescent signal when UDP-GlcNAc is hydrolyzed by NleB1 to liberate UDP. A significant reduction in luminescent signal can be acquired when NleB1 glycosylation is inhibited by the compound being tested. We obtained two most potent hits 100066N and 102644N in the primary screening. 100066N is a flavone analog that has been previously synthesized (Ahn et al. 2007). 102644N is a substituted isoxazole whose synthesis has also been described (Waldo et al. 2008). 100066N and 102644N were confirmed to inhibit NleB1 glycosylation of human GAPDH protein *in vitro* through Western blot technique. Both (100066N and 102644N) compounds can also inhibit enzyme activity of NleB1 orthologs SseK1 and SseK2. For *in vivo* studies, we found that both compounds can reduce NleB1 glycosylation of TRADD in HEK293T cells when provided 1 μ M concentration. We then tested the two compounds showing no significant toxicity to mammalian cells using MTT assay, nor did they cross react with human OGT. Our work further demonstrated the two inhibitors do not affect EHEC and *Salmonella* growth rate when provided at 500 μ M concentration, but can significantly reduce *Salmonella* replication in RAW264.7 cells 24 h after infection. These data show high potential of 100066N and 102644N for bacterial glycosyltransferase drug development.

In Chapter 3, we performed larger compound library screening for more potential small molecule targets. The previous 100066N and 102644N showed good characteristics as NleB1 inhibitors but both were hard to synthesize and difficult to resolve in DMSO. One solution to solve this issue was to look for existing drugs or compounds which were clearly defined and sold in the market. So we screened 42,498 compounds from KU-IDAD laboratory holding diversity compounds libraries: Analyticon Natural products library (n = 4,197), ChemDiv 3D Biodiversity (n = 19,528), ChemDiv Peptidomimetics (n = 12,139), FDA (n = 1,280), Selleck Bioactives (n =

3,354), and MayBridge mini (n = 2,000) for their ability to inhibit NleB1 glycosylation activity with HTS approach described previously. We obtained 200 hits with over 50 percent reduction in luminescence signal derived from liberated UDP. Among these hits, eight primary targets were from both FDA and Bioactives libraries: Thonzonium Bromide, Telbivudine, Eprosartan mesylate, NH125, Drofenine hydrochloride, Beta-Lapachone, PR-619 and sepantronium bromide (YM155). We validated the activity of these compounds in secondary screening assays and decided to focus on YM155 because some of the compounds have unfavorable properties which restricts future studies while others didn't show significant reduction of NleB1 activity in western blot assays. It is already known that YM155 is a survivin inhibitor and suppresses proliferation of various human cancer cell lines (Nakahara et al. 2007; Nakahara et al. 2011). Here we determined YM155 also works as glycosyltransferase inhibitor to inhibit EHEC NleB1, *Citrobacter* NleB, *Salmonella* SseK1 and SseK2. We performed similar assays on YM155 as previous paper to assess potential of YM155 for drug development. The IC₅₀s of YM155 for NleB orthologs were estimated at ~2 μ M from luciferase assays and Western blot assays. YM155 did not show significant toxicity on HEK293T and RAW264.7 cells, nor did it affect enteric bacteria stains growth rates at up to 250 μ M concentration. YM155 also did not cross-react with human OGT but was able to reduce *salmonella* colonization in RAW264.7 cells. YM155 has the advantage of being more soluble and can be more easily purchased than 100066N and 102644N, making it a better choice to study anti-virulence therapeutics. YM155 can be modified and repurposed drug to counter enteric pathogens infection. YM155 derivatives can be used together with antibiotics or as alternative treatment to fight against bacterial antibiotic resistance.

In Chapter 4, we gave an overview of inhibitor aspects and discussed how to identify a proper inhibitor. There are two classes of inhibitors, reversible inhibitors and irreversible inhibitors. Reversible enzyme inhibitors are further classified into four types: competitive inhibition; uncompetitive inhibition; non-competitive inhibition and mixed inhibition. Enzyme inhibitors are widely used in agricultural and medical field. They can be used for pest control, serve as antibiotics, alter metabolic signaling pathway, and treat cancer patients during chemotherapy. Mechanisms of the inhibition varies but all related to the characteristic of substrate protein. Despite discovering new inhibitory targets of natural products, there are two major methods applied in new drug discovery: High-throughput screening and rational design. HTS is rather traditional and practical method while rational design is computational method with more theoretical support. HTS is not applicable in circumstances when there is no simple protocol to test a good number of candidates or it is too costly to do so. Rational design can be more mechanism instructed but needs detailed structure information to begin with. HTS and rational design are complementary to each other, combining these two methods and achieving a deeper understanding of natural products are busting steps of modern drug discovery.

We discussed why we choose effector NleB as target, hypothesized how the three inhibitors may block NleB function, planned co-crystalize of inhibitor-enzyme complex for understanding the mechanisms and future refinement of the inhibitors, and targeting inhibitor of broader species of T3SS Gram-negative bacteria.

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