บทคัดย่อ

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ชื่อโครงการ : การป้องกันการเกิดโรค Salmonellosis ด้วยการยับยั้งโปรตีน InvC ATPase

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เชื้อ *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) เป็นแบคทีเรียที่เป็นสาเหตุของโรคทางเดินอาหารอักเสบและโรคอุจจาระร่วงในมนุษย์และปศุสัตว์ ระบบการหลั่งโปรตีนแบบที่ 3 เป็นที่ทราบกันดีว่าเป็นกลไกสำคัญที่เกี่ยวข้องกับการเกิดโรคโดยทำหน้าที่ในการนำส่งโปรตีนก่อโรคเข้าสู่เซลล์เจ้าบ้าน เชื้อ *S.* Typhimurium มีระบบการหลั่งโปรตีนแบบที่ 3 สองประเภทโดยสร้างจากกลุ่มยีนก่อโรค Salmonella Pathogenicity Islands (SPI) 1 และ 2 การศึกษาที่ผ่านมาแสดงให้เห็นว่าระบบการหลั่งโปรตีนแบบที่ 3 สามารถพบได้ในแบคทีเรียแกรมลบหลากหลายสายพันธุ์และมีโครงสร้างโมเลกุลที่คล้ายคลึงกัน ดังนั้นระบบการหลั่งโปรตีนแบบที่ 3 จึงเป็นเป้าหมายที่น่าสนใจในการพัฒนาวิธีการรักษาแบบใหม่ในการรักษาโรคติดเชื้อแบคทีเรีย การศึกษาเมื่อไม่นานมานี้รายงานว่าโมเลกุลขนาดเล็ก $C_{24}H_{17}ClN_4O_2S$ มีประสิทธิภาพในการยับยั้งโปรตีน YscN ซึ่งเป็นเอนไซม์ ATPase ของเชื้อ *Yersinia pestis* ส่งผลให้ยับยั้งการหลั่งของโปรตีนก่อโรคได้ ในการศึกษานี้ผู้วิจัยพบว่าสารดังกล่าวมีความสามารถในการยับยั้งการหลั่งของโปรตีนก่อโรคผ่านระบบหลั่งโปรตีนแบบที่ 3 ของเชื้อ *S.* Typhimurium ที่ความเข้มข้น 100 μM ได้ซึ่งส่งผลให้ความสามารถในการบุกรุกของเชื้อแบคทีเรียเข้าสู่เซลล์เพาะเลี้ยงลดลง แต่พบว่าสารนี้ไม่สามารถลดการทำงานของโปรตีน InvC ซึ่งเป็นเอนไซม์ ATPase ของระบบการหลั่งโปรตีนแบบที่ 3 ได้ อย่างไรก็ตามจากผลการศึกษาโปรตีนภายในเซลล์แบคทีเรียโดยใช้เทคนิคโปรตีโอมิกเชิงปริมาณพบว่าในแบคทีเรียที่ได้รับสาร $C_{24}H_{17}ClN_4O_2S$ มีปริมาณโปรตีน InvF ที่ทำหน้าที่ในการควบคุมการแสดงออกของโปรตีนที่สร้างจาก SPI-1 และโปรตีนก่อโรคสองชนิดคือ โปรตีน SipA และ โปรตีน SipC ลดลง ผลของงานวิจัยนี้อาจอธิบายได้ว่ากลไกของโมเลกุล $C_{24}H_{17}ClN_4O_2S$ ที่สามารถปิดกั้นการหลั่งของระบบหลั่งโปรตีนแบบที่ 3 ของเชื้อ *S.* Typhimurium เกิดจากความสามารถในการยับยั้งการสร้างโปรตีน InvF

คำสำคัญ โมเลกุลขนาดเล็ก *Salmonella* Pathogenicity Island 1 (SPI-1) ระบบหลั่งโปรตีนแบบที่ 3 และ เอนไซม์ ATPase

Abstract

Project Code : MGRG5580028

Project Title : Prevention of *Salmonella* Typhimurium pathogenesis by inhibition of InvC, Type III Secretion ATPase.

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Project Period : 2 years

Salmonella enterica serovar Typhimurium (*S.* Typhimurium) causes gastroenteritis and diarrhea in humans and food-producing animals. The Type III Secretion System (T3SS) has been known to be potent virulence mechanisms by injecting effector proteins into the cytosol of host cells. *S.* Typhimurium possesses two T3SSs located on *Salmonella* Pathogenicity Islands (SPI) 1 and 2. Previous studies showed that T3SS shared a potent virulence mechanism and molecular structure among several gram-negative bacteria. Therefore, T3SS has been identified as an attractive target to develop of novel therapeutics for treatment of bacterial infections. Recent study reported that small molecule, $C_{24}H_{17}ClN_4O_2S$ has been shown ability to inhibit *Yersinia pestis* T3SS ATPase, YscN resulting to block secretion of effector proteins. In this study, we illustrated that same compound prohibited secretion of effector proteins at 100 μM. As this result, bacterial invasion ability into epithelial cell cultures were reduced. In contrast with previous study, $C_{24}H_{17}ClN_4O_2S$ molecule did not inactivate activity of SPI-1 T3SS ATPase, InvC. However, we studied global cellular effects of. *S.* Typhimurium after treated with or without this compound using quantitative proteomic technique. These proteomic profile results showed that SPI-1 transcription regulator, InvF and two effector proteins, SipA and SipC were reduced in bacterial cells treated with compound. This may explain that action of $C_{24}H_{17}ClN_4O_2S$ molecule for blocking secretion of SPI-1 T3SS is through inhibition of InvF protein production.

Keywords : Small molecule inhibitor, *Salmonella* Pathogenicity Island 1 (SPI-1), Type III Secretion System (T3SS) and ATPase

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Introduction

Thailand is a developing nation dependent on agriculture. Productions from livestock such as pigs and chickens are commonly produced and consumed in Thailand. Chickens and pork products are the major exported food commodities apart from rice. In Thailand, there are approximately 7 million pigs and 250 million chickens on farms (Padungtod, Kadohira, & Hill, 2008). During five years, livestock farming in Thailand, particularly on swine and chicken farms, have been inspected by the department of livestock and certified to meet the standard of practice required by law. This criteria for certification includes microbial standard for feed and water used on farms (Department of Livestock Development, 2003). Despite efforts to improve standards of food hygiene in Thailand, from 1996 to 2004 the incidence of foodborne diseases increased from 137 to 248 cases/100,000 people then declined slightly to 217 cases/100,000 people in 2006. It shows that foodborne diseases affect many people. The most commonly isolated etiologic agents from patients with foodborne illness in Thailand are rotavirus, *Salmonella* and *Campylobacter* (Coker, Isokpehi, Thomas, Amisu, & Obi, 2002; Echeverria et al., 1989).

