INVESTIGATION OF ANTIVIRAL MOLECULES INHIBITING VIRAL INFECTION

Mr. Sitthichai Kanokudom

A Dissertation Submitted in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy Program in Microbiology
Department of Microbiology
Faculty of Science
Chulalongkorn University
Academic Year 2018

การศึกษาโมเลกุลต้านไวรัสซึ่งยับยั้งการติดเชื้อไวรัส

นายสิทธิชัย กนกอุดม

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต

สาขาวิชาจุลชีววิทยา ภาควิชาจุลชีววิทยา

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Field of Study	Microbiology
Thesis Advisor	Associate Professor Wanchai Assavalapsakul, Ph.D.

Accepted by the Faculty of Science, Chulalongkorn University in Partial Fulfillment of the Requirements for the Doctoral Degree

…….…….Dean of the Faculty of Science
(Professor Polkit Sangvanich, Ph.D.)

THESIS COMMITTEE

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Sitthichai Kanokudom

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PART I
THESIS CONTENT

1.1. Overview of the publications

Over the past four decades, there are several antiviral agents which have been used for the medication in human and animal. Normally, the antiviral agents are mostly used to inhibit specific viral replication step such as Tenofovir for human Immunodeficiency virus (HIV) (Arts and Hazuda, 2012), Acyclovir for herpesvirus, Entacovir for Hepatitis B virus (HBV) (Rivkin, 2005), Ledipasvir for Hepatitis C virus (HCV) (Scott., 2018) and Amantadine for influenza virus (Littler and Oberg, 2005). However, there are no antiviral agents for some viral infections such as Dengue virus (DENV) (Low et al., 2017), Bovine viral diarrhea virus (BVDV), Foot and mouth disease virus (FMDV) and Bovine herpesvirus (BoHV) in cattle (Newcomer et al., 2014), Infectious myonecrosis virus (IMNV) and Yellow head virus (YHV) in shrimp (Loy et al., 2013).

This thesis composes of 3 chapters focusing on viral inhibition with different types of antiviral agents against DENV 2, Bovine herpesvirus-1 (BoHV-1) and YHV. From the data, they showed antiviral activity of the molecules could help dealing with viral infection in each study.

Chapter I: Sitthichai Kanokudom and co-authors investigated the expression of microRNAs (miRNAs) during dengue virus serotype 2 (DENV 2) infection in human hepatocellular carcinoma (HepG2) cells. This study showed that selected candidate miRNAs were elevated or diminished upon DENV 2 infection. Only miR-21 was constantly up-regulated during 24 hr after DENV 2 infection, hence, the role of miR-21 was further investigated. The results suggested that the up-regulated miR-21 was specific to DENV 2 not DENV 4 or Zika virus (ZIKV) infection in HepG2 cell. Moreover, upon antigomir-21 (AMO-21) transfection in HepG2 cells, the miR-21 was suppressed, resulting in decreasing of DENV 2 progeny.

Charpter II: Sitthichai Kanokudom and his co-authors continued studying the role of bhv1-miRNAs derived from bovine herpes virus 1 as previously reported by Glazov et al., 2010. Three out of ten bhv1-miRNAs was selected including bhv1-miR-B6, bhv1-miR-B8 and bhv1-miR-B9 due to the complementary sequence analysis and northern blot analysis (Glazov et al., 2010). In this work, the bhv1-miRNA expressing plasmid of each candidate were constructed and performed transient transfection into bovine cell (Madin-Darby Bovine Kidney epithelial cells, MDBK). The results showed that the transient expression of bhv1-miR-B8 and bhv1-miR-B9 had partial inhibition of BoHV-1 transcript and protein in MDBK cells. In conclusion, these two miRNAs are implicated for a role in transcription and translation of BoHV-1. The miRNA based strategy may be used as a tool to control viral replication.

Chapter III: This research constructed a recombinant *Escherichia coli* which was able to express the protein named *Penaeus monodon* Yellow Head Virus receptor protein (PmYRP65). The production of recombinant protein was optimized and scaled-up for an *in vitro* neutralizing assay and following up the mortality of shrimp. This study directly incubated the YHV with recombinant PmYRP65 prior injection to shrimp. The result indicated that recombinant PmYRP65 had neutralizing activity against YHV particle affecting to lessen the viral infection in shrimp and leading to the reduction of shrimp mortality after YHV challenge. This study showed recombinant PmYRP65 is potent to neutralize YHV infection in shrimp.

1.1. Background and a significance of the study

Recently, viral infectious disease is one of the serious problems in health and economy in the world. Virus could infect an organism and cause asymptom (vector/ carrier) to a range of mild to severe diseases or until causing death in that organism. Therefore, this thesis focuses on three viral pathogens including dengue virus, bovine herpesvirus and yellow head virus, which are below described.

DENGUE VIRUS

Dengue virus (DENV) is an enveloped virus, belonging to the *Flaviviridae* family and contains a 11 kb positive-sense single stranded RNA as the genetic material (Fig. 1.1). The viral genome is translated into ten proteins including viral non-structural and structural proteins (Fig 1.2). They are important for biological properties of dengue viruses during its infection and replication. Moreover, DENV has been classified by the antigenicity which can be divided into four serotypes; serotype-1, serotype-2, serotype-3 and serotype-4. DENV is capable of transmitting to humans by mosquitoes, especially *Aedes aegypti.*

Dengue virus is a cause of hemorrhagic fever which affects the public health concern. In general, the infection causes asymptomatic disease (Runge-Ranzinger et al., 2014). However, the DENV infection can lead to a range of symptom from a mild fever to more severe diseases such as hemorrhagic fever and dengue shock syndrome (WHO, 2009). Primary infection results in the activation of adaptive immune responses. It could lead to more severity in secondary infection with heterotypic DENV infection. In secondary infection with a heterotypic dengue virus, the antibodies from the first infection are unable to neutralize the virus but trigger a process termed antibody dependent enhancement (ADE) of infection. In ADE, complexes of virus and antibody are internalized to monocytes through the Fc receptor. This process is believed to cause the significant increase of infected cells, resulting in markedly severe diseases, termed dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) where plasma loss occurs (Guzman et al., 2010). The number of the people infected with dengue virus has continuously increased, reaching approximately 390 million each year (Bhatt et al., 2013).

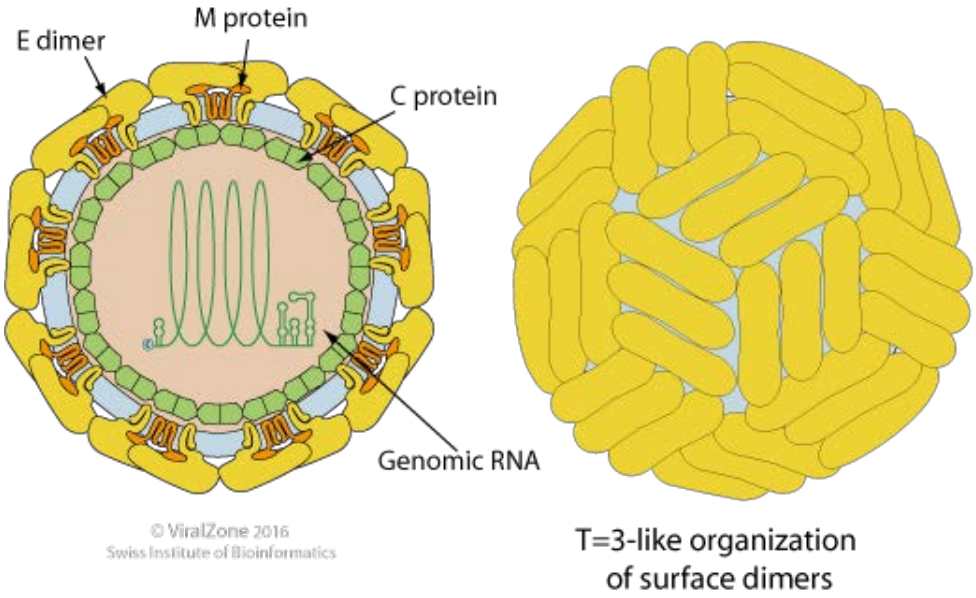

Figure 1.1. Schematic structure of *Flavivirus*. *Flavivirus* is an enveloped virus with a spherical shape (https://viralzone.expasy.org/24, last access in 19 December 2018).

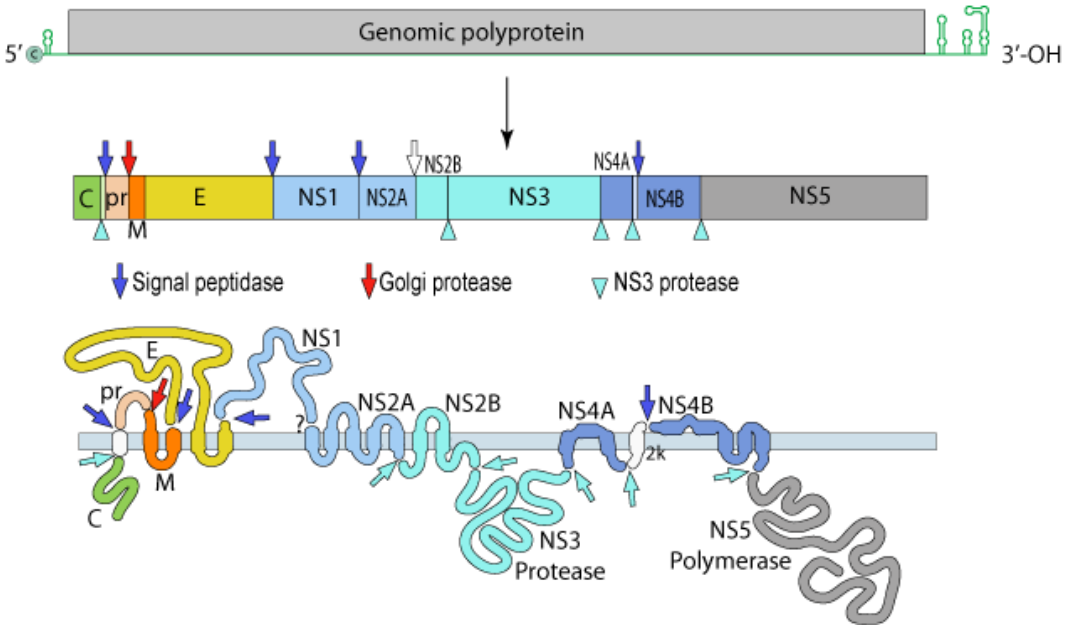

Figure 1.2. Genome organization of *Flavivirus*. The viral genomic RNA serves as a viral messenger RNA and can be translated in a polyprotein which is processed by post-translation modification using host and viral enzymes to be eleven proteins (https://viralzone.expasy.org/24, last access in 19 December 2018).

BOVINE HERPES VIRUS

Bovine herpes virus 1 (BoHV-1) is an enveloped virus that is classified in the *Varicellovirus* genus, the *Herpesviridae* family. BoHV-1 contains a 135 kb double-stranded DNA as its genome (Fig 1.3). The viral genome is predicted to have approximately 73 open reading frames (ORFs) (Fig. 1.4). These ORFs are known to encode for 33 structural and up to 15 non-structural proteins (Muylkens et al., 2007). Based on restriction enzyme digesting analysis and clinical sign observation, bovine herpes virus can be categorized into subtypes 1.1, 1.2a, 1.2b and 1.3 (Muylkens et al., 2007). BoHV-1 is the causative agent of three major syndromes are named as infectious bovine rhinotracheitis (IBR), infectious pustular vulvovaginitis (IPV) and infectious pustular balanoposthitis (IPB). Typical presenting signs include high fever leading to abnormality life threatening and reducing rate of milk production (Yates, 1982). Moreover, it can cause a variety of symptoms in cattle with high-rate abortion, infertility, conjunctivitis and encephalitis (Yates, 1982; Jones and Chowdhury, 2007). BoHV-1 can cause high severity in younger calves resulting to the high rate of fatality in new born calves. Since they survive from acute infection, it may suffer from the immunosuppression apart from latent infection (Yates, 1982). All of these symptoms lead to the significant reduction in quality and quantity of products from livestock farm industries worldwide (Miles, 2009).

Common killed and live-attenuated vaccines were wildly used to treat in cattle; however, it was still unsuccessful to long-lasting control the BoHV-1 infectious diseases. A variety of conventional vaccine was later developed, it was generally induce humoral immune responses but failure to protect animal from the infection due to weak cell-mediated immune responses (Turin et al., 1999; van Drunen Littel-van den Hurk, 2006).

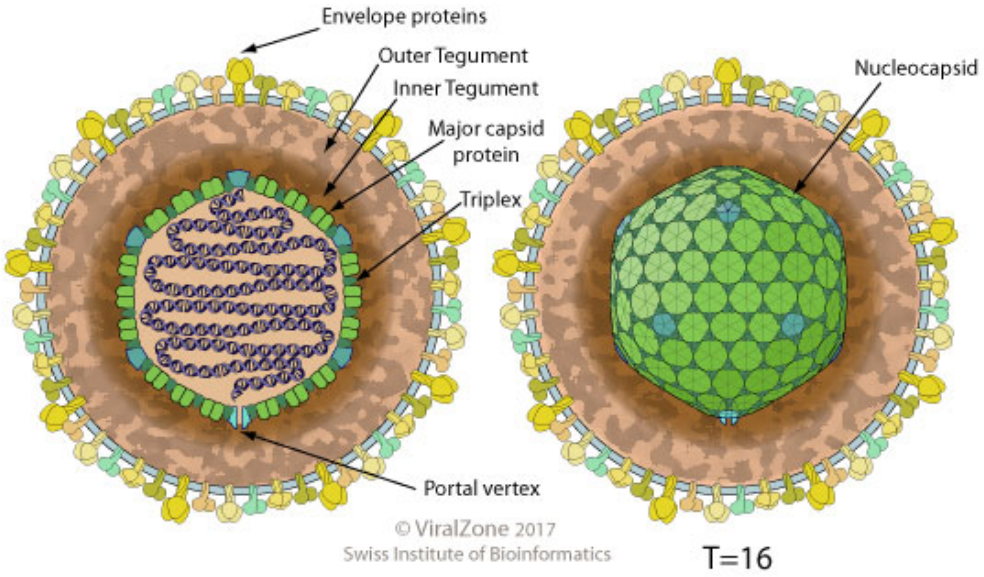

Figure 1.3. Schematic structure of *Varicellovirus*. *Varicellovirus* is an enveloped virus with a spherical to pleomorphic shape. The virion size is 150-200 nm in diameter (https://viralzone.expasy.org/179, last access in 19 December 2018).

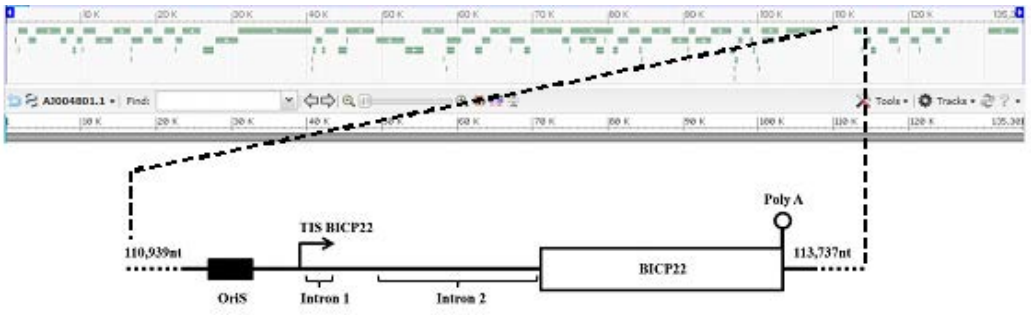

Figure 1.4. Genome organization of BoHV-1 (AJ004801). The BoHV-1 contains 135 kb dsDNA as genetic material. The selected segment contains Origin of replication (OriS) as transcriptional active area and transcription regulator BICP22 which were focused in this study (chapter II) (This picture of BoHV-1 genome was modified from GeneBank Database and Kanokudom et al., 2018a).

YELOW HEAD VIRUS

Yellow head virus (YHV) is an enveloped virus with a rod shape and is classified as a member of the genus *Okavirus,* Family *Roniviridae* and Order *Nidovirales* (Jitrapakdee et al., 2003; Sittidilokratna et al., 2002) (Fig. 1.5). The YHV particle contains a positive sense single stranded RNA as genetic material. The viral genome can act as a viral mRNAs for encoding their proteins and act as template for replication itself. Additionally, the viral genome can also be transcribed to subgenomic RNAs which can be translated to be viral structural and non-structural proteins (Fig 1.6). The sequence analysis and genome organization indicate that it is closely related to gill-associated virus (GAV) which causes shrimp disease in Australia (Cowley et al., 2000).

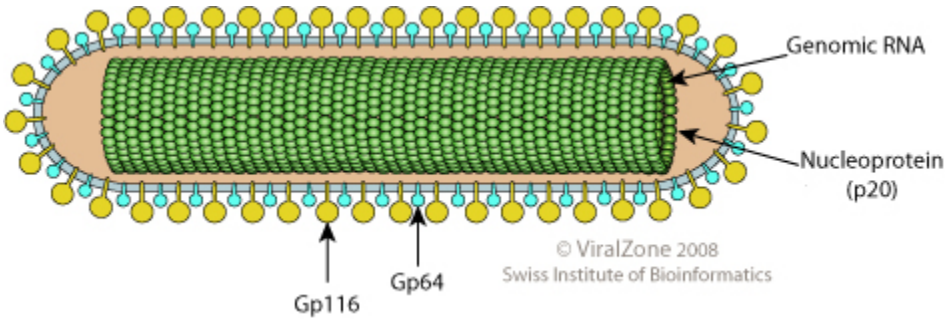

Figure 1.5. Schematic structure of *Okavirus*. *Okavirus* is an enveloped virus with rod shape. The virus size is 150-200 × 40-60 nm in diameter. (https://viralzone.expasy.org/290, last access in 19 December 2018).

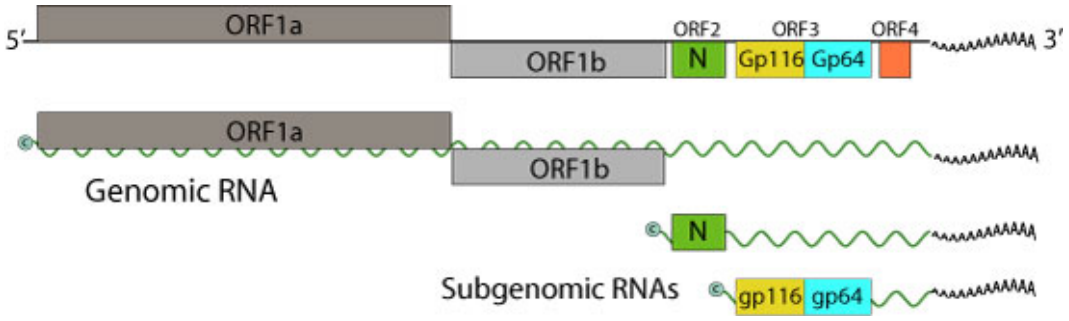

Figure 1.6. Genome organization of *Okavirus.* The viral genomic RNA serves as a viral messenger RNA and can be transcribed as subgenomic RNAs which are translated to be structural and non-structural proteins of virus (https://viralzone.expasy.org/290, last access in 19 December 2018).

