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IDENTIFICATION OF HYDRALAZINE AS AN ANTI-INFECTIVE COMPOUND IN *Pseudomonas aeruginosa*-INFECTED *C. elegans*

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2017

IDENTIFICATION OF HYDRALAZINE AS AN ANTI-INFECTIVE COMPOUND IN *Pseudomonas aeruginosa*-INFECTED *C. elegans*

by

TAN BOON KHAI

Thesis submitted in fulfillment of the requirements
for the degree of
Doctor of Philosophy

APRIL 2017

ACKNOWLEDGEMENT

Firstly, my utmost gratitude to my supervisor, Alexander Chong Shu-Chien for taking me on as your apprentice and I still remember the day we first met for project discussion. Thank you for introducing me to the door of research field and I appreciate your motivation and inspiring ideas throughout these years in supervising my development as researcher. I am indebted to all of our collaborators as this project will not progress from conception to completion without your contribution. It has been a privilege learning experience from esteemed scientist, Tan Man-Wah and I am thankful for your valuable insights and guidance in shaping the course of this work. I have been very fortunate to work side by side with my mentor, Kenny Ang during my stay in SMDC, UCSF and I could not thank you more for all the inspiration and advice for me in both academic and life. Thank you Steve and Kenny as my lifeline as you both have spent tremendous efforts in helping me during the critical time and answered to my every call whenever I needed. Despite the critical review and discussion session, I also treasure the friendship made with PeiChuan and HsinYen, whom are two esteemed and passionate scientists that always making me feel at home in Kenyon's Lab. A thank-you note goes to Awang Ahmad Sallehin for your encouragement years ago and those words give me confidence to strive forward until today.

I would like to record my appreciation to all the core facilities access granted in IPHARM, CCB, USM, UCSF, SMDC and Kenyon's Lab, along with all the supportive department staff that helped directly or indirectly. Thank you Ministry of Science, Technology and Innovation, Malaysia (R&D Initiative Fund Scheme) for funding the whole project (Project Number: 08-05-IFN-8PH002) and QB3-Malaysia Program for the work in UCSF. I would like to thank Nathan's and Kenyon's Lab for

the generosity in sharing worm strains. Some strains were provided by the CGC, which is funded by NIH Office of Research Infrastructure Programs (P40 OD010440).

Each current and former member of Alex's Lab deserves a huge credit with their warm supports and scientific discussion given throughout this project. Thanks to MK, BengSiang, ShuShen, Hainisah, Faiz, Ann, Sai, YenShan, KahLoon, SzeHuey, Wyin, WT, HanJie, Michelle, JenYang, YSing, Kelly, HungHui, Crystal, Komala, Karthi, Adele, Wai Kwan, Zulkarnain Mahmud, Badio and Valerie for all the great fun time during lab hours and of course, after lab hours, too!

In life, we never lose friend. Instead, we only learn who the true ones are. To all my friends that we have known each other for a long time, I could not imagine how it would be without your companion in this journey. DP, BW, SSLIM, CLH, MHI, JJ, LKW, CS, YZ, ALB, OKH, Chiam and Zen, thank you for all the creative efforts to assure I am always on the right track with momentum keep going. Thanks for the time spent together to find us all a balance between work and life.

I would like to dedicate my wholehearted feelings to my beloved family for their unconditional love and support at all time. Thank you for being patience and giving me the confidence I needed the most when I am taking time in finding my way. I am proud to be what I am today because of your endless love for me. And, a very special person to thank to, Annie who is always be there for me. By saying that I love you, it takes care of everything I need to say here.

TABLE OF CONTENTS

ACKNOWLEDGEMENT .... **ii**

TABLE OF CONTENTS .... **iv**

LIST OF TABLES .... **viii**

LIST OF FIGURES .... **ix**

LIST OF ABBREVIATIONS .... **xi**

ABSTRAK .... **xiii**

ABSTRACT .... **xiv**

CHAPTER 1 INTRODUCTION

1.1 Research background **1**

1.2 Aim of the study **4**

CHAPTER 2 LITERATURE REVIEW

2.1 Antibiotic resistance **5**

2.2 Anti-infective **9**

2.3 Host – pathogen interaction study **12**

2.4 *Caenorhabditis elegans* as model organism **14**

2.5 *C. elegans* features as model for immunity study **18**

2.6 Lysozyme **22**

2.7 *C. elegans* lysozyme **23**

2.8 *C. elegans lys-7* **24**

2.9 *C. elegans* innate immunity signaling pathways **25**

2.10 *Pseudomonas aeruginosa* as infectious agent **29**

2.11 High-throughput screening for anti-infectives **31**

2.12 Image-based high-throughput screening **36**

2.13 Application of lysozyme for therapeutic use **37**

CHAPTER 3 MATERIALS AND METHODS

3.1 Culture media and stock solutions **40**

3.2 Nematode and bacterial.. **41**

3.3 Culturing and maintenance of *C. elegans* **42**

3.4 *C. elegans* synchronization protocol.. **42**

3.5 Survival assay **43**

3.5.1 Slow-killing assay.. **43**

3.5.2 Lifespan assay.. **44**

3.5.2 Scoring of survival and lifespan assay.. **44**

3.5.3 Pharyngeal pumping rate **44**

3.6 qPCR **45**

3.7 Statistical analysis **47**

CHAPTER 4 HIGH-CONTENT SCREENING ASSAY: THE IDENTIFICATION OF *lys-7* IMMUNOSTIMULATING COMPOUNDS

4.1 Introduction **48**

4.2 Materials and methods **50**

4.2.1 Compound library and screening plate **50**

4.2.2 Assay automation for high-content screening.................................... **50**

4.2.3 Image acquisition, image feature extraction and image analysis **51**

4.2.4 High-Content Screening data analysis and hits identification **52**

4.3 Results **53**

4.3.1 Assay development using *C. elegans* in High-Content Screening **53**

4.3.2 High-content screening for *lys-7* up-regulating compound **60**

4.3.3 Hit validation **66**

4.4 Discussion **70**

4.5 Summary **72**

CHAPTER 5 MODE OF ACTION STUDY OF *lys-7* IMMUNOSTIMULATING COMPOUND HYDRALAZINE AS ANTI-INFECTIVE AGAINST *Pseudomonas aeruginosa* INFECTION

