Discovery of small molecule therapeutics for the Middle Eastern respiratory syndrome-coronavirus (MERS-CoV)

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2020

Gan, J. H. (2020). Discovery of small molecule therapeutics for the Middle Eastern respiratory syndrome-coronavirus (MERS-CoV). Doctoral thesis, Nanyang Technological University, Singapore.

<https://hdl.handle.net/10356/137291>

<https://doi.org/10.32657/10356/137291>

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**DISCOVERY OF SMALL MOLECULE
THERAPEUTICS FOR THE MIDDLE EASTERN
RESPIRATORY SYNDROME-CORONAVIRUS
(MERS-CoV)**

GAN HANJIE JONATHAN

SCHOOL OF BIOLOGICAL SCIENCES

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(MERS-CoV)**

GAN HANJIE JONATHAN

SCHOOL OF BIOLOGICAL SCIENCES

A thesis submitted to the Nanyang Technological
University in partial fulfilment of the requirement for the
degree of Doctor of Philosophy

2020

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Acknowledgements

Firstly, I would like to express my utmost gratitude for my supervisor, Professor Yoon Ho Sup, for his kind acceptance and guidance throughout my 4 years of study. I would also like to extend my thanks to my Thesis Advisory Committee members, Associate Professors Julien Lescar and Liu Xuewei, for their invaluable suggestions and advice on my project.

Next, I am grateful towards Dr Harikishore, for his structure-guided *in-silico* screening of FDA-approved drugs. I would like to thank my lab mates, especially Dr. Sreekanth Rajan, Dr. Ye Hong, Dr. Serap Beldar, Dr. Nguyen Quoc Toan, Dr. Toh Hui Ting, Dr. Ngow Yeen Shian, Dr. Choi Min Joo, Mr. Yoo Jun Yeob and Ms. Tanvi Parnaik for their constant support, encouragement and suggestions which helped me tremendously in my project.

I would also like to thank NTU/PPP, namely Dr. Chen Ming Wei, Ms. Dina and Ms. Shi Hui for their kind guidance and help in the area of recombinant protein expression and optimisation through the baculoviral system. Next, I would like to extend my gratitude towards Dr. Kim Seungtaek and Dr. Lee Jihye from Institut Pasteur Korea, for their kind help in conducting the live MERS-CoV studies to validate our *in-vitro* findings. I would also like to thank Dr. Lee Su Seong from A*star (IBN) for his assistance with the design and stapling of our DPP4 mimetic peptide.

I would like to express my thanks to NTU/School of Biological Sciences, for providing me with this opportunity to embark on my PhD study.

Lastly, I am grateful to my family for their constant support and encouragement throughout my studies.

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List of Abbreviations

(-)	Negative control
(+)	Positive control
6-HB	6-helical bundle
6x His/6-His	hexa-Histidine
ACE-2	Angiotensin-Converting Enzyme - 2
APS	Ammonium Persulfate
Camp	Chloramphenicol
Car	Carbencillin
CE-5	SARS-CoV 3C-like protease inhibitor
Cef	Cefaclor
Cep	Cephadrine
CoV	Coronavirus
CQ	Chloroquine diphosphate
CV	Column Volume
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic acid
DP12m	DPP4-12mer mimetic peptide
DPP4	Dipeptidyl Peptidase IV
DTT	Dithiothreitol
E	Elution
E protein	Envelope protein
ECL-HRP	Enhanced Chemiluminescent – Horseradish Peroxidase
EDTA	Ethylenediaminetetraacetic acid
ER	Endoplasmic reticulum
F	Flowthrough
FPLC	Fast Performance Liquid Chromatography
FRET	Förster Resonance Electron Transfer
Gent	Gentamicin
hDPP4	human Dipeptidyl Peptidase IV
His	Histidine
IC ₅₀	Half maximal inhibition concentration
ICTV	International Committee on Taxonomy of Viruses and Nomenclature
IPTG	Isopropyl-thiogalactoside
Kan	Kanamycin
kb	kilobases
K _D	Dissociation constant
L	Lovastatin
LB	Lysogeny broth
LPV	Lopinavir
M protein	Membrane protein
MALDI-TOF	Matrix Assisted Laser Desorption Ionisation - Time Of Flight
MERS	Middle Eastern Respiratory Syndrome
MERS-CoV	Middle Eastern Respiratory Syndrome-Coronavirus
mRNA	messenger Ribonucleic acid

MSH	Montelukast Sodium Hydrate
MW	Molecular Weight
N	Nalmefene
N protein	Nucleocapsid protein
NaCl	Sodium Chloride
NaCl	Sodium chloride
Ni ²⁺ -NTA	Nickel charged affinity resin with nitrilotriacetic acid chelating agent
ORF	Open reading frame
PBS	Phosphate-buffered saline
PCR	Polymerase Chain Reaction
PDB	Protein Data Bank
PEG	Polyethylene Glycol
Pen-Strep	Penicillin-Streptomycin
PV	Pseudovirion
R	Reserpine
RBD	Receptor binding domain
RDRP	RNA dependent RNA polymerase
ResQ	Resource Q (Anion Exchange) column
RMSD	Root of Standard
RNA	Ribonucleic acid
S	Supernatant
S protein	Spike protein
S200	Superdex-200 column
S75	Superdex-75 column
SARS	Severe Acute Respiratory Syndrome
SARS-CoV	Severe Acute Respiratory Syndrome-Coronavirus
SDS	Sodium Dodecyl Sulfate
SDS-PAGE	Sodium Dodecyl Sulfate - Polyacrylamide Gel Electrophoresis
SEC	Size Exclusion Chromatography
SF9 cells	Clonal isolate of Spodoptera frugiperda Sf21 cells
TBS	Tris-buffered saline
TBS-T	Tris-buffered saline with Tween 20
Tet	Tetracycline
TOA assay	Time of Addition assay
Trp	Tryptophan
W	Wash
WB	Western Blot
WHO	World Health Organisation
X-gal	5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside

Abstract

Middle-Eastern Respiratory Syndrome (MERS) is a coronavirus infection which affects the lower respiratory tract of infected individuals. Its high mortality rate of 34.5% led MERS to be listed as a high priority disease for research by the World Health Organisation (WHO). MERS can be both symptomatic and asymptomatic, which leads to a high rate of misdiagnosis and treatment. MERS-coronavirus (MERS-CoV) was identified as a beta-coronavirus in 2013, like Severe Acute Respiratory Syndrome-coronavirus (SARS-CoV) which resulted in a global outbreak in 2003. As there are currently no specific vaccines and therapeutics for this viral infection, there is an unmet medical need to develop drugs against MERS-CoV infection. To achieve this objective, three approaches were attempted: 1) Drug repurposing via structure-guided screening of FDA-approved drugs; 2) New fragment library screening; and lastly 3) Designing of peptide mimetics targeting MERS-CoV Receptor Binding Domain (RBD). Those attempts allowed us to identify a promising drug candidate, Montelukast Sodium Hydrate (MSH), which was previously used in the treatment of acute allergic rhinitis and asthma. Structural model predicted that MSH is engaged in the binding interface between MERS-CoV RBD and human DPP4 and consequently exerts inhibitory effects. The molecular basis of MSH in MERS-CoV infection has been studied while its potential clinical implications are discussed. Finally, validation studies on this potential therapeutic candidate were performed by employing live MERS-CoV. The results presented in this thesis provide insights into repositioning potential of existing drugs and an opportunity to design novel class of drugs to combat MERS-CoV infection.

1. Introduction

1.1 Background of Viruses

Viruses are infectious microscopic pathogens that require a host cell to undergo propagation and can target both prokaryotic and eukaryotic cells (Koonin, Senkevich, & Dolja, 2006; Lodish et al., 2000). These agents vary in size and exhibit an array of different morphologies. They are generally smaller as compared to bacterial cells and can have circular or linear RNA or DNA genomes (Baron S, 1996).

1.1.1 Discovery and Classification of virus

In 1884, the Chamberland-Pasteur filter was developed using porcelain ‘candles’ of 0.1 to 1 micron pore sizes to remove any bacteria or cells from liquid suspensions (Horzinek, 1997; Rybicki E, 2015). Using this filter, an unknown infectious pathogen in tobacco plants was discovered in 1892 by Ivanovski, and was subsequently identified as a ‘virus’ in 1898 by Beijerinck (Lustig & Levine, 1992; Rybicki E, 2015). Since then, the study of virology led to the discovery of numerous different types of viruses and the development of viral classification systems. There are generally 2 systems for the classification of viruses.

First is the Baltimore system which focuses on the difference in genomic material which were classified under different classes. Class I are double-stranded DNA viruses, Class II are single-stranded DNA viruses, Class III are double-stranded RNA viruses, Class IV are positive single-stranded RNA viruses, Class V are negative single-stranded RNA viruses, Class VI are single-stranded RNA-RT viruses and Class VII are double-stranded RNA-RT viruses (Baltimore, 1971).

The other is the International Committee on Taxonomy of Viruses (ICTV) system which focuses on the taxonomic codes. Taxon suffices are given to the different taxonomic codes. In the ICTV system, classification of viruses start from Realm (-*viria*) followed by Subrealm (-*vira*), Kingdom (-*virae*), Subkingdom (-*virites*), Phylum (-*viricota*), Subphylum (-*viricotina*), Class (-*viricetes*), Subclass (-*viricetidae*), Order (-*virales*), Suborder (-*virineae*), Family (-*viridae*), Subfamily (-*virinae*), Genus (-*virus*), Subgenus (-*virus*) and Species (ICTV, 2018).

1.1.2 General structure of virions

A typical completely-assembled infectious virus particle, known as a virion, contains its core genome (RNA or DNA), encapsulated within a protein shell known as a capsid (Homa & Brown, 1997). This capsid serves numerous functions, some of which are to protect the viral genomic content from degradation from host defences, regulation of viral replication and expression and virion movement (Kobayashi et al., 2015). The capsid proteins once expressed, self-assemble into the capsid structure determined by the viral genome. This capsid structure varies in different types of viruses and can exhibit helical or icosahedral symmetrical properties (Lidmar, Mirny, & Nelson, 2003; Vernizzi & Olvera de la Cruz, 2007). Some viruses have an additional layer of lipid molecules which form an envelope around the capsid (Aloia, Tian, & Jensen, 1993; Miles, Cassidy, Donlon, Yarkoni, & Frankel, 2015). This viral envelope is generally acquired from the host cell budding consists of membrane bound glycoproteins that assists in the attachment and entry into host cells (Aloia et al., 1993; Gelderblom, 1996; Miles et al., 2015).

1.1.3 Lytic life cycle of viruses

The first phase of a typical viral life cycle begins with the adsorption of virus to its host cell (Gescher et al., 2011). Normally, the virus has a unique epitope on the surface such as the capsid or envelope to recognise and bind to specific host cell factors (Cuestas et al., 2006). This determines the tropism of the virus (Baron, Michael, & Albrecht, 1996). The binding triggers viral entry, typically via the fusion of host cell and viral membranes, endocytosis or macropinocytosis (Banerjee et al., 2014; Kumar et al., 2016; Miles et al., 2015; Veetil et al., 2016).

As a result, the viral genetic material is released into the host cells and the virus utilises the host cell replication and protein expression mechanisms to replicate its genomic content and expression of its viral proteins (Chaikeeratisak et al., 2017). These viral proteins undergo protein folding, oligomerisation and intracellular transport, contributing to the formation of viral structures such as capsids and envelope proteins (Cong, Kriegenburg, de Haan, & Reggiori, 2017; Persson & Pettersson, 1991).

Eventually, the self-assembly of these viral proteins lead to the formation of functional and infectious viral progeny (Rong, Niu, Lee, & Wang, 2011). Newly formed viruses will then be released out of the cells by budding, exocytosis or lysis of host cells (Göttlinger, 2001; Pornillos, Garrus, & Sundquist, 2002). Viruses which typically release its viral progeny via budding or exocytosis does not directly cause the death of its infected host cell. Hence, they are labelled as cytopathic viruses (Goulding, 2019).

1.1.4 Human, animal viruses and zoonosis

Viruses can be categorised by its host tropism: Human or Animal viruses (Loeffelholz & Fenwick, 2017). Animal viruses specifically infects and causes widespread viral diseases in animals. These pathogens tend to cause damage to livestock and crops (Delwart, 2012). An example of animal virus is the foot-and-mouth disease which was discovered in 1898 (Rybicki E, 2015). This pathogen infects cattle and leads to adverse effects in animal farming. (Salguero, Sánchez-Martín, Díaz-San Segundo, de Avila, & Sevilla, 2005; Sei, Waters, Kenney, Barlow, & Golde, 2016)

Human viruses refer to types of viruses that can targets human cells as host for infection and replication. There are approximately 220 known species of human viruses to date (M. Woolhouse, Scott, Hudson, Howey, & Chase-Topping, 2012). Yellow fever virus was the first human virus that was discovered in 1901 (Gardner & Ryman, 2010; Staples & Monath, 2008). Numerous emerging human viruses were found to be multi-host pathogens that are zoonotically transmitted within animal reservoirs before host adaptation to infect humans (M. E. J. Woolhouse & Gowtage-Sequeria, 2005; M. E. J. Woolhouse, Taylor, & Haydon, 2001).

1.2 Coronavirus

Coronaviruses are enveloped positive-sense single stranded RNA viruses with spike-like protrusions. ‘Corona’ refers to the crown or halo formed by the spike glycoproteins on the surface of the virus (Li, 2016).

1.2.1 General structure of coronaviruses – Spike glycoprotein

Coronavirus particles are spherical in shape and have distinctive ‘spikes’ emanating from its surface viral envelope (Arabi et al., 2017; Fehr, Channappanavar, & Perlman, 2017). Within the virion interior, it encompasses a nucleocapsid which has a helical symmetry and its positive-sense single-stranded RNA genome (Chang, Hou, Chang, Hsiao, & Huang, 2014; McBride, van Zyl, & Fielding, 2014). Coronaviruses typically encode 4 main structural proteins in its genome, namely its Spike glycoprotein (S), Envelope (E), Membrane (M) and Nucleocapsid (N) proteins (Figure 1.1). The S protein, being responsible for host cell recognition, attachment as well as viral entry, is heavily glycosylated (Hebert, Zhang, Chen, Foellmer, & Helenius, 1997; Shen, Tan, & Tan, 2007). Therefore, it requires access into the endoplasmic reticulum (ER) of its host cells for N-linked glycosylation (Hebert et al., 1997). This S protein forms homo-trimers to constitute the Spike glycoprotein protrusions on the viral surface (Berend Jan Bosch, van der Zee, de Haan, & Rottier, 2003; Walls et al., 2017).

