

表現登革熱二型病毒 NS2, NS4 基因及探討四環黴素衍生物對登革熱病毒的

抑制作用

研究生：張瀨云

指導教授：楊昀良 博士

國立交通大學生物科技研究所

中文摘要

登革熱目前已成為一個全球性健康的議題，主要分布在熱帶及亞熱帶地區。目前統計超過一百個國家曾經爆發過登革熱疫情並且大約有二十五億的人口受到登革熱病毒的威脅。因此目前針對登革熱疫苗的研發，治療和預防的方法也是一個迫切的議題。

在登革熱病毒產生蛋白質的過程，會先產生單一個蛋白質轉譯區，再切割成十個獨立的蛋白質。其中，NS2A、NS2B、NS4A 及 NS4B 是較小的非結構型蛋白，具有疏水的特性。這四種蛋白質中，只有 NS2B 已知會參與蛋白切割酶反應，而 NS2A、NS4A、及 NS4B 尚未被鑑定出確切的生化功能。為研究登革熱病毒此四蛋白質的結構及功能，本研究嘗試將四種蛋白質在哺乳類細胞中以加上 EGFP (enhanced green fluorescence protein) 的融合蛋白(fusion protein)形式表現。成功篩選出 NS2A 及 NS2B 穩定轉植細胞株後，溶斑試驗(plaque assay)的結果顯示：表現 NS2A 的穩定轉植細胞株經登革熱病毒感染後，相較於對照組會減少 44%的溶斑數 ($P < 0.05$)。

另外先前實驗室已經篩選出某些以四環結構為主體的四環黴素衍生物，其可與外膜蛋白上的 β -OG pocket docking，也對登革熱二型病毒有初步的抑制效果。因此我將進一步利用溶斑試驗測試這些四環黴素衍生物針對登革熱病毒二型 PL046 病毒株及三型 H87 病毒株的抑制是否有 strain specificity 的效果。結果顯示，在溶斑試驗當中四環黴素衍生物對於登革熱病毒二型和三型的溶斑生成確實是有抑制的效果，其中又以 doxycycline 和 chlortetracycline 抑制的效果最為顯著。而進一步的比較發現，針對登革熱病毒二型的抑制能力比三型的抑制能力高。

Functional Expression of NS2 & NS4 of Dengue Virus 2 and Mechanism Study of Tetracycline-Derivatives as Dengue Virus Inhibitors

Student: Ching-Yun Chang

Advisor: Dr. Yun-Liang Yang

Department of Biological Science and Technology

National Chiao Tung University

Abstract

Dengue viral infections have become a global health issue in tropical and subtropical regions. There were outbreaks reported in more than 100 countries and about 2.5 billion people lived under the threats. Therefore, the developments of vaccines, treatments, or prevention measures for dengue viral infections are a pressing issue.

Dengue virus encodes 10 proteins in a single open reading frame. Among them, NS2A, NS2B, NS4A and NS4B are small non-structural proteins exhibiting hydrophobicity profiles. NS2B has been suggested to involve in protease activity while specific biological functions for NS2A, NS4A and NS4B have not been identified. With the interest to study the structure and function of these four proteins, I attempted to clone, express, and functional assay of these four genes. Their proteins were expressed as EGFP (enhanced green fluorescence protein) fusion proteins in mammalian cells. The stable cell lines of NS2A and NS2B were successfully selected and confirmed by Western blot analysis. The results showed that NS2A-EGFP effected a 44% reduction of plaque numbers compared to the negative controls, BHK-21 cells ($P < 0.05$).

Previously, our lab has identified certain tetracycline derivatives as potential inhibitors to Dengue virus, by docking into the β -OG pocket on the DV2 E protein. Hence, I set out to test various tetracycline compounds for their effect on dengue viral propagation of DV2 PL046 strain and DV3 H87 strain by plaque formation assay. All the tetracycline derivatives tested inhibited the plaque formations on DV2 and DV3 in cell culture systems, especially of doxycycline and chlortetracycline. And in comparison, the inhibitory effect on DV2 is better than that on DV3.

Acknowledgement

兩年的時間很快就過去了，這其間要感謝的人很多，第一個當然是指導教授楊昀良老師，從大三專題生到研究所畢業，算起來在老師的指導下也經歷了四年的時間，謝謝老師在這四年來的教導，除了實驗上獲得很多寶貴的經驗，也增加了很多邏輯思考與解決問題的能力!也很感謝老師給了我很多成長的機會，非常感謝老師對我的指導!!!除此之外也要感謝口試委員徐祖安老師、徐維莉老師和黃兆祺老師，對於論文的完成提供了很多寶貴的意見!