5.1 Introduction **74**
5.2 Materials and methods **78**
5.2.1 Mode of action study, survival assay, lifespan analysis **78**
5.2.2 Toxicity assay **78**
5.2.3 Developmental rate and body size measurement analysis **78**
5.2.4 Pharyngeal pumping rate **79**
5.2.5 Brood size analysis **79**
5.2.6 PA14 growth kinetic assay **80**
5.2.7 Quantification of biofilm formation **80**
5.2.8 Measurement of pyocyanin, elastase and protease production **81**
5.2.9 Bacterial Colony Forming Unit (CFU) within *C. elegans* gut **82**
5.2.10 Compound conditioning assay **82**
5.3 Results **83**
5.3.1 Hydralazine modulates *lys-7* via IGF-1 and TGF-β signaling pathways **83**
5.3.2 Hydralazine is not toxic to the worms **87**
5.3.3 Hydralazine promotes nematode survival during PA14 infection without affecting normal lifespan **91**
5.3.4 Hydralazine-mediated survival promotion during PA14 infection requires IGF-1 and TGF-β signalling pathways **93**
5.3.5 Hydralazine does not perturb bacterial cell viability and virulence factors **95**
5.3.6 Hydralazine does not affect PA14 load within *C. elegans* gut **98**
5.3.7 Compound conditioning promotes worm resistance against infection **99**
5.3.8 Anti-infective activity against *E. faecalis* and *S. typhimurium* infection **102**
5.4 Discussion **104**
5.5 Summary **108**

CHAPTER 6 CONCLUSION **110**

LIST OF REFERENCES **112**

APPENDICES **128**

LIST OF PUBLICATIONS **141**

LIST OF TABLES

		Page
Table 2.1	Examples of anti-infective strategies………………………….	**10**
Table 2.2	The examples of high-throughput screening performed using *C. elegans*. …………………………………………………	**34**
Table 3.1	The composition of culture media and reagents……………...	**40**
Table 3.2	*C. elegans* and bacterial strains used in this study…………....	**41**
Table 3.3	Primer sequences for qPCR gene expression study ……........	**46**
Table 4.1	Summary of preliminary screening………………………......	**63**
Table 4.2	Summary of hit candidates……………………………….......	**64**
Table 4.3	Hits validation by qPCR……………………………………...	**65**

LIST OF FIGURES

		Page
Figure 2.1	The timeline of antibiotic discovery………………………..	**6**
Figure 2.2	Number of approved antibiotics from 2000 to 2012 ………	**8**
Figure 2.3	The morphology of hermaphrodite and male nematode……	**15**
Figure 2.4	The life cycle of *C. elegans*……………………………...	**17**
Figure 2.5	Example of pathogens that can infect *C. elegans* and the infection routes by pathogens………………………….....	**20**
Figure 2.6	The three major evolutionarily conserved signaling pathways implicated in *C. elegans* innate immune defense..	**27**
Figure 2.7	The instruments for *C. elegans* phenotypic screening……..	**33**
Figure 4.1	The sorting accuracy and performance of COPAS Biosort...	**54**
Figure 4.2	The optimization of screening assay…………………..……	**54**
Figure 4.3	The interface of COPAS Biosort software…………………	**55**
Figure 4.4	Image and feature extraction………………………………	**56**
Figure 4.5	The assessment of assay performance……………………...	**58**
Figure 4.6	Assay performance. ………………………………………..	**59**
Figure 4.7	High-content screening data analysis………………………	**61**
Figure 4.8	Autofluorescent small molecule ………………………..….	**62**
Figure 4.9	Hits validation by qPCR analysis…………………………	**67**
Figure 4.10	Compound hydralazine induces *lys-7* expression…………..	**69**
Figure 5.1	Mode of action study using qPCR reveals that hydralazine induces *lys-7* via insulin/IGF-1 and TGF-β signaling pathways ……………………..………………………	**85**

Figure 5.2	The qPCR analysis of stress-related genes expression …….	**86**
Figure 5.3	Hydralazine is not toxic to the worm……………………….	**89**
Figure 5.4	Lifespan analysis..………………………………………..	**92**
Figure 5.5	Survival analysis of *C. elegans* during PA14 infection…….	**94**
Figure 5.6	Compound hydralazine does not perturb PA14 cell viability and virulence factors production…………………………...	**96**
Figure 5.7	Compound hydralazine does not affect PA14 bacterial load within *C. elegans* gut……………………………………….	**98**
Figure 5.8	Compound conditioning promotes the worm resistance towards PA14 killing……………………………………….	**101**
Figure 5.9	Hydralazine promotes worm resistance towards *E. faecalis* and *S. typhimurium* infection but not *S. aureus*…………….	**103**

LIST OF ABBREVIATIONS

%	Percentage
±	Plus minus
µ	Micro
°C	Degree Celcius
BHI	Brain-Heart Infusion
CTLD	C-Type Lectin Domain
CV	Crystal Violet
DAF-2	Dauer Formation isoform
DBL-1	DPP/BMP-like protein; Drosophila Decapentaplegic (Dpp) / Bone Morphogenetic Proteins (BMPs)
dH_2O	Distilled water
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
esp	Enhanced Susceptibility to Pathogen
et al	*et alii* (and other people)
FDA	Food and Drug Administration
FITC	Fluorescein Isothiocyanate
FOXO	Forkhead Box O protein
GFP	Green Fluorescent Protein
HTS	High-Throughput Screening
IGF-1	Insulin/insulin-like Growth Factor 1
L	Liter
m	Meter
M	Molar
MAPK	Mitogen-Activated Protein Kinases
MAPKK	MAPK Kinase