Yellow head virus is considered as a devastating pathogen in black tiger shrimp (*Penaeus monodon*) farming which was first pandemic since 1990 in Thailand and can cause yellow head disease (YHD). It is virulent and can infect various penaeid shrimp including *P. monodon* (Boonyaratpalin et al., 1993), *P. aztecus, P. duorarum, P. merguiensis, and P. setiferus* (Flegel et al., 1997; Lightner et al., 1998) as well as *P. stylirostris and P. vannamei* (Lu et al., 1994; De la Rosa-Velez 2006). YHV can infect both endodermal and mesodermal cells following the apparent clinical signs within a few days (Boonyaratpalin et al., 1993). Upon YHV infection, the infected shrimp will have abnormal symptoms such as swimming near the surface of pond edges, reduction in food consumption, a yellowish cephalothorax and followed by high rate of mortality within 2-4 days. Therefore, YHV can cause massive economic loss in the shrimp farm industries in Southeast Asia. The primary target cells of YHV are gill tissue, and Oka (lymphoid) cell and YHV can infect in a variety of shrimp tissues including brain, nerve cord, hepatopancreas, ovary, abdominal muscle and hemocyte (Boonyaratpalin et al., 1993).

Viral diseases are major threats toward human and veterinary public health by causing diseases and suffering economic loss. This thesis proposes three different strategies to inhibit viral infection and to prevent further viral invasion as following described.

1) miRNA-induced RNA interference

MicroRNAs (miRNAs) are small single-stranded non-coding RNAs of 21-23 nucleotides in length. miRNA can complimentarily bind to the target RNA resulting in regulation of protein expression (Felekkis and Deltas, 2006). Briefly, the long-stem primary miRNA (pri-miRNA), which is transcribed by RNA polymerase II, is processed by Drosha to yield a 70–80 nucleotide hairpin precursor of miRNA (pre-miRNA) in the nucleus. The pre-miRNA hairpin with 2-nt 3' overhang recognized by exportin 5 (Exp5) and will be transported to the cytoplasm (Yi et al., 2003; Lund et al., 2004). Further, pre-miRNA is cleaved by DICER-1 and subsequently recognized by Argonaute protein to form miRNA-RNA Induced Silencing Complexes (miR-RISCs) (Lee et al., 2006; MacRae et al., 2008). The mature single stranded miRNA in RISC (Fig. 1.7) will locate to complementary mRNA targets, resulting in gene silencing by interfering with the translational process or mRNA degradation using the RNase III activity in RISC (Hammond et al., 2000). In addition, miR-RISC complex can interact with complementary target in DNA sequence in a gene promoter (Zhang et al., 2014) as well as viral RNA (Israelow et al., 2014; Kanokudom et al., 2017; Mortimer and Doudna, 2013) in order to modulate gene expression (Felekkis and Deltas, 2006; Harfe, 2005).

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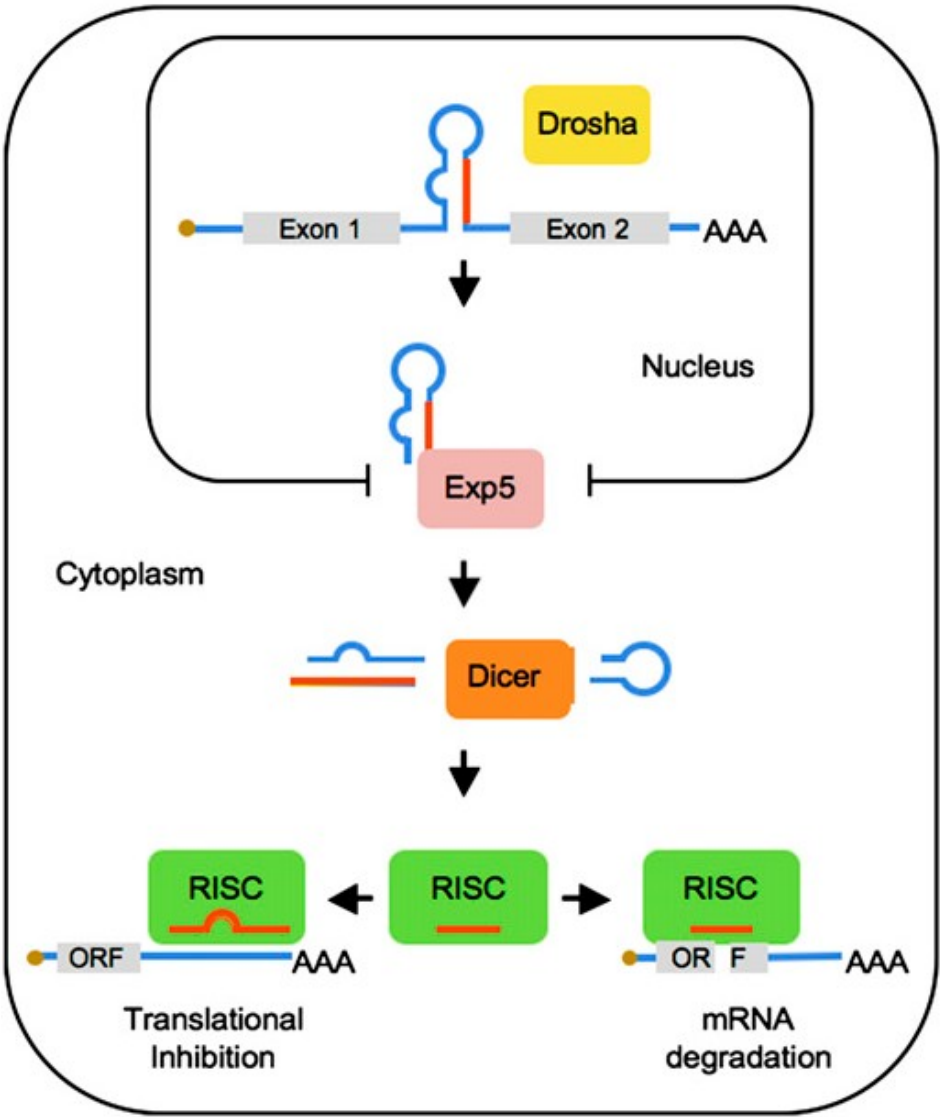

Figure 1.7. Schematic miRNA biogenesis and RNAi pathway (Umbach and Cullen, 2009).

Currently, many reports showed that viruses could encod their miRNAs, which influence to their and host biological processes (Kincaid et al., 2012). Viral encoded miRNAs could divide in to two types which are host analog miRNAs and their viral specific miRNAs. In the term of host, analog miRNAs refer to the viral encoded miRNAs, which normally has seed sequence identical with host miRNAs. The analog has important role to bind with RISC and complement to the same target transcripts (Kincaid et al., 2012). Another type are viral specific miRNAs are recently discovered and lacking to understand their function. Many researchers hypothesize that those viral specific miRNAs are important for viral prolonging longevity in host cells, evading immune response and/or regulation their replicating cycle.

At present, many reports show miRNA has a crucial role in viral replication and could be used to diminish the viral replication. Numerous evidences have suggested that miRNA could be developed as an antivirus agent for viral inhibition such as Hepatitis C virus (HCV) (Chen et al., 2013; Israelow et al., 2014; Mortimer and Doudna, 2013), Japanese encephalitis virus (JEV) (Thounaojam et al., 2014), Influenza A virus (Ingle et al., 2015) and DENV (Wu et al., 2013).

2) Recombinant viral receptor protein

Nowadays, the recombinant protein technology has been used to produce desired protein in many applications. This technology can produce high amount of protein which is inexpensive and easy to control for quality (Rosano and Ceccarelli, 2014). To obtain the recombinant protein, specific DNA or RNA template, which encodes desired protein, has to be cloned into an expression system such as *Escherichia coli,* yeasts, algae, insect cell or mammalian cell (Demain and Vaishnav, 2009; Sanchez-Garcia et al., 2016). The *E. coli* expression system is the most commonly used as a cell factory for the production of recobinant proteins due to their beneficial characteristics including fast growth rate, easy manipulation and cost-effectiveness (Rosano and Ceccarelli, 2014; Baeshen et al., 2015). In recent years, recombinant protein technology has been used in biopharmaceutical product development (Wurm, 2004; Sanchez-Garcia et al., 2016).

Many viral pathogens require an interaction between surface molecules at the first step of infection (Grove and Marsh, 2011). A viral receptor is normally defined as a cell surface molecule involved in viral entry and triggering the recoginition of immune reponses (Fig. 1.8).

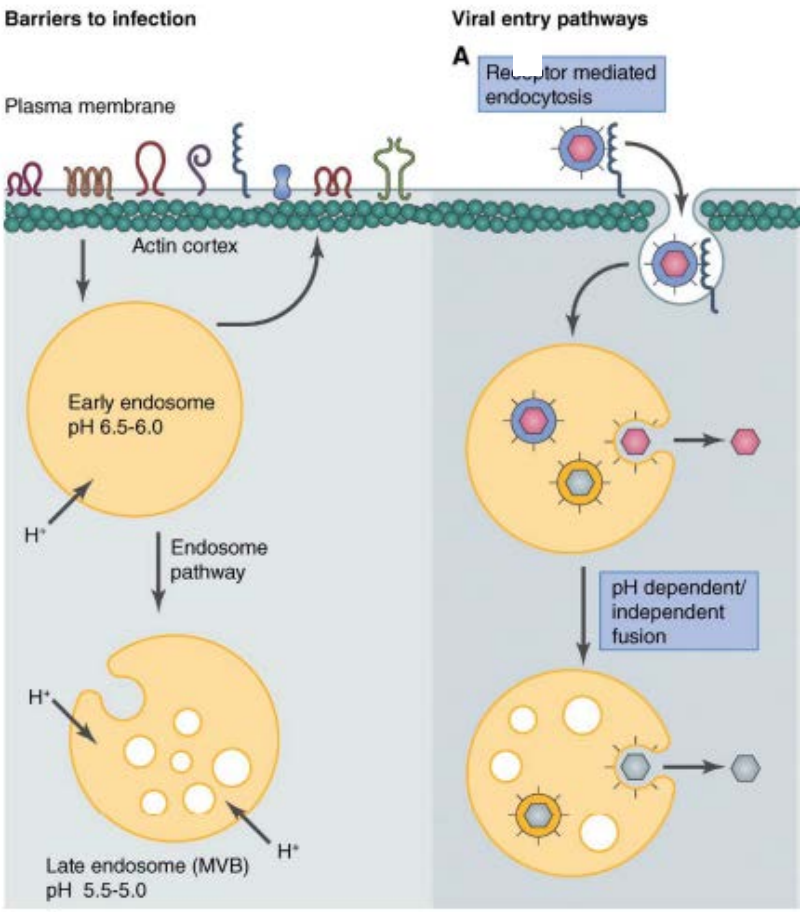

Figure 1.8. Viral entry strategies. The host cell composes barrier such as plasma membrane to restict viral entry. However, some certain enveloped virus can infect to host cell through receptor mediated endocytosis (Grove and Marsh, 2011).

Several previous studies have suggested the receptor could serve as a targets for viral infection. For examples, Mxra8, a cell adhesion molecule, involved in the entry of chukungunya virus (Zhang et al., 2018) and the transmembrane receptor protein AXL was required for infection of Zika virus (Persaud et al., 2018) as well as laminin receptor (Lamr) for White spot syndrome virus (WSSV) (Liu et al., 2016), Taura syndrome virus (TSV) and YHV (Busayarat et al., 2011). Herein, this study using recombinant protein technology overproduced a recombinant receptor to inhbibit YHV infection as shown in the previous study of recombinant PmLamr protein that could decrease shrimp mortality in a YHV challenge experiment (Busayarat et al., 2011) as well as delay WSSV infection in shrimp.

1.2. Purposes of the study

Chapter I: Due to a limit study of miRNA profiles in dengue-infected cells, this study aimed to identify miRNAs related to dengue viral replication and to verify the roles of selected miRNA (miR-21) in dengue viral replication. Moreover, a synthetic small complementary RNA was used to lessen the beneficial miRNA, affecting to the depletion of viral replication.

Chapter II: This study was carried by transient expression of three selected bhv1-miRNAs, which were bhv1-miR-B6, bhv1-miR-B8, bhv1-miR-B9, in MDBK cell and followed by BoHV-1 infection. The objective was to investigate the role of these three bhv1-miRNAs during BoHV-1 replication.

Chapter III: The recombinant protein technology was used to produce PmYRP65. The receptor gene was cloned and expressed in *E. coli* expression system. The recombinant PmYRP65 was subsequently purified and characterized. Thus, the purpose of this study was to examine the *in vitro* neutralizing activity of the receptor protein against YHV infection in shrimp.

1.3. Scope of the study

Investigation and development of new antiviral agents has been of interest in recent years. Although many antiviral agents have already been established and used as viral therapeutics, there are still issues to search for new sources, and there is no specific treatment for some viral diseases. This research focuses on different approaches to inhibit viral infection. The first topic is to identify DENV 2 induced miRNAs, both up- and down-regulated, and to further characterized the role of a beneficial miRNA (miR-21). The next topic is to construct recombinant plasmids to do transient expression of bhv1-miRNAs in bovine cell and investigated the role of bhv1-miRNAs on BoHV-1 replication. The last, the recombinant protein technology was used to produce viral receptor protein for YHV inhibition in shrimp.

1.4. Expected outcomes

The research focuses on the antiviral agents in different strategies based on miRNAs and recombinant viral receptor protein in different kinds of viruses. The works could be published in 3 international journals. The knowledge from this work could be applied to develop the effective antiviral agent against other viruses. Importantly, the knowledge of this study could motivate other researchers to investigate and develop new antiviral agents to face with other viral pathogens.

PART II

THE MANUSCRIPT OF DISSERTATION

Chapter I

miR-21 promotes dengue virus serotype 2 replication in HepG2 cells

Published in: Antiviral Research 2017. 142, 169-177.

Chapter II

Modulation of bovine herpesvirus-1 infection by virally encoded microRNAs

Published in: Virus Research 2018. 257, 1-6.

Chapter III

In vitro neutralization of yellow head virus infection in shrimp using recombinant PmYRP65 protein

Published in: Aquaculture 2018. 486, 266-270.

CHAPTER I

miR-21 promotes dengue virus serotype 2 replication in HepG2 cells

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Abstract

Infection with the mosquito transmitted dengue virus (DENV) remains a significant worldwide public health problem. While the majority of infections are asymptomatic, infection can result in a range of symptoms. MicroRNAs (miRNAs) are small non-coding RNAs that regulate gene expression through repression or degradation of mRNAs. To understand the contribution of miRNAs to DENV 2 replication, we screened a number of candidate miRNAs for variations in expression levels during DENV 2 infection of HepG2 (liver) cells. Seven miRNAs were identified as differentially expressed, and one, miR-21, was differentially expressed at all time points examined. Interestingly, miR-21 was also differentially regulated in DENV 2 infection under conditions of antibody dependent enhancement of infection, and in direct Zika virus infection, but not in DENV 4 infection. The role of miR-21 during DENV infection was further examined by treating HepG2 cells with an anti-miR-21 (AMO-21) before DENV infection. The results showed a significant reduction in DENV 2 production, clearly suggesting that miR-21 plays a key role in DENV 2 replication. To further confirm the role of miR-21 in DENV infection, a peptide nucleic acid-21 (PNA-21) construct with a nucleotide sequence complementary to AMO-21, was co-administered with AMO-21 as an AMO-21/PNA-21 complex followed by DENV 2 infection. The results showed that AMO-21 significantly reduced DENV 2 titer, PNA-21 significantly increased DENV 2 titer and the combined AMO-21/PNA-21 showed no difference from non-treated infection controls. Taken together, the results show that miR-21 promotes DENV 2 replication, and this mechanism could serve as a possible therapeutic intervention point.

Keywords: Dengue virus serotype 2 (DENV 2); HepG2; microRNA (miRNA); miRNA-21 (miR-21); Anti-miRNA-21 oligonucleotide (AMO-21); Peptide nucleic acid-21 (PNA-21)

1.1. Introduction

Despite the recent introduction of a vaccine to protect against dengue virus (DENV) infection in selected countries (WHO, 2016), there remains no specific treatment for DENV infection. While the majority of DENV infections are asymptomatic (Runge-Ranzinger et al., 2014), the infection can lead to a range of symptoms from a mild fever to the more severe forms of the disease, dengue hemorrhagic fever and dengue shock syndrome (WHO, 2009). During infection, DENV manipulates the host cell machinery to facilitate its own replication (Walsh and Mohr, 2011) and as such targeting these processes offer the potential for the development of therapeutic agents.

RNA interference (RNAi) is a process by which small, non coding RNA molecules regulate gene expression through either attenuation of mRNA transcription or by targeting mRNAs for degradation (Felekkis and Deltas, 2006). Currently, three classes of small RNAs associated with silencing pathways have been described in mammals, namely endogenous small interfering RNAs (endo-siRNAs), piwi-associated RNAs (piRNAs) and microRNAs (miRNAs). The first two classes of small RNAs are primarily involved in the repression of transposons as well as the nucleic acid of viruses (Carmell et al., 2007; Farazi et al., 2008), while the last class of small RNAs in particular regulates cellular gene expression (Felekkis et al., 2010). miRNAs are generated through transcription to generate a primary miRNAs (pri-miRNA) that is composed of exonic and intronic regions (Borchert et al., 2006; Lee et al., 2004). The stem loop region of the pri-miRNA is processed by a complex of proteins to yield 70–80 nucleotide stem loop precursor miRNAs (pre-miRNA) (Han et al., 2004; Landthaler et al., 2004; Lee et al., 2003), which are further cleaved by DICER-1 and subsequently recognized by Argonaute protein to form miRNA-RNA Induced Silencing Complexes (miR-RISCs) (Lee et al., 2006; MacRae et al., 2008). The miR-RISC complex subsequently interacts with target sequences such as mRNA transcripts or DNA sequences in gene promoters, in order to modulate gene expression. miRNAs have been shown to modulate processes including cellular development, proliferation, apoptosis, the immune system and host-pathogen interactions (Bartel, 2004; Felekkis and Deltas, 2006; Harfe, 2005).

Studies have increasingly shown that miRNAs have functional roles during viral infection. For example, Hepatitis C virus (HCV) infection leads to the induction and extracellular release of miR-122 and miR-885-5p without altering intracellular levels, whereas miR-494 accumulates intracellularly (El-Diwany et al., 2015). Moreover, miR-373 was shown to be significantly upregulated in HCV-infected primary human hepatocytes and HCV-infected liver biopsy specimens, which induces the negative regulation of the type I IFN signaling pathway by suppressing JAK1 and IRF9 (Mukherjee et al., 2015). Interestingly, the knockdown of miR-373 could inhibit HCV replication by up-regulating interferon-stimulated gene expression (Mukherjee et al., 2015). In addition, the up-regulation of miR-146a by Japanese encephalitis virus (JEV) infection in human brain microglial cells contributes to the suppression of NF-κB activity and disruption of anti-viral JAK-STAT signaling which helps the virus to evade the cellular immune response (Sharma et al., 2015).

Although much attention has been paid to studying the interplay between miRNAs and viral infection, understanding the interaction of human miRNAs and dengue virus infection remains incomplete (Escalera-Cueto et al., 2015; Qi et al., 2013; Wu et al., 2013). Therefore, this work has investigated the differential expression of miRNAs during DENV 2 infection of HepG2 (liver) cells, with the candidate miRNAs being evaluated by RT-qPCR. While several miRNAs showed evidence of regulation during DENV infection, one miRNA, miR-21, showed differential expression at all time points examined. We further evaluated the role of miR-21 during DENV infection and established that miR-21 is a positive regulator of DENV 2 replication.