實驗室的成員也是陪伴我這幾年的重要快樂因子，謝謝柏吟學姐一開始對初進實驗室什麼都不懂的我細心的指導；感謝嘉嘉學姐即使畢業了也耐心的回答我關於實驗上很多的問題，給了我很多的幫助；感謝怡瑾學姐在實驗室生活上的陪伴，也帶給我很多歡笑與寶貴的回憶。除此之外還有搞笑功力一流的志豪學長，酷酷的建李學長，超可愛的歐陽學姐，看似冷酷卻很可愛的金蓉學姐，超有行動力是個好玩咖的欣悟，又高又白的欣彬學姐，育穎學長認真的對於實驗上的指導，感謝這些學長姐們在我大學剛進實驗室時的陪伴與教導!

而在這兩年的研究所生涯當中，當然還有好好先生敏書，一起耍白痴之白痴二人組的淑萍，溫柔可愛的淑貞，坐我隔壁常常一起玩一起討論實驗的旻秀，超有魄力的佳真，傻傻可愛的妍寧，看似成熟卻心思動作超級可愛的馨儀，女人味比我厲害的毓駿，認真直爽的幸福，愛吃小上海認真負責的重延，單純可愛的禎憶，喜歡唱歌很有力的蔡鏗，我最可愛最傻最天真的女兒阿大，跟你在一起都很開心的跟你一起耍白痴一起吵鬧，大學四年加研究所兩年的好同學好室友好戰友小倩，你單純直接的個性真的太有型了，雖然常常都聽不懂你的笑點，也很感謝你一直以來的陪伴與鼓勵，雖然我好像常常兇你，可是我還是很愛你的，呵呵!還有一樣是楊家一份子的永馨，有我們就有你啦，這兩年很开心有你的陪伴，你的率真、認真、愛恨分明、看似冷酷卻超級好笑的個性總算讓我摸透拉，也很感謝你的照顧，在我想亂買東西的時候打醒我，雖然常常一起買，哈!除此之外還要謝謝育忠，總是默默的包容我，支持我，鼓勵我，陪伴我，也在研究上給我很多的意見!謝謝大家的陪伴，有你們真的很開心!!!!!!歡笑的記憶會永遠的存在!當然還有很多新竹的朋友也要謝謝你們讓我這兩年的生活過得更多采多姿!

最後還要謝謝我的家人，爸爸媽媽哥哥妹妹和阿公，謝謝你們默默的支持我，在我難過的時候陪我幫助我，給我打氣，有你們的陪伴也讓我在求學的過程中能認真的努力!謝謝!!

Contents

Abstract (Chinese)	i
Abstract (English)	ii
Acknowledgement	iii
Contents	iv
Tables	ix
Figures	x
Appendix	xiii
Abbreviation	xiv
Chapter 1. Introduction	1
1.1 Dengue virus	1
1.2 Replication of dengue virus	2
1.3 Genome of dengue virus	3
1.4 Four small non-structural proteins of dengue virus	3
1.5 Properties of NS2A	4
1.6 Properties of NS2B	5
1.7 Properties of NS4A	6
1.8 Properties of NS4B	7
1.9 Properties of E protein	9
1.10 The β -OG pocket of E as a target	10
1.11 The overview of the experimental design	11
Chapter 2. Materials and Methods	13
2.1 Materials	13
2.1.1 Virus	13
2.1.2 Cell lines	13
2.1.3 Bacterial strains	13

2.1.4 Plasmids.....	13
2.1.5 Primers.....	14
2.1.6 Chemicals, enzymes and reagents	15
2.1.7 Antibodies.....	18
2.1.8 Kits	19
2.1.9 Buffers	19
2.1.10 Media.....	20
2.1.11 Equipments	21
2.2 Methods	22
2.2.1 Transformation of <i>E. coli</i>	22
2.2.1.1 Preparation of competent cells (for chemical method).....	22
2.2.1.2 Transformation	22
2.2.2 Plasmid DNA extraction.....	23
2.2.3 Restriction enzyme digestion	23
2.2.4 Cell culture	24
2.2.5 Transfection of mammalian cell	24
2.2.6 Immunofluorescence and confocal microscopy	24
2.2.7 Selection of stable transfected cells.....	25
2.2.8 Preparation of proteins from mammalian cells.....	25
2.2.9 Western blot analysis.....	25
2.2.10 Plaque formation of stable cell lines.....	26
2.2.11 Statistical analysis	26
2.2.12 Preparation of genomic DNA from mammalian tissue	26
2.2.13 RNA extraction.....	27
2.2.14 Semi-quantitative RT-PCR-Superscript™ One-Step RT-PCR	28
2.2.15 Amplification of Dengue virus	28
2.2.16 Plaque formation assay for the inhibitory effects of compounds in DV2 and DV3	