MAPKKK	MAPK Kinase Kinase
NGM	Nematode Growth Medium
PGS	Peptone-Glucose-Sorbitol
pH	Potentiometric hydrogen ion concentration
PIA	Pseudomonas Infection Agar
qPCR	Quantitative real time – polymerase chain reaction
QS	Quorum sensing
RFU	Relative Fluorescence Units
RNA	Ribonucleic acid
RNAi	RNAI-interference
rpm	Revolutions per minute
s.d.	Standard Deviation
SMILES	Simplified Molecular-Input Line-Entry System
TGF-β	Transforming Growth Factor-β
TOL-1	Toll-like Receptor
TSA	Tryptic Soy Agar
v/v	Volume per volume
w/v	Weight per volume

PENGENALPASTIAN HYDRALAZIN SEBAGAI SEBATIAN ANTI-JANGKITAN *Pseudomonas aeruginosa* DENGAN MODEL *C. elegans*

ABSTRAK

Lisozim adalah model enzim yang penting dalam bidang penyelidikan bioperubatan. Sehubungan dengan taburan meluas dalam pelbagai tisu dan rembesan, lisozim dikenali dengan fungsi sebagai enzim pertahanan anti-bakteria manakala fungsi sampingan sebagai enzim pencernaan juga dilaporkan dalam segelintiran taksa. Antara keluarga lisozim *C. elegans*, fungsi *lys-7* telah dilaporkan dengan kepentingan imun dalam mekanisma penentangan terhadap jangkitan *P. aeruginosa*. Dengan menggunakan gabungan kaedah penyaringan kandungan tinggi berpandukan imej dan model jangkitan berasaskan *C. elegans* – patogen, sebanyak 15, 629 sebatian molekul kecil telah disaring dalam usaha pencarian calon pemodulat imun *lys-7* sebagai anti-jangkitan. Ini juga merupakan laporan pertama bahawa kajian penyaringan kandungan tinggi yang berasaskan *lys-7*::GFP. Saya mengenalpasti hydralazin sebagai molekul kecil yang mampu meningkatkan daya penentangan *C. elegans* terhadap jangkitan *P. aeruginosa* tanpa menjejaskan kebolehhidupan dan penghasilan faktor kevirulenan bakteria. Saya juga merungkaikan mekanisma hydralazin sebagai anti-jangkitan ini memerlukan laluan isyarat biologi seperti Insulin/Factor Pertumbuhan-1 bak insulin (IGF-1) dan Faktor Pertumbuhan Transformasi- β (TGF-β). Saya juga membuktikan bahawa rawatan awal dengan hydralazin dapat meningkatkan daya penentangan *C. elegans* terhadap jangkitan *P. aeruginosa*. Selain itu, hydralazin juga menunjukkan spektrum bioaktiviti yang luas dengan keupayaan merangsangkan daya hidup *C. elegans* semasa jangkitan patogen *Enterococcus faecalis* dan *Salmonella typhimurium*. Kajian ini menunjukkan ketersauran penyaringan secara fenotaip yang berpandukan imej untuk penemuan sebatian anti-jangkitan.

IDENTIFICATION OF HYDRALAZINE AS AN ANTI-INFECTIVE COMPOUND IN *Pseudomonas aeruginosa*-INFECTED *C. elegans*

ABSTRACT

Lysozymes are important enzyme models with biomedical importance. In accordance with its widespread distribution in various biological tissues and secretions, lysozyme is implicated primarily in the antibacterial defense, although an additional function as digestive enzyme has been described as well for several taxa. Using a combination of image-based high-content screening and *C. elegans* - pathogen infection model, I screened a total of 15,629 small molecule compound to isolate potential immunomodulators of *lys-7*, an immune effector implicated in *C. elegans* innate immunity during microbial infection. I reported this as the first *lys-7*::GFP based phenotypic high-throughput screening assay. I identified hydralazine as a small molecule capable of promoting *C. elegans* resistance towards *Pseudomonas aeruginosa* infection without affecting bacterial cell viability and virulence factor production. I delineated that this protective mechanism was mediated by the evolutionarily conserved signaling pathways of insulin/insulin-like growth factor 1 (IGF-1) and Transforming Growth Factor-β (TGF-β). I also further provided evidence that early stimulation of the host immune response by hydralazine could confer a survival advantage to *C. elegans* during infection. Remarkably, this immunostimulating compound also protected *C. elegans* from *Enterococcus faecalis* and *Salmonella typhimurium* infection, suggesting the broad spectrum bioactivity of hydralazine. This study demonstrated the feasibility of using an image-based phenotypic screen to search for small molecules with potential anti-infective properties.

CHAPTER 1

INTRODUCTION

1.1 Research background

The unprecedented emergence of multidrug resistant bacteria had urged the search for novel approaches to address current unmet medical needs. The rapid widespread of multidrug resistant strains had rendered most antibiotics, such as aminoglycosides, cephalosporins, fluoroquinolones and carbapenems ineffective against infection (Centers for Disease Control and Prevention, 2013). Thus, the development of anti-infective that capitalizes on the mechanism targeting host immunity or pathogen virulence factor could be a conceivable strategy in combating these antimicrobial-resistant infections (Bhavsar & Brown, 2006; Pirofski & Casadevall, 2006; Hamill et al, 2008). Without affecting microbial viability, anti-infective imposes weaker selection pressure and reduces the emergence of resistant strains (Clatworthy et al, 2007; Hancock et al, 2012; Arvanitis, 2013).

Caenorhabditis elegans is first proposed as model organism to study development and neurobiology by Sydney Brenner in 1960's (Brenner, 1974). Since then, this nematode has become a principal model organism in biological research such as ageing (Kenyon et al, 1993), genetic study, cell lineage mapping (Sulston & Horvitz, 1977) and more recently in innate immunity studies (Tan et al, 1999; Kaletta et al, 2006). This genetically tractable and amendable model organism offers an excellent screening tool for pharmacology-related agents. The microscopic size of *C. elegans* also permits a high-throughput screening manner in a whole-animal context and this high screening capacity is not achievable by using traditional mammalian models due to the enormous costs and ethical constraints (Arvanitis et al, 2013). With the

surprisingly high degree conservation of biochemical pathways between *C. elegans* and human, the study of *C. elegans* innate immunity yields valuable biology insights into molecular basis and advances the understanding towards the higher organisms.