The S protein can be further divided into 2 different regions, S1 and S2. S1 contains the receptor-binding domain (RBD) which selectively binds to host cell factors or receptors, promoting host cell specificity and attachment while S2 forms the stalk of the spike consisting of the fusion peptide, and is responsible for membrane fusion to promote viral entry (Berend Jan Bosch et al., 2003; Fehr et al., 2017; Yuan et al., 2017). Coronaviruses use a Class I fusion mechanism, where the fusion proteins are in a trimeric state in both pre- and post-fusion phases. The final form of the fusion proteins normally includes an N-terminal central α -helical coiled coil surrounded by 3 other C-terminal helices to form a

6-helix bundle (6-HB) (Gao et al., 2013; C. Wang et al., 2018; White, Delos, Brecher, & Schornberg, 2008).

Figure 1.1 General structure of Coronaviruses.

Coronaviruses typically consists of 4 major structural proteins – S, N, M, and E proteins; and its positive-sense single-stranded RNA genome. The spike-like protrusions are formed from the homo-trimeric fusion of S proteins. The RNA genome of coronaviruses associates with the N proteins to form a nucleocapsid which has helical symmetrical properties.

1.2.2 Genomic organisation

Coronaviruses hold a large genome of approximately 30 kb in size. The genome consists of a non-segmented, positive-sense single-stranded RNA that resembles an mRNA transcript, having a 5' methylated guanine cap and a 3' polyadenylated tail (Sawicki, Sawicki, & Siddell, 2007). The RNA genome generally encodes for non-structural proteins on the N-terminal region and structural proteins on the C-terminus (Figure 1.2). All coronaviruses have approximately two-thirds of its genome encoding large Open Reading Frames (ORF) 1a and 1b. These two ORFs were found to be overlapping and involved in ribosomal frameshift to express 2 polyproteins pp1a and pp1ab. Further downstream processing of the polyproteins

lead to the generation of 16 different non-structural proteins (Forni, Cagliani, Clerici, & Sironi, 2017). Some examples of non-structural proteins are the replicase, helicase and RNA-dependent RNA polymerase (RDRP) (Fehr & Perlman, 2015).

Figure 1.2: Typical genomic organisation of Coronaviruses.

N-terminal Open-reading frame 1 (ORF1) codes for non-structural proteins and C-terminal region codes for the 4 major structural proteins (van Boheemen et al., 2012). Ribosomal frameshift typically occurs between ORF1a and ORF1b to generate 2 polyproteins: pp1a and pp1ab. These 2 polyproteins in turn generate the non-structural proteins after proteolytic processing.

1.2.3 Phylogenetic classification

Coronavirus is the major group of viruses belonging to the *Nidovirales* order, under the *Coronaviridae* family, and *Coronavirinae* subfamily. The *Coronavirinae* subfamily can be further categorised into alpha, beta, delta and gamma coronavirus based on phylogenetic clustering (Payne, 2017). *Nidovirales* typically have large genomic content in each virion, with *Coronaviridae* family having the biggest genome in this order (Nakagawa, Lokugamage, & Makino, 2016; Sawicki et al., 2007). *Nidovirales* generally have a few common features in the order. Firstly, they have a highly conserved RNA genome organisation encoding for replicase in the N-terminal region and the 4 major structural proteins at the C-terminus. Secondly, ribosomal frameshifting is prevalent in viruses under

this order for the expression of the N-terminal non-structural genes, in ORF1a and 1b. Thirdly, they have a large replicase-transcriptase polyprotein that encompasses a few unique enzymatic activities. Lastly, sub-genomic mRNA transcripts with 3' poly-adenylated tails are expressed from the C-terminal structural and accessory genes (Fehr & Perlman, 2015).

1.3 Middle-Eastern Respiratory Syndrome – Coronavirus (MERS-CoV)

MERS is an acute respiratory disease, akin to its predecessor, Severe Acute Respiratory Syndrome (SARS). Both diseases were found to be caused by beta-coronaviruses of the same genus but of different lineages.

1.3.1 Background and Epidemiology of MERS-CoV

MERS emerged in 2012, causing region-wide outbreaks in Saudi Arabia, Qatar and Jordan (Sharif-Yakan & Kanj, 2014; Zaki, Van Boheemen, Bestebroer, Osterhaus, & Fouchier, 2012). As reported in the initial outbreaks, MERS was confined within the Middle Eastern region. However, reported cases of MERS surfaced in other regions such as America (Kapoor et al., 2014). Two major outbreaks of MERS were reported – one in Saudi Arabia in 2014 and the other in the Republic of Korea in 2015 (K. CDC, 2015; Park et al., 2017; WHO, 2018b). Since its emergence, MERS has progressed from a regional to a global outbreak, affecting as many as 27 different countries (WHO, 2018c).

To date, there are 2,449 laboratory-confirmed cases and at least 845 deaths resulting from MERS (WHO, 2019c). The high mortality rate observed amongst infected patients, led the World Health Organization (WHO) to classify MERS as a disease which has 'high epidemic potential' and 'a great cause of concern' (WHO, 2019a). Additionally, MERS was labelled as a disease which demands

priority and rapid research and developments of therapeutics and vaccines (WHO, 2018a). Therefore, it is paramount to study and understand the mechanisms and pathways involved in this respiratory disease for therapeutic development.

MERS-infections could be asymptomatic to symptomatic in nature (Carias et al., 2016; Moon & Son, 2017; Song et al., 2018). Infected patients typically exhibit 'cold-like symptoms' such as fever, diarrhoea, body aches, nausea, runny or blocked nose (WHO, 2018b). As a result, misdiagnosis of MERS-infections is common, and can lead to the increased spread of this disease (SPH, 2016). Additionally, asymptomatic patients could have been silent carriers which contributed to the transmission of this infection (Adalja, 2014). Previous research on MERS identified a novel highly pathogenic coronavirus that is responsible for this infection. Therefore, it was coined as MERS-Coronavirus (MERS-CoV) (WHO, 2018b).

1.3.2 Phylogenetic studies of MERS-CoV

Two distinct species of bats (*Neoromicia capensis* and *Vespertilio superans*) and one species of camels (*Camelus dromedaries*) were found to be phylogenetically linked with MERS-CoV. (Conzade et al., 2018; Ithete et al., 2013; Yang et al., 2014). Thus, these animals might be natural reservoirs for the growth and spread of MERS-CoV. Previous studies highlighted the possibility that there might be zoonotic transmission between natural host species that could have contributed to viral evolution (Han, Yu, & Yu, 2016). Additionally, recombination between previously unlinked DNA in members of beta-coronavirus was found to be prevalent (Z. Zhang, Shen, & Gu, 2016). Therefore, a hypothesis was that this

might have led to increased virulence and adaptation to infecting humans and evading host immunity.

MERS-CoV falls under the classification of beta-coronavirus genus, similar to SARS-CoV (Fehr & Perlman, 2015). Both viruses were found to be structurally similar in previous studies. (N. Wang et al., 2013). However, their binding partners are distinct due to differences in binding specificity resulting from their surface Spike (S) glycoproteins (B. J. Bosch, Smits, & Haagmans, 2014; CSMEC, 2004).

1.3.3 SARS-CoV and MERS-CoV

SARS-CoV emerged in November 2002 in China and was later identified as a cause of global concern due to a high number of infected cases. Within a year, the total number of lab-confirmed cases and deaths were 8,098 and 774 respectively. This amounts to a mortality rate of approximately 9.6% of infected cases (CDC, 2004; WHO, 2003). On the other hand, MERS-CoV emerged later in 2012 and led to 2,449 lab confirmed cases and 845 deaths. This adds up to a high mortality rate of approximately 34.5% (CDC, 2004; WHO, 2018a, 2018b, 2019b, 2019c). As such, the WHO labelled MERS-CoV as one of the critical pathogens for research and development of vaccines and therapeutics (WHO, 2017, 2018a).

MERS-CoV and its predecessor, SARS-CoV share the same phylogenetic cluster of beta-coronavirus genus (Fehr & Perlman, 2015). However, due to their different lineages, SARS-CoV being of lineage B and MERS-CoV being in lineage C, they exhibit significant differences in RNA dependent RNA

polymerase, S and N proteins. As a result of their distinctive S protein sequences, viral tropism is affected as well (Lau et al., 2013).

Analysis of SARS-CoV and MERS-CoV RBDs suggest that both viruses share structural similarities with critical differences in the receptor binding subdomains. MERS-CoV consists of longer β -chains while SARS-CoV has distinctly shorter chains and longer loops (N. Wang et al., 2013). In addition, MERS-CoV RBD consists of 2 short α -helices and 8 β -sheets within the structure, while SARS-CoV RBD has 4 short α -helices and 6 shorter β -sheets. These slight differences in the RBD might have contributed to the distinct difference in host cell specificity, as SARS-CoV targets the Angiotensin-converting enzyme-2 (ACE-2) while MERS-CoV binds to Dipeptidyl-peptidase IV (DPP4 or CD26) (B. J. Bosch et al., 2014; Chinese SARS Molecular Epidemiology CSMEC, 2004; N. Wang et al., 2013).

1.3.4 MERS-CoV life cycle and possible antiviral strategies

MERS-CoV infection cycle begins with viral attachment and entry into host cells. In this phase, the Spike glycoprotein (S) plays an important role in the recognition and attachment to the host cell DPP4 receptors via its RBD in the S1 subunit (N. Wang et al., 2013; Yuan et al., 2017). Binding between the MERS-CoV RBD and host cell DPP4 triggers endocytosis, promoting viral entry. After this, low pH-mediated membrane fusion occurs via a Class I fusion mechanism. This is carried out by the fusion peptide present in the S2 subunit of the S protein (Berend Jan Bosch et al., 2003). Upon membrane fusion and release of viral genome into the host cells, the virus utilizes the host cell machinery to undergo replication and viral protein expression. After which, spontaneous initiation of viral self-assembly will take place within the host cell before the maturation of viral

progeny. Lastly, the release of viral progeny from the host cells via budding will be carried out (Lou, Sun, & Rao, 2014; Lu et al., 2013). A schematic illustration of the whole viral infection cycle is shown (Figure 1.3).

As there are many stages of the viral life cycle, where specific processes are required to take place for viral progression, many anti-viral strategies can be developed by targeting them. The primary objective of anti-viral therapeutics is to inhibit the progression of the infection to induce a protective response in the body against the virus.

Figure 1.3: Schematic representation of the MERS-CoV infection cycle

The key components of the viral life cycle of MERS-CoV include attachment and viral entry, viral replication and protein expression, assembly and release of progeny viruses. Upon viral

attachment, endocytosis is triggered to promote viral entry via endosomes. pH-mediated endosomal release of the viral genome occurs after entry to allow the RNA genome to undergo translation and replication in the cytoplasm and nucleus respectively. After which, the viral proteins are expressed and N-glycosylated via the ER pathway. Then, the different viral protein components self-assemble and bud off from the host membrane to release the newly synthesized viral progeny.

1.4 Key proteins involved in MERS-CoV host tropism

The host cell specificity of MERS-CoV is predominantly determined by the viral attachment factor present in the Spike glycoprotein (B. J. Bosch et al., 2014). Recent studies have narrowed down a domain which is critical for recognition and host attachment to 204 amino acids (N. Wang et al., 2013).

1.4.1 Receptor-binding domain of MERS-CoV

The S glycoprotein can be divided into 2 subunits: S1 and S2. Both subunits are responsible for the facilitation of viral entry. RBD can be found in the S1 subunit, which is critical for the recognition and binding to its complementary host cell receptor. On the other hand, the S2 subunit contains the Class I fusion peptide which is crucial in pH-mediated membrane fusion between virus and host cells to facilitate viral genome release into the host cells (Berend Jan Bosch et al., 2003; Fehr & Perlman, 2015). Recent research narrowed down the MERS-CoV RBD to 204 amino acids in the centre region of the S1 subunit. This specific motif was found to be critical for host cell recognition and attachment as a single mutation on some of the key residues led to significant reduction in viral entry (N. Wang et al., 2013).

1.4.2 Host cell receptor -Dipeptidyl peptidase IV

Previous studies identified Dipeptidyl-Peptidase 4 (DPP4) as the specific host cell receptor to MERS-CoV. DPP4 is an antigenic enzyme that plays key roles in apoptosis, signal transduction and immune regulation. It is a 766 amino acid long protein that has a theoretical molecular mass of 88.3 kDa. It was found to be an intrinsic membrane protein that requires numerous N- and O-glycosylation for translocation to the apical membrane (Alfalah, Jacob, & Naim, 2002; Ikushima et al., 2000). Previous investigations into inhibition of this binding between the MERS-CoV RBD and host DPP4 determined that DPP4 inhibitors were ineffective (Raj et al., 2013). Further structural insights into this binding interface revealed that MERS-CoV RBD interacts and binds to the β -propeller region instead of targeting the main catalytic site of the host DPP4. Specifically, the MERS-CoV RBD was found to contact DPP4 residues: Arg336 and Lys267 on blade 4; Arg317 and Gln344 on blade 5 of the β -propeller region (Figure 1.4) (N. Wang et al., 2013).

Figure 1.4: MERS-CoV RBD-hDPP4 binding interface

MERS-CoV RBD was found to contact hDPP4 at the β -propeller region (PDB: 4L72). (A) Tyr499 and Asp539 on RBD interacts with Arg336 and Lys267 found in the Blade 4 of the DPP4 β -propeller domain respectively. (B) Asp510 and Glu513 on RBD interacts with Arg317 and Gln344 found in the Blade 5 of the DPP4 β -propeller

1.4.3 Binding interface analysis of MERS-CoV RBD and hDPP4

As the structure of the MERS-CoV RBD and hDPP4 complex was previously identified and published in the PDB database (4L72), it was used in our binding interface analysis. For this analysis, the PDBsum platform was used (Laskowski, Jabłońska, Pravda, Vařeková, & Thornton, 2018). The predicted binding pocket of MERS-CoV RBD was analysed and highlighted in yellow (Figure 1.5A). Potential interacting residues between the short DPP4 helical region and the RBD pocket was identified using this software and their interactions were approximated (Figure 1.5B). The interface area was calculated to be 1,957 Å², with 1,028 Å² and 929 Å² contributed by hDPP4 and MERS-CoV RBD respectively. This interface region was found to consist of 2 salt bridges, 5 hydrogen bonds and 78 non-bonded contacts (Figure 1.5E). A short helical region of the hDPP4 protein: Pro-290 to Ile-295 was found to contribute significantly to the interface, of which a deletion mutation resulted in approximately 200 Å² decrease in interface area from each protein (Figure 1.5C-E). This short helical region fitted in the binding pocket on the MERS-CoV RBD was further examined using computational screening to identify any potential compounds which can fit into this pocket like this helix.