1.2. Materials and Methods

1.2.1. Cells and culture conditions

The human liver cancer cell line HepG2 (ATCC® HB-8065™) was cultured in Dulbecco's modified Eagle's medium-DMEM (HyClone™ Thermo Scientific, USA) with 10% heat-inactivated fetal bovine serum (FBS; Gibco^{TM} Invitrogen), at 37 °C, 5%CO_2. The human lung carcinoma epithelial cell line A549 (ATCC® CCL-185™) was cultured in MEM (HyClone™ Thermo Scientific, USA) with 5% FBS at 37 °C. The human monocytic cell line U937 (ATCC CRL-1593.2) was cultured in RPMI 1640 medium (RPMI; Gibco^{TM} Invitrogen) supplemented with 10% FBS. The mosquito cell C6/36 (ATCC® CRL-1660™) was cultured in MEM (HyClone™ Thermo Scientific, USA) with 10%FBS at 28 °C. The monkey kidney cell LLC-MK2 (ATCC® CCL-7™) was cultured in DMEM with 5% FBS at 37 °C, 5%CO_2. All media were supplemented with 100 units/ml of penicillin and 100 μg/ml of streptomycin (HyClone™ Thermo Scientific, USA).

1.2.2. Viruses

Dengue virus serotype 2 (DENV 2) strain 16681 and dengue virus serotype 4 (DENV 4) strain 1036 were propagated in C6/36 at a multiplicity of infection (MOI) of 1 at 28 °C for 6 days (Sakoonwatanyoo et al., 2006) after which the culture medium was centrifuged to remove cell debris and the supernatants were stored as stock virus at −80 °C. The viral titer was determined by plaque assay on LLC-MK2 cells as previously described (Panyasrivanit et al., 2011; Sithisarn et al., 2003). Zika virus (ZIKV) strain SV0010/15 used in this study was obtained from the Armed Forces Research Institute of Medical Sciences (AFRIMS) and The Department of Disease Control, Ministry of Public Health, Thailand. It was passaged 7 times through C6/36 cells.

1.2.3. Oligonucleotides

The anti-miRNA-21 oligonucleotide (AMO-21) employed in this study was modified at the 2′-OH position to generate 2′O-methylation in the ribose residue of RNA (Supplementary Table S2.1.1, Integrated DNA technologies, Coralville, IA). AMO-21 was resuspended in nuclease-free water to obtain a 10 μM final concentration stock which was stored at −80 °C until required.

Peptide nucleic acid-21 (PNA-21, Supplementary Table S2.1.1.) was synthesized as previously described (Vilaivan et al., 2011; Vilaivan, 2015) to obtain 15 nucleotides in length of which all the sequence was identical to the first 15 nucleotides of hsa-miR-21-5p (MIMAT0000076). It was further modified at the N-terminus with a fluorescent label (FAM) to assist detection by fluorescence microscopy. HPLC-purified PNA-21 was subsequently resuspended in nuclease-free water to obtain a 10 μM final concentration stock which was stored at −80 °C until required.

1.2.4. miR expression profile during DENV 2 replication

To investigate miR expression profiles, HepG2 cell were cultured and infected with DENV 2 as previously described (Suksanpaisan et al., 2009; Thepparit et al., 2004). Briefly, HepG2 cells were seeded into 6-well plates at a density of 10^6 cells/well and then cultured at 37 °C, 5%CO_2 overnight. Cells were mock infected or infected with DENV 2 at MOI of 10 for 2 h. Then, the culture medium was replaced with fresh medium and cells cultured for 6, 12, 24 and 48 h post infection (h.p.i.). At appropriate time points, all cells were harvested and total RNA extracted using the RiboZol™ RNA Extraction Reagent (AMRESCO, LCC., USA). RNA was quantified using a Nanodrop 2000 (Thermo Scientific™, USA) or a Quantity One (BIORAD Laboratory Inc., USA).

To determine miR expression, 150 ng of total RNA was used as the template for synthesizing cDNA with specific primers (Table S2.1.1) using RevertAid™ Premium Reverse Transcriptase (Thermo Fisher Scientific inc., USA) as described by others (Varkonyi-Gasic et al., 2007). miRNAs were amplified with specific primers (Table S2.1.1) by quantitative PCR (qPCR) using IQ™ SYBR Green Supermix (BIORAD Laboratory Inc., USA). The C_T values obtained from RT-qPCR were used to determine relative gene expression using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). All data was normalized against U6 small RNA. All nucleotide sequences of miRNAs are given in the supplementary information (Table S2.1.1).

To test the whether the miR-21 expression is specifically induced by DENV 2, two other viruses, DENV 4 and ZIKV were used to infect HepG2 cells at MOI of 20 and A549 cells at MOI of 2, respectively for 24 h following which total RNA was collected and analyzed for miR-21 using RT-qPCR as described above.

To determine whether miR-21 expression was specifically induced by DENV 2 under antibody-dependent enhancement (ADE) of infection, infection of U937 cells was undertaken as previously described (Klomporn et al., 2011). Briefly, DENV 2 (16681) at MOI 20 or DENV 4 at MOI 1 was mixed with a 1:200 final dilution of a pan-specific anti-dengue E protein monoclonal antibody (HB114) in RPMI medium and incubated at 4 °C for 1 h with constant agitation. Then, the virus-antibody complexes were added to 2×10^6 U937 cells in RPMI-1640 without FBS and incubated at 37 °C for 2 h with constant agitation. After incubation, the cells were resuspended to a final concentration of 3×10^5 cells/ml in RPMI-1640 supplemented with 10% FBS and further incubated at 37 °C 5%CO_2 until required. At 2 days post-infection, expression of miR-21 was determined by RT-qPCR as described above.

Table S2.1.1. Primer and oligonucleotide sequence in this study

No.	Name	Sequence (5′-3′)
1	Universal R	GTGCAGGGTCCGAGGT
2	U_6 RT	TATGGAACGCTTC
3	U_6 F	CTCGCTTCGGCAGCACA
4	U_6 R	ACGCTTCACGAATTTGCGTGTC
5	hsa-mir-21-5p RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTC GCACTGGATACGACTCAACA
6	hsa-mir-21-5p F	GCCCGTAGCTTATCAGACTGA
7	hsa-mir-106b-5p RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTC GCACTGGATACGACATCTGC
8	hsa-mir-106b-5p F	GGGGCTAAAGTGCTGACAGT
9	hsa-mir-128-3p RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTC GCACTGGATACGACAAAGAG
10	hsa-mir-128-3p F	CTCTATCACAGTGAACCGGT
11	hsa-mir-148a-3p RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTC GCACTGGATACGACACAAAG
12	hsa-mir-148a-3p F	CAAGGTCAGTGCACTACAGAA
13	hsa-mir-30b-5p RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTC GCACTGGATACGACAGCTGA
14	hsa-mir-30b-5p F	CTCCTTGTAAACATCCTACAC
15	hsa-mir-125a-5p RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTC GCACTGGATACGACTCACAG
16	hsa-mir-125a-5p F	CTTTTTCCCTGAGACCCTTTAAC
17	hsa-mir-146a-5p RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTC GCACTGGATACGACAACCCA
18	hsa-mir-146a-5p F	CCTTGTGAGAACTGAATTCCA

19	AMO-21	2′OMe UCAACAUCAGUCUGAUAAGCUA
20	PNA-21	FAM-O-*TAGCTTATCAGACTG*-LysNH_2
21	GAPDH_1057F	CCCACTCCTCCACCTTTGAC
22	GAPDH_1155R	TGTTGCTGTAGCCAAATTCGT
23	MY88_576F	TGAAGCAGCAGCAGGAGGAG
24	MYD88_684R	AGGGGGTCATCAAGTGTGGT
25	IRAK1_786F	ATGCTGTGAAGAGGCTGAAG
26	IRAK1_943R	GTACACCAGGCAGTAGAAGC

(Underline = sequence complement to miRNA, Italic = sequence similar to miRNA - sequence from mirbase.org)

1.2.5. Nucleic acid binding properties of PNA-21

To ensure the complementary binding of nucleic acid between PNA-21 and AMO-21, AMO-21 alone (A), PNA-21 alone (P) and AMO-21 mixed with PNA-21 (AP) were prepared in culture medium and were analyzed by a gel mobility shift assay using 10% polyacrylamide gel electrophoresis (Das et al., 2012). After electrophoresis, the gel was stained with ethidium bromide.

1.2.6. Repression of miR-21 expression by AMO-21

To suppress miR-21 in HepG2 cells, reverse transfections were undertaken using the DharmaFECT4 reagent (Thermo Fisher Scientific Inc., USA) according to the manufacturer's protocol. Briefly, the requisite oligonucleotides and 1 µl of DharmaFECT4 were prepared in 100 µl of DMEM. All transfections were undertaken in a final volume of 500 µl with either 100 nM of AMO-21(A), 200 nM of PNA-21(P) or mixture of AMO-21 and PNA-21 (AP). After a 40-min incubation at room temperature, transfection mixtures were added to a suspension of 1×10^5 HepG2 cells, and then the cell-complex mixtures were seeded to single wells of a 24 well-plate and cultured under standard conditions for 24 h. At 24 h post transfection, transfected HepG2 cells were harvested to extract total RNA and to determine the

amount of miR-21 using qPCR as described above. In a parallel experiment cytotoxicity of the synthetic oligonucleotides was investigated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assays.

1.2.7. Expression of MYD88 and IRAK1 transcripts after miR-21 repression

To investigate whether miR-21 promotes DENV replication directly or indirectly by suppressing the type I interferon response, the expression of myeloid differentiation primary response 88 (MYD88) and Interleukin-1 receptor-associated kinase 1 (IRAK1) was investigated by RT-qPCR. Briefly, 150 ng of total RNA from transfected HepG2 cell was converted to cDNA by using ReverseAid™ Reverse Transcriptase (Thermo Fisher Scientific inc., USA). One microliter of cDNA was used as template to quantitate by qPCR using gene-specific primers (Table S2.1.1). The C_T values were normalized with glyceraldehyde-3-phosphate dehydrogenase (GADPH) and determined as previously described.

1.2.8. Influence of AMO-21 on DENV 2 replication

Approximately 10^5 HepG2 cells were reverse-transfected with appropriate concentrations of transfection mixtures as described above. At 24 h post transfections, the transfected cells were infected with DENV 2 at MOI of 10 for 2 h. At 24 h.p.i., the supernatants were harvested for determination of the DENV titers by standard plaque assay as previously described (Panyasrivanit et al., 2011).

1.2.9. Fluorescent microscopy

HepG2 cells were either transfected or non-transfected with oligonucleotides as described above (Method 1.2.7). At 24 h post-transfection, cells were washed twice with PBS. Transfected HepG2 cells were fixed with 4% formaldehyde for 10 min and then washed twice with PBS. Cells were mounted with anti-fade Mowiol reagent (EMD Chemical Inc, Germany) and observed under a fluorescent microscope.

1.2.10. Statistical analysis

All analyses were performed using the GraphPad Prism program version 5.03 (GraphPadSoftware Inc., CA). Data were compared by Student's t-test or One-way ANOVA. The p-value < 0.05 (*); p-value < 0.01 (**); p-value < 0.001 (***) were considered to be statistically significant. Results are expressed as means ± SD from at least three independent replicates.

1.3. Results

1.3.1. Regulation of miRNAs in DENV 2 infected HepG2 cells

To identify miRNAs with a possible role in DENV infection, a panel of miRNAs selected based on prior knowledge (miRbase and literature search) were evaluated for amplification from HepG2 cells. A number of candidate miRNAs failed optimization for reasons including multiple bands (e.g. miR-30a, miR-122, miR-155, miR-149, miR-218, and miR-375a) and failure to amplify (e.g. miR-23b and miR-221). However, seven miRNAs, namely miR-21, miR-106b, miR-128, miR-148a, miR-30b, miR-125a and miR-146a all gave optimized amplification profiles. To investigate the expression profile of these miRNAs during DENV infection, HepG2 cells were infected with DENV 2 or mock infected and at 6, 12, 24 and 48 h.p.i. the expression levels of 7 miRNAs assessed by RT-qPCR. The result revealed that all seven miRNAs were differentially expressed during infection (Fig. 2.1.1). The first four miRNAs (miR-21, miR-106b, miR-128 and miR-148a) generally showed up-regulation at 24 h.p.i. (Fig. 2.1.1A), while the three remaining miRNA (miR-30b, miR-125a, and miR-146a) were generally down-regulated at 12 h.p.i. (Fig. 2.1.1B). Surprisingly, only one miRNA, miR-21, exhibited significant changes in terms of gene expression level at every time point examined (Fig. 2.1.1A). Therefore, miR-21 was selected for further investigation into its function during DENV 2 infection.

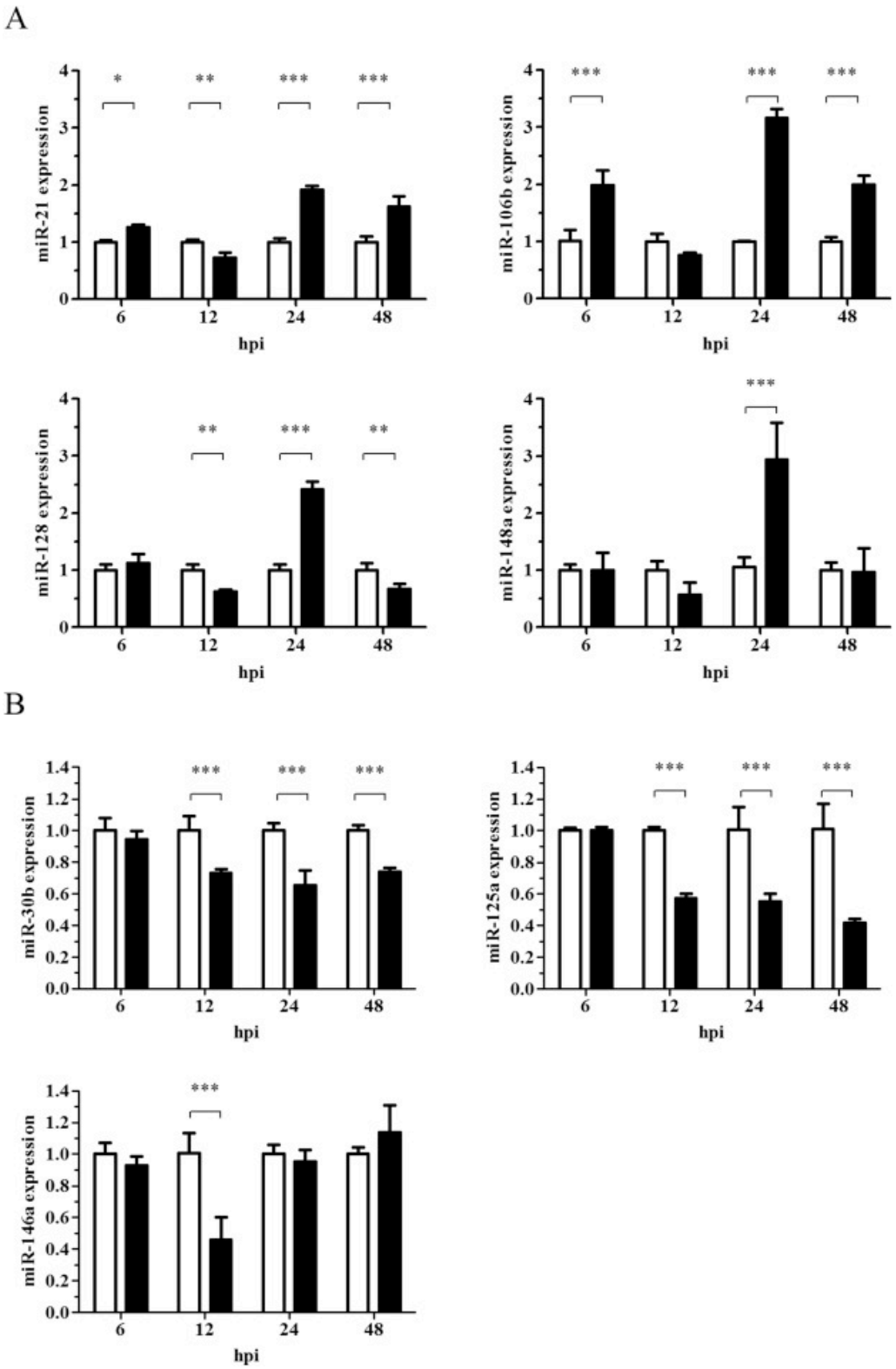

Figure 2.1.1. miRNA expression profile upon DENV 2 infection. HepG2 cells were infected with DENV 2 at MOI of 10 for 6, 12, 24 and 48 h, respectively (see below).

Figure 2.1.1. miRNA expression profile upon DENV 2 infection. HepG2 cells were infected with DENV 2 at MOI of 10 for 6, 12, 24 and 48 h, respectively. miRNA expression was quantitated by RT-qPCR. (A) miRNAs (miR-21, miR-106b, miR-128 and miR-148a) showing up regulation at 24 h.p.i and (B) miRNAs (miR-30b, miR-125a and miR-146a) showing down-regulation at 12 h.p.i. Experiments were undertaken independently in triplicate. Data are shown as means with SD. The *p*-value < 0.05 (*); *p*-value < 0.01 (**); p-value < 0.001 (***) were considered to be statistically significant.

1.3.2. Specificity of miR-21 up-regulation by DENV 2

To determine the specificity of the up-regulation of miR-21, total RNA of HepG2 cells infected with DENV 2, DENV 4 and ZIKV was isolated and used as a template for monitoring expression of miR-21. qPCR analysis showed that miR-21 was significantly up-regulated during DENV 2 infection (Fig. 2.1.2), consistent with the previous results (Fig. 2.1.1A). In ZIKV infection miR-21 was significantly up-regulated, albeit it to a lower extent than DENV 2, while no significant regulation of niR-21 was seen in DENV 4 infection (Fig. 2.1.2A).

To determine whether the specific up-regulation of miR-21 also occur under conditions of ADE infection, U937 cells were infected with DENV 2 and DENV 4 separately under a previously established protocol (Klomporn et al., 2011). Results showed that consistent with direct infection of HepG2 cells, miR-21 was significantly up-regulated in ADE mediated infection of U937 cells during DENV 2 infection, but not in DENV 4 infection (Fig. 2.1.2B).

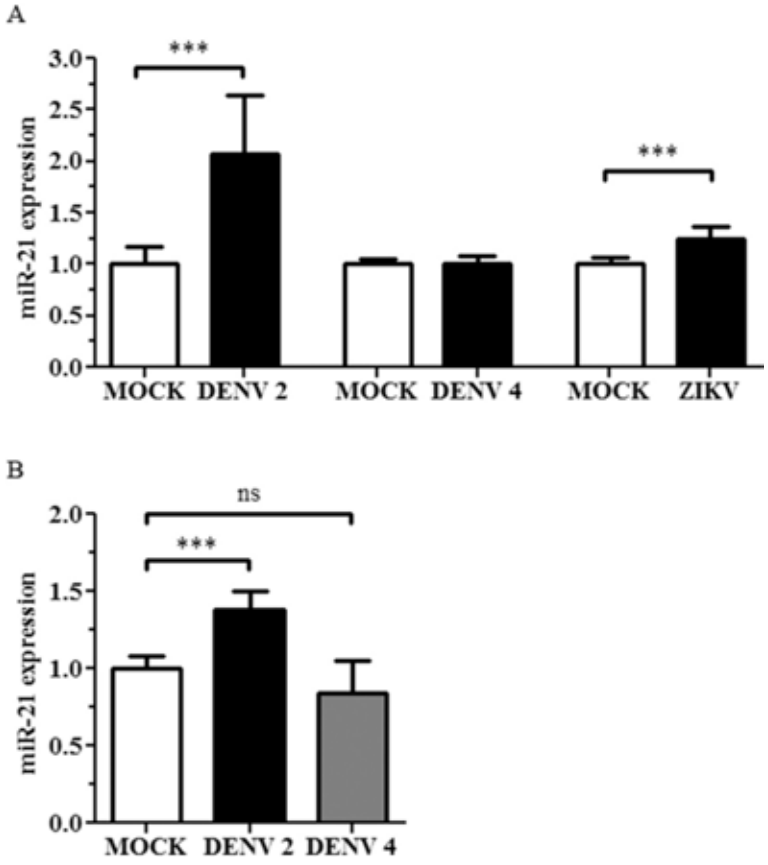

Figure 2.1.2. miR-21 expression profile in different viral infected cells. (A) HepG2 cells were infected with DENV 2 (MOI of 10) or DENV4 (MOI of 20) for 24 h, whereas A549 cells were infected ZIKV (MOI of 2) for 24 h. (B) U937 cells were infected with DENV 2 (MOI of 20) or DENV 4 (MOI of 1) under conditions of ADE of infection for 48 h. miR-21 expression was quantitated by RT-qPCR. Experiments were undertaken independently in triplicate. Data are shown as means with SD. The *p*-value < 0.05 (*); *p*-value < 0.01 (**); *p*-value < 0.001 (***) were considered to be statistically significant.