Pseudomonas aeruginosa is a clinically important pathogen responsible for nosocomial infection and infamous for the arising morbidity, mortality and healthcare costs worldwide. The multidrug resistance of *P. aeruginosa* poses great challenges to the healthcare community and this problem is worsen by the bottleneck faced by the pharmaceutical industries in developing new drugs combating this infectious agent. The clinical isolate of *P. aeruginosa* UCBPP-PA14 (hereafter annotated as PA14) is identified as multi-host pathogen capable to infect mouse, plants and insects (Rahme et al, 1995). PA14 is thus commonly used as reference strain as this highly virulent strain affords for a wide host spectrum for studies of virulence mechanisms (Mahajan-Miklos et al, 2000). Following the establishment of *C. elegans* – pathogen infection model (Tan et al, 1999), *C. elegans* are reported to be susceptible to a number of human pathogens including *P. aeruginosa* and it appears that the genetic factors involved in the mammalian pathogenesis are also required for *C. elegans* model (Marsh and May, 2012). Particularly, I focus my study on lysozyme, an immune effector implicated in *C. elegans* defense during *P. aeruginosa* infection. Lysozyme was first described in chicken egg white (Laschtschenko 1909) but was not officially named for its lytic bioactivity until 1922 by Alexander Fleming (Fleming 1922). Following then, a wide range of research were performed on lysozyme, including protein structure study (Blake et al. 1965, Phillips, 1967), the use of lysozyme as disease marker for diagnostic purpose (Osserman & Lawlor 1966; Glynn, 1968; Pruzanski & Saito, 1969), the enzymatic function of lysozyme (Jolles & Jolles, 1984), protein binding (Baniahmad et al. 1991; Madhusudan & Vijayan 1992; Bonifer et al, 1997) and lysozyme gene

regulation (Short et al, 1996). Of note, owing to its bacteriolytic characteristic to hydrolyze 1,4-β-linkages between N-acetylmuramic acid and N-acetyl-D-glucosamine residues within peptidoglycan, lysozyme is implicated as an important immune defense effector against bacterial infection (Jolles & Jolles, 1984). More recently, the host-pathogen interaction studies using *C. elegans* as infection model has revealed the importance of lysozymes in pathogen resistance (Mallo et al, 2002; Evans et al, 2008; Kurz & Ewbank, 2003; Marsh et al, 2011). Of note, Evans et al (2008) described PA14 unique virulence mechanisms to overcome *C. elegans* host immune defense by suppressing lysozyme (*lys-7*, specifically) expression. Thus, the search for such lysozyme modulating agent would be interesting to investigate if such modulating agent could be used as anti-infective to promote host resistance against infection.

Although lysozyme has been intensively studied over the decades, only few research had been done in the search for lysozyme modulating agents from crude extracts or semi-purified natural products. Most of these works were performed by using laborious experimental procedures with limited screening throughput (Zhou et al, 2011; Dharmalingam et al, 2012; Kandasamy et al, 2012; Kong et al, 2014). With the increasing needs for therapeutic products, it is imperative to employ innovative methodologies to improve the robustness, efficacy and sensitivity of a screening assay in the search for relevant compounds.

Thus, this work describes an image-based high-content screening for lysozyme immunomodulating agents, with *P. aeruginosa* selected as the representative pathogen to evaluate the potential of small molecules as efficacious anti-infectives. Ultimately, this strategy could discover novel therapeutics in addressing the fundamental challenges in drug development.

1.2 Aim of the study

Recognizing the biomedical importance of lysozyme, I aim to perform a high-throughput screening assay for lysozyme immunomodulating compounds. I hypothesize that a lysozyme immunostimulating compound could be used as an anti-infective agent to mediate host immune response and promote host resistance towards pathogen infection.

To achieve these goals, this thesis examines the following objectives:

1. To establish an automated, image-based screening assay in 384-well microplate format by using *lys-7*::GFP transgenic *C. elegans* as screening subject.
2. To perform a high-content screening of small molecule chemical libraries containing ~16, 000 compounds to identify candidates that induce *C. elegans lys-7* GFP expression.
3. To examine *lys-7* stimulating compound efficacy as anti-infective using *C. elegans – P. aeruginosa* survival assay.
4. To perform preliminary mode-of-action study of immunostimulating compound by investigating the involvement of signaling pathways in mediating compound-induced *lys-7* expression.

CHAPTER 2

LITERATURE REVIEW

2.1 Antibiotic resistance

The discovery of penicillin by Sir Alexander Fleming in 1928 was recognized as one of the major advances in human medical history. Since then, the antimicrobial drug development had been actively pursued for this wonder drug to treat various infectious diseases. Different classes of antibiotics target the essential physiological and biochemical processes within bacteria to achieve antimicrobial activity, such as the interference of bacterial cell wall synthesis, cell membrane permeability, DNA and RNA synthesis, protein synthesis and folic acid metabolism (Leung, 2011). Regrettably, the development of tolerance and resistance in microbial population is predictable and inevitable for any antimicrobial compound discovered (**Figure 2.1**) (Davies & Davies, 2010). The antibiotic resistance within microbial community is achieved through four main mechanisms *i.e.*: the alteration of target site, drug inactivation through enzyme-catalyzed destruction, the reduction of drug bioaccumulation (through active efflux pumps or decreasing drug permeability) and alteration of metabolic pathway (Leung, 2011). In addition to this immunity and bypass, the microbial intrinsic characteristic (by genetic mutations) and acquired resistance traits (via horizontal genetic transfer) exacerbated the rapid widespread of antibiotic resistance among microbial community.