A

B

C

D

Figure 1.5: Binding Interface Analysis of RBD-DPP4 complex

(A) Illustration of the short DPP4 helical region (highlighted in dotted circle) sitting in the predicted binding pocket on RBD (yellow). (B) PDBSum predicted interactions based on the protein interface analysis. Residues highlighted with a red asterisk represents residues critical for binding between the DPP4 helix and RBD pocket. (C) Illustration of the deletion mutation of the short DPP4 helical region (highlighted in dotted circle) in the predicted binding pocket on RBD (yellow). (D) Results predicted interactions by PDBSum after deletion mutation. (E) Comparison of interface interactions between the native DPP4 and deletion mutant (Δ290-295) with information on the total number of salt bridges, hydrogen bonds and non-bonded contacts before and after deletion.

1.4.4 Development of vaccines and therapeutics against MERS-CoV

There are currently no approved vaccines or drugs for the treatment of MERS-infections (WHO, 2018b, 2018c). Quarantine of MERS-infected patients is

conducted to minimize the spread of this virus. In addition, due to the lack of specific drugs for MERS, previously used drugs against SARS are being administered in lower dosages to reduce any potential side effects (Dyall et al., 2014). Although improvements were observed in MERS-infected patients, the side effects of such drugs were adverse. For example, the use of ribavirin could lead to ribavirin-induced anaemia and have potential carcinogenic effects, as observed in animal studies using *in vitro* mouse lymphoma assay (NCBI, 2019). Research on vaccine development is being carried out globally (Modjarrad, 2016). However, as vaccines are not absolute in its effectivity in preventing a viral infection, it should be coupled with drug treatments and options to mitigate the impacts of this viral infection. Therefore, the discovery and generation of more effective therapeutic options for the treatment of MERS-infections should be implemented concurrently. Hence, the clinical situation vis-à-vis MERS-CoV infection is exacerbated by the lack of vaccines and specific therapeutic drugs.

1.5 Aim of project

This thesis predominantly focuses on the discovery of small molecules targeting the first step of viral infection: viral attachment and entry via receptors on the host cell. The main objective is to prevent or disrupt the attachment of the virus surface Spike glycoproteins to their specific receptors on the host cell surface and as a result, inhibit any downstream infection of host cells. Therefore, binding and inhibition studies on complex formation between MERS-CoV RBD and its host cell hDPP4 receptor were carried out. This project focuses on 3 different approaches of drug discovery: 1) Drug re-purposing via structure-guided screening of FDA-approved drugs, 2) New fragment library screening and 3) Designing of peptide mimetics against MERS-CoV RBD. Of these 3 different

approaches, previous structure-guided *in-silico* screens and analysis on FDA-approved drugs by Dr. Harikishore and Dr. Sreekanth revealed seven potential hits. From these seven hits, MSH was predicted to fit and fill the MERS-CoV RBD pocket well and would be further studied in this thesis under Approach 1.

2. Materials and Methods

2.1 MATERIALS

2.1.1 Chemicals and Drugs used

All chemicals were purchased from Sigma Aldrich (St Louis, MO) unless otherwise stated. Information on the chemicals used in this study are listed in Table 1.

Table 1: List of chemicals with their respective companies and locations

Chemicals	Company	Location
Bacto-Yeast Extract, Bromophenol Blue, Glycerol, Triton X-100	Affymetrix	Santa Clara, CA
Glycine	Axil Scientific	Singapore
Bacto-Tryptone, Bacto-Agar	BD Biosciences	San Jose, CA
Precision Plus Protein All Blue, Dual Color Protein Standards	Bio-Rad	Hercules, CA
Kanamycin sulfate, Gentamicin, Fetal Bovine Serum/Heat inactivated FBS, OPTI-MEM I Reduced Serum Medium, 0.05 % Trypsin-EDTA, Penicillin-Streptomycin, SF900III Serum Free Medium (SFM) Grace's Insect Medium	Gibco	Carlsbad, CA
Carbenicillin, Isopropyl-beta-D-thiogalactopyranoside (IPTG), Dithiothreitol (DTT)	GoldBio	St Louis, MO
Sodium Dodecyl Sulfate (SDS)	Hoefer Inc	Holliston, MA
Dulbecco's Modified Eagle Medium (DMEM)	Hyclone	Carlsbad, CA
Absolute Ethanol 99.5-99.8 %, Isopropanol, Sodium Chloride (NaCl)	Merck Millipore	Whitehouse Station, NJ
Tris-base, Dual-Luciferase® reporter assay system	Promega	Madison, WI
WST-1 cell proliferation reagent, cOmplete His-tag Purification Ni ²⁺ resin	Roche	Basel, CH
Chloroform, TRIzol Reagent, Lipofectamine 3000, <i>taq</i> and <i>pfu</i> polymerases, Cellfectin II, GeneRuler 1 kb DNA ladder	Thermo Fisher Scientific	Waltham, MA
WesternBright Enhanced Chemiluminescent	Advansta	Menlo Park, CA
Mini-prep DNA Isolation Kit	Axygen	Corning, NY
anti-MERS-CoV-S1 center, N-ter antibodies	Sino-Biological	Beijing, CN
Goat anti-Rabbit IgG-HRP antibodies	Santa-Cruz Biotechnology	Dallas, TX

All drugs used are dissolved in DMSO unless otherwise stated. Information on drugs used in this study are listed in Table 2.

Table 2: List of drugs with their respective chemical structures and companies

Drug	Structure	Company
Lopinavir (LPV)		Selleckchem
Chloroquine diphosphate (CQ)		Sigma Aldrich
SARS 3C-like protease inhibitor (CE-5)		Korea Research Institute of Chemical Technology (KRICT)
7, 8 dihydroxyflavone (7,8-DHF)		Microsource
Montelukast Sodium Hydrate (MSH)		Sigma Aldrich
Cefaclor		Sigma Aldrich
Nalmefene		Sigma Aldrich
Cephadrine		Sigma Aldrich
Lovastatin		Sigma Aldrich
Reserpine		Sigma Aldrich
Carvediol		Sigma Aldrich

2.1.2 Bacterial Strains, Insect and Mammalian Cell Lines

DH5α cells were primarily used for plasmid amplification. *BL21(DE3)*, *BLR(DE3)pLysS* and *Origami(DE3)* were used in bacterial expression screens. *DH10EMBaY-GFP* cells acquired from EMBL (Heidelberg, DE) were used for transformation and transposition of a donor plasmid for bacmid generation. *HEK 293T* human embryonic kidney cells were obtained from American Type Culture Collection (ATCC) (Manassas, VA) and were used in the production of MERS-CoV Spike pseudovirions. *Vero E6* cells acquired from ATCC (Manassas, VA) were used in pseudovirion infection studies. SF-9 *Spodoptera Frugiperda* cells were kindly gifted by Ms Dina from NTU/PPP.

2.1.3 Vectors and Primers

pFastbac-Dual-RBD and pFastBac-Dual-DPP4 plasmids were kindly gifted by Professor Wang Xinquan. pCMV-MERS-CoV Spike plasmid was synthesised and purchased from Sino-Biological (Beijing, CN). HIV-1 based lentiviral packaging plasmid: psPAX2, and pLenti CMV puro LUC (w168-1) containing a firefly luciferase reporter gene was kindly gifted by Didier Trono (Addgene, MA). Primers were designed for the main purpose of identification and verification of transposition during bacmid generation for MERS-CoV RBD and hDPP4. pUC-M13 primers were used to determine the length of transposed insert from the donor plasmid to the *lacZα* insertion site on the bacmid (Table 2).

Table 3: Sequence of primers used

Primer	Sequence (5' to 3')
MERS-CoV RBD Forward	CTCGCGGATCCGTCGATGCTACTAGTAAAT
MERS-CoV RBD Reverse	CACGCAAGCTTATCGCTAATGGTGGTGATGGTGGTG
hDPP4 Forward	ATCCAGCTCGAGATCGCGATGGCGTTCAAGAGT
hDPP4 Reverse	CGCTGGTACCGGATCCTAATGGTGGTGATGGTGGTG
pUC-M13 Forward	CCCAGTCACGACGTTGTAAAACG
pUC-M13 Reverse	AGCGGATAACAATTTCACACAGG

2.1.4 Cell culture media and Antibiotic stocks

Mammalian culture media for maintenance: RPMI-1640 and DMEM were supplemented with 10 % FBS and 1 % Pen-Strep. Insect cell media for maintenance of *Sf9* cells, SF900 III SFM was supplemented with 1 % Heat-Inactivated FBS and 0.5 % Pen-Strep. For transfection purposes, Opti-MEM I Reduced Serum Medium and Grace's Insect Medium were used for mammalian and *Sf9* cells respectively. For antibiotic stocks, Kanamycin sulfate stock was prepared in water at a concentration of 30 mg/mL for bacterial cell growth and 50 mg/mL for blue-white colony screening plates. Carbenicillin stock was prepared at 100 mg/mL concentration in water. Tetracycline stock was prepared at 10 mg/mL stock concentration in 70% ethanol. X-gal (prepared in DMSO) and IPTG stock concentration was set at 100 mg/mL and 1 M respectively.

2.1.5 Media for bacterial culture

2.1.5.1 Luria-Bertani (LB) Broth

1 L of LB broth was prepared by dissolving 5 g of NaCl, 5 g of Bacto-Yeast extract and 10 g of Bacto-Tryptone in MiliQ water. The LB broth was then sterilised via autoclaving and left to cool down to room temperature before use.

2.1.5.2 LB Agar plates

For normal LB agar plates, 17 g of Bacto-Agar was added to 1 L of LB broth and autoclaved. After which, the LB agar was left to cool to approximately 50 to 60 °C before adding the respective antibiotics and distributed into petri dishes for solidification and use. The plates were stored in 4 °C and pre-warmed in 37 °C for 1 hour on the day of use. For the blue-white colony screening plate, Kanamycin sulfate, Tetracycline, Gentamicin, X-gal and IPTG were added at a final concentration of 50 µg/mL, 10 µg/mL, 7 µg/mL, 100 µg/mL and 100 mM respectively.

2.1.6 Buffers and reagents

2.1.6.1 Buffers used

Buffers used for protein purification were prepared according to Table 2, filter-sterilised and degassed before use. For the SDS-PAGE running buffer, a 5x stock was prepared and diluted prior to each use.

Table 4: List of Buffers and their corresponding compositions[#]

Buffers used	Buffer composition
Ni ²⁺ -NTA Lysis buffer	50 mM Tris-HCl pH 8.0, 150 mM NaCl
Ni ²⁺ -NTA Elution buffer	50 mM Tris-HCl pH 8.0, 150 mM NaCl, 500 mM Imidazole pH 8.0
FPLC Buffer (Tris)	25 mM Tris-HCl pH 8.0, 30 mM NaCl, 0.01% NaN ₃
ResQ Buffer A	50 mM Tris, 0.01% NaN ₃
ResQ Buffer B	50 mM Tris, 500 mM NaCl, 0.01% NaN ₃
SDS-PAGE Running Buffer	25 mM Tris, 200 mM Glycine, 0.5% (w/v) SDS
Transfer Buffer	25 mM Tris, 200 mM Glycine, 0.5% (w/v) SDS, 20% (v/v) Methanol
1x Tris-Buffered Saline – Tween 20 (TBS-T)	25 mM Tris-HCl pH 7.4, 3 mM KCl, 140 mM NaCl, 0.1% Tween-20

[#] All buffers used in purification processes such as Ni²⁺-NTA, Size exclusion and anion exchange chromatography were filtered and degassed before usage.

2.1.6.2 Reagents used

Reagents used in our study were prepared accordingly as shown in Table 3. The 6x SDS-loading dye and 0.4% Trypan Blue solution were prepared, filter-sterilised and aliquoted into 1 mL vials for use. As the 6x SDS-loading dyes contain DTT, the unused aliquots were stored in -20 °C.

Table 5: List of Reagents with their corresponding compositions

List of Reagents used	Reagent Composition
6x SDS-loading dye	300 mM Tris (pH 6.8), 50% Glycerol, 12% (w/v) SDS, 600 mM DTT, 0.2% Bromophenol blue
Coomassie Blue staining solution	0.25% Coomassie-Brilliant Blue R-250, 50% Methanol, 10% Glacial Acetic acid
Destaining solution	45% Methanol, 10% Glacial Acetic acid
0.4% Trypan Blue solution	0.4% w/v Trypan Blue, 1x PBS

2.2 METHODS

2.2.1 Transformation and Transposition

Transformation was performed by first adding 200 ng of plasmids (pFastBac DUAL DPP4, psPAX2, pLenti CMV puro LUC (w168-1), or pCMV-MERS-Spike) to 50 μ L of cells (*BL21(DE3)*, *BLR(DE3)pLysS*, *Origami(DE3)*, *DH5 α* , or *DH10EMBacY-GFP* cells) and incubating on ice for 10 minutes. After which, the cells were subjected to a heat shock at 42 °C for 45 seconds and 2 minutes incubation on ice. 250 μ L of LB broth was then added to each tube and the mix was incubated at 37 °C, shaking at 200rpm for 1 hour. The contents of each tube were then plated out on LB agar plates with specific antibiotics according to the different plasmids used. (Carbenicillin plates for psPAX2, pLenti-CMV-puro-LUC-(w168-1) and pCMV-MERS-Spike; Kan/Tet/Gent/IPTG/X-gal plates for pFastBac DUAL DPP4). The LB agar plates were then incubated in a static incubator at 37 °C for 16 hours.

For transposition, instead of 16 hours incubation time, it was extended to 48 hours to allow the X-gal selection to take place. Upon 48 hours of incubation, cells

which have undergone transposition via *Tn7* sites would disrupt the *lacZ* cassette, which in turn prevents the expression of β -galactosidase enzyme. Due to the absence of this enzyme, the substrate, X-gal would not be hydrolysed to form 5-bromo-4-chloro-indoxyl, which would dimerise and produce an insoluble blue compound known as 5,5'-dibromo-4,4'-dichloro-indigo (Merck, 2019). As a result, the colonies on the plate with transposed gene fragments would appear white instead of blue. After 48 hours, white colonies were picked, diluted in 100 μ L of LB broth and re-plated on fresh Kan/Tet/Gent/IPTG/X-gal plates to confirm that the previously picked white colonies were 'true white' instead of false positives for another 48 hours (Invitrogen, 2015).