Foodborne infections from *Salmonella* spp. in developing countries are thought to be due to foodborne contaminations. Pre-slaughter pigs and chickens have been incriminated as the major sources of *Salmonella* contaminations of chickens and pork products at later stages in food processing chain (Rasrinaul et al., 1988). In 2006, Padungtod and Kaneene (Padungtod & Kaneene, 2006) reported that the prevalence of *Salmonella* isolation from pre-slaughter pigs (6%) and chickens (4%). However, serological testing of pigs yielded a much higher sero-prevalence (60%) (Patchanee et al., 2002). In addition, pork and chicken meat samples were collected from the same area during the same period of time, the prevalence of *Salmonella* contamination in both pork and chicken meat increased significantly. In Thailand, a survey of raw meat preparations showed up to 56% *Salmonella* contamination in Nham (raw pork sausage) and 19% contamination in Labbdib (raw minced meat salad). Moreover, a foodborne salmonellosis outbreak was associated with vegetable consumption (Padungtod & Kaneene, 2006).

The genus *Salmonella* is composed of motile bacteria that belong to the family Enterobacteriaceae: facultative anaerobic Gram-negative bacilli, 2 to 3 μm x 0.4 to 0.6 μm in size. There are two main clinical diseases associated with *Salmonella* infection: the systemic disease typhoid fever is caused by *Salmonella enterica* serovar Typhi (*S*. Typhi) and non-thyphoidal salmonellosis–a gastrointestinal disease also known as enteritis. In this experiment, I focus in *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) which is common cause of enterocolitis and diarrhea disease in several mammalian species (Murray & Baron, 2007). *S.*

Typhimurium causes disease in animals and it can be generally transmitted to human *via* food derived from infected live stock. In humans, the infection manifests as intestinal disease ranging from gastroenteritis, usually lasting 3-5 days, to a more debilitating enteritis lasting 2-3 weeks (Ina, Kusugami, & Ohta, 2003). In animals, *S.* Typhimurium induced inflammation focuses on different parts of gut. For example, in calves, inflammation is observed in the terminal ileum and the proximal colon (enterocolitis) (R.M. Tsolis et al., 1999).

S. Typhimurium colonizes the apical epithelium of the intestine, induce invasion associated virulence machinery and elicit significant inflammatory changes including focal and diffuse PMN infiltrate, crypt abscesses, epithelial necrosis, edema and fluid secretion. *In vitro*, PMN recruitment to cultured epithelial monolayers occurs *via* the induction of interleukin-8 (IL-8) by *Salmonella* proximate to the apical epithelium. The ability of *Salmonella* to cause intestinal disease correlated to their ability to attract PMN across T84 cell monolayers. In the first 1-3 h of infection, *S.* Typhimurium enrolls neutrophils. After that, over the next 8-10 h there is extensive neutrophil migration which results and the secretion of protein-rich exudates into the intestinal lumen. The diarrheal symptoms occur approximately 8-72 h after bacterial colonization (Tsolis, Adams, Ficht, & Baumler, 1999).

For molecular pathogenesis of *S.* Typhimurium, bacteria contain two virulence-associated gene clusters for *Salmonella* pathogenesis, *Salmonella* Pathogenicity Island 1 and 2 (SPI-1 and SPI-2). Both of these SPIs encode type III secretion systems (T3SSs) (Galan, 2001). The functions of two T3SSs are the translocation of virulence factors or effector proteins from the bacterial cytoplasm into the host cell, thus functioning as “molecular syringes” (Cornelis, 2006). SPI-1 and SPI-2 express at different times during infection. Whereas the SPI-1 is required for the initial interaction of *Salmonella* with intestinal epithelial cells, the SPI-2 encoded system is required for systemic infection. The SPI-1 has been shown to be essential factor for invasion of non-phagocytic cells, induction of intestinal inflammatory responses and diarrhea, as well as colonization of the intestine (Wallis & Galyov, 2000). Contrasting, the SPI-2 has important role in bacterial survival in macrophages (Coburn, Li, Owen, Vallance, & Finlay, 2005).

At least 13 effector proteins are delivered by the SPI-1 type III secretion system. The translocated effectors alter key host-cell functions including signal transduction, cytoskeletal architecture, membrane trafficking and cytokine expression. These processes command the host cell to engulf bacteria into the host cell cytosol by bacterial-mediated endocytosis or macropinocytosis (Hayward & Koronakis, 1999). In the intracellular stages of the infection process, bacteria form the large *Salmonella*-containing vacuoles (SCVs). This process is modulated by the function of the SPI-2 T3SS, which expresses ~3 h after infection (Hensel, 2000).

A variety of gram-negative pathogens (*Yersinia*, *Salmonella*, *Erwinia* and *Pseudomonas*) use type III secretion systems (T3SS) as highly adapted virulence mechanism. Functions of T3SS of pathogenic bacteria are in the assembly of both flagella and virulence-associated organelles, secretion of proteins across both membranes independently of the *sec*-pathway and without the need for a periplasmic intermediate or proteolytic processing (Cornelis & Van, 2000). Because of their shape and their ability to directly translocate proteins to the cytoplasm of a host cell, they are also referred to as “injectisomes” or “molecular needle” (Cornelis, 2006). The T3SS consists of three types of proteins: (i) structural components of the injection apparatus (ii) effector (secreted) proteins and (iii) regulatory factors controlling expression of structural and effector proteins (Figure 1) (Galan & Collmer, 1999).

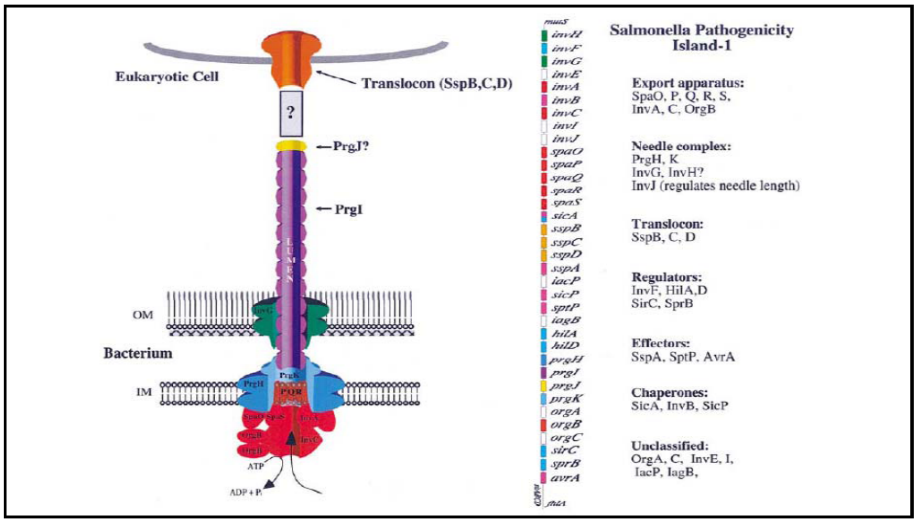

Figure 1. Schematic model of the S. Typhimurium and categorization of SPI-1-encoded proteins. Figure taken from (Kimbrough & Miller, 2002).