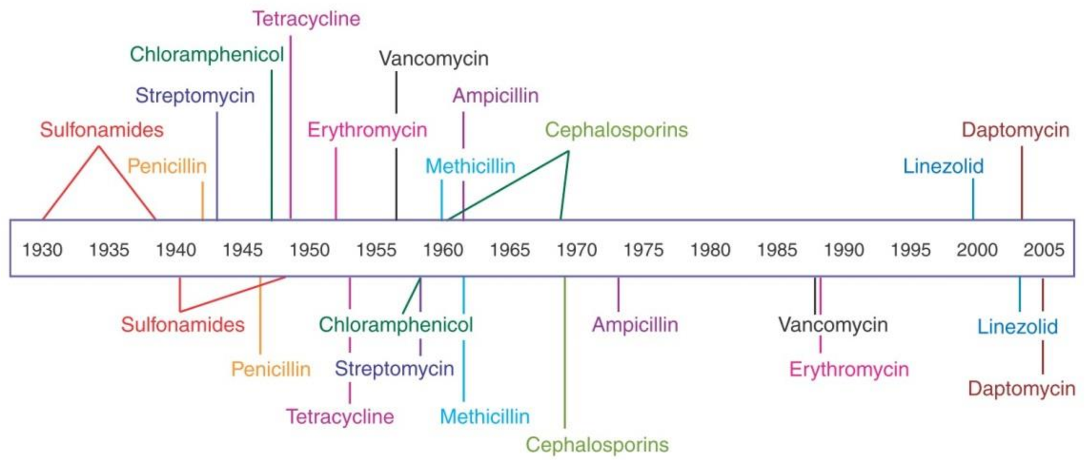

Figure 2.1: The timeline of antibiotic discovery (depicted above timeline) and the timeline when the antibiotic resistance was first reported (depicted below timeline). (Source: Clatworthy et al, 2007)

The emergence of multidrug resistant bacteria strains is an alarming threat to public health as these microbes are resilient towards most antibiotic treatments, impairing the effectiveness of antimicrobial chemotherapy. The pipeline for new effective antibacterial agents would be endless as a successful treatment of infection requires effective antibiotic therapy and yet the antibiotic resistance emerged at a faster pace than it can be controlled. With only 22 new antibiotics discovered since 2000 (**Figure 2.2**), the declining number of approved antimicrobial drugs in recent decades had been a worrisome concern (Butler et al, 2013). Apart from the long timescale required for drug development, the disengagement of most pharmaceutical companies from antimicrobial research due to risks-and-benefits consideration and regulatory challenges further contributed to this unprecedented bottleneck in drug development (Carlet et al, 2012). At present, only several large pharmaceutical companies remain active in the antibacterial discovery programs, namely GSK, Novartis, AstraZeneca, Sanofi-Aventis, Merck and Pfizer, compared to 18 pharmaceutical companies registered back in 1990 (Butler et al, 2013).

It appears that the current drug development for new classes of antibiotics could not meet the increasing needs (Clatworthy et al, 2007). Thus, the development of novel strategies to combat microbial infectious disease becomes imperative before current therapeutic measures dried out, placing the future patients in menace.

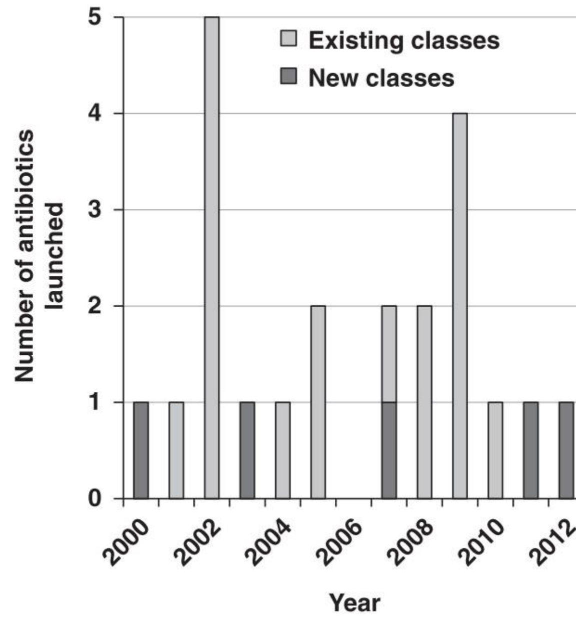

Figure 2.2: Number of approved antibiotics launched from 2000 to 2012. Only five new first-in-class antibiotics have been described, namely linezolid (approved 2000), daptomycin (approved 2003), retapamulin (approved 2007), fidaxomicin (approved 2010) and bedaquiline (approved 2012). (Source: Butler et al, 2013)

2.2 Anti-infective

Acknowledging the challenges faced by pharmaceutical industries in antibiotic discovery programmes and the difficulties in keeping up with the rapidly evolving microbial resistance (Ewbank & Zugasti, 2011), non-antibiotic-based approaches are proposed as alternative therapeutic options to address the current needs for effective treatment against infectious diseases. These alternative therapies include vaccines, probiotic therapy, phage therapy, immune stimulation and virulence factor neutralization (Butler et al, 2013).

The possible next-generation anti-infective with mode of action via immune stimulation or virulence factor attenuation has become the spotlight of scientific attention (Bhavsar & Brown, 2006; Arvanitis et al, 2013). The development of anti-infective that capitalizes on mechanism to improve host immune defense or disrupt pathogen virulence factor is a conceivable strategy in combating pathogenesis (Bhavsar & Brown, 2006; Pirofski & Casadevall, 2006; Hamill et al, 2008).

Table 2.1: Examples of anti-infective strategies.

Strategies	Mode of action	Natural products / compound	Application	References
Attenuation of virulence factor	Anti-Quorum Sensing (QS) activity	Cinnamaldehyde analog	Against *Vibrio* spp.	Brackman et al, 2011
	Attenuation of pyocyanin production	Raloxifene	Against *P. aeruginosa*	Ho Sui et al, 2012
	Inhibition of staphyloxanthin	Indole and derivatives (7-benzyloxyindole)	Against *S. aureus*	Lee at al, 2013
	Inhibition of biofilm formation	CCG-203592	Against *S. aurues*	Ma et al, 2012
Immunomodulation	p38 MAPK pathway and transcription factor ATF-7	RPW-24 Small molecule	Against *P. aeruginosa*	Pukkila-Worley et al, 2012
	Immunomodulation of *lys-7*	*Swietenia macrophylla* extract	Against *P. aeruginosa*	Dharmalingam et al, 2012
	p38 MAPK pathway and β-catenin signaling pathway	Probiotic *Lactobacillus* NCFM	Against Gram positive bacteria	Kim et al, 2012
	Microbiota-induced immune protection via p38 MAPK pathway	Non-pathogenic soil bacteria *B. megaterium* and *P. mendocina*	Against *P. aeruginosa*	Montalvo-Katz et al, 2013