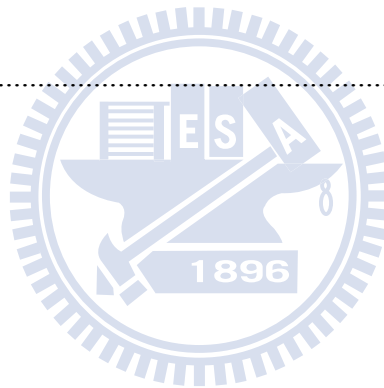
propagation	29
Chapter 3. Functional expression of nonstructural proteins NS2A, NS2B, NS4A, and NS4B of dengue virus type 2 PL046 strain	
(I) Result	30
3.1 Construction of pNS2A-EGFP, pNS2B-EGFP, pNS4A-EGFP, and pcDNA3-D24B-EGFP expression plasmids	30
3.2 Expression and subcellular localization of pNS2A-EGFP, pNS2B-EGFP, pNS4A-EGFP, and pcDNA3-D24B-EGFP in mammalian cells, BHK-21	31
3.2.1 Confocal fluorescence microscope analysis	31
3.2.2 Assay of the transient expression of pNS2A-EGFP, pNS2B-EGFP, pNS4A-EGFP, and pcDNA3-D24B-EGFP in BHK-21 by Western blot analysis	31
3.3 Selection of stable transfected cells of pNS2A-EGFP, pNS2B-EGFP, pNS4A-EGFP, pcDNA3-D24B-EGFP, and pEGFP-N2	32
3.3.1 Fluorescence microscopy and Western blot analysis of the selected stable cell lines	32
3.3.2 PCR analysis on the genomic DNA of the selected stable cell lines of NS2A-EGFP, NS2B-EGFP, NS4A-EGFP, and NS4B-EGFP	33
3.3.3 RNA expression of the stable cell lines of 2A-EGFP, 2B-EGFP, 4A-EGFP, and 4B-EGFP	33
3.4 Construction of pNS2B-EGFP(pro), pNS4A-EGFP(pro), and pcDNA3-D24B-EGFP(pro) expression plasmids	34
3.5 Expression of pNS2B-EGFP(pro), pNS4A-EGFP(pro), and pcDNA3-D24B-EGFP(pro) in mammalian cells, BHK-21	35
3.6 Selection of stable transfected cells of pNS2B-EGFP(pro), pNS4A-EGFP(pro), and pcDNA3-D24B-EGFP(pro)	35
3.7 Plaque formation on stable cell lines	36
(II) Discussion	37

3.8 Construction of pNS2A-EGFP, pNS2B-EGFP, pNS4A-EGFP, and pcDNA3-D24B-EGFP expression plasmids	37
3.9 Expression of pNS2A-EGFP, pNS2B-EGFP, pNS4A-EGFP, and pcDNA3-D24B-EGFP in mammalian cells, BHK-21	37
3.9.1 Confocal fluorescence microscopy analysis.....	37
3.9.2 Transient expression of pNS2A-EGFP, pNS2B-EGFP, pNS4A-EGFP, and pcDNA3-D24B-EGFP in BHK-21	38
3.10 Selection of stable transfected cells of pNS2A-EGFP, pNS2B-EGFP, pNS4A-EGFP, pcDNA3-D24B-EGFP, and pEGFP-N2	38
3.10.1 Western blot analysis of the selected stable cell lines	38
3.10.2 Materials and RNA expression confirmation of the selected stable cell lines of NS2A-EGFP, NS2B-EGFP, NS4A-EGFP, and NS4B-EGFP	39
3.11 Construction of pNS2B-EGFP(pro), pNS4A-EGFP(pro), and pcDNA3-D24B-EGFP(pro) expression plasmids	40
3.12 Expression of pNS2B-EGFP(pro), pNS4A-EGFP(pro), and pcDNA3-D24B-EGFP(pro) in mammalian cells, BHK-21	41
3.13 Selection of stable transfected cells of pNS2B-EGFP(pro), pNS4A-EGFP(pro), and pcDNA3-D24B-EGFP(pro).....	41
3.14 Plaque formation on stable cell lines.....	42
(III) Conclusion.....	43

Chapter 4. Biological assay of inhibitor candidates to dengue virus type 2 and dengue virus type 3