The T3S mechanism has been described Akeda *et al*. (Akeda & Galan, 2005). The size of the pore of T3SS is estimated to be ~28 A°, whereas the chaperone-effector complex is substantial, up to 6,200 A° in some cases. Therefore, the chaperone-effector complex must be stripped of the chaperone and the effector protein unfolded by T3SS ATPase located in basement of T3S machinery (Akeda & Galan, 2005; Gauthier & Finlay, 2003). In *S.* Typhimurium, T3SS ATPase, InvC is able to recognize both the chaperone and the complex with its substrate. Before effector secretion, the chaperone-effector complex binds with InvC ATPase. Then, InvC releases the chaperone from its cognate substrate and induces the global unfolding of effector protein. Finally, the unfolding effector is secreted through the central channel of T3SS-associated needle

complex (Akeda & Galan, 2005). Recently, the proton motive force (PMF) has been suggested to be involved in the secretion of proteins through T3SS (Galan, 2008).

An additional issue of concern in foodborne bacterial infections is antimicrobial resistance. One study in Vietnam and Thailand reported a higher proportion of *Salmonella* isolated in Thailand with resistance to antimicrobial agents used for treatment of gastrointestinal infection, with 28% and 59% of isolates resistant to ampicillin and tetracycline, respectively. Resistance to new agents such as ciprofloxacin (0.5%) and azithromycin (5%) has already found in Thailand (Isenbarger et al., 2002).

There is increasing of antibiotic-resistant *Salmonella* strains. It will be crucial to develop new antimicrobial agents. Strategies that rely on existing drugs and targets, which affect the viability of bacteria all face the same problem i.e., that bacteria quickly develop drug resistance under the huge selective pressure applied. The generation of drugs that specifically target pathogenic properties without killing bacteria is a strategy that might decrease the chance of bacterial resistance emerging against these drugs (Keyser, Elofsson, Rosell, & Wolf-Watz, 2008). As these reasons, T3SS is a attractive target for new anti-bacterial drugs. As described previously, T3SSs are conserved in a variety of gram-negative animal and plant pathogenic and symbiotic bacteria and deliver a set of effectors (unique for each bacterial pathogen) into host cells. Moreover, T3SSs are evolutionally and functionally related to the flagellar export apparatus (Gophna, Ron, & Graur, 2003). The type III secretion ATPase of *Salmonella*, InvC shares significant amino acid sequence similarly to the β subunit of F0F1 ATPase. This study showed that the result from mutagenesis and functional analysis demonstrating Lys-165 is required for the InvC ATPase activity (Akeda & Galan, 2004). In flaggellar T3SS, FliI, an ATPase homologous to InvC has been crystallized and solved the structure in the presence of ADP. It reported that Lysine in amino acid position 188 involve in ATPase activity of FliI (Imada, Minamino, Tahara, & Namba, 2007). Recently, small molecules could inhibit of T3SS ATPase activity of *Yersinia pestis*, YscN below 40 μM concentration. In addition, small molecules prevent secretion of the effector protein from *Y. pestis* into bacterial medium and mammalian cells. From docking conformational model indicated that these molecules tightly bind inside the hydrophobic clave of YscN result loss of ATPase function (Swietnicki et al., 2011). Additional, in this study showed that N-[2-[4-(benzimidazol-1-yl)anilino]-2-oxoethyl]-3-chloro-1-benzothiophene-2-carboxamide ($C_{24}H_{17}ClN_4O_2S$) compound was capable of inhibiting both effector protein secretion and ATPase activity in *Y. pestis*. Moreover, this compound was also non-toxic to the mammalian cells due to a low potential to target human enzyme (Swietnicki et al., 2011). I reasoned that if ATPases for T3SS among gram-negative bacteria are similarity, then I compared amino acid sequences of

FliI, YscN and InvC from *S.* Typhimurium or *Y. pestis*. The alignment result demonstrated that there are significant amino acid sequence similarities among three ATPases and also showed highly conserved in catalytic amino acid (Figure 2).Thus, in this experiments, I hypothesize that $C_{24}H_{17}ClN_4O_2S$ compound would also has inhibitory effects to InvC ATPase in *S.* Typhimurium. However, I found that $C_{24}H_{17}ClN_4O_2S$ compound did not show ATPase inhibitor activity, but this compound prohibited expression of main SPI-1 transcription regulator, InvF.

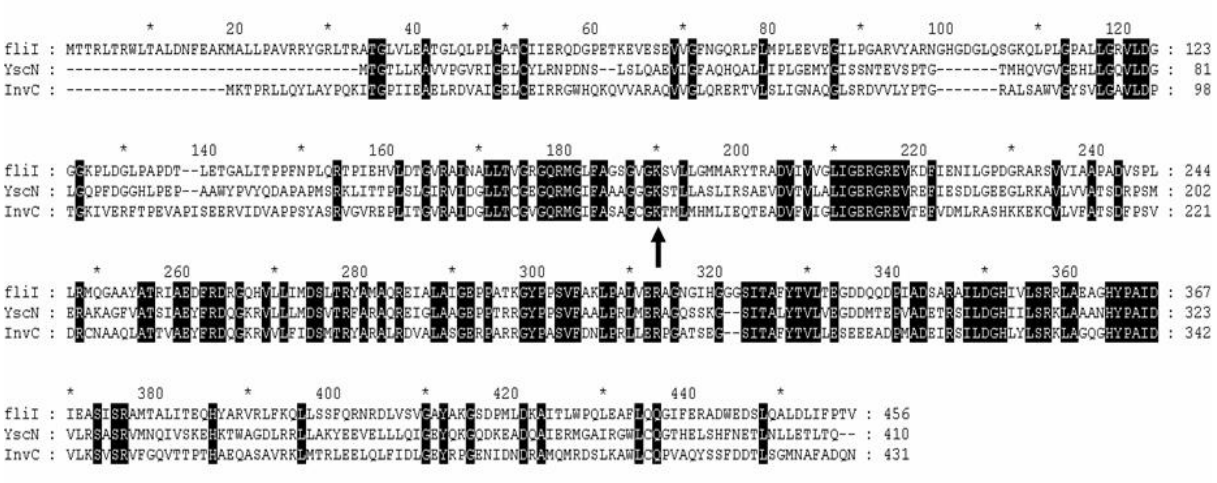

Figure 2. Amino acid sequence comparison of FliI, YscN and InvC ATPases. Black boxes denote identity. Arrow indicates amino acid identified as active site.

Objectives

To study the inhibitory effects of small molecule, $C_{24}H_{17}ClN_4O_2S$ to SPI-1 T3SS ATPase InvC including InvC ATPase inhibition, *salmonella* host cell invasion, and blocking effector proteins secretion.

Materials and Methods

Materials

1. The small molecule N-[2-[4-(benzimidazol-1-yl)anilino]-2-oxoethyl]-3-chloro-1-benzothiophene-2-carboxamide ($C_{24}H_{17}ClN_4O_2S$) (Figure 3) was purchased from commercial vendor Enamine and Life Chemicals (USA).

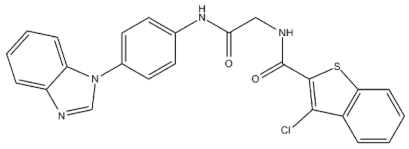

Figure 3. Structure of $C_{24}H_{17}ClN_4O_2S$ compound.