Strategies	Mode of action	Natural products / compound	Application	Reference
Immunomodulation and attenuation of virulence factor	Induction of lysozyme, thaumatin and saponin; Attenuated production level of lipase, protease and pyocyanin	Tasco® *Ascophyllum nodosum* extract	Against *P. aeruginosa*	Kandasamy et al, 2012

2.3 Host – pathogen interaction study

The host - pathogen interaction best describes the strategies evolved by both the host and microbes to outcompete each other in the rally to survive. The host harnesses immunity system encompassing at least three major responses, namely pathogen recognition, immune signaling triggering and immune effectors responses to ward-off pathogen infection (Mallo et al, 2002; Gravato-Nobre and Hodgkin, 2005; O'Rourke et al, 2006). On the other hand, pathogens have as well deployed a repertoire of evasion and invasion strategies to subvert host immune barriers, including the interference of host signaling pathway and the suppression of host immune effectors (Tan, 2001; Kurz & Ewbank, 2003; Finlay & McFadden, 2006; Evans et al, 2008). Thus, the study of host – pathogen interaction at cellular and molecular level could provide better understanding of these underlying mechanisms, including the evolutionarily conserved immune signaling pathways (*i.e.*: which signaling pathways are involved and what are the transcriptional changes?) and the host-specificity of microbial pathogenesis (*i.e.*: what are the virulence factors deployed by pathogen? Are these host-specific or universal virulence factors?). These valuable insights could then be expanded into drug development for the discovery of novel antimicrobials (Rahme et al, 1995; Garvis et al, 2009).

The establishment of experimental host – pathogen system using invertebrate host has then become a rapid emerging research themes such as *Arabidopsis thaliana*, *Drosophila melanogaster* (Jennings, 2011) and *Caenorhabditis elegans* (Tan et al, 1999) to facilitate the genetic dissection of the underlying mechanism (Kim et al, 2002; Schlaich, 2011). These invertebrate model organisms often share several advantages over the mammalian models, including economical experimental setting, ease of genetic manipulation and amenability for high-throughput techniques without raising

ethical concerns intrinsic to vertebrate animal research (Zhou et al, 2011). These invertebrate models could not completely replace mammalian model as preclinical model to predict drug safety in human, it is however useful for preliminary research to address fundamental questions and valuable as a quick platform for discovery purpose, such as gene function study and pioneer medical research (Kaletta & Hengartner, 2006).

The evolutionarily conservation of genetic and biochemical pathways between invertebrates and mammalian models are useful for drug discovery programs that define disease models based on a molecular basis. The introduction of *C. elegans* – pathogen interaction model (Tan et al, 1999) had made use this simple non-vertebrate model organism to facilitate the understanding of pathogenesis and innate immunity. Despite bridging the knowledge gap between the *in vitro* assays and mammalian model, *C. elegans* – pathogen interaction model can also be expanded as a cost-effective and rapid screening tool for novel antimicrobial agents, in which the drug-and-target interaction can be interpreted *in vivo* for intervention efficacy and mechanism of action study (Kaletta & Hengartner, 2006; Arvanitis et al, 2013).

Model organisms are important in modeling biological question during preliminary research phase. Through the understanding of mechanisms and function at a simple level *i.e.*: invertebrate organism, this fundamental knowledge could be extended to a more complex level like mammalian models. Through the in-depth study of innate immunity of a diverse species, the understanding of evolutionarily conserved mechanisms could help researchers to explore further into evolutionary origins and molecular mechanisms, which ultimately could lead to the therapeutic advances in human research (Kim et al, 2002).

2.4 *Caenorhabditis elegans* as model organism

Since the introduction of *C. elegans* by Sydney Brenner in 1960's as the model organism to study animal behavior and development, *C. elegans* has been actively researched to address various important biological questions (Brenner, 1974). *C. elegans* is a simple multicellular, transparent microscopic nematode (about 1mm in adult size) that offers several experimental advantages as model organism. Although *C. elegans* is a relatively simple microorganism, it contains about 1000 somatic cells with highly differentiated anatomy such as muscles, epidermis nervous system, gonad and gastrointestinal tract. Under normal growth condition in laboratory, *C. elegans* feeds on *E. coli* OP50 and are maintained on agar medium in Petri dish. The population consists of hermaphrodite predominantly, with only 0.1% being male in the wild-type population (**Figure 2.3**). A self-fertilizing hermaphrodite worm can give rise to 300 progeny whereas a male-fertilized hermaphrodite could produce up to 1000 progeny (Strange, 2006). This feature is particularly unique for genetics study (*e.g.*: mutation research), whereby a self-fertilizing hermaphrodite could yield a homozygous population without the caveat for crossing.

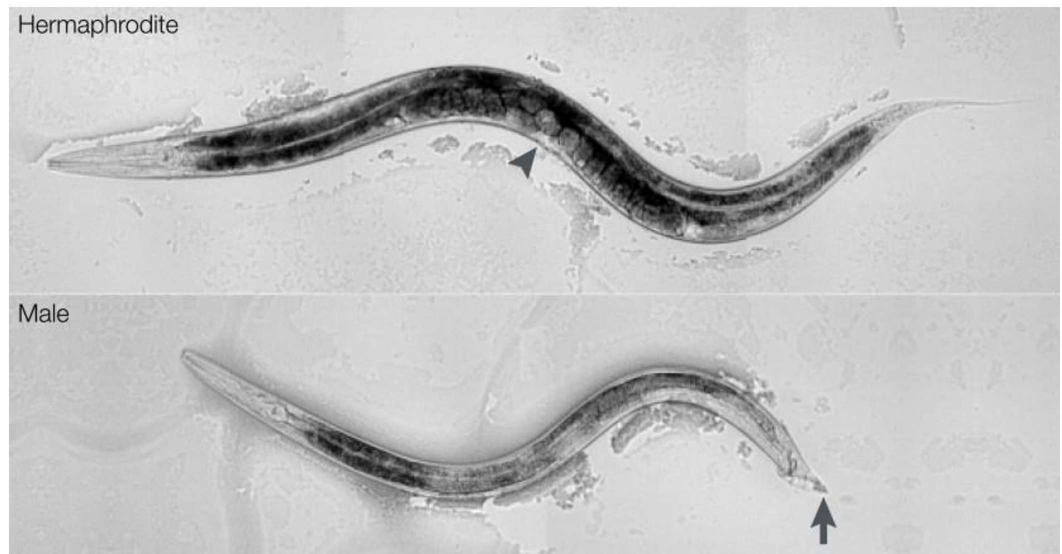

Figure 2.3: The morphology of hermaphrodite and male nematode. The arrowhead (top panel) indicates the vulva of a hermaphrodite while the arrow (bottom panel) indicates the male nematode with fan-like tail. (Source: Jorgensen & Mango, 2002)