1.3.3. miR-21 promotes DENV 2 replication

To determine the role of miR-21 during DENV infection, we first established whether an anti-miR-21 oligonucleotide (AMO-21) with a nucleotide sequence complementary to the mature hsa-miR-21-5p was able to reduce expression of miR-21. HepG2 cells were therefore transfected with 50 and 100 nM of AMO-21, and the expression of miR-21 quantified at 24 h post-transfection by RT-qPCR. The results showed that transfection with AMO-21 significantly reduced expression of miR-21 by approximately half (Fig. 2.1.3A), showing that AMO-21 can suppress miR-21 expression. To analyze the expression of possible miR-21 target genes, MYD88 and IRAK1, the relative expression of MYD88 and IRAK1 was measured by using RT-qPCR. Although AMO-21 could suppress miR-21 (Fig. 2.1.3A), the MYD88 and IRAK1 transcripts were not significantly changed after transfection in HepG2 cells (Fig. 2.1.3B and C, respectively). These results indicated that the MYD88 and IRAK1 are not directly affected as a result of miR-21 repression.

In order to determine if miR-21 is functionally involved in DENV 2 replication, AMO-21-transfected HepG2 cell was infected with DENV 2 in parallel with mock transfected cells, and at 24 h.p.i., the culture media were collected to determine DENV 2 titer by standard plaque assay. As shown in Fig. 2.1.3D, viral titers showed a significant and dose-dependent decrease in response to infection in the presence of AMO-21 (Fig. 2.1.3D).

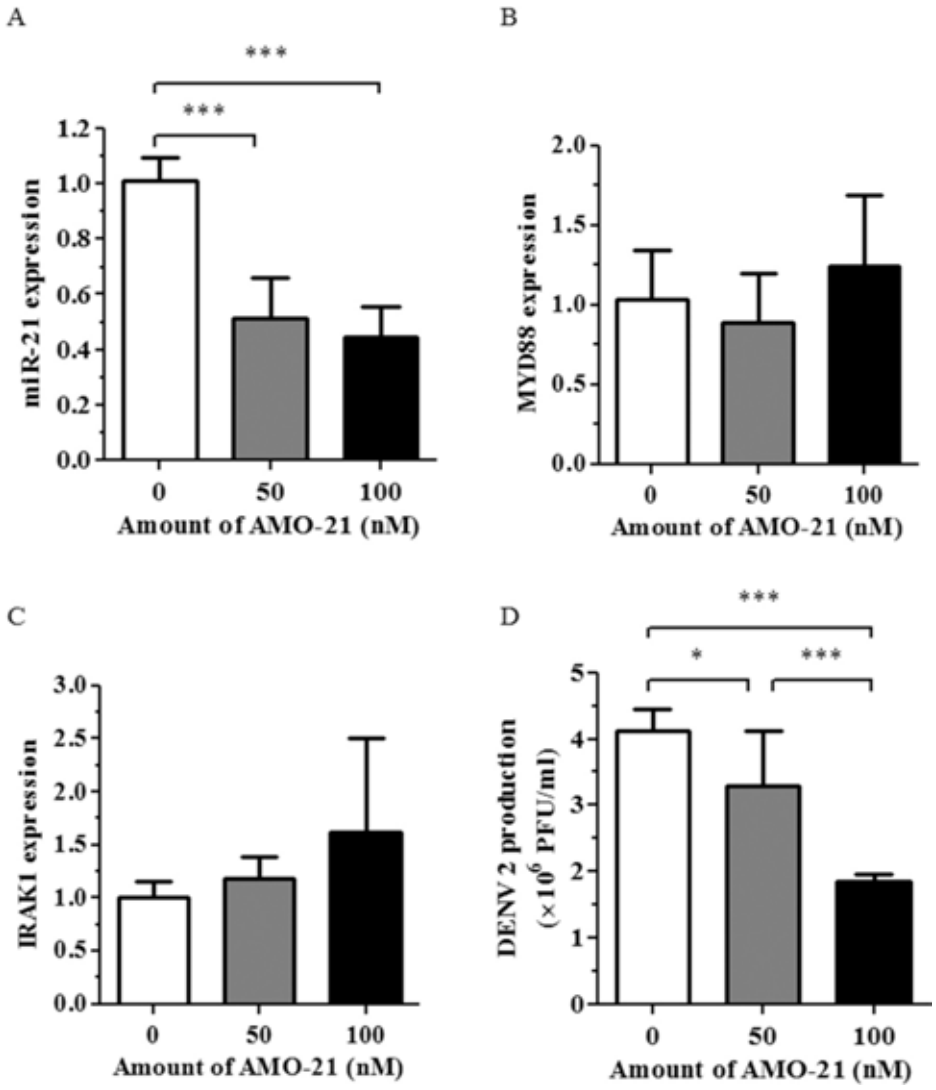

Figure 2.1.3. Inhibition of DENV 2 replication by AMO-21. HepG2 cells were reverse-transfected with AMO-21 (0, 50 and 100 nM) for 24 h before DENV 2 infection. (A) miR-21 expression, (B) MYD88 expression and (C) IRAK1 expression of transfected HepG2 cells were assessed by RT-qPCR. (D) DENV 2 production was assessed by plaque assay. Experiments were undertaken independently in triplicate with duplicate plaque assay. Data are shown as mean with SD. The p-value < 0.05 (*); p-value < 0.01 (**); p-value < 0.001 (***) were considered to be statistically significant.

1.3.4. Transfection of AMO-21, PNA-21, and AMO-21/PNA-21 in HepG2

Peptide nucleic acids (PNAs) are polynucleotides where the sugar-phosphate backbone has been substituted with electrostatically neutral peptide backbones (Nielsen, 2010). Many variants of PNA exists, and the PNA used in this work is the conformationally constrained pyrrolidinyl PNA with (2R,4R)-proline-2-aminocyclopentanecarboxylic acid backbone (acpcPNA) that reportedly shows stronger and more specific nucleic acid binding than the original PNA (Vilaivan and Srisuwannaket, 2006; Vilaivan, 2015). The peptide nucleic acid-21 (PNA-21) was synthesized to have a sequence complementary to AMO-21. To confirm that PNA-21 could hybridize with AMO-21, polyacrylamide gel electrophoresis (PAGE) was used to determine the mobility shift of the AMO-21/PNA-21 hybrid. As shown in Fig. 2.1.4, PNA-21 could specifically anneal to AMO-21 as shown by the mobility shift of the band (AP lane) at the position close to 50 bp. We next determined that the AMO-21/PNA-21 complex and PNA-21 per se can enter into HepG2 cell after transfection. As shown by fluorescent microscopy, specific internalization of AMO-21/PNA-21 and PNA-21 into HepG2 cells was observed, while no internalization of AMO-21 alone was seen (Fig. 2.1.5A). Additionally, there is no signal observed with either PNA-21 and AMO-21/PNA-21 in the absence of the transfection reagent (Fig. 2.1.5B). These results indicated that synthetic PNA-21 can bind to AMO-21 and that the resultant complex can enter into HepG2 cells after transfection.

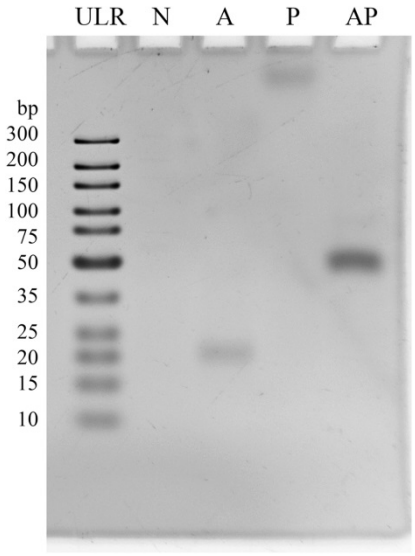

Figure 2.1.4. Nucleic acid binding property of PNA-21. Oligonucleotide medium which used for transfection in HepG2 cells was analyzed by PAGE, lane N is no oligonucleotide control, lane A is 100 nM of AMO-21, lane P is 200 nM of PNA-21 and lane AP is a mixture of AMO-21 and PNA-21.

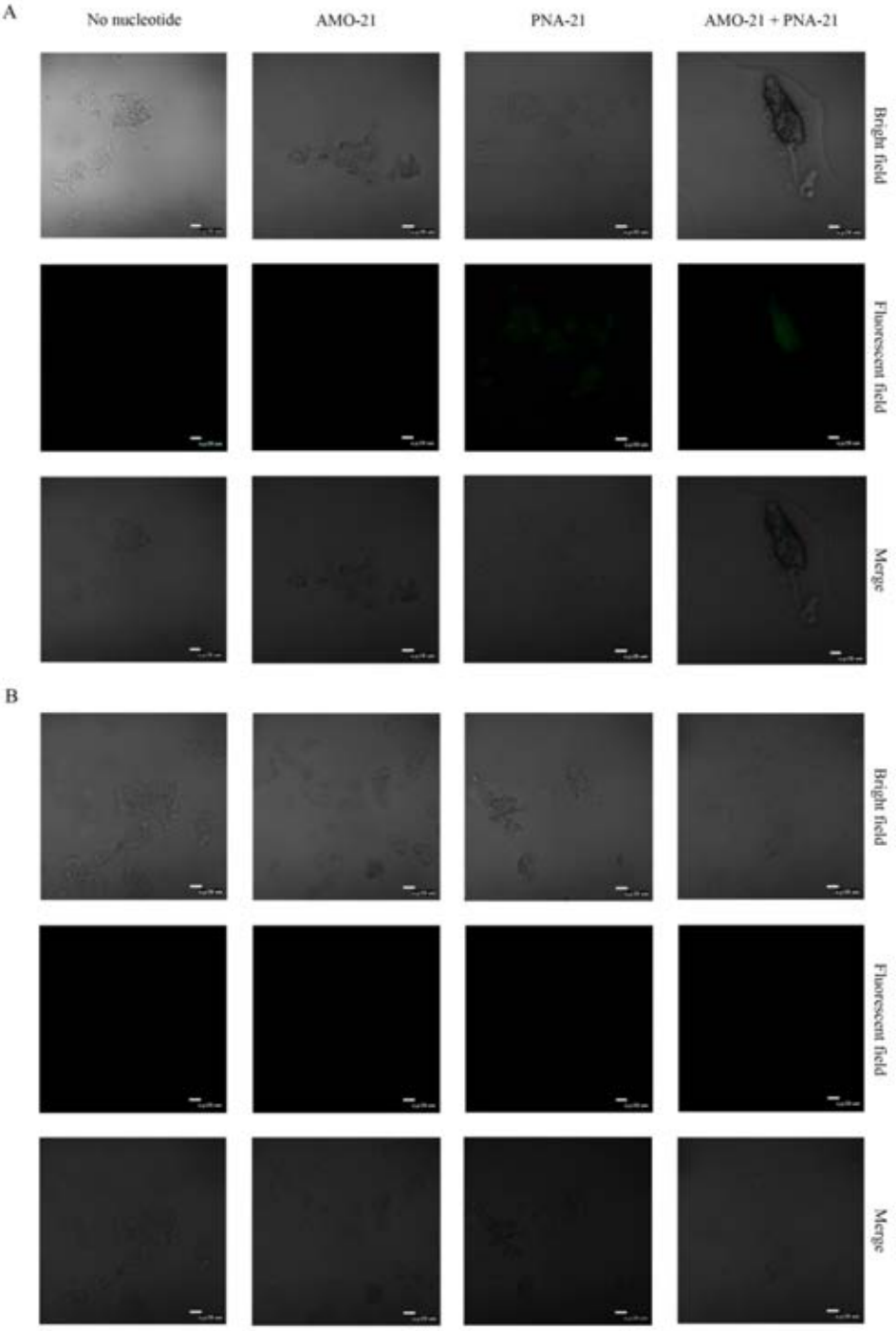

Figure 2.1.5. Fluorescence microscopy of transfected HepG2 cells. HepG2 cells were either transfected (A) or non-transfected (B) with no nucleotide, AMO-21, PNA-21 and AMO-21/PNA-21 complex and were subsequently observed under a fluorescence microscope. Representative images are shown.

1.3.5. Specific inhibition of AMO-21 by PNA-21 in the DENV 2 infected HepG2 cell

To rule out any possibility that the oligonucleotide and the peptide nucleic acids were deleterious to cells, AMO-21 (A), PNA-21 (P), and AMO-21/PNA-21 (AP) were transfected into HepG2 cells, which were then and assayed for cell cytotoxicity. The results showed that AMO-21, PNA-21, and AMO-21/PNA-21 did not exert significant cytotoxicity towards HepG2 cells (Fig. 2.1.6A). To determine if PNA-21 can specifically inhibit AMO-21, HepG2 cells were transfected with AMO-21, PNA-21, and AMO-21/PNA-21 separately. At 24 h post-transfection, cells were collected and levels of miR-21 quantitated by RT-qPCR. As shown in Fig. 2.1.6B, miR-21 expression in AMO-21 treated cells was significantly reduced, whereas miR-21 expression in PNA-21 treated cells was significantly increased. AMO-21/PNA-21 treated cells showed a slight, but non-significant increase in levels of miR-21 (Fig. 2.1.6B).

Finally, HepG2 cells transfected either with AMO-21, PNA-21 or AMO-21/PNA-21 were subsequently infected with DENV 2 at MOI 10. At 24 h.p.i., the culture media were collected and virus titer measured by standard plaque assay. Results (Fig. 2.1.6C) showed that AMO-21 treatment significantly reduced DENV titer to about 50%, consistent with the reduction in miR-21 expression (Fig. 2.1.6B), while PNA-21 significantly increased DENV titer, again consistent with the expression of miR-21. AMO-21/PNA-21 treatment had no significant effect on DENV titer, again consistent with the expression of miR-21 (Fig. 2.1.6).

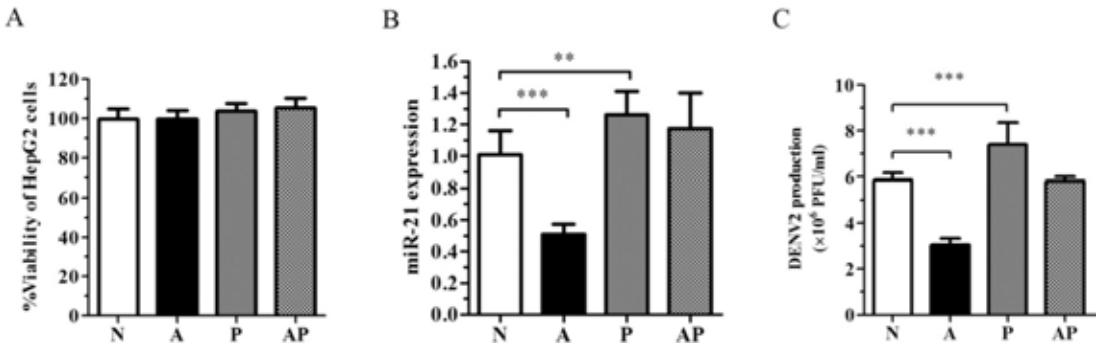

Figure 2.1.6. Influence of miR-21 and AMO-21 on DENV 2 production in HepG2 cells. HepG2 cells were mock transfected (test N), or transfected with 100 nM of AMO-21 (test A), 200 nM of PNA-21 (test P) and a mixture of AMO-21/PNA-21 (test AP), respectively. The percentage viability after treatment (A), miR-21 expression (B) and DENV 2 production (C) were determined. Experiments were undertaken independently in triplicate with duplicate plaque assay. Data are shown as mean with SD. The *p*-value < 0.05 (*); *p*-value < 0.01 (**); *p*-value < 0.001 (***) were considered to be statistically significant.

1.4. Discussion

Since their discovery in *C. elegans* in 1993 (Lee et al., 1993), miRNAs have been shown to regulate a number of biological processes, and more than 2000 human miRNAs have been identified (Friedlander et al., 2014). miRNAs are known to exert their effects through regulation of mRNA translation (Felekkis et al., 2010) as well as by interacting with mRNA binding proteins (Ciafrè and Galardi, 2013) resulting in the modulation of numerous biological processes (Felekkis et al., 2010; Harfe, 2005).

The role of miRNAs in DENV infection remains poorly described. Previous studies have shown contradictory mechanisms, with specific miRNAs either enhancing (Wu et al., 2013) or suppressing (Zhu et al., 2014) DENV replication, and expression of specific miRNAs may be either increased (Qi et al., 2013; Wu et al., 2013; Zhu et al., 2014) or decreased (Castillo et al., 2016; Qi et al., 2013) during DENV infection. miRNAs that have been shown to suppress DENV replication include Let-7c (Escalera-Cueto et al., 2015) and miR-30e* (Zhu et al., 2014) which are both up-regulated during DENV infection whereas miR-133a (Castillo et al.,

2016) and miR-233 (Wu et al., 2014) are decreased during DENV infection. miR-146a has been shown to be increased in DENV infection and expression facilitates DENV replication (Wu et al., 2013).

In this study we have shown that miR-21 is significantly increased upon DENV 2 infection and that expression of this miRNA promotes DENV replication, similar to the effect of miR-146a (Wu et al., 2013). Despite the limited number of studies of the role of miR-21 in terms of viral infection, our results are in congruence with the results of others as miR-21 was found to be up-regulated in serum from patient infected with DENV 1 (Ouyang et al., 2016). Interestingly, miR-21 was shown to be significantly increased in ZIKV infection, but not in DENV 4 infection. Moreover, miR-21 was also shown to be significantly up-regulated in ADE mediated infection of monocytic U937 cells by DENV 2, but not by DENV 4. This would suggest that miR-21 has a broad involvement in flaviviral infections, but that this can be modulated by virus and strain type factors. Particularly for DENV the specific up-regulation of miR-21 in both primary and secondary DENV 2 infection, but not for DENV 4 under any conditions, may help explain the observed variations in pathogenicity seen between different DENVs (Rico-Hesse et al., 1997).

Recent studies have also shown that higher expression of miR-21 helped HCV mute the innate immune response by modulating IFN-α through inhibition of MYD88 and IRAK1, resulting in increased levels of the virus in the human hepatoma cell line Huh7 (Chen et al., 2013). In contrast, in our study, MYD88 and IRAK1 were not regulated by the repression of miR-21 via transfected AMO-21 in HepG2 cells, suggesting that this pathway is not the primary way in which miR-21 regulates DENV 2 replication in HepG2 cells. In addition to a role in HCV infection, miR-21 levels were significantly increased after infection by EBV, and miR-21 was responsible for facilitating EBV replication through enhancing pAKT expression (Anastasiadou et al., 2015; Rosato et al., 2012).