2. Bacterial strains and plasmids used in this study (Table 1)

Table 1. Cell line, bacterial strains and plasmids used in this study

Cell line, Bacterial strains and plasmids	Properties	Source or references
Cell line		
HT-29	Colon cancer cell line	Lab stock
***S.* Typhimurium strains**		
SL1344	Wild-type	SGSC
SW002	*invC::Kan*^{R}	Lab stock
SL1344 sipA-strep tag	Wild-type containing *sipA::strep tag*	This study
***E. coli* strains**		
BL21	F^-, *omp*T, *hsdS*β ($r_\beta^m\beta$), *dcm*, *gal*, *lon*	Novagen
Plasmids		
pGEX-2T	Expression vector with GST fusion, Amp^R	GE Healthcare
pGWC	pGEX-2T derivative encoding full-length InvC	This study
pGEX-Ast	pGEX-2T derivative encoding full-length SipA tagging with Strep-tag	This study
PKD46	Lambda-red recombinase expression plasmid ; Amp^R	(Datsenko & Wanner, 2000)
pCP20	FLP recombinase expression plasmid ; Amp^R	(Datsenko & Wanner, 2000)

Methods

1. Inhibition of *salmonella* host cell invasion by $C_{24}H_{17}ClN_4O_2S$ compound

The gentamicin protection assay was used for measuring bacterial invasion.

1.1 HT29 preparation

HT29 cells monolayers ($2x10^4$cells/well) will be grown at 37°C in 5% CO_2 in DMEM medium supplemented with 10% heat-inactivated fetal bovine serum.

1.2 Bacterial cell preparation

S. Typhimurium strain SL1344 and strain SW002 were grown in Luria-Bertani for 12 h at 37°C, then diluted 1:100 into fresh LB medium supplement with or without inhibitor and grown for another 4 h with 200 rpm shaking. Approximately $3x10^6$ colony forming units (CFU) of bacterial culture were harvested by centrifugation, after that supernatant were removed and gently resuspended the bacterial pellet with DMEM medium.

1.3 Infection of mammalian cells

Bacterial resuspension treated with inhibitor were added directly to the cell-culture supernatant. After incubation of 15 minutes to allow bacterial entry into cells, monolayers were washed with PBS and DMEM containing gentamicin to each well for killing any remaining extracellular bacteria. After washing cells, cells were lysed with solubilization buffer. Finally, the number of intracellular bacteria was determined by plating onto LB agar plates.

2. Blocking of effector proteins secretion in a bacterial cell culture by $C_{24}H_{17}ClN_4O_2S$

2.1 Preparation of bacterial secretion and SDS-PAGE analysis

S. Typhimurium strain SL1344 and strain SW002 were grown in Luria-Bertani medium for 12 h at 37 ºC, then diluted 1:100 into fresh LB. The inhibitor was added to the culture medium at various concentrations. A negative control is DMSO without inhibitor. Bacterial cultures were then allowed to grow for 4 h with 200 rpm shaking. Culture supernatants were separated by centrifugation at 6,000 rpm for 10 min and filter-sterilized (0.2 μm pore-size). Subsequently, proteins will be precipitated with 10% v/v, trichroloacetic acid (TCA). The precipitated sample was separated on SDS-PAGE. The gel was stained with Coomassie blue staining.

3. Confirmation of inhibition of SipA effector protein using *S. Typhimurium* containing *sipA* gene tagging with strep-tag strain

3.1 Construction of S. Typhimurium containing *sipA* gene tagging with strep-tag strain

S. Typhimurium containing *sipA* gene tagging with strep-tag strain was constructed by protocol of Datsenko *et al*. (Datsenko & Wanner, 2000). Firstly, a double-stranded DNA cassette containing *sipA* franked with homologous sequences and antibiotic resistance marker was amplified by PCR with standard procedure using previously constructed pGEX-Ast plasmid as a template. PCR product was treated with *DpnI* restriction enzyme at 37 ºC for 2 hours following to purified by Hi Yield™ Gel/PCR DNA Fragment Extraction kit (RBC Bioscience, Taiwan). Cassette was introduced into *S.* Typhimurium SL3261 containing pKD46 plasmids electrocompetent cell. Electrocompetent cells were cultured on LB agar containing 50 µg/ml Kanamycin. Single colonies were picked into PCR tube for colony PCR in numerical order for detection of correct chromosomal integration in *Salmonella* strain. Kanamycin resistance (Kan^R) gene was deleted from bacterial chromosome using pCP20 plasmid containing FLP recombinase enzyme. DNA sequence was validated by DNA sequencing. Expression of SipA-Strep tag fusion protein was determined by western blotting with Strep-tag antibody.

3.2 Blocking of SipA effector proteins secretion in a bacterial cell culture by $C_{24}H_{17}ClN_4O_2S$

S. Typhimurium strain SipA-strep tag strain was grown in Luria-Bertani medium for 12 h at 37 ºC, then diluted 1:100 into fresh LB. The inhibitor was added to the culture medium at various concentrations. A negative control is DMSO without inhibitor. Bacterial cultures were then allowed to grow for 4 h with 200 rpm shaking. Culture supernatants were separated by centrifugation at 6,000 rpm for 10 min and filter-sterilized (0.2 µm pore-size). Subsequently, proteins will be precipitated with 10% v/v, trichroloacetic acid (TCA). The precipitated sample was separated on SDS-PAGE and determined of SipA effector protein secretion using western blotting with Strep-tag antibody.

4. Bacterial cell effects analysis by GeLC-MS

Bacterial cells growing in LB medium with or without inhibitor were lysed and electrophoresed on 12% SDS-PAGE. After protein bands were excised, the gel plugs were dehydrated with 100% acetonitrile (ACN), reduced with 10mM DTT in 10mM ammonium bicarbonate at room temperature for 1 h and alkylated at room temperature for 1 h in the dark in the presence of 100mM iodoacetamide (IAA) in 10mM ammonium bicarbonate. After alkylation,

the gel pieces were dehydrated twice with 100% ACN for 5 min. To perform in-gel digestion of proteins, 10 µl of trypsin solution (10 ng/µl trypsin in 50% ACN/10mM ammonium bicarbonate) was added to the gels followed by incubation at room temperature for 20 min, and then 20 µl of 30% ACN was added to keep the gels immersed throughout digestion. The gels were incubated at 37^{O}C for a few hours or overnight. To extract peptide digestion products, 30 µl of 50% ACN in 0.1% formic acid (FA) was added into the gels, and then the gels were incubated at room temperature for 10 min in a shaker. Peptides extracted were collected and pooled together in the new tube. The pool extracted peptides were dried by vacuum centrifuge and kept at -80^{O}C for further mass spectrometric analysis.

LC-MS/MS analysis of digested peptide mixtures was performed using a Waters SYNAPT™ HDMS™ system. The 1D-nanoLC was carried out with a Waters nanoACQUITY UPLC system. Four microlitre of tryptic digests was injected onto the RP analytical column (20 cm x 75 µm) packed with a 1.7 µm Bridged Ethyl Hybrid (BEH) C18 material (Waters). Peptides were eluted with a linear gradient from 2% to 40% acetonitrile developed over 60 minutes at a flow rate of 350 nl/min. This was followed by a 15 min period of 80% acetonitrile to clean the column before returning to 2% acetonitrile for the next sample. The effluent samples were electrosprayed into a mass spectrometer (Synapt HDMS) for MS/MS analysis of peptides and then generated the spectral data for further protein identification against database search.