The short generation time of *C. elegans* (about 2.5 days from an embryo to a fertile adult) offers a rapid experimental manipulation (**Figure 2.4**). With an average lifespan of 3 – 4 weeks under optimal laboratory condition, this feature also facilitates the study of developmental biology, embryogenesis and post-embryonic development within a short period. Indeed, the mapping of cell lineage during embryogenesis and post-embryonic has been made available (Sulston & Horvitz, 1977; Sulston et al, 1983). In addition, the transparent worm body also permits real-time observation of cellular processes. Upon the introduction of Green Fluorescent Protein (GFP) as an expression reporter, biological activities can now be studied without any invasive methods in the context of an intact living organism (Corsi, 2006). Furthermore, gene knockdown technique through RNA-interference (RNAi) that can be easily achieved through microinjection or feeding delivery method also facilitates gene function study in *C. elegans* (Hull & Timmons, 2004; Boutros & Ahringer, 2008).

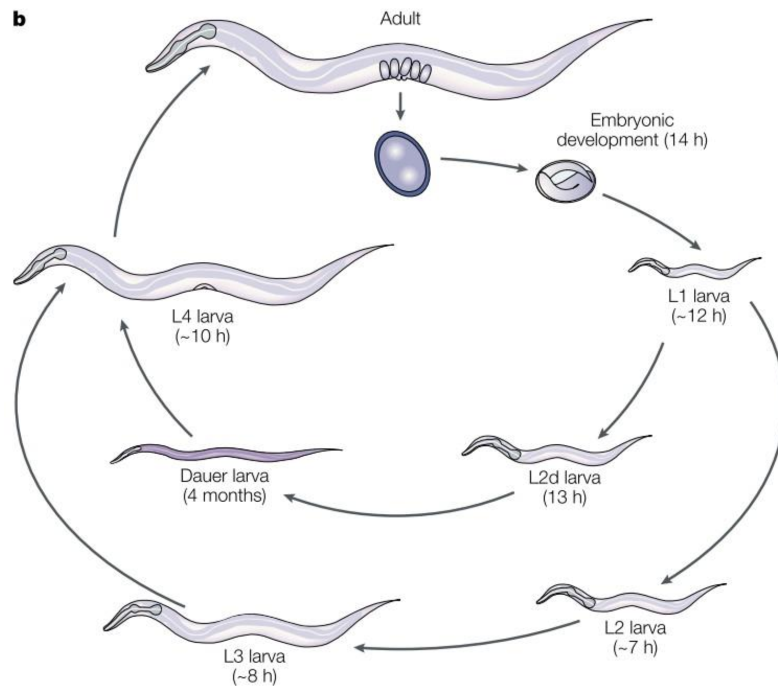

Figure 2.4: The life cycle of *C. elegans*. The nematode undergoes four larval stages from L1 to L4 before they turn into gravid adult worms. The number within bracket indicates the development timing for each stage. (Source: Jorgensen & Mango, 2002)

With its genome completely sequenced by 1998, *C. elegans* has provided a wealth of bioinformatics resources to the scientific communities, which can be accessed online at http://www.wormbase.org (Corsi et al, 2015). With these intrinsic characteristics and advantages, *C. elegans* proves to be a relatively cost-effective and yet a powerful model organism in multiple fields of biological research, such as neurobiology, developmental, aging and genetics study (Ewbank & Zugasti, 2011; Portal-Celhay et al, 2012). More recently, *C. elegans* has been introduced into microbial pathogenesis and host innate immunity research, and expanded to drug discovery program.

2.5 *C. elegans* features as model for immunity study

Being the soil dweller in the wild, *C. elegans* is susceptible to a wide range of pathogen, including bacteria and fungi. However, it appears that *C. elegans* depends solely on their genetically-encoded innate immunity as defense mechanisms; this lacking of an adaptive immune system nevertheless allows the study of innate immunity without the complication of adaptive immunity.

Three major mechanisms are described for *C. elegans* immune defense against invading agents (Engelmann & Pujol, 2010). Firstly, the avoidance behavior that involves olfactory sensory to trigger nematode aversive response from noxious pathogen. Research finding suggested that *C. elegans* can discriminate and avoid noxious bacterial food source through “odour learning” in an asymmetric chemosensory neurons-dependent manner. This behavioral avoidance thus prevents nutrients uptake from potential pathogenic source and greatly minimizes the exposure to lethal risk factor. The second line of defense is the worm cuticle that serves as a resilient physical barrier against invading pathogens. This chitinous worm exoskeleton provides strong resistance towards puncturing, avoiding open wound for pathogen

invasion. As *C. elegans* ingests bacteria as its nutrition source, the pharyngeal grinder also function as a defensive barrier in which ingested bacteria can be destroyed through mechanical disruption. Lastly, the inducible innate immunity that involves complex signaling cascades to regulate the expression and secretion of immune effectors, such as lectins, lysozymes, antibacterial factors for pathogen clearance (Engelmann & Pujol, 2010; Marsh and May, 2012).