2.2.2 Miniprep plasmid isolation from *DH5 α* cells

Plasmid isolation was conducted following the Axygen® Axyprep™ Plasmid Miniprep kit protocol (Corning, NY). Firstly, 1 single colony from the bacterial agar plates containing the plasmid of interest was selected and inoculated into 5 mL LB broth and grown overnight for 16 hours at 37 °C at 200 rpm. On the next day, the 5 mL overnight culture was centrifuged at 3,000x g for 10 minutes at 4°C to pellet down the cells. After which, the cells were resuspended in 250 μ L of Buffer S1 containing RNase A by pipetting up and down. 250 μ L of Lysis buffer S2 was then added and the tube was gently inverted 4 to 5 times to mix well. Then, 350 μ L of Neutralisation Buffer S3 was added within 2 minutes and the tube was gently inverted for 5 to 6 times. The mixture was then centrifuged at 12,000x g for 10 minutes in 4°C.

The supernatant was collected and transferred into a Mini-prep column in a 2 mL micro-centrifuge tube for binding to the column. The tube was then spun at 12,000x g for 1 minute. The filtrate in the tube was discarded and the column was

placed back in the tube. 700 μ L of wash buffer W2 was added into the column and centrifuged at 12,000x g for 1 minute. To elute the plasmids from the column, 50 μ L of autoclaved water was added to the centre of the column and incubated at room temperature for 2 minutes before spinning at 12,000x g for 1 minute.

2.2.3 Small scale bacterial expression screen of recombinant proteins

For a 50 mL bacterial expression, a 2 mL starter culture was prepared. A single colony containing the plasmid of interest was picked from the agar plate and inoculated into the 2 mL culture containing the specific antibiotics and grown overnight for approximately 16 hours at 37 °C and 200 rpm. 500 μ L of the overnight culture was then added to 50 mL autoclaved LB broth with the relevant antibiotics and grown at 37 °C for 2.5 hours before the Optical Density was measured at 600 nm wavelength (OD_{600}). Once the OD_{600} of the culture reaches 0.5 to 0.6, 50 μ L of IPTG was added to the 50 mL culture to induce protein expression. The culture was then incubated in the specific temperature for 3 to 4 hours before harvesting. After 3 to 4 hours incubation, the culture was centrifuged at 3,500x g for 15 minutes to pellet down the cells. The cell pellet was resuspended in 10 mL of lysis buffer and sonicated on ice at 3 seconds on, 1 second off pulse for 15 minutes at 24 % amplitude. 1 mL of the sonicated sample was harvested and centrifuged at 14,000x g for 30 minutes. The supernatant fraction was collected, and the pellet was resuspended in 150 μ L of lysis buffer. Both the supernatant and the pellet fractions were analysed using SDS-PAGE for solubility of protein expressed.

2.2.4 Bacmid Isolation via Isopropanol precipitation

DH10EMBacY-GFP cells containing the transposed constructs were inoculated into 5 mL LB broth supplemented with Kan/Tet/Gent antibiotics and incubated

in 37 °C at 200 rpm for 24 hours. The 5 mL overnight cultures were then centrifuged at 13,000x g for 1 minute to pellet down the cells. After which, the supernatant was discarded, and the cells were resuspended in 300 µL of Resuspension buffer containing RNase A. 300 µL of Lysis buffer was then added into the sample and mixed well. Next, 300 µL of Neutralisation buffer was added and the sample was incubated on ice for 10 minutes before centrifugation at 13,000x g for 10 minutes was performed to pellet down the cells. The supernatant was transferred to a clean 1.5 mL eppendorf tube and 800 µL of isopropanol was added and mixed well.

The supernatant in isopropanol was incubated on ice for 30 minutes for the precipitation of DNA. After which, the sample was centrifuged at 13,000x g for 10 minutes in 4 °C and the supernatant was discarded. The DNA pellet formed at the base of the tube was then washed twice with 500 µL of ice cold 70 % ethanol, with centrifugation at 13,000x g for 10 minutes at 4 °C after each wash and left to dry for 15 to 30 minutes. The pellet was dissolved in 40 µL of autoclaved water and the bacmid purity as well as concentration was analysed using NanoDrop.

2.2.5 Polymerase Chain Reaction (PCR)

The samples (plasmids or bacmids) were subjected to PCR screening to ensure that the gene of interest present. For this screening, a 50 µL PCR mix of forward and reverse primers specific to the gene or insertion site, dNTPs, Taq polymerase reaction buffer, Magnesium chloride (MgCl₂) and Taq polymerase was prepared for each sample, topped up with autoclaved water. The specific volumes of each constituents were as followed: 36.5 µL of autoclaved water, 1.5 µL forward primers, 1.5 µL reverse primers, 1.5 µL dNTPs, 1 µL DNA template (plasmids or bacmids), 5 µL of 10x Taq reaction buffer, 2.5 µL of MgCl₂ and 0.5 µL of Taq

polymerase. The mix was placed and ran in a thermocycler at the following conditions: 95 °C for 5 minutes (denaturation phase), 30 cycles of 95 °C for 15 seconds, approximately 55 to 65 °C (annealing temperature depends on primers' melting temperature) for 30 seconds and 72 °C for 2 minutes (depends on length of construct, Taq operates at 1 kb per second), and lastly 72 °C for 10 minutes.

2.2.6 DNA Gel Electrophoresis

After the PCR amplification, a 1 % agarose gel was prepared due to the size of the DNA construct of interest. For the agarose gel, Gel-Red was used for visualization under UV radiated light. Generally, 50 mL of gel would be prepared for 1 electrophoretic run. 0.5 g of agarose was first weighed and mixed with 50 mL of 1x TAE buffer. The gel mix was then heated in a microwave oven till the agarose fully dissolves in the buffer. The mix was cooled by running water till approximately 50 °C before addition of 5 µL of Gel-Red staining solution. The gel mix was swirled gently to ensure even distribution of the Gel-Red solution and poured into the gel tray with the well-comb for solidification of the agarose gel. For the DNA samples for analysis, 2 µL of 6x Nucleic acid purple loading dye (NEB, MA) was added to 10 µL of PCR samples and ran at 110 V for approximately 45 minutes. The gel was then analysed using an Agarose Gel Documentation system.

2.2.7 Culturing of *Sf9* cells

A frozen vial of *Sf9* cells in the -80 °C freezer was thawed rapidly in a 37 °C water bath and subsequently, the thawed cells were added to 25 mL of SF900III media containing 1 % Heat-Inactivated Fetal Bovine Serum (HI-FBS) (Life Technologies, USA) and 0.5 % Penicillin-Streptomycin (Pen/Strep) solution. The

cells were then incubated in a 27 °C shaking incubator at 150 rpm for 2 to 3 days before cell viability and cell count was checked using a hemocytometer.

For this procedure, 5 µL of cells were diluted in 45 µL of 0.4 % Trypan Blue solution. (Sigma Aldrich, USA) 10 µL of cells in Trypan Blue solution was added on a hemocytometer and placed under a light microscope. The cells would then be counted and the cell count is calculated using the dilution factors and volume of cells used. If there are many dead cells, the culture would be centrifuged at 1,500x g for 5 minutes to pellet down the cells and then the cell pellet would be resuspended in 30 mL of fresh SF900III media with 1 % HI-FBS and 0.5 % Pen/Strep. As the doubling time of *Sf9* cells is approximately 24 hours, the cells were checked and split every 2 to 3 days depending on the viability and cell concentration.

2.2.8 Transfection of bacmids into *Sf9* cells

For the transfection of bacmids, 4 sets of 6-well plates were used – 1 for each bacmid construct, RBD and DPP4. Firstly, 5×10^5 cells were added to each well and incubated at room temperature in the biosafety cabinet for 15 to 30 minutes for attachment. While the cells were incubating, the transfection reagents were prepared. For each well, 200 µL of Grace's Insect Medium was added with 4 µL (2µg) of bacmid and 8 µL of Cellfectin II transfection reagent. However, 2 of the wells were labelled for negative control – one for no bacmid, the other for no Cellfectin. Therefore, in these 2 wells, the bacmids or cellfectin reagents were replaced with Grace's Insect Medium. The transfection mix were prepared and mixed well. Then, these mixes were incubated at room temperature for 15 to 30 minutes in the biosafety cabinet.

The *Sf9* cell attachment was analysed using a light microscope. Subsequently, the media was aspirated and the unbound *Sf9* cells were washed off with 1 mL of Grace's Insect Medium for each well. Then, 1.5 mL of Grace's Insect Medium was added into each well before the transfection mix was added drop wise. The 2 plates were then incubated in 27 °C for 3 to 5 hours before the media was changed back to 2 mL of SF900III media containing 1 % HI-FBS and 0.5 % Pen/Strep. After which, the plates were left in 27 °C for approximately 5 to 7 days and the viral supernatant was harvested when the cells looked well infected under the light microscope. Harvesting of the viral supernatant was performed by first resuspending the cells briefly by pipetting up and down and transferring the sample to a falcon tube. The samples were then centrifuged at 1,500x g for 5 minutes to pellet down the cells. The supernatant containing the P₀ baculovirus was then collected and placed in a clean 15 mL falcon tube and stored in 4 °C.

2.2.9 Amplification of baculovirus

Firstly, *Sf9* cells were seeded in a 125 mL flask at a density of approximately 2.5×10^6 cells/mL, topped up to 50 mL with SF900III media supplemented with 1 % HI-FBS and 0.5 % Pen-strep. 50 µL of P₀ baculovirus was added to 50 mL culture for amplification of baculovirus and incubated in 27 °C at 130 rpm, protected from light, for 60 to 72 hours. This is due to the light sensitive nature of baculovirus. After 60 to 72-hour incubation, the culture was then centrifuged at 1,500x g for 5 minutes to pellet down the cells and debris. The supernatant was collected, labelled as P₁ baculovirus stock, and kept in 4 °C, protected from light. The P₁ baculovirus stock was then further amplified to P₂ and then P₃ following the above-mentioned protocol. P₃ baculovirus stock is the highest passage before usage for protein expression as further amplification might lead to a high rate of

deleterious mutations in baculoviruses (Kool, Voncken, Van Lier, Tramper, & Vlak, 1991). Therefore, P₃ stock was used for subsequent protein expression studies.

2.2.10 Protein Expression optimisation screens using P₃ baculovirus stock

Small cultures of approximately 20 to 25 mL were used for protein expression optimisation screens. For a 25 mL culture, a cell density of approximately 2.5×10^6 cells/mL were seeded and varying volumes of P₃ baculovirus stocks were used for infection. Additionally, incubation period was also optimised. Generally, incubation for protein expression screens were harvested and tested at 3- and 4-days post infection at 27 °C, protected from light, and 130 rpm. After harvesting, Ni²⁺-NTA purification will be carried out to remove impurities. SDS-PAGE will be performed to check for the presence of the target protein expression.

2.2.11 Protein Expression in *Sf9* cells

Similar to protein expression screens, *Sf9* cells were seeded in a 2 L flask at a density of 2.5×10^6 cells/mL. Next, the optimal volume of P₃ baculoviruses from previous screens were added into the cell culture. The culture was then incubated in a shaking incubator protected from light at 27 °C at 130 rpm and for the optimal incubation period of either 3 or 4 days. After the incubation period, the infected culture was then harvested by centrifugation at 9,000x g for 15 minutes. The supernatant containing the secreted proteins were then collected and used for downstream purification and assays.

2.2.12 Immobilised Metal-Ion Affinity Chromatography (Ni^{2+} -NTA Purification)

The secreted proteins (MERS-CoV RBD or hDPP4) both contain a 6-His tag. This tag allows the purification of proteins via the binding to Ni^{2+} resin (Roche, CH). The Ni^{2+} resin was first washed with approximately 15 to 20 column volume (CV) of autoclaved water and then equilibrated with 2.5 mL of lysis buffer. Subsequently, the supernatant containing the secreted proteins was collected and incubated with Ni^{2+} beads for 1 hour on a shaking incubator at 90 rpm in 4 °C. After which, the sample was passed through a gel filtration column. The flow through was collected and the beads were then washed with 15 CV of lysis buffer to remove any non-specific proteins present in the sample. The proteins were subsequently incubated with 10 mL elution buffer for 15 minutes and eluted thereafter. 50 μL of each fraction – Supernatant (before Ni^{2+} -NTA), Flowthrough, Wash and Elution were collected for SDS-PAGE analysis.

2.2.13 Gel Filtration Chromatography (FPLC)

For gel filtration chromatography, the HiLoad Superdex-75 16/60 column (GE Healthcare, CT) was used for MERS-CoV RBD. Firstly, the column was pre-equilibrated with 130 mL of FPLC buffer. The elution from Ni^{2+} -NTA purification was then concentrated from 10 mL to 1.5 mL. The concentrated sample was centrifuged at 16,000x g for 10 minutes at 4°C. Prior to the injection of sample, the sample loop was washed and equilibrated with 5 mL of FPLC buffer. After which, the sample was injected and the FPLC system was started. The proteins were monitored via Ultra-violet (UV) absorption at 280 nm. The flow rate was set at 0.4 mL/minute and pressure alarm was fixed at 0.5 MPa. Upon completion of the run, fractions containing the RBD sample were collected

based on the UV absorption curve generated by the system, concentrated using Amicon® Ultra 15 mL centrifugal unit (Merck Group, DE) to approximately 1.5 mL for 2nd injection into the Superdex 200 Increase 10/300 GL (GE healthcare, CT). For hDPP4 samples, 2 rounds of S200 runs were performed instead of S75.