Mass lists in the form of Mascot generic files were created and used as the input for Mascot MS/MS Ions searches of the National Center for Biotechnology Information nonredundant (NCBI nr) database (www.matrixscience.com). Default search parameters used were the following: Enzyme = trypsin, max. missed cleavages =1; fixed modifications = carbamidomethyl (C); variable modifications = oxidation (M); peptide tolerance ±1.2 Da; MS/MS tolerance ±0.6 Da; peptide charge = 1+, 2+ and 3+; instrument = ESI-QUAD-TOF.

5. Inhibition of InvC ATPase by $C_{24}H_{17}ClN_4O_2S$ compound

5.1 *invC* gene cloning

Synthetic primers corresponding to CDS of the *S.* Typhimurium *invC* gene were used in PCR reaction for amplification of *invC* gene. The PCR products were cloned into pGEX-2T between *BamH*1 and *EcoR*1 sites. The final construct is coded for N-terminal GST tag. The recombinant clone was transformed into *E. coli* BL21 expression strain. The bacterial containing recombinant plasmid was verified by SDS-PAGE to express the desired protein.

5.2 Protein expression and purification

To express the InvC protein, a single colony containing recombinant plasmid was inoculated in 3 mL of LB with ampicillin medium. The cell was grown overnight at 37^{O}C with shaking at 200 rpm. The next day, the cell culture was diluted 1:100 into 2 L. of fresh medium and incubated at 37^{O}C with shaking until the cell density reached OD_{600} = 0.4. At this time, a solution of IPTG was added and the cell was incubated for an additional 2 hr. Cells were harvested by centrifugation.

To purify proteins, the cell paste was resuspended in chilled protein binding buffer supplemented with complete EDTA-free protease inhibitor cocktail. The bacterial suspension was homogenized using sonicator, the debris was removed by centrifugation, the supernatant was filtered through a sterile 0.45 μm filter and was purified on AKTA prime automated protein purification chromatography system using Glutathione column. Protein concentration was determined with Protein Assay kit with bovine serum albumin as a standard. The purified protein was distributed into aliquots and stored at -70^{O}C.

5.3 ATPase enzyme activity assays

ATPase activity of InvC ATPase was determined using ADO-Glo^{TM} MAX Assay (Promega, USA). The principle of this assay is to determine concentration of ADP after ATPase catalytic ATP. Reagent in this kit reconverses ADP to ATP and measuring by luciferase/luciferin reaction. The light generated correlates with the amount of ADP generated in the ATPase assay, which is indicative of ATPase activity. Inhibitor compound serially diluted 1:10 in DMSO and assayed with InvC ATPAse. Only InvC ATPase was used as control.

6. Statistical Analysis

All statistical analyses were performed using GraphPad Prism 7 (GraphPad Software, San Diego, CA). The data are presented as mean and standard deviation (SD). Student t-test was used to consider the statistical significance at *P* value of <0.001.

Results

1. $C_{24}H_{17}ClN_4O_2S$ is concentration-dependent inhibition of SPI-1 T3S effector proteins but did not affect the growth of *S.* Typhimurium

I investigated this compound affected the growth of bacterium. The result showed there were no difference between various concentrations of compound and DMSO solvent control (Figure 4). For SP-1 effector protein inhibition, $C_{24}H_{17}ClN_4O_2S$ inhibited the appearance of SPI-1 effector proteins, SipA and SipC in *S.* Typhimurium culture supernatant at concentration 100 μM. Additionally, the amount of FliC protein secretion decreased at 100 μM of compound (Figure 5). This result related with immunoblotting to detect SipA strep-tag protein in supernatant of *S.* Typhimurium containing *sipA* gene tagging with strep-tag strain (Figure 6). It indicated that inhibition effect on SPI-1 secretion proteins depending on concentration of $C_{24}H_{17}ClN_4O_2S$ molecule. However, $C_{24}H_{17}ClN_4O_2S$ was not due to affecting the growth of *S.* Typhimurium.

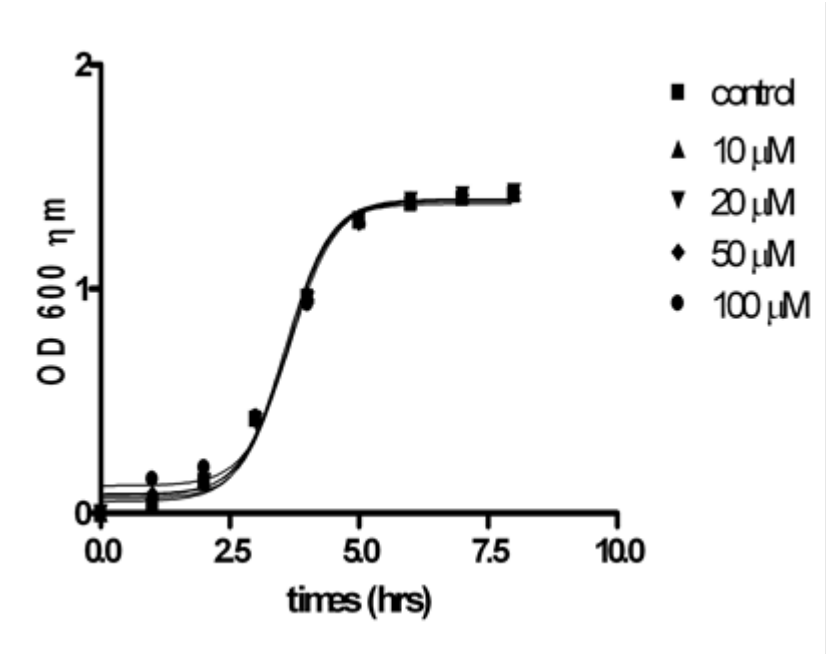

Figure 4. Effect of $C_{24}H_{17}ClN_4O_2S$ substance on growth curve of S. Typhimurium at various concentrations. Control was bacteria culturing in media with DMSO.

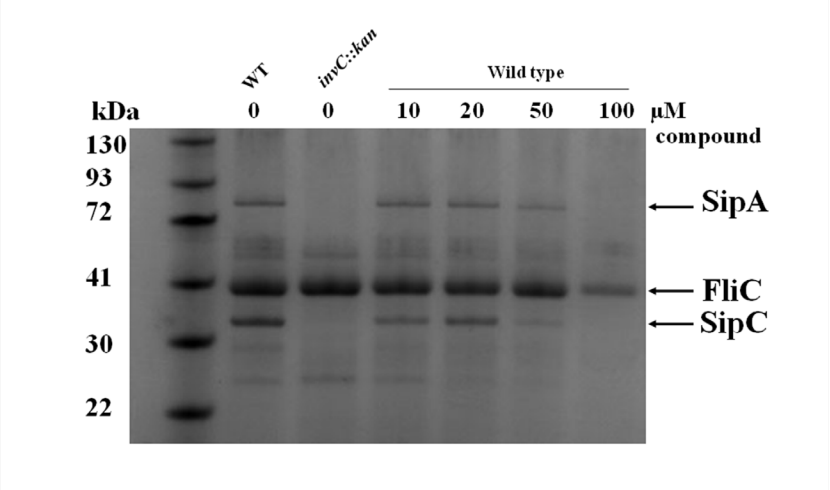

Figure 5. SDS-PAGE of bacterial supernatants. Wild-type Salmonella were grown in presence of DMSO or various concentration of compound, and then culture supernatants were isolated. Samples were separated by SDS-PAGE, and were strained with SimplyBlue™ SafeStain (Life technology, USA.) comparing with culture supernatants from SPI-1 deficiency strain, *invC::kan*.