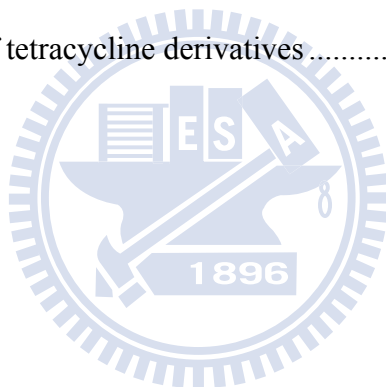
(I) Result	44
4.1 Candidate compounds on DV propagation.....	44
4.2 Sequence analysis of the detergent-binding pocket in envelope protein between DV2 PL046 strain and DV3 H87 strain	45
4.3 <i>In vivo</i> plaque formation assay	45

4.3.1 Tetracycline	46
4.3.2 Doxycycline.....	46
4.3.3 Chlortetracycline	46
4.3.4 Rolitetracycline.....	47
4.3.5 Kanamycin.....	47
4.3.6 9-amino-1,2,3,4-tetrahydroacridine hydrochloride hydrate	48
4.3.7 Berberine	48
(II) Discussion	49
(III) Conclusion.....	52
Reference	53



Tables

Table 1 Contents of the SuperScript™ One-Step RT-PCR reaction.....	58
Table 2 The thermal cycle program.....	58
Table 3 Contents of the PCR reaction	58
Table 4 Condition for the PCR reaction for EGFP.....	58
Table 5 The relative percentage of plaque numbers of the stable cell lines.....	59
Table 6 Comparison of the amino acids of the β -OG-binding hydrophobic pocket in E protein between dengue virus type 2 PL046 strain and dengue virus type 3 H87 strain.....	60
Table 7 Chemical structures and IC ₅₀ s for the tetracycline derivatives.	61
Table 8 2D and 3D structures for the tetracycline derivatives	62
Table 9 Pharmacokinetics of tetracycline derivatives.....	64



Figures

1.1 World distributions of dengue viruses and their mosquito vector, <i>Aedes aegypti</i> , in 2008	65
1.2 The flavivirus replication cycle	65
1.3 Schematic representation of flavivirus genome organization and polyprotein processing	66
1.4 Nucleotide mapping of NS2A, NS2B, NS4A, and NS4B in dengue virus type 2 genome	66
1.5 NS2B hydrophobicity plots and a hypothetical model of NS2B-NS3pro association with membranes.....	67
1.6 Schematic structure of the DV polyprotein	68
1.7 Model for the membrane topology of DV NS4A	68
1.8 Model for the membrane topology of DV 2K-NS4B	69
1.9 Structure of the dimer of dengue E soluble fragment (sE) in the mature virus particle ...	69
1.10 Structure of dengue 2 E protein.....	70
1.11 Molecule structure of the compound P02.....	70
1.12 Molecule structure of the compound 6.....	70
3.1 Map of pEGFP-N2. The EGFP fragment was obtained by PCR reaction with primers EGFP-F and EGFP-R	71
3.2 Maps of pNS2A-EGFP, pNS2B-EGFP, pNS4A-EGFP, and pcDNA3-D24B-EGFP.....	72
3.3 Restriction enzyme digestions of pNS2A-EGFP, pNS2B-EGFP, pNS4A-EGFP, and pcDNA3-D24B-EGFP.....	73
3.4 Expression and colocalization of NS2A-EGFP, NS2B-EGFP, NS4A-EGFP, and NS4B-EGFP with the cellular marker protein, calnexin	74
3.5 Commassie blue staining and Western blot analysis of transiently expressed nonstructural proteins expressed in BHK-21	75
3.6 Fluorescence microscopy and Western blot analysis for stable transfected cells (BHK-21) of EGFP	76