2.2.14 Anion Exchange Chromatography (ResourceQ)

After 2 rounds of gel filtration using S75 and S200, if the sample still contained slight impurities, a ResourceQ column (GE Healthcare, CT) would be used (Appendix I). For this column, 2 buffers were used: ResQ Buffer A containing no NaCl and ResQ Buffer B with high NaCl for elution of sample. Like that of the gel filtration chromatography, the injection loop was first washed with 5 mL of ResQ buffer A and the column was pre-equilibrated with ResQ buffer A. The proteins were monitored via Ultra-violet (UV) absorption at 280 nm. The flow rate was set at 0.75 mL/minute and pressure alarm was fixed at 1.5 MPa. Initial sample injection flow rate was lowered to 0.1 mL/minute to allow binding to the resin. During the elution phase, a NaCl gradient was configured, starting from 50 % ResQ Buffer B to 100 % ResQ Buffer B over 25 mL of elution. Upon completion of the run, fractions containing the RBD sample were collected based on the UV absorption curve generated by the system for SDS-PAGE analysis.

2.2.15 SDS-PAGE Analysis

SDS-PAGE was performed to obtain the Ni²⁺-NTA purification profile, identify the FPLC fractions which contain the purified protein of interest and to test for the purity of a protein sample. For both MERS-CoV RBD and hDPP4, 12 % SDS gels were used. 6x loading dye containing DTT was first added to break disulfide bridges present in the protein samples. The samples were then placed on a heat block at 95 °C for approximately 10 minutes to denature the proteins. After which,

they were briefly centrifuged for 3 seconds before being loaded into the wells of the SDS gel. SDS-PAGE was then started at 160 V for 55 minutes. Upon completion of the run, the gel was then stained with coomassie blue staining solution for 1 hour before being de-stained for 1 hour with de-staining solution. To further reduce background in the SDS gels, the gels were de-stained in distilled water overnight before the visualization of results.

2.2.16 Western Blotting

Expression of MERS-CoV Spike and RBD protein samples were visualized by Western Blotting with an anti-MERS-CoV S1 Center region antibody (Sino-Biological, CN). Firstly, SDS-PAGE was carried out as mentioned above. Upon completion of the run, the gels were used for the transfer of proteins to a nitrocellulose membrane at 100 V for 1 hour. Next, the membrane was blocked in 5 % skimmed milk for 1 hour. The membrane was then probed with the protein specific primary antibodies prepared in 5 % skimmed milk, overnight.

The membrane was subsequently washed with 1x TBS-T thrice, where each wash was incubated on a shaker at room temperature for 10 minutes. After which, the membrane was probed with specific secondary antibodies conjugated to an enzyme – Horseradish Peroxidase (HRP). The secondary antibodies selected as dependent on the type of primary antibodies used previously. For instance, if a primary antibody generated from a mouse model was used, the secondary antibody should be an anti-mouse antibody conjugated with HRP. HRP was used as a reporter enzyme such that when it was exposed to the substrate, it would cleave the substrate to generate a chemiluminescent signal that could be detected using a photographic film or computer programs.

2.2.17 Structure-guided *in-silico* screening of FDA approved drug library

This *in-silico* screening of FDA approved drug library dataset was conducted by Dr. Harikishore. Firstly, the protein subunits of the MERS-CoV RBD-hDPP4 complex structure were first prepared by removing heteroatoms, adjusting charges, potentials, bond orders and missing atoms with CHARMM force field and energy minimized with heavy atom constraint for 5000 steps of conjugate gradient algorithm in Biovia Discovery Studio 4.0 (Dassault Systèmes, 2016), before it was used for further molecular docking simulation studies.

The 3-dimensional coordinates of the FDA approved drugs were obtained from Zinc database server (Sterling & Irwin, 2015). These ligands were energy minimized using the smart minimizer algorithm and used in molecular docking studies using GOLD, CCDC suite of programs (Jones, Willett, Glen, Leach, & Taylor, 1997). Default parameters for docking were employed to generate docking poses for the ligands in FDA approved library due to the absence of previously identified inhibitors. All modelling and simulation studies were carried on a Linux Workstation.

2.2.18 Thermal Shift Assay for ligand screening

Thermal shift assay was performed for the purified MERS-CoV RBD samples with the 7 previously identified ligands. Firstly, purified MERS-CoV RBD samples were centrifuged at 16,000x *g* for 10 minutes to pellet down any aggregates. The protein sample was diluted in 25 mM Tris-HCl (pH 8.0), 30 mM NaCl and 0.01 % NaN₃ to a final concentration of 10 µM per well in a Framestar® 96, semi-skirted PCR plate (4titude, Surrey). After which, ligands diluted in DMSO were added into each well at a protein to ligand ratio of 1:5. For the control wells, DMSO was added in place of the ligands. 5x SYPRO Orange dye

(Life Technologies, CA) was then added into each well. The plate was then sealed with a MicroAmp® Optical Adhesive Film from Applied Biosystems (Life Technologies, CA) and loaded into the Applied Biosystems® 7500 Real-Time PCR System. The system was initiated with a temperature increase from 25 °C to 99 °C, with a ramp in 1 °C per minute. The absorption and emission wavelengths were measured at 490 and 575nm respectively. The data was normalized and fitted to a Boltzmann sigmoidal model.

2.2.19 Tryptophan (Trp) Quenching Experiments

Trp Quenching assay was performed on the purified MERS-CoV RBD samples with the 7 previously identified ligands. Firstly, purified MERS-CoV RBD samples were centrifuged at 16,000x g for 10 minutes to pellet down any aggregates. The protein sample was diluted in 25 mM Tris-HCl (pH 8.0), 30 mM NaCl and 0.01% NaN₃ to a final concentration of 5 µM per well in a Corning® 96-well, flat bottom black plate (Corning Inc, NY). After which, ligands diluted in DMSO were added into each well at a protein to ligand ratio of 1:5. For the control wells, DMSO was added in place of the ligands. Subsequent screens were carried out for a protein to ligand ratio of 1:5, 1:10, 1:20, 1:30, 1:40 and 1:50. For the control wells, DMSO was added in place of ligands. The plate was then loaded onto a Tecan Safire²™ microplate reader (Tecan Group Ltd, NY). The absorption wavelength was set at a range of 280 to 290nm and the emission range was specified between 290 to 430nm respectively. Data was obtained via the Magellan software (Tecan Group Ltd, NY) and was fitted onto a non-linear regression model.

2.2.20 Crystal screening

Primary screening was performed using the Crystal Gryphon (Art Robbins Instruments LLC, CA) and MOSQUITO (TTPLabtech, UK) crystallisation platforms with the RockImager and RockMaker (Formulatrix®, MA) automated imaging system, available at the NTU Institute of Structural Biology (NISB). For this primary screen, 7 mg/mL of MERS-CoV RBD was pre-incubated with MSH at a ratio of 1:10 (protein-to-ligand) for 24 hours at 4 °C, which was subjected to crystal screening at 18° C using the Rigaku Wizard Classic (Rigaku Reagents Inc, CA) and Index HT (Hampton Research, CA) screen kits. Initial hits were observed in 25 % Polyethylene glycol 3,350 (PEG 3,350), 0.1 M Bis-Tris pH 5.5 and 0.2 M Ammonium Sulfate. Optimisation to yield better crystals was carried out by varying PEG 3,350, Bis-Tris pH range and the concentration of samples. The RBD+MSH co-crystals grew up to 0.2 mm³ - 0.3 mm³ in 10 to 14 days, while apo crystals of RBD were also grown under similar conditions to be used for soaking experiments.

2.2.21 Culturing of Mammalian cells

HEK 293T and *Vero E6* cells were grown and maintained on a 10 cm³ culture plate in DMEM medium with high glucose and 4 mM L-glutamine, supplemented with 10 % (v/v) fetal bovine serum (FBS) and 1 % (v/v) 100x Penicillin-Streptomycin solution. For the passaging and maintenance of *HEK 293T* and *Vero E6* cells, the media would first be aspirated and the cells washed once with 1 mL of 1x PBS to remove any remaining culture medium. After which, 2 mL of 1x Trypsin-EDTA solution would be added to the cells and the plate would be swirled to ensure even distribution of the solution. The plate will then be incubated in 37 °C for approximately 5 to 10 minutes till the cells detached. 6 mL

of DMEM media supplemented with 10 % FBS and 1 % Pen-strep will be added to the cells to rinse off any loosely attached cells. The cells would then be transferred to a 15 mL falcon tube and centrifugated at 500x *g* for 3 minutes to pellet down the cells. After which, the supernatant would be decant and 8 mL of DMEM media supplemented with 10% FBS and 1% Pen-strep would be added to resuspend the cell pellet. 0.5 mL of the resuspended cells would be added dropwise to a new 10 cm³ culture plate containing 9.5 mL of DMEM media with 10% FBS and 1% Pen-strep. The plate would then be swirled gently to ensure even distribution of cells before incubation in 37°C. Cells were incubated for 3 to 4 days before the next passaging.

2.2.22 MERS-CoV Spike Pseudovirion (PV) generation

As the usage of the isolated stock of MERS-CoV would be classified as a level 3 biosafety concern, due to safety reasons, we were unable to perform assays on them. As a result, MERS-CoV Spike pseudovirions were generated as a means for *in vitro* testing of inhibition efficacy of the compounds. A typical PV is a viral particle containing the primary backbone of a modified HIV-1 virus and an envelope or spike glycoprotein of the virus of interest. For instance, in this study, the spike glycoprotein of interest would be the MERS-CoV Spike protein. A key advantage for using PV is the inability of the virions to propagate as the plasmids used are self-inactivating. Therefore, upon infection by the PV, the infected cells expresses the luciferase enzyme encoded in the transfer vector, which can be monitored by the luciferase assay.

The generation of PV was carried out in *HEK 293T* cells, according to the Addgene lentiviral production protocol (Addgene, 2016). For this protocol, a 3 plasmids system was used. The 3 plasmids were psPAX2, pLenti CMV puro LUC

(w168-1) and pCMV-MERS-Spike. psPAX2 is the packaging vector which consists of the primary HIV-1 backbone genes such as HIV-1*gag*, HIV-1*pol*, HIV-1*pro*. pLenti CMV puro LUC (w168-1) is the transfer vector which holds a luciferase reporter gene which will be incorporated into the host cells upon infection with the PV. Lastly, pCMV-MERS-CoV Spike plasmid encodes the MERS-CoV Spike glycoprotein, under the control of the cytomegalovirus (CMV) promoter, which will be inserted into the virion surface as shown (Figure 2.1).

Figure 2.1: Schematic illustration of MERS-CoV Spike pseudovirion generation.

A 3-plasmid system was used for the generation of the MERS-CoV Spike PV. For the HIV-1 backbone, a packaging vector psPAX2 was used. The pCMV-MERS-Spike plasmid provides the Spike glycoprotein for the assembly of the PV. The pLenti CMV puro LUC (w168-1) vector is the transfer plasmid that contains a luciferase reporter gene that will be packaged within the virion interior which can be used to monitor subsequent PV infections.

Firstly, a 6 well plate was coated with 700 μ L of Poly-L-lysine per well and placed on a shaker at room temperature for 15 minutes. After which, the Poly-L-lysine solution was removed, and the wells were rinsed with 1 mL of 1x PBS per well twice and left to dry in a biosafety cabinet for 1 hour. *HEK 293T* cells were

then seeded at an approximate seeding density of 3×10^5 cells per well and the plate was incubated in a static incubator at 37 °C overnight for the cells to attach. On the following day, 2 sets of tubes were prepared. The first set contained the plasmids with P3000 reagent in Opti-MEM and the other set contained the Lipofectamine 3000 transfection reagent in Opti-MEM. Negative controls with no transfection reagent or no plasmids were prepared as well.

The 2 sets of tubes were incubated at room temperature for 5 minutes. After which, the contents of the 2 sets of tubes were mixed together and incubated at room temperature for 30 minutes to allow the DNA-lipid complexes to form. The transfection mix containing the plasmids and transfection reagent was then added dropwise to the *HEK 293T* cell monolayer. The plate was incubated in 37 °C overnight. The transfection mixes were removed and replaced with 2 mL of DMEM with high glucose and 4 mM L-glutamine, without the addition of fetal bovine serum (FBS) nor penicillin-streptomycin (Pen-Strep) per well. The plates were incubated in 37 °C. After 72 hours since the addition of the transfection mixes, the media containing the budded pseudovirions were harvested by centrifugation at 1,500x g for 5min. The supernatant was collected and aliquoted into 500 µL cryovials and flash-frozen in liquid nitrogen for storage in -80 °C.

2.2.23 Detection of MERS-Spike glycoproteins and functionality tests of PV

Once the MERS-Spike PV was generated, some assembly and functionality tests were conducted to ensure the PV was well packaged for subsequent infection assays. Firstly, the presence of the MERS-Spike glycoprotein was checked in the viral supernatant. For this test, the viral supernatant was concentrated approximately 25x, from 1 mL to 40 µL. After which, the sample was used for

Western Blot analysis with anti-MERS-S1-center antibodies. If the spike glycoprotein was detected in the sample, it suggested that the PV might have been assembled and budded off from the host cell, as the cells were not lysed, and the Spike glycoproteins was detected in the media instead.

Further tests on the functionality of the PV was checked during the end-point dilution assay for viral titre calculations. Serial dilutions of the MERS-Spike PV were prepared from 10^0 to 10^{-7} . The diluted PV were all used for subsequent infections on *Vero E6* cells and incubated for 48 hours. This infection assay was performed in duplicates to ensure replicability of results. After the 48-hour incubation, the supernatant was discarded and replaced with 20 μ L of 10x diluted passive lysis buffer in autoclaved water. The plate was then placed on a shaker for 30 minutes for the cells to completely lyse. Then, 100 μ L of Dual-Luciferase® reagent was added into each well following the Dual-Luciferase® assay protocol (Promega, 2019). Luminescence readings were measured using the Tecan Safire²™ microplate reader (Tecan Group Ltd, NY).

2.2.24 Cell viability assay using WST-1 reagent on Vero E6 cells

After functionality and assembly tests on the MERS-Spike PV were completed, the toxicity of our compounds was tested on *Vero E6* cells before infection with PV. For this test, a cell viability assay using WST-1 was carried out. *Vero E6* cells were first seeded on a Nunc™ 96-well, flat bottom transparent plate (Nalge Nunc Int, NY) at approximately 5×10^3 cells/well. Triplicates per concentration of the compound was prepared. The cells were incubated in 37 °C overnight for adhesion to the plate. Serial dilutions of the compound to be tested would then be prepared from 10 μ M to 10 mM concentrations. After which, the media from the 96-well plate would be replaced with fresh DMEM containing 10 % FBS and 1

% pen-strep and the varying concentrations of the compound would be added dropwise to each well. The plate containing the compounds and *Vero E6* cells would then be incubated in 37 °C for 48 hours. Upon 48-hour incubation, the media will be aspirated and replaced with the WST-1 reagent and incubated in 37 °C for approximately 45 minutes for the metabolic activity of the cells to take place and react with the reagent. After the incubation, the WST-1 reagent in wells with live cells will typically turn orange. The readings will be measured using the Tecan Safire²™ microplate reader (Tecan Group Ltd, NY). Results obtained would be normalised and transformed to percentage-based, with reference to the negative control. The IC₅₀ will then be determined from the results.