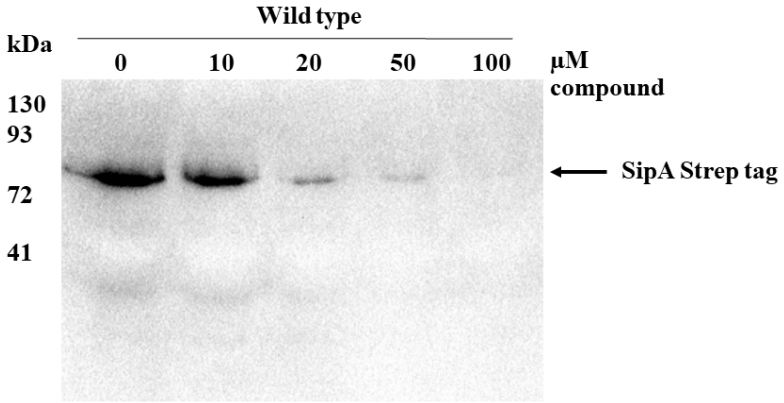

Figure 6. Western blotting of bacterial supernatants. *Salmonella* strains containing Strep-tag on C-terminus of SipA protein were grown in presence of DMSO or various concentration of compound, and then culture supernatants were isolated. SipA tag recombinant protein was detected with a monoclonal strep-tag antibody.

2. $C_{24}H_{17}ClN_4O_2S$ markedly inhibited the invasion of S. Typhimurium into HT29 cells

As described previously, $C_{24}H_{17}ClN_4O_2S$ has inhibited the secretion of SPI-1 effectors, we wished to test this compound had blocking effect on the invasion of *Salmonella* into host cells. Gentamicin protection assay was used to assess the invasion effect. In gentamicin assay, $C_{24}H_{17}ClN_4O_2S$ compound at concentration 100 μM significantly inhibited the invasion of *Salmonella* into HT29 cells ($P<0.001$) compared to solvent control (Table 2 and Figure 7). *Salmonella* with SPI-1 deficiency strain, *invC::kan* was used as control strain.

Table 2 Colony forming units of intracellular bacteria in HT29 cells

strains	CFU of intracellular bacteria
Untreated bacteria I	15600
Untreated bacteria II	12600
Untreated bacteria III	15800
Compound treated bacteria I	6000
Compound treated bacteria II	5740
Compound treated bacteria III	6800
SPI-1 deficiency strain I	100
SPI-1 deficiency strain II	134
SPI-1 deficiency strain III	156

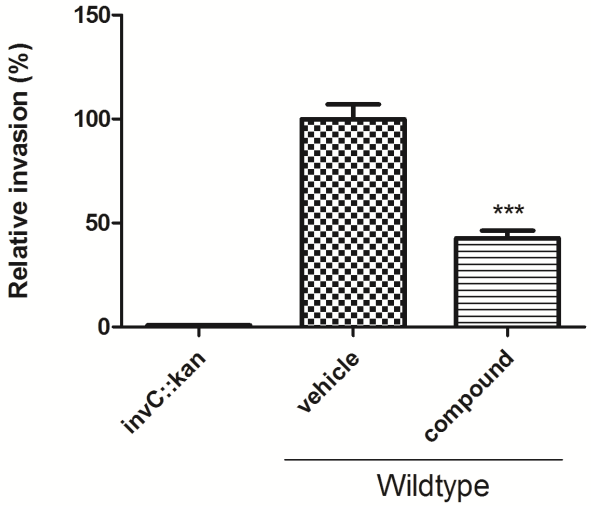

Figure 7. Percentage of relative invasion of bacteria after treated with compound. HT-29 cells were infected with wild-type Salmonella after treated with or without 100 μM. of compound at MOI of 100. The SPI-1 negative strain, *invC::kan* was used as positive control. *** Statistical significant difference between DMSO vehicle control and compound group ($p<0.001$). Results presented at three independent experiments and error bars indicated standard deviations from the means.

4. Production of GST-InvC ATPase recombinant protein

Before ATPase enzyme activity assays, I constructed full-length of invC gene fusing with GST under control of tac promoter. Firstly, we amplified the full-length of *invC* gene from genomic DNA of *S.* Typhimurium strain SL1344 by standard PCR condition using CF primers set including BamHI and EcoRI restriction enzyme sites. After that PCR product was digested with BamHI/EcoRI and cloned into BamHI/EcoRI digested pGEX-2T expression vector, giving pGWC.

The recombinant plasmid pGWC express the full-length InvC fused with GST under control of IPTG inducible tac promoter. The *E.coli* containing plasmid pGWC was induced with 1 mM of IPTG for 3 h. Bacteria was harvested by centrifugation, then, bacterial cells were lysed by sonication. The bacterial lysate was mixed with glutathione agarose beads. After washing, the elution was analyzed by SDS-PAGE. The purification of GST-InvC fusion protein was shown approximately 72 kDa on SDS-PAGE (Figure 8). Finally, these purified proteins were concentrated using MWCO 30 kDa Vivaspin column (Merck, USA). The concentration of GST-InvC fusion protein was about 10 mg/mL.

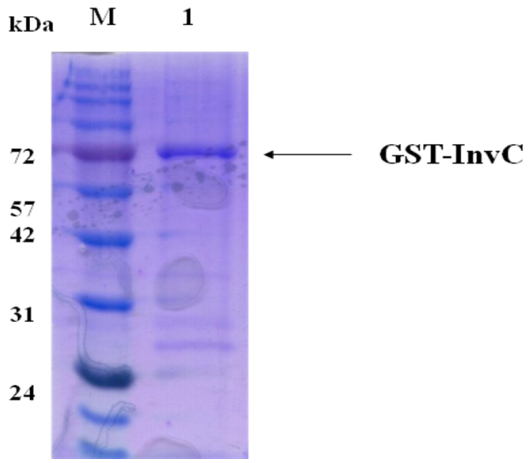

Figure 8. Analysis of purified GST-InvC fusion protein.

Lane M, molecular weight maker; lane 1, purification of GST-InvC recombinant protein

The GST-InvC fusion protein was determined the optimal concentration for degradation of ATP. The optimal concentration of GST-InvC recombinant protein to degrade ATP molecules is about 10 μg/uL or about 140 μM. (Figure 9) In further experiment, I used 10 μg of InvC ATPase protein for each catalytic reaction.