Under laboratory growth condition, infection-like condition can be achieved in this bacterophagus *C. elegans* by substituting the relatively innocuous bacterial food source, *E. coli* OP50 with the desired choices of pathogen, such as *P. aeruginosa* (Tan et al, 1999), *E. faecalis* (Garsin et al, 2001; Sifri et al, 2002), *Staphylococcus aureus* (Sifri et al, 2003; Bae et al, 2004), *Salmonella enterica* (Aballay and Ausubel, 2001; Tenor et al, 2004), *Burkholderia pseudomallei* (O'Quinn et al., 2001; Gan et al., 2002) and *Serratia marcescens* (Kurz et al., 2003). With this introduction of pathogen invasion through feeding delivery, *C. elegans* intestinal gut is the major interface where infection takes place for most known human pathogens, although with some exceptions whereby the primary infection sites are at *C. elegans* vulva, anus or epidermal cuticle (**Figure 2.5**).

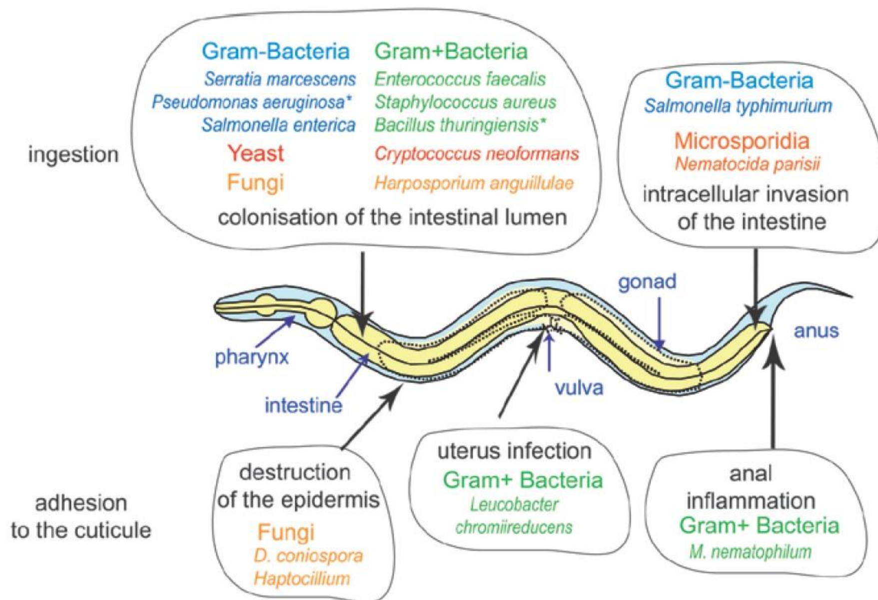

Figure 2.5: Example of pathogens that can infect *C. elegans* and the infection routes by pathogens. Asterisk (*) denotes the pathogen that produces toxin to kill host. Most known human pathogens colonize and persist within *C. elegans* intestinal gut to establish infection. (Source: Engelmann & Pujol, 2010)

The effectiveness of *C elegans* immunity and pathogenesis can be assessed by studying the function of *C. elegans* survival over infection time. The progression of motility, physiological and immunesenescence, pathogen burden within *C. elegans* can be easily examined non-invasively. Following the complete sequencing of *C. elegans* genome made available in 1998, *C. elegans* homologs have been identified for about 60-80% of human genes (Corsi, 2006; Mondoux et al, 2010). Remarkably, approximately 40% genes associated with human disease appears to have homologs in *C. elegans* genome (Corsi, 2006). More importantly, *C. elegans* are also susceptible to most human pathogens (Kirienko et al, 2013), further supporting *C. elegans* as a suitable model to study infectious disease at genetic and molecular basis. This sequenced genome also offers *C. elegans* amenability in genetic manipulation to generate a large mutant library, in which hypersensitive or resistant mutant strains are available for research.

Since the first description of *P. aeruginosa* as an infection model (Tan et al, 1999), *C. elegans* has become a facile *in vivo* model to facilitate host-pathogen interaction studies such as *E. faecalis* (Garsin et al, 2001; Sifri et al, 2002), *Staphylococcus aureus* (Sifri et al, 2003; Bae et al, 2004), *Salmonella enterica* (Aballay et al., 2000; Aballay and Ausubel, 2001; Tenor et al, 2004; Tenor et al, 2008), *Burkholderia pseudomallei* (O'Quinn et al., 2001; Gan et al., 2002) and *Serratia marcescens* (Kurz et al., 2003). The experimental systems, most if not all, involve the intestinal infection of *C. elegans* with human microbial pathogen (Shivers et al, 2008). With these strikingly high degree of conservation of biochemical pathways between nematodes and human, *C. elegans* – pathogen experimental system has become an ideal model in uncovering novel drug targets (Giacomotto and Segalat, 2010). By varying choices of pathogen, microarray and candidate gene studies reported the

pathogen-specific gene expression changes within *C. elegans* upon infection (Evans et al, 2008), facilitating the identification of antimicrobial effectors in *C. elegans*.

2.6 Lysozyme

The bacteriolytic phenomenon may have been described earlier than we thought. However, the discovery of this lytic element was only first introduced as "lysozyme" by Alexander Fleming in early 1922 (Laschtschenko, 1909; Rettger & Sperry, 1912; Fleming, 1922).

Lysozymes are ubiquitously present in numerous phylogenetically diverse forms of life, ranging from virus, fungi to plants and animals (Schulenburg & Boehnisch, 2008). Lysozyme is characterized by its ability to hydrolyse β-1,4-glycosidic linkages between N-acetylmuramic (NAM) and N-acetylglucosamine (NAG) in peptidoglycan of bacterial cell walls. Owing to this bacteriolytic bioactivity, lysozyme is widely recognized as an important immune effector against various invading pathogens. In mammals, lysozymes constitute a key component in the first line of defense whereby they are found in mucosal and epithelial secretion such as tears, saliva, milks and mucus (Bachali et al, 2002; Callewaert and Michiels, 2010). Lysozymes are also present in the organs of ruminants (true stomach) and marine bivalves (digestive gland and gills), displaying their functional diversity as digestive enzymes, in addition to their primary immune function (Bachali et al, 2002; Van Herreweghe & Michiels, 2012). This secondary function is further suggested as an intracellular effector of ruminants to digest and exploit gastrointestinal microbes for nutrition, despite to prevent the uncontrolled microbial growth within intestine (Nickel et al, 1998).