2.2.25 MERS-Spike PV infection assay

Vero E6 cells were first seeded on a Greiner CELLSTAR® 96-well white flat bottom plate (Greiner Bio-One, AT) at a density of 5×10^3 cells/mL and incubated overnight at 37 °C for cell adhesion to the plate. MERS-Spike PV was diluted accordingly for subsequent infection at a MOI of 2. The compound to be tested was prepared and added to the MERS-Spike PV at varying final concentrations of 10, 15 and 20 µM under the non-toxic concentrations according to previous cell viability results. After which, the compound was pre-incubated with the PV for 1 hour at room temperature before addition to the seeded *Vero E6* cells. The plate was then incubated in 37 °C for 48 hours before harvesting for luciferase assay. After 48 hours, the cells were harvested, lysed and Dual-Luciferase® reagent was added following the Dual-Luciferase assay protocol (Promega, 2019). Luminescence readings were measured using the Tecan Safire²™ microplate reader (Tecan Group Ltd, NY).

2.2.26 Time of Addition (TOA) assay using live MERS-CoV

Live MERS-CoV experiments were conducted by Dr. Kim Seungtaek and Dr. Lee Jihye from Institut Pasteur Korea, in a Biosafety Classification 3 (BSL3) laboratory (Facility license number: KCDC-09-3-03). *Vero E6* cells were seeded at 1.2×10^4 cells/well in a 96-well plate and incubated at 37 °C overnight for attachment. The negative control, 7,8-DHF was used at a concentration of 25 µM and the positive controls: Chloroquine (CQ), Lopinavir (LPV) and CE-50 were used at 100 µM, 25 µM and 50 µM respectively. The concentration of MSH was fixed at a high 25 µM for initial screens to observe for any potential inhibitory effects. Seven different time-points of MSH addition was set at 1-hour intervals between each time-point, from -1 to +5 hours with reference to the viral adsorption at 4 °C (Figure 3.14A). MERS-CoV/KOR/KNIH/002_02_2015 was used for viral adsorption at 0 hours at a MOI of 5. The end-point was fixed at 7 hours post infection (hpi) for harvesting of cells. Upon harvesting, cells were fixed with 4 % volume by volume (v/v) paraformaldehyde, washed with 0.25 % Triton X-100 and probed with 0.5 µg/mL anti-MERS-CoV Spike antibody (Sino-Biological, CN). Secondary antibody used was 1 µg/mL Alexa Fluor 488 anti-IgG (Molecular Probes, OR) with 1 µg/mL Hoechst 33342 (Molecular Probes, OR). Imaging and analyses were conducted on the PerkinElmer (PE) Operetta platform (PerkinElmer, MA).

2.2.27 Viral entry inhibition studies using live MERS-CoV

Similar to the TOA assays, *Vero E6* cells were seeded at 1.2×10^4 cells/well in a 96-well plate and incubated at 37 °C overnight for attachment. The positive controls, CQ and LPV were added at a top concentration of 150 µM and 50 µM respectively, at 2-fold dilutions per point for 10 different concentrations. MSH

was tested at a top concentration of 10 μ M, at a 1.5-fold dilution per point for 10 different concentrations. MERS-CoV/EMC/2012 was inoculated at a MOI of 0.06 for these assays. 4 different conditions were used: Pre-incubation, Co-treatment, Pre-treatment and Post-treatment. The end-point was determined at 24 hours and the cells were harvested for imaging and quantitative analyses as mentioned in Section 2.2.25: TOA assays.

3. Results

3.1 Expression, Purification and Optimisation of recombinant proteins

3.1.1 Bacterial expression system

For expression of recombinant proteins used in binding and structural studies, the bacterial expression system is generally preferred due to its higher yield (Gräslund et al., 2008). However, there were no information regarding bacterial expression of recombinant MERS-CoV RBD proteins in recent publications. Instead, they employed the use of the baculoviral expression system (Invitrogen, 2015; Life Technologies, 2015) using *Sf9* insect cells, a clonal isolate of *Spodoptera frugiperda* cells (*Sf21*). Hence, we performed preliminary solubility screens for our recombinant RBD proteins in both bacterial and baculoviral expression systems.

For bacterial expression, our MERS-CoV RBD gene construct was first cloned into a pSUMO vector containing a SUMO-6xHis tag (Harvard, 2018) with the help of a URECA student, Ms. Yong Jing Yen. This vector was used as SUMO-tag was found to enhance the solubility of the recombinant protein expressed (Marblestone et al., 2006). After which, the plasmid containing the RBD construct was then transformed into chemically competent *DH5α* cells for amplification of plasmids for sequence verification (Figure 3.1A). Upon sequence confirmation of the RBD construct, the plasmids were then transformed into competent *BL21(DE3)*, *BLR(DE3)pLysS* and *Origami(DE3)* *E. coli* cells for bacterial expression screens. These 3 bacterial strains were selected based on availability and function. *BL21(DE3)* cells are the most commonly used strain for general protein expression, *BLR(DE3)pLysS* cells are suitable for the expression

of toxic genes and *Origami(DE3)* cells are good for constructs containing cysteines and multiple disulfide bonds (Doron, 2015).

From our bacterial expression screens, the recombinant MERS-CoV RBD proteins were observed in the inclusion bodies (Figure 3.1B-D). This suggests that the RBD sample expressed was insoluble and would require unfolding and refolding for purification purposes. Further reduction in protein induction temperature did not lead to any improvements in generating soluble proteins (Figure 3.1E). Although this system generates high yields of recombinant proteins, these unfolding and refolding processes involve risks of producing non-functional proteins which are unusable for downstream studies (Díaz-Villanueva, Díaz-Molina, & García-González, 2015; Wingfield, 2015). Therefore, this expression system was not favoured.

A

```
#=====
#
# Aligned_sequences: 2
# 1: EMBOSS_001
# 2: EMBOSS_001
# Matrix: EBLOSUM62
# Gap_penalty: 10.0
# Extend_penalty: 0.5
#
# Length: 204
# Identity:   204/204 (100.0%)
# Similarity: 204/204 (100.0%)
# Gaps:       0/204 ( 0.0%)
# Score: 1077.0
#
#=====
```

EMBOSS_001	117	ECDFSPLLSGTPPQVYNFKRLVFTNCNYNLT	166
EMBOSS_001	1	ECDFSPLLSGTPPQVYNFKRLVFTNCNYNLT	50
EMBOSS_001	167	AIASNCYSSLILDYFSYPLSMKSDLSVSSAGPISQFNYKQSF	216
EMBOSS_001	51	AIASNCYSSLILDYFSYPLSMKSDLSVSSAGPISQFNYKQSF	100
EMBOSS_001	217	ATVPHNLTITKPLKYSYINKSRLSDDRTEVPQLVNAVQYSPCVSIVP	266
EMBOSS_001	101	ATVPHNLTITKPLKYSYINKSRLSDDRTEVPQLVNAVQYSPCVSIVP	150
EMBOSS_001	267	STVWEDGDYRKQLSPLEGGGWLVASGSTVAMTEQLQMGFGITVQYGTDT	316
EMBOSS_001	151	STVWEDGDYRKQLSPLEGGGWLVASGSTVAMTEQLQMGFGITVQYGTDT	200
EMBOSS_001	317	NSVC	320
EMBOSS_001	201	NSVC	204

B **C** **D**

E

Figure 3.1: Cloning and Expression screens in *E. coli* cell lines.

(A) Sequence verification of MERS-CoV RBD construct cloned into pSUMO vector. Expression screens using SDS-PAGE analysis and verification by Western Blot probed with anti-MERS-CoV S1 center antibody in (B) *BL21(DE3)* cells, (C) in *BLR(DE3)pLysS* cells (D) and in *Origami2(DE3)* cells. (E) Low temperature screen for *BL21(DE3)* and *Origami2(DE3)* cells. S represents supernatant or soluble fraction, P represents pellet or insoluble fraction. 3/4hpi refers to 3 or 4 hours post infection harvest for infection screens.

3.1.2 Baculoviral expression system

Glycosylation was previously found to play an important role in viral replication, starting from host cell receptor recognition and interaction to viral maturation and assembly (Peck et al., 2017; Shen et al., 2007). As glycosylations were found to be important in the receptor-ligand binding for viral entry, recent publications reported the use of *Sf9* cells for their protein expression (Yu et al., 2015; S. Zhang et al., 2018). Unlike *E. coli* cells, *Sf9* cells are of insect origin and can undergo post translational modifications, which are required for our recombinant proteins. As secretion peptides were used in the constructs to enhance solubility of the proteins, the resultant recombinant proteins would be secreted into the media for purification. Therefore, the cell pellets after harvesting would not be collected for

recombinant protein purification as the proteins in the cells would not be completely glycosylated yet.

3.1.2.1 Molecular cloning and generation of baculovirus

pFastBac Dual vector containing hDPP4 was firstly transformed and transposed into the *lacZα* insertion site of the *DH10EMBacY-GFP* cells in a single step. Colonies with successful transposed vectors must be distinguished from transformants, as both would be able to grow in triple antibiotics agar plate. Thus, a blue-white colony screening was performed using X-gal and IPTG. If only transformation took place, the colonies would turn blue after 48 hours. However, if both transformation and transposition has taken place, the colonies remain white (Invitrogen, 2015; Merck, 2019). This allows the stricter selection of colonies, reducing the probability of any false positives. Initial positive colonies were selected and re-streaked on a fresh triple antibiotics blue-white screening plate for double confirmation.

Positive colonies were grown in LB with triple antibiotics for bacmid isolation. Isolated bacmids were tested using PCR with gene specific primers to detect the presence of the 2 gene constructs. However, although the gene specific primers showed the presence of the gene constructs in the samples (Figure 3.2A), it was insufficient to confirm that the transposition has taken place in the insertion site. Therefore, pUC-M13 primers specific to the *lacZα* site were used to ensure that the gene constructs were transposed at this specific site. If the constructs were found to be of the right molecular weight, it suggests that the intended gene has been incorporated well (Invitrogen, 2015). The hDPP4 transposition experienced much difficulties. As a result, successful transposition of the hDPP4 gene insert was achieved after repeated attempts and optimisation. For the bacmid PCR

screening, the negative control without any insert would show a 300 bp band and hDPP4 insert would be approximately 4,800 bp (Figure 3.2B).

Isolated bacmids were transfected into *Sf9* cells after PCR verification of transposition into the *lacZα* site. Upon harvesting the baculovirus stock from the media, the cells were observed under fluorescence microscopy for the presence of GFP signals. As *DH10EMBacY-GFP* cells were used for the bacmid generation, a GFP reporter gene was cloned into the bacmid backbone by Dr. Ufuk. Therefore, when the bacmid proteins are expressed in the transfected *Sf9* cells, GFP will be expressed. As there were GFP signals observed only in the transfected sample, it showed that the bacmid proteins were expressed and in turn, baculovirus was produced in *Sf9* cells (Figure 3.2C).

From previous published data, the MERS-CoV RBD was found to be 204 residues long in the MERS-CoV S1 region (N. Wang et al., 2013). Therefore, from the extended MERS-CoV RBD construct kindly supplied by Prof. Wang Xinquan, we decided to truncate it to the 204 residues length as reported. However, due to the presence of a C-terminal cysteine residue which was found to be involved in a disulfide bridge in the published MERS-CoV RBD structure, 4 extra residues from the extended construct were kept to minimise any effects on the disulfide bridge formation. This truncated MERS-CoV RBD was cloned and its P₁ baculovirus was kindly prepared by NTU's Protein Purification Platform (NTU/PPP). For this construct, the original gene insert was cloned into another vector: pFastBac-Sec-NH, while excluding the flanking regions in the extended MERS-CoV RBD construct (Figure 3.2D). The low-titre P₁ baculovirus stock was further amplified before use for expression screens and downstream studies.

E

Figure 3.2: Molecular cloning of MERS-CoV RBD and hDPP4 constructs

(A) Detection for presence of hDPP4 gene insert after transformation and transposition into *DH10EMBaY-GFP* cells. The theoretical size of hDPP4 construct is 2.2 kb. (B) Analysis of transposition of hDPP4 into bacmid DNA. The theoretical sizes of the negative control and hDPP4 are 300 bases and 4.8 kb respectively. (C) Detection of GFP signals in the generation of P₁ hDPP4 baculovirus stock. Scale bar on the bottom right represents 20 μ m. (D) Design of the truncated MERS-CoV RBD for cloning by NTU/PPP. (E) hDPP4 plasmid construct layout. Lanes in the DNA gel are labelled L for protein ladder/standard, (-) for negative control and D for DPP4.

3.1.2.2 Expression screens

P₁ baculovirus stocks were amplified in *Sf9* cells for another 2 passages to generate a high-titre P₃ baculovirus stock. The P₃ baculovirus stocks were then used in expression screens at virus to culture ratios of 1:5, 1:10, 1:25 and 1:50, and the small-scale cultures were purified using Immobilised Metal Affinity Chromatography (IMAC), more specifically, nickel charged affinity resin with nitrilotriacetic acid chelating agent (Ni²⁺-NTA) due to the 6xHis tags cloned into the protein constructs. Infection ratios that produced the highest protein yield were then selected for large scale protein expression for downstream studies. One major disadvantage of the baculoviral expression system is the significantly lower protein yields as compared to the bacterial system. Therefore, numerous optimisation screens were attempted to achieve the highest yield, especially with the viral quality and amount of baculovirus used for infection for expression.

For the viral quality, if the viral stock is suboptimal, the subsequent expression rates will be drastically reduced, leading to low yields and wastage of culture media. As a result, different batches of the same baculovirus were screened with different transfection incubation times. After the optimisation screens, a specific incubation time of 7 days of transfection incubation period was selected for future baculovirus preparations and the optimal amount of virus for each construct was determined. MERS-CoV RBD and hDPP4 has an approximate size of 28 and 85 kDa respectively (Figure 3.3A).