Previous studies have shown that miRNAs may modulate DENV infection through either directly targeting the DENV genome (Escalera-Cueto et al., 2015) or by targeting innate immune response genes (Chen et al., 2014; Wu et al., 2013; Zhu et al., 2014). While the mechanism of how miR-21 enhances DENV replication remain unknown, it is possible that miR-21 may directly target NS1 sequences in the DENV 2 genome (Miranda et al., 2006) and Table S2.1.2. Interestingly, this particular region of the DENV 2 genome showed 100% conservation in more than 100 DENV 2 isolates when subjected to a BLAST search as of August 2016, reflecting the strong conservation of these sequences. The importance of miRNAs binding to the viral genome and enhancing replication has been described for other viruses. For example, the binding of miR-122 to the 5′ region of the HCV genome promotes HCV genome stability and replication (Israelow et al., 2014; Mortimer and Doudna, 2013). In addition, miR-485 binds to the genomic IAV-PB1 of influenza virus in order to promote viral replication (Ingle et al., 2015). Interestingly, we observed that the miR-21 expression level was not affected by a 2 fold increase in the dose of AMO-21 (Fig. 2.1.3A), although there was a 50% decrease in the titer of DENV 2 (Fig. 2.1.3D), suggesting that AMO-21 could directly affect DENV 2 replication, and additionally given that there is an increase of miR-21 in response to transfection with PNA-21, it is possible that there is an endogenous inhibitor, such as an anti-miR-21 mediating some of the effect.

Table S2.1.2. Interactive prediction

miR Name	Target name	First position of predicted target site	Folding energy (in -Kcal/mol)	Heteroduplex	*p* value
miR-21	DENV2_16881	2733	-12.10	GCGGCCUCAGCCCACUGAGCUG AGUUGUAGUCAGACUAUUCGAU	0.0276

In summary, we have shown that miR-21 mediates DENV 2 infection by enhancing DENV replication. Knock down of miR-21 with AMO-21 reduced virus replication, suggesting that AMO-21 is a promising antiviral therapeutic agent against DENV infection.

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In charpter I showed that many host miRNAs expression were up- or down-regulated during viral replication and some influenced on the replication of virus during demonstration with pre-treatment of antigomir. Next, we would also investigate an effect of virally derived miRNAs on their replication. The virally encoded miRNA cassettes were performed as a tool the evaluated the function or effect of the virally derived miRNAs on viral replication as shown in chapter II.

CHAPTER II

Modulation of bovine herpesvirus 1 infection by virally encoded microRNAs

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Abstract

Bovine herpesvirus 1 (BoHV-1), is a member of the subfamily *Alphaherpesvirinae* in the order *Herpesviridae* and is a ubiquitous pathogen of cattle responsible for significant economic loss worldwide. The BoHV-1 genome encodes at least 10 BoHV-1 microRNA (miRNA) genes, whose functions remain poorly understood. This study sought to understand the role of three BoHV-1 miRNA genes, Bhv1-miR-B6, Bhv1-miR-B8 and Bhv1-miR-B9, which are located proximal to the BoHV-1 origins of replication (*OriS*). Therefore, plasmids expressing the precursor miRNA hairpins for the Bhv1-miR-B6, Bhv1-miR-B8, and Bhv1-miR-B9 genes were constructed and transfected into Madin-Darby bovine kidney cells prior to BoHV-1 infection. Interestingly, transient expression of either Bhv1-miR-B8 or Bhv1-miR-B9 in Madin-Darby bovine kidney cells prior to infection resulted in partial suppression of BoHV-1 replication, quantified through estimating levels of glycoprotein C mRNA and protein levels. Putative interactions between the mature miRNA bhv1-miR-B8-3p and bhv1-miR-B9 and BoHV-1 transcripts were identified providing plausible pathways for these molecules to affect virus replication. Therefore, these two miRNAs are implicated in the post-transcriptional regulation of BoHV-1 transcripts important for virus replication and could be used to limit BoHV-1 replication.

Keywords: Bovine herpesvirus 1 (BoHV-1), microRNA, virus replication, transfection

MicroRNAs (miRNAs) are small noncoding RNA molecules which range from 19 to 24 nucleotides in length. The biogenesis of miRNAs initiates with nuclear transcription of primary miRNA (pri-miRNA) transcripts (Borchert et al., 2006). Subsequently the stem-loop pri-miRNAs are processed by a complex of Drosha-RNaseIII and DGCR8 to generate precursor miRNAs (pre-miRNA) (Denli et al., 2004; Gregory et al., 2004; Lee et al., 2003). The pre-miRNAs are subsequently transported by exportin 5 to the cytoplasm where the terminal hairpin loop is cleaved by dicer to generate duplex miRNAs containing a two nucleotide overhang at the 3′ terminus (Yi et al., 2003). One or both strands of the duplex miRNAs are subsequently recruited by argonaute protein, leading to formation of miRNA-RNA induced silencing complexes (Lee et al., 2006; MacRae et al., 2008), which then function as post-transcriptional regulators (Felekkis et al., 2010). Several studies have shown that cellular miRNAs can directly interact with viral genomes promoting viral replication. Examples of this include, hsa-miR-485 in influenza A virus replication (Ingle et al., 2015), hsa-miR-122 in hepatitis C virus (HCV) replication (Israelow et al., 2014; Mortimer and Doudna, 2013), hsa-miR-21 in the replication of HCV (Chen et al., 2013) and dengue virus serotype 2 (Kanokudom et al., 2017). Some cellular miRNAs indirectly support viral replication, for example hsa-miR-23a can reduce levels of interferon regulatory factor 1, consequently enhancing human herpesvirus 1 (HHV-1) replication (Ru et al., 2014). Additionally, many viruses including HHV-1 (Han et al., 2016; Jiang et al., 2015), HHV-2 (Umbach et al., 2010), Epstein-Barr virus (EBV) (Zhu et al., 2009) and Kaposi's sarcoma-associated herpesvirus (Qin et al., 2010) encode miRNAs that regulate either host or viral genes post-transcriptionally (Cui et al., 2006; Parameswaran et al., 2010; Umbach and Cullen, 2009).

Bovine herpesvirus 1 (BoHV-1) is associated with several important diseases of cattle, including bovine respiratory disease which causes high economic losses in cattle industries throughout the world. BoHV-1 is a member of the subfamily *Alphaherpesvirinae* of the order *Herpesviridae*. The BoHV-1 genome is a double-stranded DNA molecule of approximately 135,300 bp in length. There are three recognised BoHV-1 genotypes, BoHV-1.1, BoHV-1.2a and BoHV-1.2b based on genomic restriction endonuclease profiles and associated clinical syndromes (Engels et al., 1981; Metzler et al., 1985). The BoHV-1.1 genotype is considered the most virulent genotype, associated with severe/fatal respiratory disease and abortion (Muylkens et al., 2007). While the BoHV-1.2b genotype has historically been associated with mild reproductive disease, evidence has been reported of this genotype being linked to severe respiratory disease (Smith et al., 1995; Fulton et al., 2015). Recent studies using complete genome sequence comparisons support the genotypic segregation of BoHV-1 strains and have enabled differentiation of vaccine strains from field strains (Fulton et al., 2015, 2016; Chothe et al., 2018).

Typical of the alphaherpesviruses, after the initial productive infection BoHV-1 can establish latent infections with the neuronal cell bodies of the peripheral nervous system of cattle. Studies have demonstrated the *in vitro* and *in vivo* expression of two miRNAs involved the maintenance of BoHV-1 latency, including the down regulation of BoHV-1 transcription factor BCIP0 (Jaber et al., 2010; da Silva and Jones, 2012). Glazov et al. (2010) reported that the BoHV-1 genome encodes and expresses at least ten miRNA genes, Bhv1-miR-B1 to Bhv1-miR-B10, during *in vitro* productive infections. The study demonstrated that the precursor miRNA (pre-miRNA) of these genes are processed into 12 mature miRNAs. However, the functional roles of the BoHV-1 miRNAs expressed during productive infections and any association with virulence are yet to be elucidated, but is an emerging area of interest for members of this virus family (Mahony, 2015; Bhela and Rouse, 2017).

The aim of the current study was to determine the impact of prior expression of Bhv1-miR-B6, Bhv1-miR-B8 and Bhv1-miR-B9 on the *in vitro* replication capacity of BoHV-1. It was hypothesized that if the BoHV-1 miRNAs were involved in the regulation of BoHV-1 genes, the presence of these miRNA prior to infection could interrupt the normal gene cascade and negatively affect BoHV-1 replication.

The regions of the BoHV-1 genome corresponding to the BoHV-1 pre-miRNAs of interest, defined by Glazov et al. (2010), were generated using complementary oligonucleotides (Supplemental Table S2.2.1). The oligonucleotide cassettes were cloned into plasmid pTD273 for expression under the control of the bovine 7SK promoter (Supplemental methods; Lambeth et al., 2006). The resulting plasmids encoding the pre-miRNA for Bhv1-miR-B6, Bhv1-miR-B8, Bhv1-miR-B9 were designated pmiR-B6, pmiR-B8, and pmiR-B9, respectively. Expression of the BoHV-1 mature miRNA of interest was confirmed by transfecting each plasmid into Madin-Darby bovine kidney (MDBK) cells and reverse-transcriptase quantitative real-time PCR (RT-qPCR) analysis to detect the mature forms of the four BoHV-1 miRNAs of interest (Supplemental methods; Glazov et al., 2010). The miRNA bhv1-miR-B6 was detected only in MDBK cells transfected with pmiR-B6 (Table 2.2.1). The miRNAs bhv1-miR-B8-5p and bhv1-miR-B8-3p were only detected in MDBK cells transfected with pmiR-B8 (Table 2.2.1), while the miRNA bhv1-miR-B9 was only detected in MDBK cells transfected with pmiR-B9 (Table 2.2.1).

Table 2.2.1. RT-qPCR detection of the BoHV-1 microRNAs of interested in MDBK cells transfected with plasmids encoded the BoHV-1 pre-miRNA. The average threshold cycle values (n = 3) with one standard deviation are shown for each viral miRNA and for the bovine control miRNA, bta-miR-25.

Transfected plasmid	miRNA target	Threshold Cycle
pTD273	bhv1-miR-B6	not detected
	bhv1-miR-B8-5p	not detected
	bhv1-miR-B8-3p	not detected
	bhv1-miR-B9	not detected
	bta-miR-25	26.61 ± 0.09
pmiR-B6	bhv1-miR-B6	36.34 ± 0.09
	bta-miR-25	26.41 ± 0.05
pmiR-B8	bhv1-miR-B8-5p	34.1 ± 0.13
	bta-miR-25	26.54 ± 0.19
pmiR-B8	bhv1-miR-B8-3p	33.49 ± 0.13
	bta-miR-25	26.33 ± 0.06
pmiR-B9	bhv1-miR-B9	42.48 ± 0.37
	bta-miR-25	26.34 ± 0.10

To determine the effects of BoHV-1 miRNA expression prior to BoHV-1 infection, MDBK cells were transfected with one of the pre-miRNA expression plasmids. At 24 hr post-transfection the cells were infected at a multiplicity of infection (MOI) of 0.1 with a recombinant BoHV-1 that constitutively expresses green fluorescence protein (GFP), derived from the BoHV-1 infectious clone, pBACBHV-37 (Mahony et al., 2002). The progression of the BoHV-1 infection was monitored for cytopathic effects (CPE) and GFP expression using light and fluorescent microscopy, respectively at 6 hr, 12 hr and 18 hr post infection (PI).

At 6 hr PI, no discernible CPE or GFP expression was observed in any of the BoHV-1 infected MDBK cell monolayers (data not shown). At 12 hr PI, characteristic BoHV-1 CPE and GFP expression were observed in the transfected/infected MDBK cell monolayers, although no differences were evident between the treatments (data not shown). At 18 hr PI, the intensity of GFP expression in MDBK cells transfected with the plasmids encoding the BoHV-1 pre-miRNAs was reduced in comparison to the control pTD273-transfected cells (Fig. 2.2.1A). This effect was most evident in the cells transfected with pmiR-B8 and pmiR-B9. No GFP fluorescence was evident in the non-transfected/uninfected MDBK cells (Fig. 2.2.1A). These effects on GFP fluorescence were consistent across three replicate experiments, suggesting that the replication of BoHV-1 in the MDBK cells transfected with the pre-miRNA encoding plasmids was reduced compared to the cells transfected with parental vector, pTD273.

To further investigate these effects, the MDBK cell monolayers were harvested at 18 hr PI and divided into two samples. One sample was used for total RNA extraction and RT-qPCR analysis for the BoHV-1 mature miRNAs of interest and the glycoprotein C (gC) transcript (Supplemental methods), while the remaining sample was used for western blot analyses of the BoHV-1 gC polypeptide (Supplemental methods). The cell culture supernatants were also retained for virus titre determinations (Supplemental methods).

The expression of the BoHV-1 miRNAs, bhv1-miR-B6, bhv1-miR-B8-5p, bhv1-miR-B8-3p, and bhv1-miR-B9 in the transfected/infected cells were quantified by RT-qPCR and compared with the levels of each miRNA in infected MDBK cells transfected with pTD273. The results showed that the levels of bhv1-miR-B6 and bhv1-miR-B8-3p were significantly higher, by 1.21-fold and 1.31-fold, respectively as compared to the pTD273 control (Fig. 2.2.1B). No significant changes in the levels of bhv1-miR-B8-5p were detected (Fig. 2.2.1B). In contrast, the expression of miRNA bhv1-miR-B9 was significantly less, decreased to 0.54-fold, compared to the infected MDBK cells transfected with pTD273 (Fig. 2.2.1B). These patterns of BoHV-1 miRNA expression in the transinfected/infected MDBK cells were consistent across two of three replicate experiments. As the GFP expression was suggestive of reduced BoHV-1 replication in the cells, it was anticipated that the overall levels of the BoHV-1 miRNAs would be reduced in infected MDBK cells transfected with pmiR-B6, pmiR-B8, and pmiR-B9 compared to those transfected with pTD273. However, this effect was only observed for infected cells transfected with pmiR-B9 (Fig. 2.2.1B). The significant increases in levels of bhv1-miR-B6 and bhv1-miR-B8-3p detected were most likely due to the cumulative expression of these miRNAs from BoHV-1 and the respective expression plasmids introduced into the MDBK cells, rather than up-regulation of the virally encoded genes.

The RT-qPCR analysis of the BoHV-1 gC transcript demonstrated significant reductions in the infected cells transfected with pmiR-B8 and pmiR-B9 compared to the gC transcript levels in the infected cells transfected with pTD273 (Fig. 2.2.1C). This effect was consistent in two of three replicate experiments. While the gC transcript levels in BoHV-1 infected cells transfected with pmiR-B6 also appeared to be reduced, this effect was not significant (Fig. 2.2.1C). The western blot analysis supported the gC RT-qPCR results, with lower levels of the gC protein detected in the pmiR-B8 and pmiR-B9 transfected/BoHV-1 infected cells as compared to pTD273 transfected/infected cells (Fig. 2.2.1D), while similar levels of the gC protein were detected in infected cells transfected with pmiR-B6 and pTD273 (Fig. 2.2.1D).

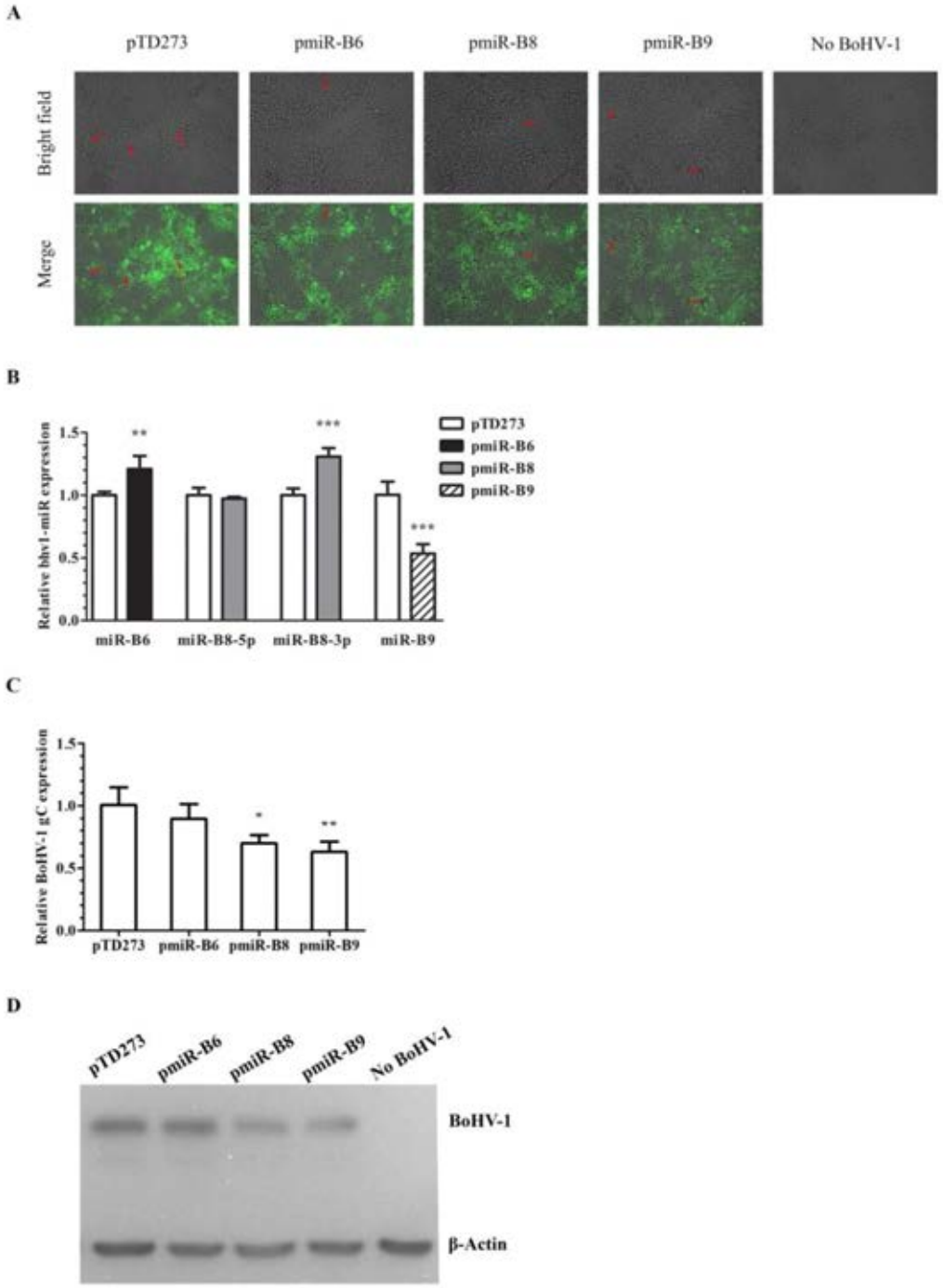


Figure 2.2.1. The effects on virus replication of prior expression of bovine herpesvirus 1 (BoHV-1) microRNAs in MDBK cells at 18 hr post-infection (See below).