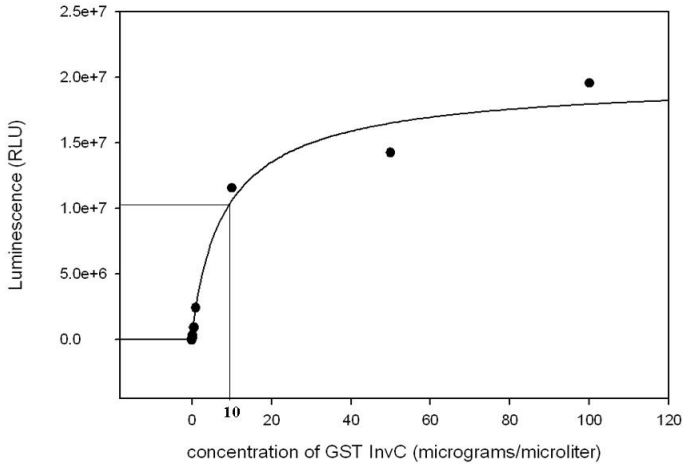

Figure 9. Graph illustrates relation between concentration of GST-InvC fusion protein and intensity of luminescence (RLU).

5. T3S inhibition effect of $C_{24}H_{17}ClN_4O_2S$ did not caused by inactivated InvC ATPase

From previous results showed that compound was able to block for SPI-1 T3S. The mechanism of action of $C_{24}H_{17}ClN_4O_2S$ on the secretion SPI-1 effectors was investigated. From literature has shown that this compound is able to inhibit both effector protein secretion and T3S ATPase, YscN activity in *Y. pestis*. From amino acid sequences, there show similarity between T3S ATPase from *Y. pestis* and *S.* Typhimurium. Thus, I hypothesized that this compound may have inhibitory activity to SPI-1 T3S ATPase, InvC in *Salmonella*. I determined the InvC ATPase inhibitor activity of various concentration compound using ADO-Glo^{TM} MAX Assay. The result showed that at concentration 10, 20, 50 and 100 μM of compound did not reduce amount of luminescence intensity (RLU) producing with InvC ATPase catalytic reaction (Figure 10). It indicated that this compound had not inhibitive effect for *Salmonella* InvC ATPase enzyme.

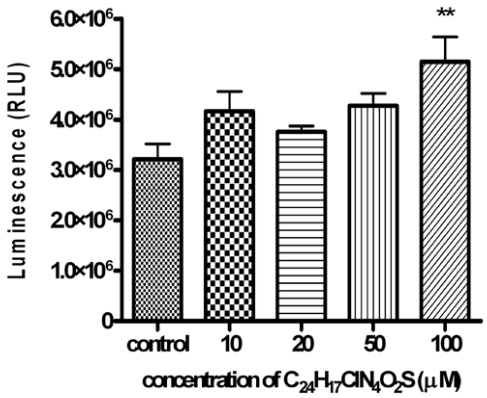

Figure 10. Intensity of luminescence after adding various concentration of compound in InvC ATPase reaction. Control was reaction containing only InvC ATPase enzyme. Results presented at three independent experiments and error bars indicated standard deviations from the means.

6. T3SS transcription regulator, InvF was disappeared in $C_{24}H_{17}ClN_4O_2S$ treated bacteria

To further explore the action of $C_{24}H_{17}ClN_4O_2S$ compound. We performed a proteomic analysis for identification the mechanism of compound inhibition on *S.* Typhimurium SPI-1 T3SS. As shown in table 3, results from proteomic profiles illustrated that bacterial cells treated with 100 μM of compound showed significantly decreased quantification of *S.* Typhimurium AraC-family regulator of type III secretion system, InvF protein. Moreover, level of effector proteins, SipA and

SipC dramatically dropped in compound treated bacteria. These results provided evidence that this compound can affect the production of multiple SPI-1 effectors by inhibition of translation or increasing degradation of regulator of type III secretion system protein, InvF.

Table 3. Absolute selected protein quantification from proteomic analysis comparison between cellular proteins of *S.* Typhimurium were grown in presence of DMSO and 100 μM compound.

Accession number	Protein name	DMSO	Compound treated
gi|52212981	AraC-family regulator of type III secretion system, InvF	5.39963	0
gi|618634010	Pathogenicity island 1 effector protein, SipA	4.821184	0
gi|975296	SipC	6.675706	0

Discussion

Infectious diseases are a major public health problem worldwide. The disease has a high rate of morbidity and mortality comparing with non-infectious. Treatment of bacterial infections is necessary to get antibiotics for killing bacteria and patients recovering. However, the frequent occurrence of pathogens that are resistant to traditional antibiotics becomes a great health problem. Therefore, development of new antimicrobial drug is required. Antibiotics replacement therapies target on bacterial pathogenesis. The Type 3 Secretion System (T3SS) is one of the most interesting targets for drug discovery. Because this system is main mechanism for bacterial virulent proteins transportation into host cells (Salmond & Reeves, 1993). Moreover, T3SS has found in gram-negative bacteria that causes disease in humans, such as *Salmonella* (Salmond & Reeves, 1993), *Shigella* (Cornelis & Van, 2000), *Pseudomonas* (Cornelis & Van, 2000), enteropathogenic *Escherichia coli* (EPEC) (Jarvis et al., 1995), *Vibrio* (Cornelis & Van, 2000), *Yersinia* (Schesser Bartra et al., 2019), and *Chlamydia* (Dai & Li, 2014). Several compounds from synthesis or natural product were discovered as T3SS inhibitors (Pendergrass & May, 2019; Swietnicki et al., 2011; Tao, Fan, et al., 2019; Tao, Tian, et al., 2019). Here, I demonstrated that the small molecule, $C_{24}H_{17}ClN_4O_2S$ had inhibitory effect on secretion of SPI-1 effector proteins and invasion of *Salmonella* into host cells without interfered against growth of bacteria. Thus, $C_{24}H_{17}ClN_4O_2S$ compound may be potential candidate for a novel T3SS inhibitor and be less likely to induce the resistance of *Salmonella* to this compound.

Previous study showed that the $C_{24}H_{17}ClN_4O_2S$ compound was capable of inhibiting both effector protein secretion and ATPase activity in *Y. pestis* (Swietnicki et al., 2011). Moreover, this compound was also non-toxic to the mammalian cells due to a low potential to target human enzyme (Swietnicki et al., 2011). In this study, this molecule had ability to inhibit only effector proteins secretion from *Salmonella* SPI-1. But activity of SPI-1 T3SS ATPase enzyme of *S.* typhimurium, InvC did not decreased. It assumed that this molecule affected T3S ability in *S.* typhimurium by different pathways. I identified mechanism of action of compound on SPI-1 TSS using proteomic analysis. Protein quantification from mass spectrophotometry indicated that untreated bacteria differed 3 SPI-1 T3SS related proteins from compound treated group. Two of them were SPI-1 T3SS effector proteins, SipA and SipC. Another protein was SPI-1 regulator protein, InvF protein. InvF is a transcriptional activator encoding by SPI-1. This protein is required for activation of the expression of SPI-1 T3SS effectors encoded both inside and outside of SPI-1 (Darwin & Miller, 1999; Eichelberg & Galan, 1999). The activity of InvF requires SicA, which also encodes within SPI-1 (Darwin & Miller, 2000, 2001). However, control of expression of InvF

is under control by central regulator of SPI-1, HilA (Dieye, Dyszel, Kader, & Ahmer, 2007; Ellermeier, Ellermeier, & Slauch, 2005). The expression of *hilA* is influenced by environmental changes, such as osmolarity, pH, and oxygen tension (Bajaj, Lucas, Hwang, & Lee, 1996). Moreover, expression of *invF* gene is improved by upregulating of FliZ and HilD (Aurass et al., 2018). The global regulatory system, ArcAB also promotes the expression of *invF* gene (Lim et al., 2013; Pardo-Este et al., 2018).