For the truncated MERS-CoV RBD prepared by NTU/PPP, their general protocol was followed where the P₃ baculoviral stock was screened differently. According to this protocol, the harvesting period was fixed at 3 days post infection and the volume of baculovirus screened were 20 mL virus/L, 30 mL virus/L and 40 mL virus/L. Similar to the previous constructs for RBD and DPP4, the optimal conditions for protein expression was determined by the expression screens. For the truncated RBD construct, its approximate size on SDS-PAGE analysis was 26 kDa (Figure 3.3B).

B**Recombinant MERS-CoV RBD expression screens**

Figure 3.3: Expression screens for recombinant hDPP4 and MERS-CoV RBD in *Sf9* cells.

Expression screens for recombinant (A) hDPP4 and (B) MERS-CoV RBD protein samples using *Sf9* cells. A 25 mL culture of 2.5×10^6 cells/mL seeding density was prepared for each screen. Following different protocols for the 2 protein samples, the hDPP4 screens were conducted using a baculovirus-to-culture ratio while the MERS-CoV RBD screens was following a fixed volume per litre set-up. For each case, the best ratio or volume of baculovirus required was selected from these screens and used for subsequent large-scale expressions. L represents protein ladder/standards, (-) is the uninfected sample, S is for supernatant, F is flowthrough, W is for wash and E is for elution.

3.1.2.3 Large scale purification of recombinant proteins

After the 3 days incubation period for protein expression, the supernatant containing the secreted proteins was collected for Ni^{2+} -NTA affinity purification. As the protein samples all contain 6xHis tags, it could be pulled down using the Ni^{2+} resin and eluted with imidazole thereafter. The elution fractions were then concentrated and injected into a column for size-exclusion chromatography. Fast-performance liquid chromatography (FPLC) runs were performed till the protein sample is pure. MERS-CoV RBD and truncated RBD samples are typically eluted

between 65 to 75 mL from the HiLoad Superdex 75 16/60 column and then around 15 to 17 mL in the Superdex 200 Increase 10/300 GL column. hDPP4 samples, on the other hand, are eluted between 11 to 13 mL from the Superdex 200 Increase 10/300 GL column (Figure 3.4A, B). As for the MERS-CoV RBD samples, if 2 rounds of FPLC were unable to remove the impurities, an anion-exchange column was used (Appendix I).

Figure 3.4: Size exclusion chromatography profiles of recombinant MERS-CoV RBD and hDPP4 proteins.

(A) First round of size exclusion chromatography performed on MERS-CoV RBD elution using a Superdex 75 (S75) column. The fraction size corresponds to approximately 30 kDa and produced a 26 kDa band in our SDS-PAGE analysis. This suggests that MERS-CoV RBD samples are in the monomeric form. (B) Secondary run of MERS-CoV RBD fractions using a

Superdex 200 Increase 10/300 GL column. (C) First round of size exclusion chromatography for hDPP4 purification using a Superdex 200 Increase 10/300 GL column. The fraction size corresponds to approximately 160 kDa but produced an 85 kDa band in our SDS-PAGE analysis. This suggests the formation of hDPP4 homodimers. (D) Secondary purification using the same S200 column. Samples were collected from each run, concentrated and analysed on SDS-PAGE. Purified recombinant protein samples were then used for protein identity verification and downstream binding studies.

3.1.3 Functionality tests for purified recombinant proteins

Purified protein samples were first subjected to protein identity (Protein ID) verification via Mass Spectrometry: Matrix-Assisted Laser Desorption/Ionization – Time of Flight (MALDI-TOF) (Figure 3.5A, B). The functionality of the 2 protein samples were then analysed using an analytical Superdex 200 Increase 10/300 GL (S200) column. As these 2 proteins are able to bind together to form a complex structure (Lu et al., 2013; N. Wang et al., 2013), if the purified samples were functional, a complex should be formed after a 2-hour incubation at 4°C. This would then translate to a shift in the peaks formed in the S200 column to a lower fractionation volume. MERS-CoV RBD and hDPP4 samples were incubated for functionality test first. The truncated RBD construct was also tested with the hDPP4 sample. Both samples were shown to be functional, suggesting that they were all well-folded and glycosylated during *Sf9* expression (Figure 3.5C).

A

Rank	Protein Name	Accession No.	Protein MW	Protein Score	Protein Score C. I. %	Total Ion Score	Total Ion C. I. %
1	Dipeptidyl peptidase 4 OS=Homo sapiens GN=DPP4 PE=1 SV=2	sp P27487 DPP4_HUMAN	88906.7	302	100	114	100

B

Rank	Protein Name	Accession No.	Protein MW	Protein Score	Protein Score C. I. %	Total Ion Score	Total Ion C. I. %
1	Spike glycoprotein OS=Middle East respiratory syndrome-related coronavirus (isolate United Kingdom/	sp K9N5Q8 SPIKE_CVEMC	151710.3	129	100	121	100

C

Figure 3.5: Mass Spectroscopy Protein Identity (Mass Spec Protein ID) verification and functionality tests of recombinant protein samples.

(A) Mass Spectroscopy verification of recombinant hDPP4 sample confirming the Protein ID and species of DPP4. (B) Mass Spectroscopy verification of recombinant MERS-CoV RBD sample showing a 100% score. Full reports of DPP4 and MERS-CoV RBD protein ID verification in Appendix II. (C) Samples eluted from the analytical S200 column were analysed using SDS-PAGE. Recombinant MERS-CoV RBD and hDPP4 protein samples were incubated for 3 hours at 4°C before injection into the column. Peak 1 elution from the incubated protein samples showed the presence of both RBD and DPP4 protein bands (approximately 85 kDa and 26 kDa) indicating complex formation between the 2 protein samples.

3.2 Approach 1: Drug repurposing via structure-guided screening of FDA-approved drugs

From previous *in-silico* screens on the binding pocket of MERS-COV RBD performed by Dr Harikishore, seven ligands were identified as potential hits. These ligands were further studied by *in vitro* thermal shift assay, but the results obtained were not optimal, likely due to protein stability (Appendix III). Instead, label-free Intrinsic Fluorescence (Tryptophan) quenching experiments which do not require any prior incubation were conducted. Although DPP4 protein is a direct interacting partner of MERS-CoV RBD, it cannot be used as a positive control due to the presence of numerous Trp residues present in the large DPP4 protein sample. These residues would provide fluorescence contributions that will create false positive or negative data. Therefore, as chloroquine disphosphate (CQ) was previously re-purposed and used for supportive treatment in hospitals for MERS-infected patients, it was initially used as a positive control for binding assays (Cong et al., 2018).

3.2.1 Intrinsic Fluorescence (Tryptophan) quenching experiments

Intrinsic Fluorescence quenching experiments rely on the change in the microenvironment of the Trp residues near the binding region upon protein-ligand interactions (Akbar, Sreeramulu, & Sharma, 2016). As there are only 2 Trp residues, Trp-535 and Trp-553, near the binding pocket on the MERS-CoV RBD, this quenching experiment is suitable for binding studies on the RBD sample (Figure 3.6A). This experiment detects the fluorescence emission the unbound protein sample as the baseline and focuses on the detection of any reduction/quenching of these fluorescence signals after ligand binding. As protein-ligand interactions take place, upon excitation of the sample at 280 to 290

nm, some of this energy is transferred from the protein to the ligand in close proximity via Förster Resonance Energy Transfer (FRET) (Ghisaidoobe & Chung, 2014; Marras, Kramer, & Tyagi, 2002). Therefore, the resulting emission between 300 to 430 nm from the protein is lowered. Based on this principle, the ligands were tested with MERS-CoV RBD.

Before the screens could be initiated, a control run was performed with compounds only in the protein buffer to identify any potential external fluorescence contribution from the compounds. Typically, there should not be any fluorescence emission from the compounds as they generate noise during the screening process, creating false positives or false negatives. From the control run, Carvediol was shown to have high intrinsic fluorescence emission without the addition of any protein samples (Figure 3.6B). Therefore, it was excluded from the screening process. After the control run was done, an approximate ratio of protein to compounds was pre-set at 1:5 and the screen was performed with the remaining six compounds. A final protein concentration of 5 μ M was used for each compound screen. CQ was found to produce a low level of quenching effect when incubated with MERS-CoV RBD sample, suggesting that CQ might not interact strongly with the viral protein to exert any inhibitory effect. Hence, it was not used in further quenching experiments. Preliminary findings showed that of the six compounds, Montelukast sodium hydrate (MSH), Cefaclor (Cef) and Nalmefene (N) were able to produce a slightly greater quenching effect, although the data obtained was not statistically significant. This implies that there might be some interaction between these ligands and the purified RBD samples, but further tests were required to confirm these findings (Figure 3.6C, D; Table

5). Therefore, dose dependent responses of these three potential hits were subsequently performed for downstream analyses.

Figure 3.6: Preliminary binding screens of previous computational hits with Intrinsic Fluorescence (Tryptophan) Quenching experiments.

(A) Negative control runs of ligands only to check for any innate intrinsic qualities exhibited by the compounds. (B) Summary of screening of computational hits, performed uniformly at a protein-to-ligand ratio of 1:5. Individual quenching curves of (C) Montelukast Sodium Hydrate

(MSH), (D) Nalmefene (N) and (E) Cefaclor (Cef). Preliminary findings suggest possible interaction between MERS-CoV RBD and three ligands: MSH, N and Cef. One-way ANOVA statistical analysis was performed with Dunnett's Post-hoc test to compare all columns with the control column. N=3, *** $p < 0.001$.

3.2.2 Dose-dependent titration and saturation experiments

As the initial screens were carried out at a protein-to-ligand ratio of 1:5, a dose dependent screen consisting of increasing ligand concentrations from 1:5 to 1:40 was designed to study any potential dose dependent interaction between the protein and compounds. Further experiments on the compound: N and Cef revealed that the protein-compound interaction was weak (Figure 3.7A-C Table 5). However, MSH was found to interact with the RBD sample, producing not only a statistically significant dose dependent quenching response to increasing compound concentration, but also a shift in wavelength ($\lambda_{\max}$) (Figure 3.7D, E). This suggested a strong conformational change upon binding between RBD and MSH such that it resulted in the change in polarity of the environment surrounding the Trp residues (NCBI; Wriggles, 2019). The dissociation constant (K_D) for the interaction between MERS-CoV RBD and MSH was approximated to be $62.99 \pm 7.62 \mu\text{M}$ (Figure 3.7F).

A

B

C

D

Figure 3.7: Dose dependent titration for the three potential hits identified from preliminary screening.

(A) Summary of dose dependent titration of the three previously identified ligands. Individual quenching curves of (B) N, (C) Cef and (D) MSH. (E) Tryptophan quenching chart for dose dependent titration of MERS-CoV RBD with MSH showing a consistent and dose-dependent quenching effect upon increasing MSH concentration. (F) Saturation curve of MERS-CoV RBD with increasing concentrations of MSH and its calculated dissociation constant (K_D) value of $62.99 \pm 7.62 \mu\text{M}$. One-way ANOVA statistical analysis was performed with Dunnett's Post-hoc test to compare all columns with the control column. $N=3$, ** $p < 0.01$, *** $p < 0.001$.

Table 6: Fluorescence quenching results of the seven ligands identified by *in silico* screening

Compound	Chemical structure	% Quenching [±]	Highest % quenching [*]	Dose-dependency [*]	Statistical significance [*]
Carvediol [#]		NA	NA	NA	NA
Reserpine		-	NA	NA	NA
Lovastatin		9.16	NA	NA	NA
Cephadrine		6.00	NA	NA	NA
Nalmefene		22.85	24.17	Yes	No
Cefaclor		19.19	32.73	No	No
Montelukast Sodium Hydrate		38.71	79.12	Yes	Yes

[±] Quenching was calculated at a 1:5 protein-to-ligand ratio for preliminary screens. [#] Carvediol was excluded from the screening due to its own intrinsic fluorescence. ^{*} represents further dose-dependent titration conducted on selected ligands, namely Nalmefene, Cefaclor and Montelukast Sodium Hydrate.

3.2.3 Structure elucidation attempts

Preliminary binding experiments suggested interactions between MERS-CoV RBD and MSH. Additional computational docking of MSH with MERS-CoV RBD predicted that MSH fills the RBD pocket well (Figure 3.8B and C). From the docking data, the binding free energies of RBD with DPP4 peptide and MSH were found to be -8.14 kcal/mol and -9.6 kcal/mol respectively. This indicates that MSH binds stronger than the DPP4 peptide to MERS-CoV RBD (Figure 3.8). Therefore, structural studies to understand the precise mode of interaction of

RBD-MSH complex were taken up. As the recombinant proteins were expressed using the baculoviral system, it is difficult to perform specific labelling using isotopes for NMR experiments for either structure determination or ligand titration (Hansen et al., 1992). Hence, crystallographic studies were utilised instead.

Figure 3.8: Computational docking of MERS-CoV RBD and MSH.

(A) A short helical peptide of hDPP4 from residues Ala-289 to Gly-296 was docked in the pocket of MERS-CoV RBD. Computational modelling of MSH in the binding pocket of MERS-CoV RBD revealed that (B) MSH can fit into the pocket, and (C) fill the pocket well, which suggests

that MSH might be to interact with RBD around this pocket region. Images were obtained from PDB: 4L72.

3.2.3.1 Preliminary crystal screening

As the recombinant protein yield from the baculoviral expression system posed a major issue in our study, the preliminary crystallisation screening was carried out using the Gryphon (Art Robbins Instruments) and MOSQUITO (TPPLabTech) platforms at the NTU Institute of Structural Biology (NISB). The Gryphon platform was used to load low volumes of 56 μ L well solutions from commercial crystallisation screening kits such as Rigaku Wizard Classic 1 and 2, and Hampton Research Index Screen 1 and 2. The MOSQUITO platform was utilised to dispense 100 nL of RBD-MSH, where MSH was pre-incubated with RBD at 4 °C for 24 hours prior to co-crystallisation trials. The 96-well crystallisation plate was prepared for the sitting-drop diffusion set up, tagged and stored in the RockMaker and RockImager (Formulatrix) at 18 °C. The RockMaker and RockImager platform conducts periodic image acquisition of the plates and allows UV screening to distinguish between protein and salt crystals. From this preliminary screen, many crystals under different well conditions were obtained. For the 2 best crystals formed in the screens, a UV imaging was performed to check for possibilities of non-protein crystals (Figure 3.9A, B) (Desbois, Seabrook, & Newman, 2013). Both wells were found to be protein crystals. In accordance to the screening results, a specific condition of 25 % PEG 3,350, 0.2 M Ammonium Sulfate and 0.1 M Bis-Tris pH 5.5 was selected for downstream optimisations.