Figure 2.2.1. The effects on virus replication of prior expression of bovine herpesvirus 1 (BoHV-1) microRNAs in MDBK cells at 18 hr post-infection. MDBK cells were transfected with plasmids pmiR-B6, pmiR-B8, pmiR-B9 or the parent vector, pTD273. After 24 hr the cells were infected with BoHV-1 which expresses GFP. (A) Cell morphology and BoHV-1 derived GFP expression at 18 hr post-infection (PI) observed using light and fluorescent microscopy (Magnification × 100). Arrows indicate cytopathic effects and viral plaque formation characteristic of BoHV-1 infection. (B) Relative expression of the BoHV-1 miRNAs of interest compared to bta-miR-25 in the transfected/infected MDBK cells at 18 hr PI. (C) Relative expression of the BoHV-1 glycoprotein C transcript compared to 18S rRNA transcript in the transfected/infected MDBK cells at 18 hr PI. (D) Western blot detection of the BoHV-1 glycoprotein C polypeptide in the transfected/infected cells at 18 hr PI. Where appropriate data are shown as the mean with error bars representing one standard deviation. The p-value < 0.05 (*); p-value < 0.01 (**); p-value < 0.001 (***) were statistically significant.

In agreement with the other datasets, the viral titres suggested reduced amounts of infectious BoHV-1 in the cell supernatants harvested at 18 hr PI. from cells transfected with the plasmids encoding the Bhv1-miR-B8 and Bhv1-mir-B9, although these differences were not significant (Fig. 2.2.2). The reduction in viral titre was most consistent in the cells transfected with pmiR-B9.

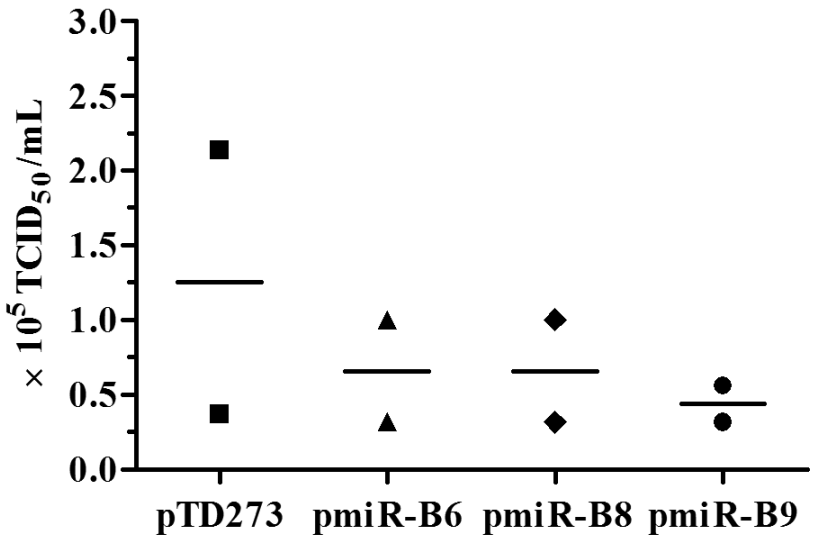


Figure 2.2.2. The bovine herpesvirus 1 titres ($TCID_{50}$/mL) from MDBK cells at 18 hr PI. Prior to infection the MDBK cells were transfected with one of four plasmids encoding viral pre-microRNAs for Bhv1-miR-B6 (pmiR-B6), Bhv1-miR-B8 (pmiR-B8) or Bhv1-miR-B9 (pmiR-B9) or the parental plasmid (pTD273). The mean viral titres are shown from two replicate experiments.

To investigate possible mechanisms of how these BoHV-1 miRNAs could reduce virus replication, putative RNA:RNA binding sites within the BoHV-1 genome were identified using RNAhybrid software (Supplemental methods; Rehmsmeier et al., 2004). The RNAhybrid analysis identified several putative binding sites for bhv1-miR-B8-3p and bhv1-miR-B9 (Fig. 2.2.3A). Two putative binding sites upstream of the *OriS* region and one binding site within the BICP22 transcript were identified for both miRNAs of interest (Fig. 2.2.3B, i-vi).

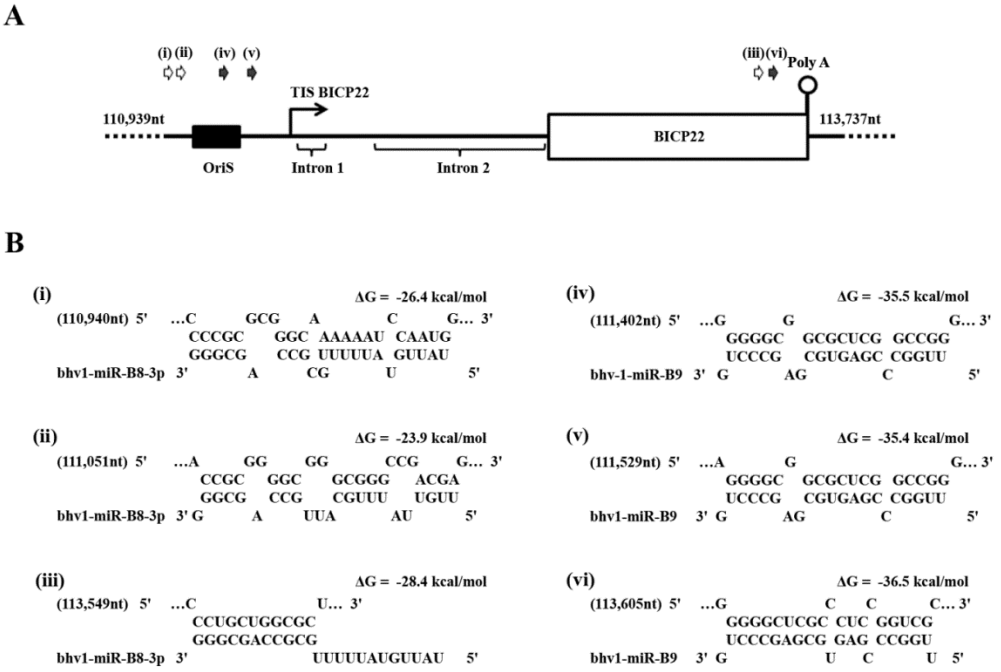


Figure 2.2.3. Schematic representation of the location and predicted recognition sites for the bovine herpesvirus 1 miRNAs, bhv1-miR-B8-3p and bhv1-miR-B9 within the internal repeat region of the viral genome. (A) Schematic representation of the location of the putative BoHV-1 miRNA binding sites proximal to the origin of replication (*OriS*). Binding sites for bhv1-miR-B8-3p (black arrows) and bhv1-miR-B9 (white arrows). (B) The predicted RNAhybrid base pairings between the viral miRNAs and the transcripts are shown with the minimum free energy values (ΔG). The nucleotide positions within the BoHV-1 genome (AJ004801) are shown in parentheses.

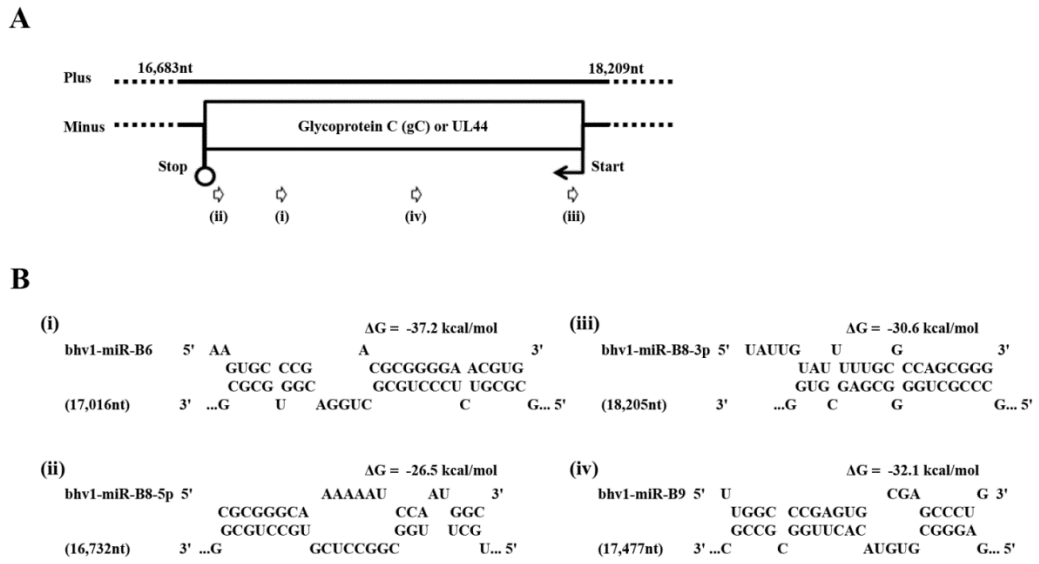
It is not readily apparent how bhv1-miR-B8-3p or bhv1-miR-B9 could affect BoHV-1 replication through the sites associated with the *OriS* region. The regions surrounding human herpesvirus 1 (HHV-1) origins of replication are known to be transcriptionally active (Nguyen-Huynh and Schaffer, 1998; Wong and Schaffer, 1991). In addition, long non-coding transcripts (Ori_SRNA_1 and Ori_SRNA_2) are expressed across the *OriS* during *in vitro* replication of HHV-1 replication (Voss and Roizman, 1988). More recently, Tombacz et al. (2017) have identified transcripts overlapping all of the orgins of replication of HHV-1, suggesting that transcription and/or the transcripts could play important, though yet to be defined, roles in herpesvirus replication. As the transcripts associated with the origins of replication are non-coding, any targeting of these transcripts by bhv1-miR-B8-3p and/or bhv1-miR-B9 would not impact on virus replication by translational repression. However, miRNAs can also facilitate target transcript degradation, providing a plausible pathway for bhv1-miR-B8-3p and bhv1-miR-B9 to affect the abundance of non-coding transcripts (Jin and Xiao, 2015). Further studies are required confirm interactions between these miRNAs and any transcripts associated with the BoHV-1 *OriS* and if such interactions do occur, what the impact is on virus replication.

Potential binding sites for bhv1-miR-B8-3p and bhv1-miR-B9 were also identified within the transcript of BICP22 (Fig. 2.2.3). BICP22 is a transrepressor protein of BoHV-1 promoters, such as the immediate early promoter of the BICP4/BICP0 gene, early promoter of the BICP0 gene, and the late promoter of the gC gene (Koppel et al., 1997). The ICP22 homologue of HHV-1, HICP22, is known to be non-essential for *in vitro* replication, although, HHV-1 viruses with mutated hICP22 genes are reported to produce less infectious virus (Fox et al., 2017). The HICP22 polypeptide is a multifunctional protein, which includes an important role in the modification of the host cell RNA polymerase II to preferentially transcribe viral genes (Rice et al., 1995; Long et al., 1999). HICP22 also acts to recruit host transcription factors for the expression of several viral genes expressed in the late phase of the viral gene cascade. The results of the current study suggest that prior expression of bhv1-miR-B8-3p and/or bhv1-miR-B9 in BoHV-1 infected MDBK cells may have interfered with the translation of BICP22, much earlier than occurs in

the normal replication cycle, subsequently prematurely reducing the transcription of its target late genes. The result of these interactions being reduced BoHV-1 replication. Additional studies are required to confirm the capacity of bhv1-miR-B8-3p and/or bhv1-miR-B9 to directly interact with the BICP22 transcript and the determine impacts on the translation of BICP22.

The results of the current study are similar to those reported by Han et al. (2016) who demonstrated that ectopic expression of the HHV-1 miRNAs, hsv1-miR-H28-3p and hsv1-miR-H29-5p, in human (HEp-2, HEK293T) or monkey (Vero) cells prior to HHV-1 infection reduced viral mRNAs, viral proteins and virus yields. The authors hypothesised that hsv1-miR-H28-3p and hsv1-miR-H29-5p, which are expressed as late genes during HHV-1 infection, were a host/pathogen adaptation to control the production of infectious virus to increase the likelihood of host to host transmission. While the temporal expression patterns of the BoHV-1 miRNA genes are yet be determined, bhv1-miR-B9 exhibited high level expression in infected MDBK cells (MOI = 10) at 6 hr PI indicating that it may be a late gene of the BoHV-1 gene expression cascade (Glazov et al., 2010). The similar expression kinetics of bhv1-miR-B9 suggest it may play an analogous role to hsv1-miR-H28-3p or hsv1-miR-H29-5p in BoHV-1 replication.

Putative binding sites for bhv1-miR-B6, bhv1-miR-B8-5p, bhv1-miR-B8-3p and bhv1-miR-B9 were also identified in the gC transcript (Supplemental Fig. S2.2.1). However, as the gC polypeptide is not required for *in vitro* replication of BoHV-1 (Robinson et al., 2008), any interactions between the BoHV-1 miRNA are considered unlikely to have affected BoHV-1 replication. As with the putative BoHV-1 miRNAs and transcripts interactions, modulation of the gC transcript requires experimental confirmation. This is clearly demonstrated by the putative interaction between bhv1-miR-B6 and gC transcript, as similar levels of gC polypeptide were detected in the western blot analysis of infected cells transfected with pmiR-B6 (Fig. 2.2.1D; Fig S2.2.1).



Supplemental Figure S2.2.1. Schematic representation of the location and predicted recognition sites for the bovine herpesvirus 1 miRNAs within the glycoprotein C (gC) transcript. (A) Location of the putative BoHV-1 miRNA binding sites with the gC transcript. (B) The predicted RNAhybrid base pairings between the viral miRNAs and the transcripts are shown with the minimum free energy values (ΔG). The nucleotide positions within the BoHV-1 genome (AJ004801) are shown in parentheses.

Some variation was observed between the replicate experiments in this study which is attribted to the low transfection efficiency of MDBK cells, reported to range from 1% to 10% (Tautz et al., 1999; Mahony et al., 2005; Osorio and Bionaz, 2017). As a consequence, few MDBK cells would have been both transfected and infected in each of the replicate experiments. Therefore the true effects of the prior expression of the BoHV-1 miRNAs of interest may have been underestimated in the current study. One way to resolve this issue would be to use a BoHV-1 susceptible cell-line with a higher transfection efficiency to ensure that the majority of cells are expressing the miRNA of interest at the time of infection. This approach was not used in the current study, as the alternate cell-lines that were available, with known higher transfection efficiences, were not of bovine origin and aberrant processing of the BoHV-1 pre-miRNA due to host species differences could not be excluded.

In conclusion, this is the first report to demonstrate that the transient expression the BoHV-1 miRNAs, bhv1-miR-B8-3p and bhv1-miR-B9, prior to infection can suppress BoHV-1 replication. Further research is warranted to fully elucidate the role of these miRNAs in BoHV-1 replication and their target transcripts, as this information will not only improve the understanding of this important bovine pathogen, but may also inform the development of effective control strategies.

Acknowledgments

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Supplemental methods

Virus and Cells

A bovine herpesvirus 1 (BoHV-1) which constitutively expresses the green fluorescent protein (GFP) was using in this study. The construction and characterization of this modified virus was constructed using the pGETrec recombineering process as described by Mahony et al. (2002). Briefly, the virus was rescued from the BoHV-1 infectious clone, pBACBHV-37, following the insertion of a GFP expression cassette using (Mahony et al., 2002). The virus was propagated using Madin-Darby Bovine Kidney (MDBK) cells (ATCC number CCL-22; Madin and Darby, 1958) using standard techniques. The MDBK cells were cultured in Earle's minimal essential medium (MEM) containing non-essential amino acids, 1× GlutaMAX™ Supplement (Thermo Fisher Scientific), 25 mM HEPES and 5% (v/v) fetal calf serum at 37°C in a 5% CO_2 atmosphere.

Construction of BoHV-1 pre-miRNA expression plasmids

The miRNA-expressing plasmids were constructed using the plasmid, pTD273, which can facilitate the expression of small RNA transcripts using the bovine 7SK small nuclear RNA promoter by RNA polymerase III as described by Lambeth et al. (2006). Briefly, oligonucleotides corresponding to each strand of the BoHV-1 pre-miRNA for bhv1-miR-B6, bhv1-miR-B8 or bhv1-miR-B9, as define by Glazov et al. (2010), were synthesized with the addition bases on the 5′ and 3′ termini to facilitate ligation into pTD273 digested with *Kpn* I and *Eco* RI sites (Supplemental Table S2.2.1). Additional nucleotides were also added to the oligonucleotides to place an RNA polymerase III terminator sequence motif to 3′ termini of the BoHV-1 pre-miRNAs. The equimolar amounts of the oligonucleotides for each BoHV-1 pre-miRNA were annealed by heating to 95 ^{o}C for 10 min and allowed to slowly cool to room temperature. The annealed cassettes were ligated into pTD273 digested with *Kpn* I and *Eco* RI using standard procedures. The correct insertion of the BoHV-1 pre-miRNA sequences into pTD273 were confirmed by conventional dideoxy terminator sequencing.

Transfection of MDBK cells and BoHV-1 infection

MDBK cells were seeded in six-well plates 24 hr prior to the experiment and allowed to reach 80% confluence. Then, the MDBK cells were transfected with the miRNA-expressing plasmid (500 ng) using Lipofectamine 2000 according to the manufacturer's instructions (Thermo Fisher Scientific). At 24 hr post-transfection MDBK cells were infected with the recombinant BoHV-1 at a multiplicity of infection (MOI) of 0.1 for 18 hr. All cell monolayers were observed using light and fluorescence microscopy at 6 hr, 12 hr and 18 hr post infection (PI).

RNA extraction

For total RNA extraction, at 18 hr PI the MDBK cells were washed with once with stertile phosphate buffered saline and then harvested by adding 700 mL of QIAzol lysis reagent (QIAGEN). The total cell lysate was processed using the miRNeasy kit according to the manufacturer’s instructions (QIAGEN). The purified total RNA was quantified using a NanoDrop spectrophotometer and either used immediately or stored at -70 °C until required.

RT-qPCR detection of BoHV-1 miRNA

For miRNA detection, total RNA (500 ng) was converted to cDNA using 50 nM of each stem-loop RT primers (Supplemental Table S2.2.1) and preheated at 65 °C for 5 min. After chilling on ice, the cDNA synthesis mix of SuperScript RT (Thermo Fisher Scientific) was added. The reaction was performed at 16 °C for 30 min, following 60 cycles of 30 °C for 30 sec; 42 °C for 30 sec; 50 °C for 1 sec. Finally, the reaction was inactivated at 85°C for 5 min. Then, 2 mL of cDNA were amplified using QuantiTect Multiplex PCR NoROX kit (QIAGEN) with specific primers and probes (Supplemental Table S2.2.1) for the BoHV-1 miRNA of interest. The qPCR cycling was performed using Rotor-Gene Q with the following conditions: preheated at 50 °C for 2 min and denatured at 95 °C for 10 min following 45 cycles of 92 °C for 15 sec and 60 °C for 15 sec. After cycling, data were acquired using the default settings of the Roto-Gene Q software (Version 2.3.1, Build 49) using an arbitrary threshold of 0.05.The expression levels of the bovine miRNA, bta-miR-25,

was determined for total RNA preparations using the procedure described for the BoHV-1 miRNAs and the specific primers shown in Supplemental Table S2.2.1.

The resulting RT-qPCR cycling data were used to calculate the relative change in expression of each BoHV-1 miRNA using delta-delta threshold cycle (Ct) methodology as described by Livak and Schmittgen (2001) using the samples from the BoHV-1 infected MDBK cells transfected with the parental plasmid, pTD273, as the calibrator sample and the bta-miR-25 as the reference gene.