To date, many T3SS inhibitor from chemical synthesis (Swietnicki et al., 2011; Tao, Fan, et al., 2019; Tao, Tian, et al., 2019) or natural product (Pendergrass & May, 2019) have been screened for developing novel anti-virulence agents, such as salicylidene acylhydrazides (Dahlgren, Zetterstrom, Gylfe, Linusson, & Elofsson, 2010; Kauppi, Nordfelth, Hagglund, Wolf-Watz, & Elofsson, 2003; Nordfelth, Kauppi, Norberg, Wolf-Watz, & Elofsson, 2005), N-phenyl-benzamide (Dahlgren, Kauppi, Olsson, Linusson, & Elofsson, 2007), caminosides (Kimura et al., 2011; Linington et al., 2002), Aurodox (McHugh, O'Boyle, Connolly, Hoskisson, & Roe, 2019), Cytosporone B (Li et al., 2013) and so on. T3SS inhibitors changed in gene expression of T3SS regulator proteins, effector protein and/or needle apparatus to present their inhibition of T3SS. Interestingly, form my results suggested that $C_{24}H_{17}ClN_4O_2S$ compound may not affect protein for SPI-1 T3SS apparatuses and SPI-1 regulators protein level excepting InvF. Therefore, $C_{24}H_{17}ClN_4O_2S$ compound may influences only protein InvF resulting to inhibit the secretion of SPI-1 effectors (Figure 11). The mechanism of action of compound affecting InvF will be required to further investigate in future. The mechanism supposed to be reducing transcription of *invF* gene, increasing InvF protein degradation or inhibition of InvF binding to SPI-1 promotor.

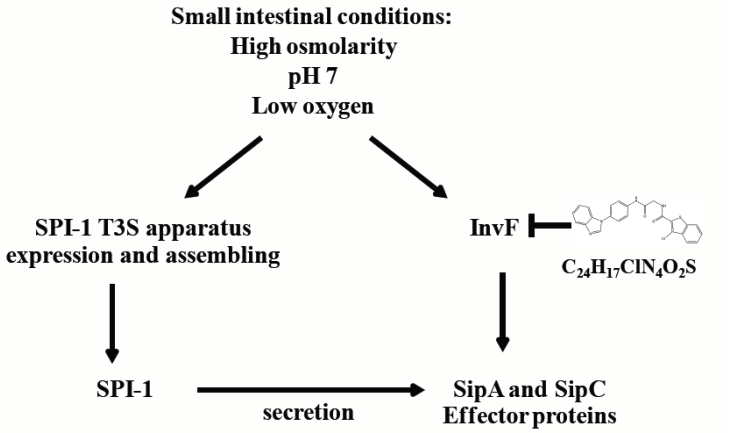

Figure 11. Inhibition pathways of $C_{24}H_{17}ClN_4O_2S$ molecule on secretion of SPI-1 effectors. Arrow lines indicated transcription activation. Blunt line indicated inhibition.

Conclusion

Anti-T3S ATPAse in *Yersinia*, $C_{24}H_{17}ClN_4O_2S$ was identified as anti-T3SS in *Salmonella* in different mechanism. This compound affect protein level of SPI-1 regulator protein, InvF in bacterial cells. Lacking of intracellular InvF exerts inhibitory effect on SPI-1 effector proteins secretion. The activities of this molecule against *Salmonella* make it potential candidate, likely combined with other agents, to use as a novel anti-salmonellosis agents.

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Appendix

เอกสารสรุปผลการวิจัยสำหรับการประชาสัมพันธ์

โรคติดเชื้อเป็นปัญหาสำคัญทางสาธารณสุขของทั่วโลกและรวมทั้งของประเทศไทย โดยโรคดังกล่าวมีอัตราการเจ็บป่วยและอัตราเสียชีวิตสูงในลำดับต้นๆ เมื่อเปรียบเทียบกับโรคไม่ติดเชื้ออื่น การรักษาโรคติดเชื้อแบคทีเรียมักจำเป็นต้องได้รับยาต้านจุลชีพเพื่อฆ่าเชื้อแบคทีเรียทำให้เชื้อก่อโรคลดลงอย่างรวดเร็วและทำให้ผู้ป่วยฟื้นและอาการหายขาดโดยเร็วที่สุด อย่างไรก็ตามเนื่องจากความชุกที่เพิ่มขึ้นของสายพันธุ์แบคทีเรียที่ดื้อต่อการรักษาด้วยยาต้านจุลชีพและการพัฒนายาต้านจุลชีพชนิดใหม่แต่ละชนิดที่ต้องใช้เวลานาน ดังนั้นจึงทำให้มีการวิจัยเพื่อค้นหาเป้าหมายใหม่ในการรักษาด้วยยาต้านจุลชีพโดยเป้าหมายนั้นๆต้องไม่จำเป็นต่อการมีชีวิตของแบคทีเรียแต่ต้องเกี่ยวข้องกับกลไกการก่อโรค ระบบการขนส่งโปรตีนของแบคทีเรียแบบที่ 3 (Type 3 Secretion System, T3SS) เป็นเป้าหมายหนึ่งที่ได้รับความสนใจในการใช้เป็นเป้าหมายในการพัฒนายา เนื่องจากระบบการขนส่งดังกล่าวเป็นระบบการขนส่งโปรตีนหลักที่ขนส่งโปรตีนก่อโรคเข้าสู่เซลล์โฮสต์ โดยผลการวิจัยของคณะผู้วิจัยพบว่าสารเคมี N-[2-[4-(benzimidazol-1-yl)anilino]-2-oxoethyl]-3-chloro-1-benzothiophene-2-carboxamide ($C_{24}H_{17}ClN_4O_2S$) มีความสามารถในการยับยั้งการหลั่งโปรตีนก่อโรคที่หลั่งผ่านระบบการขนส่งโปรตีนของแบคทีเรียแบบที่ 3 ของเชื้อ *Salmonella* Typhimurium ส่งผลให้เชื้อแบคทีเรียมีการหลั่งโปรตีนก่อโรคได้ลดลงและเข้าสู่เซลล์เจ้าบ้านได้ลดลงฤทธิ์ จากผลการวิจัยดังกล่าวแสดงให้เห็นว่าสารดังกล่าวน่าจะมีการต่อยอดเพื่อนำไปใช้เป็นยาต้านจุลชีพได้ในอนาคต

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