3.7 Fluorescence microscopy and Western blot analysis for stable transfected cells (BHK-21) of NS2A-EGFP	77
3.8 Fluorescence microscopy and Western blot analysis for stable transfected cells (BHK-21) of NS2B-EGFP	78
3.9 Fluorescence microscopy and Western blot analysis for stable transfected cells (BHK-21) of NS4A-EGFP	79
3.10 Fluorescence microscopy and Western blot analysis for stable transfected cells (BHK-21) of NS4B-EGFP	80
3.11 PCR analysis of genomic DNA of the selected stable cell lines of NS2A-EGFP, NS2B-EGFP, NS4A-EGFP, and NS4B-EGFP	81
3.12 Semi-quantitative RT-PCR of RNA from stable cell lines NS2A-EGFP, NS2B-EGFP, NS4A-EGFP, and NS4B-EGFP	82
3.13 Construction maps of pNS2B-EGFP(pro), pNS4A-EGFP(pro), and pcDNA3-D24B-EGFP(pro).....	83
3.14 Construction maps and results of restriction enzyme digestion of pNS2B-EGFP(pro), pNS4A-EGFP(pro),and pcDNA3-D24B-EGFP(pro).....	84
3.15 Sequence analysis of pNS2B-EGFP(pro), pNS4A-EGFP(pro), and pcDNA3-D24B-EGFP(pro) at the start of EGFP tag	85
3.16 Commassie blue staining and Western blot analysis of proteins pNS2B-EGFP(pro), pNS4A-EGFP(pro), and pcDNA3-D24B-EGFP(pro) expressed in BHK-21	86
3.17 Western blot analysis and fluorescence microscopy for stably transfected cells (BHK-21) of NS2B-EGFP(pro), NS4A-EGFP(pro), and NS4B-EGFP(pro)	87
3.18 Plaque formation assay on stable cell lines, NS2A-EGFP, NS2B-EGFP(pro), NS4B-EGFP(pro), pcDNA3, and EGFP	88
4.1 Alignment of amino acids at envelope protein of DV2 PL046 strain and DV3 H87 strain	89

4.2 Effect of tetracycline on DV2 plaque formation using BHK-21 mammalian cells.....	90
4.3 Effect of tetracycline on DV3 plaque formation using BHK-21 mammalian cells.....	91
4.4 Effect of doxycycline on DV2 plaque formation using BHK-21 mammalian cells.....	92
4.5 Effect of doxycycline on DV3 plaque formation using BHK-21 mammalian cells.....	93
4.6 Effect of chlortetracycline on DV2 plaque formation using BHK-21 mammalian cells ...	94
4.7 Effect of chlortetracycline on DV3 plaque formation using BHK-21 mammalian cells ...	95
4.8 Effect of rolitetracycline on DV2 plaque formation using BHK-21 mammalian cells.....	96
4.9 Effect of rolitetracycline on DV3 plaque formation using BHK-21 mammalian cells ...	97
4.10 Effect of kanamycin on DV2 plaque formation using BHK-21 mammalian cells.....	98
4.11 Effect of kanamycin on DV3 plaque formation using BHK-21 mammalian cells.....	99
4.12 Effect of 9-amino-1,2,3,4-tetrahydroacridine hydrochloride hydrate on DV2 plaque formation using BHK-21 mammalian cells.....	100
4.13 Effect of 9-amino-1,2,3,4-tetrahydroacridine hydrochloride hydrate on DV3 plaque formation using BHK-21 mammalian cells.....	101
4.14 Effect of berberine on DV2 plaque formation using BHK-21 mammalian cells	102
4.15 Effect of berberine on DV3 plaque formation using BHK-21 mammalian cells	103
4.16 The cell morphology of BHK-21 when adding the different concentrations of 9-amino-1,2,3,4-tetrahydroacridine hydrochloride hydrate with no virus	104
4.17 The cell morphology of BHK-21 when adding the different concentrations of berberine	105
4.18 The percentage of cell numbers of BHK-21 when adding the different concentrations of 9-amino-1,2,3,4-tetrahydroacridine hydrochloride hydrate and berberine.....	106

Appendix

Appendix 1 Map of pcDNA3	107
Appendix 2 Maps of pNS2A-HAHis, pNS2B-HAHis, pNS4A-HAHis, and pcDNA3-D24B-HAHis	108
Appendix 3 Effect of tetracycline on DV2 plaque formation using BHK-21 mammalian cells	109



Abbreviation

APS	Ammonium Persulfate
DF	Dengue fever
DHF	Dengue hemorrhagic fever
DMSO	Dimethyl sulfoxide
DSS	Dengue shock syndrome
DV	Dengue virus
E protein	Envelope protein
EDTA	Ethylene diamine tetra-acetic acid
ER	Endoplasmic reticular
FBS	Fetal bovine serum
JEV	Japanese encephalitis virus
KUNV	Kunjin virus
MEM	Minimum Essential Medium
MOI	Multiplicity of infection
NS protein	Nonstructural protein
PBS	Phosphate buffer saline
PMSF	Phenyl methyl sulfonyl fluoride
TBEV	Tick-borne encephalitis virus
TEMED	N, N, N', N',-tetramethylenediamine
Tween-20	Polyoxyethene-sorbitan monolaurate
vRNA	Viral RNA
WNV	West Nile virus
YFV	Yellow fever virus