RT-qPCR detection of bovine reference transcript and BoHV-1 mRNA

For BoHV-1 gene expression analysis, total RNA (500 ng) was converted to cDNA in 20 µl using 50 ng of random hexamers (N_6) and preheated at 65 °C for 5 min. The reaction mixture was chilled on ice, and then the reaction buffer containing SuperScript RT enzyme (Thermo Fisher Scientific) was added. The reaction was performed at 25 °C for 10 min; 50 °C for 50 min and then inactivated at 85 °C for 5 min. Then, of cDNA (2 µL) was amplified to qPCR by using QuantiTect Multiplex PCR NoROX kit (QIAGEN) with primers and probes (Supplemental Table S2.2.1). The qPCR cycling was performed using Rotor-Gene Q with the following conditions: preheated at 50 °C for 2 min and denatured at 95 °C for 10 min following 45 cycles of 92 °C for 15 sec and 60 °C for 60 sec. After cycling, data were acquired using the default settings of the Roto-Gene Q software (Version 2.3.1, Build 49) using an arbitrary threshold of 0.05.

The bovine reference transcript, 18S rRNA, was detected using the Eukaryotic 18S rRNA Endogenous Control assay according to the manufacturer's instructions (ThemoFisher Scientific) using 500 ng of total RNA.

The resulting qPCR cycling data were used to calculate the relative change in expression of the BoHV-1 glycoprotein C transcript using delta-delta threshold cycle (Ct) methodology as described by Livak and Schmittgen (2001) using the bovine 18S rRNA transcript as the reference gene.

Western blot analysis

Denaturing total proteins (10% of total protein) from each cell sample were separated by discontinuous SDS-PAGE with a 10% separating gel using the MiniProtean® II cell (Bio-Rad) according to the manufacturer's instructions with Laemmli buffer system (Laemmli, 1970). After separating, the proteins were transferred to PVDF membrane using a Mini Trans-Blot® cell (Bio-Rad) according to the manufacturer's instructions at 30V, 4 °C overnight. After transfer, the membrane was blocked for 1 hr with PBS containing 5% skim milk powder and 0.05% Tween-20 (Blotto), and subsequently incubated with either the monoclonal antibody, G2, anti-BoHV-1 gC monoclonal antibody G2 (at the dilution 1:2000) (VMRD, Collins et al., 1984) or anti-β-actin antibody (A5441, Sigma) (at a dilution of 1:5000) as the primary antibody diluted in Blotto. The membrane was washed three times with PBS containing 0.05% Tween-20 (PBST) for 10 min. A secondary antibody, anti-mouse-IgG-HRP, diluted 1:5000 in Blotto solution was then incubated with the membrane for 1hr. The membrane was washed three times with PBS containing 0.05% Tween-20 (PBST) for 10 min. After washing, the membrane was developed using the Clarity™ Western ECL Blotting Substrate (BioRad) according to manufacturer's instructions and exposure to X-ray film.

Virus titration

Virus titers were estimated using the 50% endpoint tissue culture infectious dose ($TCID_{50}$) method. Briefly, MDBK cells (24 well-plates) at 90% confluence were infected with 100 μL of BoHV-1 culture medium, serially diluted (10^{-1} to 10^{-8}) in OptiMEM and the cells incubated for 5 days. The cells were fixed with 3.7% formaldehyde for 30 min and stained with 0.05% crystal violet for 5 min. The presence or absence of BoHV-1 cytopathic effects (CPE) were then recorded for each dilution using light microscopy and the data used to calculate the $TCID_{50}$/mL as described by Reed and Muench (1938).

Identification of BoHV-1 miRNA binding sites

BoHV-1 miRNA binding sites was analyzed using RNAhybrid 2.2 software (Rehmsmeier et al., 2004). The IE1.7 transcript including OriS and BICP22 (110,939 to 113,737 nt) of BoHV-1 (AJ004801) and with either bhv1-miR-B8-3p (MIMAT0013971) or bhv1-miR-B9 (MIMAT0013972) were analyzed for RNA hybridization by RNAhybrid 2.2 software with default parameters. The results were shown in the duplex RNA hybrid structure between targeting RNA and miRNA with the minimal free energy or ΔG value. Moreover, the first position of the target sequence was marked. Whereas, the analysis utilised the reverse strand of the BoHV-1 genome, corresponding to the predicted glycoprotein C (gC) gene (16,683 – 18,209, BoHV-1 genomic accession AJ004801) as the target RNA.

Supplemental Table S2.2.1. Oligonucleotides used in this study.

Oligonucleotide	Sequence (5′-3′) [1]	Target name and/or function
bhv1mir-6_sense	CGCGGAGGGCAAGTGCCCGACGCGGGGAACGTGGCGGCGGAC GCCAGCGCCGCGTCTCCGGCGCCGGGTCCTGGCCCTCCGCGTT TTTTGGAA	Expression cassette for pre-miRNA-B6
bhv1mir-6_antisense	aattTTCCAAAAAACGCGGAGGGCCAGGACCCGGCGCCGGAGAC GCGGCGCTGGCGTCCGCCGCCACGTTCCCCGCGTCGGGCACTT GCCCTCCGCGgtac	
bhv1mir-8_sense	CCCCCGCGCGGGCAAAAAATCCAATGGCGGATAATCGAGAGC TATTGTATTTTTGCGCCAGCGGGGGTTTTTTGGAA	Expression cassette for pre-miRNA-B8
bhv1mir-8_antisense	aattTTCCAAAAAACCCCCGCTGGCGCAAAAATACAATAGCTCTC GATTATCCGCCATTGGATTTTTTGCCCGCGCGGGGGgtac	

bhv1mir-9_sense	GGCCTCCAGCGTTCGCACAAAGCTCAATAAGTTTATATATATA TTATTGGCCCGAGTGCGAGCCCTGGGACCTTTTTTGGAA	Expression cassette for pre-miRNA-B9
bhv1mir-9_antisense	aattTTCCAAAAAAGGTCCCAGGGCTCGCACTCGGGCCAATAAT ATATATATAAACTTATTGAGCTTTGTGCGAACGCTGGAGGCCgt ac	
miR-B6-RT	GTTGGCTCTGGTGCAGGGTCCGAGGTATTCGCACCAGAGCCAA CCACGTT	qPCR detection of bhv1-miR-B6
miR-B6-FWD	CGGCGGAAGTGCCCGACGCGGGG	
miR-B8-5p-RT	GTTGGCTCTGGTGCAGGGTCCGAGGTATTCGCACCAGAGCCAA CGCCATT	qPCR detection of bhv1-miR-B8-5p
miR-B8-5p-FWD	CGGCGGCGCGGGCAAAAAATCC	

miR-B8-3p-RT	GTTGGCTCTGGTGCAGGGTCCGAGGTATTCGCACCAGAGCCAACCCCGCT	qPCR detection of bhv1-miR-B8-3p
miR-B8-3p-FWD	CGGCGGTATTGTATTTTTGCGCC	
miR-B9-RT	GTTGGCTCTGGTGCAGGGTCCGAGGTATTCGCACCAGAGCCAACCAGGGC	qPCR detection of bhv1-miR-B9
miR-B9-FWD	CGGCGGTTGGCCCGAGTGCGA	
Universal_REV	GTGCAGGGTCCGAGGT	Universal Reverse primer for miRNA qPCR

UPL LNA probe #21(Roche)	CAGAGCCA	Universal miRNA probe
bta-miR-25-RT	GTTGGCTCTGGTGCAGGGTCCGAGGTATTCGCACCAGAGCCAA CTCAGAC	qPCR detection of bta-miR-25
bta-miR-25-FWD	CCCCCATTGTCTCG	
BHV-FWD	ATGTTAGCGCTCTGGAACC	qPCR detection of the BoHV-1 glycoprotein C transcript.
BHV-REV	CTTTACGGTCGACGACTCC	
BHV-probe(Cy5)	ACGGACGTGCGCGAAAAGA	
18S rRNA primer/ probe (JOE)	Eukaryotic 18S rRNA Endogenous Control (Thermo Fisher Scientific)	18S rRNA

[1] Underlined nucleotides encode the RNA polymerase II terminator sequence motif; Lowercase nucleotides create compatible termini for cloning of the annealed oligonucleotides into pTD273 digested with *Kpn* I (gtac) and *Eco* RI (aatt).

The first-two chapters elucidated the miRNA molecule which suggested that both host and virally encoded miRNAs had influenced to viral replication processes. The result found that the modulation of expression level of miRNAs (Charpter I and II) could interfere the production of virus *in vitro*. To investigate and study new antiviral therapeutics, the chapter III designed to study the possible way to produce antiviral agent. Hence the receptor protein acts as a target for viral entry, this study produce the recombinant shrimp receptor protein of yellow head virus then purify this protein prior test neutralizing property of the purified protein to yellow head virus.

CHAPTER III

In vitro neutralization of yellow head virus infection in shrimp using recombinant PmYRP65 protein

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Abstract

Yellow head virus (YHV) is a major pathogen in the Southeast Asia shrimp aquaculture industry especially in Thailand, and YHV associated mortality results in significant economic loss. Although appropriate farm management strategies can decrease the YHV infection rate in shrimp, an epidemic of the virus remains, with no specific anti-YHV therapy. Previous reports have identified PmYRP65 as a protein that mediates YHV entry into susceptible cells, and studies using RNA interference have shown that systemic administration of double-stranded RNAs (dsRNAs) directed to PmYRP65 are able to provide protection against infection. This study sought to determine whether recombinant PmYRP65 protein (rPmYRP65) would be able to act as a competitive binding protein to neutralize YHV infection. To undertake this a cDNA encoding PmYRP65 was cloned, and rPmYRP65 was expressed, purified and characterized for its ability to neutralize YHV infection before injection into shrimp. The results showed that rPmYRP65 protein could significantly inhibit YHV infection by its receptor characteristic, through acting as a competitor to bind YHV. This study showed that rPmYRP65 has potential applications for neutralizing YHV infection of shrimp.

Keywords: Yellow head virus (YHV); Shrimp; Recombinant PmYRP65 protein; Neutralization; Infection

3.1. Introduction

In recent years the aquatic animal farming industries have dramatically expanded partly due to the increasing demand from the growing human population. This massive expansion of aquaculture together with an emphasis on high population-density farming has led to outbreaks of a number of diseases (Crane and Hyatt, 2011). In the shrimp farming industry in the Americas and Asia, viral diseases have had a significant impact on aquaculture (Walker and Mohan, 2009) and since it first emerged in Thailand in 1990, Yellow head virus (YHV) has been considered a devastating pathogen (Boonyaratpalin et al., 1993). YHV is a rod shaped-enveloped virus which contains a positive sense singlestranded RNA and is classified as a member of the genus *Okavirus*, Family *Roniviridae*, and Order *Nidovirales* (Jitrapakdee et al., 2003; Sittidilokratna et al., 2002). The virus rapidly spreads in shrimp ponds inducing high mortality in a number of penaeid shrimp species (Boonyaratpalin et al., 1993; Flegel et al., 1997; Lightner et al., 1998; Lu et al., 1994; De la Rosa-Velez et al., 2006). To enter host cells, viruses often utilize a protein expressed on the surface of the cell to mediate entry of the virus into the cell. These viral binding proteins (VBPs) are normally referred to as receptors, and a number of shrimp virus receptors have been identified including fibronectin for viral hemorrhagic septicemia virus (Bearzotti et al., 1999), integrin for white spot syndrome virus (WSSV) (Li et al., 2007) and laminin receptor (Lamr) for WSSV (Liu et al., 2016), Taura syndrome virus (TSV) and YHV (Busayarat et al., 2011). However, a number of other VBPs have been identified that do not function as receptors including actin for TSV (Senapin and Phongdara, 2006) and PmRab7 protein for Laem-Singh virus and YHV (Ongvarrasopone et al., 2010; Posiri et al., 2016). Several previous studies have shown that recombinant VBPs proteins can bind to the virus and inhibit infection. Examples include recombinant *Penaeus monodon* (Pm) Rab7 inhibiting WSSV (Sritunyalucksana et al., 2006; Thagun et al., 2012) as well as PmChitin-Binding Protein (PmCBP), *Marsupenaeus japonicus* (Mj)-β-integrin, MjLectin-A, and MjLectin-B preventing WSSV infection (Chen et al., 2009; Li et al., 2007; Song et al., 2010). Importantly,

recombinant PmLamr protein could decrease shrimp mortality in a YHV challenge experiment (Busayarat et al., 2011) as well as delay WSSV infection (Liu et al., 2016).

The yellow head virus receptor protein (PmYRP65) was first identified as a *Penaeus monodon* lymphoid cell-expressed receptor for YHV implicated in YHV entry into susceptible cells (Assavalapsakul et al., 2006). A subsequent study that knocked down PmYRP65 in shrimp using an RNA interference strategy showed that suppression of PmYRP65 transcription resulted in protection against infection (Assavalapsakul et al., 2014). However, it has not yet been established as to whether recombinant PmYRP65 protein would be able to neutralize YHV infection in shrimp. This study therefore sought to produce purified recombinant PmYRP65 and determine whether this was able to neutralize YHV infection of shrimp, and the results support this approach as a viable strategy to reduce YHV infection and to lessen mortality.

3.2. Materials and methods

3.2.1. Construction of a recombinant PmYRP65 expression plasmid

Total RNA was extracted from the dissected gills of a black-tiger shrimp using RiboZol™ RNA extraction reagent (AMRESCO, LCC., USA). Briefly, the gills were ground in Ribozol reagent and then incubated at room temperature for 5 min. Then chloroform was added, and the solution was mixed by vortex for 15 s. After incubation for 15 min at RT, the solution was centrifuged at 12,000 ×g for 15 min at 4 °C. The upper phase was transferred to a new tube, and the total RNA was precipitated by adding an equal volume of isopropanol followed by washing with 75% ethanol. The RNA pellet was re-suspended in RNase-free water. One microgram of total RNA was used as a template to produce cDNA using RevertAid™ reverse-transcriptase (Thermo Fisher Scientific Inc., USA) used according to the manufactures protocol. Briefly, total RNA and oligo(dT) primer were denatured at 70 °C for 10 min and then immediately placed on ice, following which reverse transcription was performed at 37 °C for 5 min, 42 °C for 90 min, then 70 °C for 5 min. One microlitre of cDNA was used for PCR amplification using 1×buffer, 4 mM of MgSO4, 0.2 μM dNTPs, 0.2 μM of PmYRP65_XhoI_F

(GGGGCTCGAGAAAAGACCGGAGGATAAGCCGGCGCAGAAGAAAGCCAAAG GTGCCAAGTCTGCCGAGGCAAAGG), 0.2 μM of PmYRP 65_XbaI_R (GGGGTCTAGATCATCAGCATCTTCATCATCATCG), and 1 U of Vent DNA polymerase (Thermo Fisher Scientific Inc., USA) at 94 °C for 1 min, 67 °C for 30 s, 72 °C for 2 min for 35 cycles and followed by a final incubation at 72 °C for 10 min. The expected PCR product was purified and doubled digested with *Xho* I and *Xba* I (Thermo Fisher Scientific Inc., USA) at 37 °C for 16 h. The digested DNA fragment was ligated into pCold™I DNA (Takara Bio Inc., Shiga, Japan) that has been digested with the same restriction enzymes and the ligation mix was subsequently transformed into *E. coli* DH5α. The recombinant plasmids were verified by selective medium, rapid size screening, restriction enzyme digestion analysis and further confirmed by DNA sequencing (1st Base DNA, Malaysia). The correct plasmid was then transformed into *E. coli* Rosetta-gami for PmYRP65 protein expression.

3.2.2. Expression and purification of rPmYRP65 protein

A single colony of a recombinant clone was cultured in LB medium containing 100 μg/μl of ampicillin and 34 μg/μl of chloramphenicol at 30 °C until the optical density (OD) at 600 nm reach 0.4 after which culture was induced with isopropyl-β-D-thiogalactopyranoside (IPTG) at a final concentration of 0.3 mM followed by culture for 3 h. The induced bacteria were collected by centrifugation and re-suspended in PCL buffer [PBS (286 mM NaCl, 8 mM Na2HPO4, 2.6 Mm KCl, 1.4 mM KH2PO4), 1 mM DTT and 1% (w/v) SDS, pH 7.4] at 10 ml per 100 OD600 cell. The cell suspension was lysed by sonication until clear and then centrifuged at 8000 ×g at 4 °C for 10 min, after which the supernatant was passed through a 0.45 μM syringe filter, and the recombinant protein was immediately purified by a HisTrap column (GE Healthcare Life Sciences, USA) equilibrated with PCW binding buffer (PBS and 0.1% Sarkosyl (w/v), pH 7.4). After loading the filtrate on the column, the column was washed twice with 4 column volumes of PCW. The bound proteins were eluted by gradient elution with PCE (PCW buffer containing 60, 80, 100, 200, 300 and 400 mM imidazole). The remaining proteins were eluted by stripping buffer (PCW buffer containing 50 mM EDTA). All fractions

were collected and analyzed by 10% SDS-PAGE and western blot analysis. The elution buffer in the selected fraction (300 mM imidazole) was replaced with PBS using an Amicon® Ultra-4 (Merck Millipore Ltd., Ireland) centrifugal filter and the amount of protein was determined by the Bradford assay (BIO-RAD Laboratories Inc., CA) at 595 nm with a BSA standard. The samples were separated by 10% SDS–polyacrylamide gel electrophoresis on parallel gels, following which one gel was stained with Coomassie brilliant blue solution while the proteins in the second gel were transferred to a polyvinylidene difluoride (PVDF) membrane using a Mini Trans-Blot electrophoresis transfer cell (Bio-Rad Laboratories, Richmond, CA) at 120 A for 120 min. After transfer, the membrane was blocked to prevent nonspecific binding by immersion in blocking solution (PBS, 5% skim milk, 0.5% Tween-20) at room temperature for 5 min twice with gentle agitation. Then the membrane was incubated with 1:5000 dilution of a mouse anti-hexahistidine monoclonal antibody (R&D Systems Inc., Japan) in blocking solution at room temperature for 2 h, followed by a 1:5000 dilution of horseradish peroxidase-conjugated goat anti-mouse IgG1 antibody (Thermo Fisher Scientific Inc., USA). Signals were detected by autoradiography. The expected band of approximately 65 kDa was extracted from a 10% SDS-PAGE gel and after in-gel tryptic digestion peptides were analyzed by LC-MS/MS. The MS/MS spectra were searched against the MSDB nonredundant protein database (Imperial College, London UK) using the MASCOT search engine (Matrix Science, London, United Kingdom).

3.2.3. YHV neutralization assay using rPmYRP65 protein

L. vannamei shrimp (approximately 300–400 mg) were acclimated at 28 °C for 1–2 days prior to being divided into four groups for hemocoel injection with 1) PBS only, 2) rPmYRP65 alone (1.5 μg), 3) 5×10^{-8} LD_{50} YHV (kindly provided by Chaimongkon D., Mahidol University, Nakorn Pathom, Thailand) and 4) rPmYRP65 (1.5 μg) with 5×10^{-8} YHV. For the last group, rPmYRP65 was pre-incubated with YHV on ice for 1 h before injection. On day 3 post injection, the shrimp gills were collected, and RNA extracted to monitor YHV genome levels by semi-quantitative RT-PCR (n =8) as described below. The remaining shrimp (n = 10) were kept under standard conditions,

with mortality recorded daily for 10 days. Moreover, to verify that rPmYRP65 was specifically neutralizing YHV, bovine-serum albumin (BSA) was used as an irrelevant protein to mix with YHV before injection in a similar procedure as previously described. In a further experiment, 5 $\times 10^{-7}$ LD_{90} of YHV was mixed with 24 μg of rPmYRP65. After pre-incubation on ice for 1 h, the mixture was injected into shrimp, in parallel with shrimp injected with 5 $\times$ 10^{-7} LD_{90} of YHV alone. All shrimp samples were collected to assess YHV genome levels on day two post injection (n =12).

3.2.4. Semi-quantitative RT-PCR

One microgram of total RNA was preheated with random primers (N6) at 70 °C for 5 min then placed immediately on ice for 2 min before reverse transcription using ReverseAid RT (Thermo Fisher Sciences, USA) at 25 °C for 5 min, 42 °C for 90 min and 65 °C for 5 min. One microliter of cDNA reaction was used as a template for PCR comprising of 0.2 μM of Actin_F (GACTCGTACGTGGGCGACGAGG),
0.2 μM of Actin_R (AGCAGCGGTGGTCATCTCCTGCT),
0.067 μM of YHV_hel_F (CAAGGACCACCTGGTACCGGTAAGAC),
0.067 μM of YHV_hel_R (GCGGAAACGACTGACGGCTACATTCAC),
1× Taq buffer, 2 mM of MgCl2, 0.2 μM dNTPs and 1 U of Taq DNA polymerase (Thermo Fisher Scientific Inc., USA) with 25 cycles of 94 °C for 10 s, 55 °C for 30 s, 72 °C for 1 min, followed by 72 °C for 5 min. The PCR products were separated on 1.5% agarose gels by electrophoresis and visualized using Quantity One (BIORAD Laboratory Inc., USA). Relative band intensities of YHV and actin amplicons were monitored by the Scion Image software 4.0.2 (Scion Corporation, Maryland) and then analyzed using the GraphPad Prism Program version 5.03 (GraphPad Software Inc., CA).

3.2.5. Statistical analysis

Relative band intensities were compared using the nonparametric Student *t*-test. Results are displayed as scatter dot plots with the median indicated, with each spot representing single shrimp. Cumulative mortality was compared by Newman-Keuls multiple comparison tests. A *P*-value< 0.05 was considered to be statistically significant.

3.3. Results

3.3.1. Construction of recombinant PmYRP65 expression plasmid

To construct a recombinant PmYRP65 expression plasmid, the 1.5 kb of PmYRP65 coding sequence was amplified by RT-PCR and ligated into the pColdI expression vector at *Xho* I and *Xba* I sites. The recombinant PmYRP65 plasmid was verified by DNA sequencing, which confirmed identity to the PmYRP65 sequence (GenBank Accession: AAZ22828). The recombinant PmYRP65 plasmid was transformed into *E. coli* Rosetta-gami for recombinant PmYRP65 protein expression.

3.3.2. Expression and purification of rPmYRP65 protein

The *E. coli* Rosetta-gami containing the recombinant PmYRP65 plasmid was cultured at 30 °C for 3 h with induction by 0.3 mM IPTG. To determine solubility induced cells were suspended in PCL buffer and lysed by sonication. The soluble and insoluble fractions were resolved by SDS-PAGE. The result showed that the expected protein band of 65 kDa was mainly located in the soluble fraction, and comparison with total lysate suggested that> 95% of the protein was in the soluble fraction (data not shown). Therefore, the soluble fraction was passed through a 0.45 μM filter and the protein purified using a His-trap column. The results of the elution profile showed that the expected 65 kDa-protein could be observed to elute in the 300 mM imidazole fraction (Fig. 2.3.1A–B, lane P). This purified recombinant PmYRP65 protein was further confirmed by LC-MS/MS analysis and the MASCOT program (MASCOT value of 138).

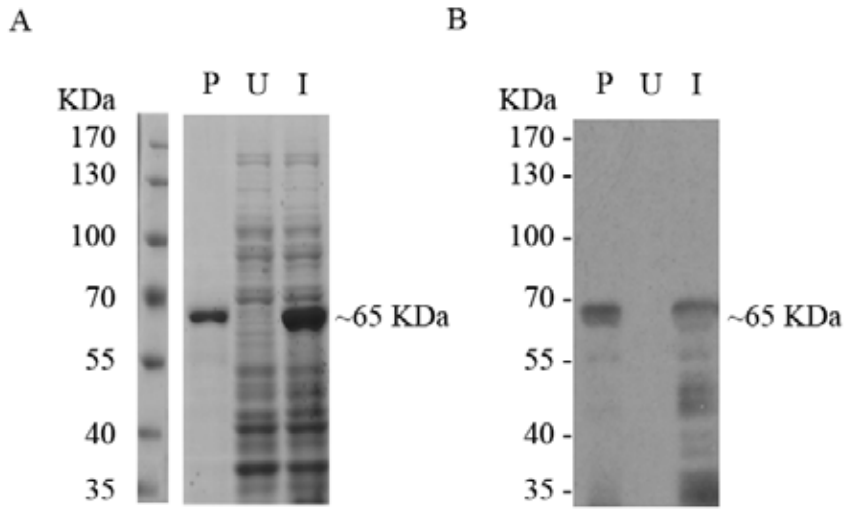

Figure 2.3.1. SDS-PAGE profile and western-blot analysis of recombinant PmYRP65 production from *E. coli* Rosetta-gami using the pColdI system. The rPmYRP65 band was observed at approximately 65 kDa in both SDS-PAGE (A) and western-blot analysis using an anti-histidine monoclonal antibody (B). Lane P, purified rPmYRP65; Lane U, uninduced bacterial lysate; Lane I, induced bacterial lysate.

3.3.3. YHV neutralization assay using rPmYRP65 protein

To investigate whether recombinant PmYRP65 protein could neutralize YHV, an *in vitro* neutralization assay was performed by co-injection of YHV with rPmYRP65 protein in the parallel injection of either YHV in PBS, PBS only or rPmYRP65 protein only. At 3 days post-injection, total RNA from gills was extracted from shrimp in the co-injection of YHV with PBS and YHV with rPmYRP65 protein groups and used as a template for semi-quantitative RT-PCR. The results showed that the YHV genome could be detected in six out of seven shrimp injected with co-injection of YHV with PBS, and five shrimp died on the third day (Fig. 2.3.2A, PBS co-injection). In contrast, the YHV genome was detected as low-intensity bands in only three out of eight shrimp injected with a co-injection of YHV with rPmYRP65, and only one shrimp died at the third day

(Fig. 2.3.2A, YRP co-injection). Normalization of the YHV signal with actin showed that YHV genome signal was 4.39 fold less intense in the shrimp co-injected with rPmYRP65 and YHV as compared to the shrimp injected with PBS and YHV (Fig. 2.3.2B). Additionally, the remaining shrimp were continually observed for mortality for up to ten days. The results showed that co-injection of YHV with rPmYRP65 treatment could inhibit YHV as shrimp mortality was reduced approximately 30% when compared to the YHV infected only group (P-value < 0.001) (Fig. 2.3.2C). No mortality was seen in either the PBS or rPmYRP65 protein alone groups. To confirm the in vitro neutralizing property of rPmYRP65, the experiment was conducted by co-injection of YHV with either PBS or BSA or rPmYRP65. At 3 days post-injection, total RNA from gills was extracted from each shrimp and used as a template for semi-quantitative RT-PCR. The result showed that the YHV band could be detected in six out of ten shrimp on the third day in the PBS and BSA groups. In contrast, the YHV band could not be observed in the rPmYRP65 group (Fig. 2.3.2D). According to semi-quantitative RT-PCR, the normalized band intensity of rPmYRP65 group was less intense when compared with PBS and BSA groups (Fig. 2.3.2E). These results confirm that rPmYRP65 can significantly neutralize YHV infection (P-value < 0.05). The remaining shrimp were observed for mortality for ten days. The result showed that rPmYRP65 treatment strongly inhibited YHV as shrimp mortality was not observed as compared to the PBS group (P-value < 0.005) and BSA group (P-value < 0.001) Fig. 2.3.2F).

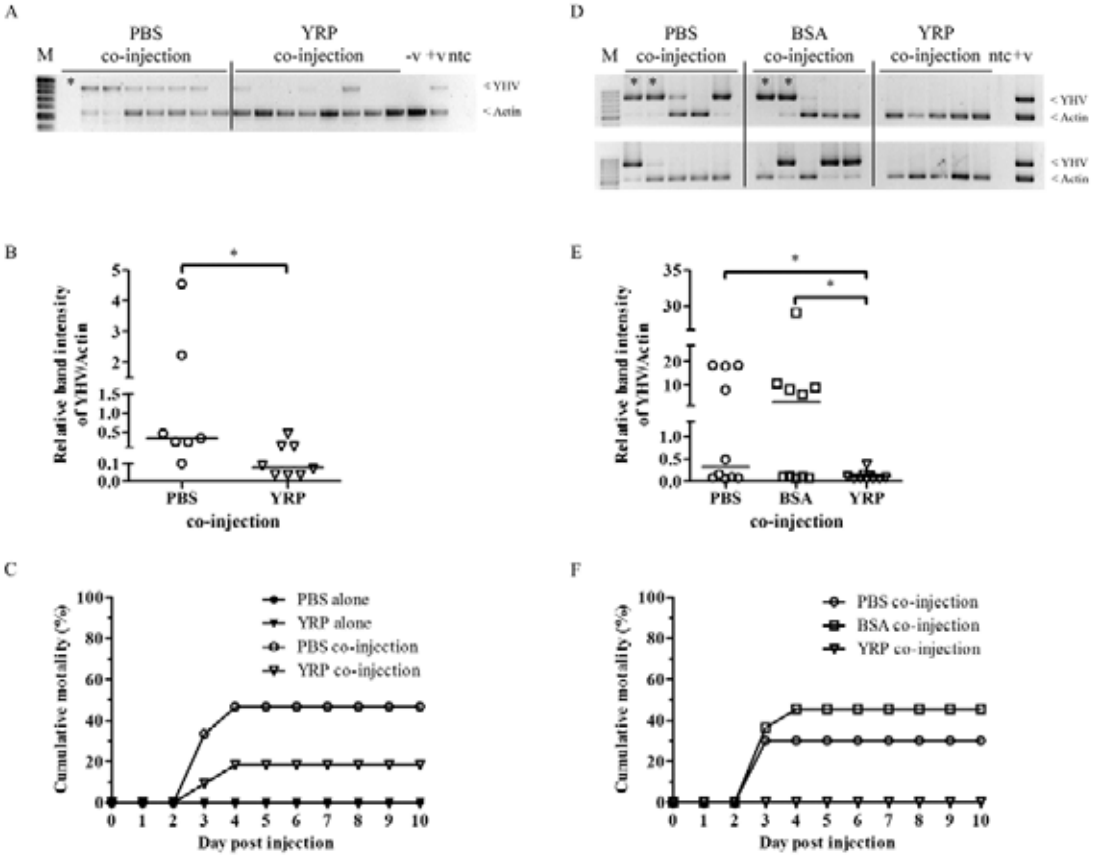

Figure 2.3.2. Neutralization assay of YHV with rPmYRP65 protein. Shrimp were co-injected with a mixture of YHV (5×10^{-8} LD_{50}) and rPmYRP65 (1.5 μg) (YRP co-injection), BSA (1.5 μg) (BSA co-injection) or with YHV alone (PBS co-injection). Individual shrimp were collected to monitor the YHV genome by multiplex RT-PCR (A, D). The relative band intensity of YHV/ actin for individual shrimp together with the median is shown (B, E). The cumulative mortality of shrimp during YHV challenge was observed for 10 days (C, F). Lane M, 100 bp marker; PBS, control group; YRP, rPmYRP65 treatment group; −v, mock-infected shrimp; +v, YHV-infected shrimp; ntc, no temple PCR control; * degraded/dead sample.

To confirm the ability of rPmYRP65 to neutralize YHV infection in high dose, shrimp were injected with either a tenfold higher titer of YHV (5×10^{-7} LD_{90}) co-injected with the sixteen fold higher amount of rPmYRP65 (24 µg). After two days all shrimps were harvested and the level of the YHV genome assessed by semi-quantitative RT-PCR. Interestingly, the result showed almost complete suppression of YHV by rPmYRP65 treatment as no YHV band could be observed (Fig. 2.3.3A, Low panel). The relative band intensity of YHV/actin shows that rPmYRP65 treatment significantly lowered levels of YHV in tissues as compared to control YHV only injected shrimp (*P*-value < 0.001) (Fig. 2.4.3B).

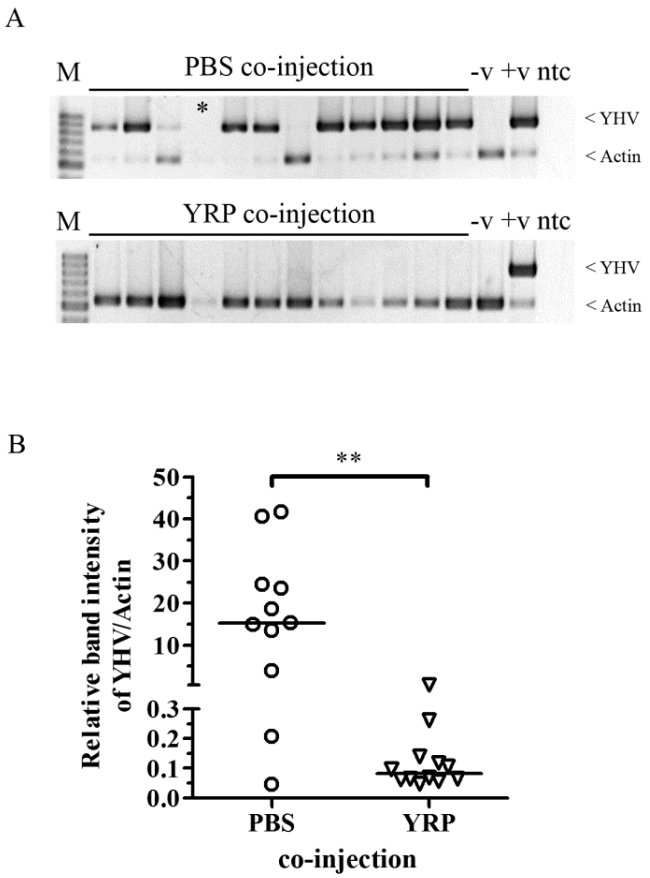

Figure 2.3.3. Neutralization assay of YHV with rPmYRP65 protein (see below).

Figure 2.3.3. Neutralization assay of YHV with rPmYRP65 protein. Shrimp were co-injected with a mixture of YHV (5×10^{-7} LD_{90}) and rPmYRP65 (24 μg) (YRP co-injection) or with YHV alone (PBS co-injection). Individual shrimp were collected on day 2 post challenge to monitor the YHV genome by multiplex RT-PCR (A). Relative band intensity of YHV/actin for individual shrimp together with the median is shown (B). Lane M, 100 bp marker; PBS, control group; YRP, rPmYRP65 treatment group; −v, mock-infected shrimp; +v, YHV-infected shrimp; ntc, no temple PCR control; * degraded sample was not included in the analysis.

3.4. Discussion

YHV is a pathogenic virus that causes a highly virulent and acute disease associated with high mortality in the black-tiger shrimp *Penaeus* (*Penaeus*) *monodon* (Boonyaratpalin et al., 1993). However, YHV can also cause high levels of mortality in farmed Pacific white-leg shrimp, *Penaeus* (*Litopenaeus*) *vannamei* (Senapin et al., 2010). Due to the widespread presence of this virus in Thailand and elsewhere, there is a need for novel anti-viral strategies to reduce economic losses caused by this virus. Recombinant protein technology is a powerful approach to produce high amounts of protein. The *E. coli* expression system is the most commonly used cell factory for the production of therapeutic proteins as this system has the potential for high level expression, is fast growing and requires inexpensive components and with the available technology this system is easy to manipulate (Rosano and Ceccarelli, 2014; Baeshen et al., 2015). A number of studies have used recombinant protein technology for the production of therapeutic proteins (Wurm, 2004) such as enzymes (Ni et al., 2016), cytokines (Jian et al., 2016) and anti-viral agents (Jiang et al., 2012; Nangola et al., 2012; Li et al., 2016; Yu et al., 2014). A previous study identified PmYRP65 as a *P. monodon* receptor protein for YHV (Assavalapsakul et al., 2006). RNA interference induced knock-down of host expressed PmYRP65 using dsRNA successfully inhibited YHV infection in black-tiger shrimp primary Oka cells *in vitro* (Assavalapsakul et al., 2006) and in black-tiger shrimp *in vivo* (Assavalapsakul et al., 2014). In this study, the coding sequences for PmYRP65 were cloned into the pCold™I *E. coli* expression system, and

protein production was optimized and scaled-up to generate sufficient protein for an in vitro neutralization and mortality assay in a YHV challenge study. The results indicated that rPmYRP65 was able to inhibit YHV infection and reduce shrimp mortality through neutralizing the infectious YHV virion (Fig. 2.3.2A–F). This is in agreement with other studies that have shown that VBPs can inhibit viral infection in shrimp. For example, PmRab7 which directly binds to VP28 could neutralize WSSV infection through inhibition of viral endocytosis (Sritunyalucksana et al., 2006; Thagun et al., 2012). Similarly, PmLamr can bind to the YHV capsid protein resulting in reduced mortality from YHV challenge (Busayarat et al., 2011). This experimental *in vitro* study showed that rPmYRP65 could neutralize YHV prior to infection of shrimp resulting in reduced mortality as a consequence of YHV infection.

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PART III

THESIS CONCLUSION

1. Conclusion

This thesis was to investigate different types of antiviral agents against viral infection which can be concluded into each chapter by

Chapter I was to identify DENV 2 regulated miRNAs in HepG2 cell and their application. The results suggested that the up-regulated miR-21 following DENV 2 infection promoted viral replication in HepG2 cell. However, the up-regulation of miR-21 was slightly seen with ZIKV infection, whereas there was no significant change after DENV 4 infection at 24 h. The result suggested that miR-21 was considerably specific for DENV 2 replication in HepG2 cell.

Chapter II was to study the role of selected bhv1-miRNAs in the bhv1-miR transient expression in the bovine cell. MDBK cell was transfected with the plasmid carrying either bhv1-miR-B6, bhv1-miR-B8 or bhv1-miR-B9 and following infected by BoHV-1. The result showed that the bhv1-miR-B8-3p was over-expressed whereas bhv1-miR-B9 was approximately half-reduced in BoHV-1 infected-MDBK cell. In summary, the transient expression of BoHV-1 miRNAs of bhv1-miR-B8 and bhv1-miR-B9 constructs can suppress BoHV-1 replication in infected cells.

Chapter III was to determine whether recombinant PmYRP65 protein (rPmYRP65) would be able to act as a competitive binding protein to neutralize YHV infection. The recombinant plasmid carrying *PmYRP65* gene was constructed and expressed in *E. coli*. The rPmYRP65 was then purified for use in *in vitro* neutralizing study by mixing with YHV before injection into shrimp. The results demonstrated the rPmYRP65 could significantly inhibit YHV infection. This study implies that the rPmYRP65 can serve as anti-viral protein to inhibit YHV infection which can be applied in shrimp farming industries.

2. Delimitation and limitation of the study

None

3. Suggestion for future work

Chapter I: The future work should identify and characterize target of miR-21 which involves in DENV 2 replication. Furthermore, other miRNAs regulating DENV 2 replication should also be investigated in HepG2 cell.

Chapter II: Further research may consider and fully elucidate the role of these miRNAs in BoHV-1 infected MDBK cell. This insight could improve the antiviral activity of these miRNAs for BoHV-1 replication.

Chapter III: Feeding shrimp with induced recombinant clone to inhibit YHV infection should be examined. Further, the region which interacts with YHV particle should be examined and then synthesized to test the neutralizing activity against YHV infection.

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Publications

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