Figure 3.9: Preliminary crystals of recombinant MERS-CoV RBD with MSH at a protein-to-ligand ratio of 1:10.

Bright field and UV images of recombinant MERS-CoV RBD with MSH different well conditions, (A) 1.0 M Ammonium sulfate, 0.1 M HEPES pH 7.0, 0.5 % w/v Polyethylene glycol 8,000 and (B) 0.2 M Ammonium sulfate, 0.1 M BIS-TRIS pH 5.5, 25 % w/v Polyethylene glycol 3,350. UV irradiated images were used to distinguish between salt and protein crystals, as only protein crystals appear bright under UV light.

3.2.3.2 Optimisation of co-crystals

After the selection of the specific precipitant, optimisation was carried out systematically by varying the (a) well volume, (b) pH of Bis-Tris, (c) sample and PEG 3,350 concentrations. During the screening process, the pH of Bis-Tris was tested from 5.0 to 7.0 with 0.5 increments. It was observed that larger crystals

appear at higher pH conditions, between 6.5 to 7.0. Subsequently, concentration of PEG 3,350 was varied from 18 % to 30 % and 22.5 % to 30 % PEG 3,350 was found to yield single crystals. As there were too many nucleation sites leading to many thin crystals, the sample concentration and well volume were optimised. A final concentration of 7 mg/mL MERS-CoV RBD and a well volume of 1 mL was used. After optimisation, a narrow range of Bis-Tris buffer pH, concentration of PEG 3,350, protein sample and well volume were selected to obtain consistent diffraction quality crystals (Figure 3.10).

To obtain the crystal structure of MSH bound MERS-CoV RBD, apart from the co-crystallisation attempts, soaking trials were also carried out. As for the soaking method, apo MERS-CoV RBD crystals were grown and incubated with ligands by varying the time of soaking and concentration of ligand (Appendix IV). Diffraction was carried out for the soaked and co-crystals at the National Synchrotron Radiation Research Center (NSRRC) facility, Hsinchu, Taiwan at 100 K on beamline TPS05A, followed by analysis (Appendix IV) by Dr. Sreekanth. The ligand density could not be observed despite the crystals diffracting to good resolution limits (approximately 2 Å). Therefore, we were unable to determine the structure of MERS-CoV RBD with MSH. However, we were able to clearly identify the electron densities of the glycosylation sites in the crystal structure, confirming that the protein is in their functional form.

Figure 3.10: Images of RBD-MSH co-crystals

MERS-CoV RBD-MSH co-crystals, pre-incubated at a protein-to-ligand ratio of 1:10, were formed under a general well condition of 0.2 M Ammonium Sulfate, 0.1 M Bis-Tris pH 6.5 to 7.0 and 22.5 % to 30% Polyethylene glycol 3,350.

3.2.4 Analytical gel filtration (Superdex 200) chromatography

From the fluorescence quenching experiments, binding between MSH and RBD was observed. Next, we investigated the potential inhibitory effects of MSH. For this test, we used the Superdex 200 Increase 10/300 GL column for analysis. Firstly, negative control runs were prepared: MERS-CoV RBD only, hDPP4 only and RBD + DPP4 complex. After which, the samples were incubated and analysed using the column. 3 independent runs of the following samples were prepared: RBD + MSH + DPP4 (1:10:1), RBD + DPP4 + MSH (1:1:10) and DPP4 + MSH + RBD (1:10:1). Protein-MSH incubations were approximately 24 hours and RBD-DPP4 incubations were kept at 2 hours (Lu et al., 2013).

These different set-ups were performed to study the target of MSH inhibition. For instance, if a shift of elution profile was observed in the RBD + MSH + DPP4 (1:10:1) run but not in the DPP4 + MSH + RBD (1:10:1), it suggests that MSH targets RBD for its inhibition rather than DPP4. In addition, the RBD + DPP4 +

MSH (1:1:10) was performed to check if MSH could potentially exert effects to dissociate the RBD-DPP4 complex. From these runs, a slight shift in elution profiles was observed in all samples, which were consistent in repeated runs with different batches of recombinant protein samples (Figure 3.11). The slight shift from the complex peak towards the individual DPP4 peak suggested that there might be some complex being dissociated or not binding to each other after MSH was added to the sample. Therefore, it revealed the possibility that there might be some weak but distinct potential inhibitory effect exerted on the RBD-DPP4 complex formation by MSH.

Figure 3.11: Analytical gel filtration experiments to study the inhibitory effect of MSH on RBD-DPP4 complex formation.

3 independent samples were prepared to study the inhibitory effect of MSH on RBD-DPP4 complex formation: (A) RBD + MSH + DPP4, (B) RBD + DPP4 + MSH and (C) DPP4 + MSH + RBD. A slight shift of the complex with MSH (red), from the complex peak (black) towards the 'DPP4 only' peak (green) was observed in the 3 samples. This observation was consistent in

repeated experiments, suggesting the possibility of a weak inhibitory effect on complex formation exerted by the addition of MSH.

3.2.5 MERS-CoV Spike pseudovirions (PV) studies

For cell-based assays on viral entry, live MERS-CoV are normally preferred. However, live viruses such as MERS-CoV typically requires a biosafety level 3 certification to handle (CDC, 2019). Additionally, recent publication revealed successful generation of Nanoparticle-based virus-like particles of MERS-CoV RBD that could potentially act as a recombinant vaccine (Y.-S. Kim et al., 2018). However, due to the complex nature of this work, it was not used in this study. Therefore, due to the biosafety concerns, MERS-CoV Spike pseudovirions were generated and used instead (Cronin, Zhang, & Reiser, 2005; Grehan, Ferrara, & Temperton, 2015). Pseudovirions are non-replicable viral particles which contain the surface glycoproteins of interest for infection studies. Therefore, these PV are not contagious and does not pose any potential biosafety concerns and health risks (Addgene, 2019).

3.2.5.1 Generation, assembly and functional tests for Spike PV

For the generation of PV carrying the MERS-CoV Spike glycoproteins, we employed a 3-plasmid system: packaging vector, envelope vector and transfer vector (Addgene, 2016). The packaging vector used was psPAX2 (Trono Lab), consisting of the HIV-1 backbone genes such as *Gag*, *Pol*, *Tat* and *Rev*. The envelope vector used was the pCMV-MERS Spike plasmid for expression of the MERS-CoV Spike glycoproteins for viral assembly onto the surface of the PV. The transfer vector used was pLenti CMV puro LUC w168-1 (Trono Lab) which consists of a firefly luciferase gene flanked by HIV-1 psi (Ψ) packaging signal

and HIV-1 Rev response element (RRE) for packaging into the PV interior (Figure 2.1). As this luciferase reporter gene is packaged in the virions, upon infection and virion entry, the luciferase gene would be expressed using host cell translational machinery. This allows the detection and quantification of luminescence signals emitted from the infected cells during infection assays (Sehr et al., 2013; Wu et al., 2013).

Upon generation of the PV, the presence of MERS-Spike glycoproteins was tested in the supernatant. Firstly, acetone precipitation of the sample was performed to precipitate the spike glycoproteins for detection purposes. In the preliminary detection using dot-blot with a random protein sample as a negative control and probed with anti-MERS S1 center antibody (Sino-Biological, CN), the spike glycoprotein was shown to be present (Figure 3.12A). However, the same sample did not produce a band when detected using western blot. This revealed a potential issue with the solubility of the precipitated sample. Therefore, 2 vials of viral supernatant were concentrated approximately 50 times, from 1 mL to 20 μ L. 6x SDS loading dye was then added to the concentrated viral sample and ran on an SDS-gel for western blot analysis. Western blot analysis on the concentrated sample showed a strong band when probed with the same antibody used previously (Figure 3.12B). The detection of MERS-CoV spike glycoproteins in the supernatant was critical as the glycoproteins expressed in cells should not be released out of cells unless they were assembled on the surface of the PV. Transfection of pCMV-MERS Spike plasmid without the packaging and transfer vectors was also carried out simultaneously and the media was concentrated and analysed with western blot as a negative control.

The functionality of the viral supernatant in infection of cells was then tested on cells. *Vero E6* cells were chosen for subsequent infection screens as they are normally used in the growth and maintenance of live MERS-CoV (Coleman & Frieman, 2015). The viral infection could be detected using a luciferase assay as the PV were generated with a luciferase reporter gene in the virion interior. Therefore, only cells infected with the PV were able to express the luciferase enzymes for detection (Figure 3.12C). Additionally, soluble MERS-CoV RBD was pre-incubated with the *Vero E6* cells prior to infection with the MERS-spike pseudovirions and results exhibited significant decrease in luciferase activity (Figure 3.12C). This data suggested that the pseudovirion infection was specific to MERS-CoV. For this test, an end-point dilution experiment was conducted to estimate the viral titre as well as to show the capacity of the PV for subsequent infections. From this experiment, TCID₅₀ was used for viral quantification and the viral stock was found to infect *Vero E6* cells but have a low titre of approximately 1.0×10^3 PFU/mL. Therefore, subsequent infections employed a Multiplicity of Infection (MOI) of 1.5.

Figure 3.12: Functional and assembly tests of MERS-CoV Spike Pseudovirions

(A) Detection of the presence of MERS-spike protein in the viral supernatant after acetone precipitation using the anti-MERS-S1 antibody. A random protein was used as a negative control. (B) Western blot of 50x concentrated viral supernatant showing a single band at the approximate size of MERS-Spike protein. The transfected media of the pCMV-MERS Spike plasmid without packaging and transfer vectors was used as the negative control. (C) Infectivity screens of viral supernatant for assembly and functionality tests and specificity for luciferase detection and quantification. One-way ANOVA statistical analysis was performed with Dunnett's Post-hoc test to compare all columns with the control column. N=3, * $p < 0.05$.

3.2.5.2 Toxicity screening for MSH

As MSH was previously found to bind to MERS-CoV RBD and possibly exert a slight inhibitory effect on the RBD-DPP4 complex formation, it was subjected to cell-based PV assays for verification. However, before the compound could be used, the toxic dose of MSH must be determined in *Vero E6* cells. Therefore, a cell viability assay using WST-1 reagent was performed on *Vero E6* cells with MSH in increasing concentrations from 0.1 μM , 0.5 μM , 1 μM , 10 μM , 20 μM , 50 μM , 100 μM . The highest non-toxic dose of MSH was found to be $20.086 \pm$

0.001 μM (Figure 3.13A, B), which is consistent with literature where MSH was found to be non-toxic up to 9 $\mu\text{g/mL}$ or approximately 16.047 μM (Igde & Yazici, 2012). Thus, following this viability assay, the highest dosage of MSH used in subsequent experiments was fixed at 20 μM to reduce the possibility of MSH toxicity affecting our assay results.

3.2.5.3 Pseudovirion infection assay screens with MSH

The infection assay was conducted on *Vero E6* cells and 3 concentrations of MSH were tested: 10 μM , 15 μM and 20 μM . Luciferase activity was measured using the Dual-Luciferase® assay system (Promega, 2019) and the results were analysed. The infection assay revealed significant inhibition of PV entry at 15 μM and 20 μM MSH. At these concentrations of MSH addition, a significantly lower luminescence activity was recorded as compared to the infected cells without MSH addition. This directly correlates to lower level of luciferase enzyme expression in the cells. As the PV carry the luciferase gene in the virion interior, if there is a reduction in virion entry, the luciferase enzyme expression would be affected. Therefore, this decrease in luciferase activity was postulated to be due to a reduction MERS-CoV Spike PV entry (Figure 3.13C).

Figure 3.13: Cell viability and Infection assay of MSH on *Vero E6* cells.

(A) Cell viability assay using WST-1 reagent of MSH on *Vero E6* cells, with the cut-off indication at 50% viability. (B) Viability curve and estimated highest non-toxic concentration to $20.086 \pm 0.00122 \mu\text{M}$. (C) Toxicity screens for MSH dosages to be used in subsequent infection assays. (D) Infection assays using MERS-Spike PV at a MOI of 1.5, with addition of varying concentrations of MSH. One-way ANOVA statistical analysis was performed with Dunnett's Post-hoc test to compare all columns with the control column. $N=3$, ** $p < 0.01$, *** $p < 0.001$.

3.2.6 Live MERS-CoV tests at Institut Pasteur Korea

From our PV infection assays, MSH seems to be showing an inhibitory effect on viral entry. However, the assay is not an accurate reflection of the live MERS-CoV infection as only the interactions between the spike glycoprotein and the host cell receptor was considered. Therefore, to further study this potential inhibitory effect of MSH more accurately, we employed the help of Dr. Kim Seungtaek's Zoonotic Virus Lab from Institut Pasteur Korea. Dr Kim's team consists of clinical experts who are experienced with the South Korean MERS-CoV outbreak in 2015. Hence, they possess live MERS-CoV specimens which were isolated from patients to assist in further testing.

3.2.6.1 Preliminary screening of MSH using live MERS-CoV

Preliminary assays were conducted in a time of addition (TOA) assay with 7,8-Dihydroxyflavone (7,8-DHF), a natural flavone which does not play any role in this MERS infection as a negative control, and 3 previously administered compounds against MERS infection as the positive controls on *Vero E6* cells. MSH was used at a fixed high concentration of 25 μ M as an initial screen for any inhibitory effect (Figure 3.14A). This assay revealed strong inhibition of viral infection of MSH as compared to previously used supportive drugs with low specificity to MERS such as CQ, Lopinavir (LPV) and the SARS-CoV 3C-like protease inhibitor, CE-5 (Figure 3.14B). In comparison with these previously used compounds for MERS-CoV infections, MSH was shown to be effective in reducing viral entry and progression even at the + 4-hour time-point of a 7-hour post infection harvest time, as compared to the + 1-hour time-point for CQ and + 3-hour time-points for CE-5 and LPV. This demonstrated that MSH can exert an inhibitory effect at a faster rate compared to CQ, CE-5 and LPV, suggesting higher potency against MERS infection.

However, this assay also revealed an increase in the toxicity of MSH when coupled with MERS-CoV infection. In comparison with the most toxic compound of the 3 positive controls: LPV where at 0-hour time-point, most of the cells were dead, MSH had cell death up to + 1-hour time-point. This means that although the potency of MSH is higher than the other 3 previously used compounds, the toxicity is higher as well. This increased toxicity of MSH might have affected our results as an increase in cell death might have led to a decrease in luciferase activity as well. As a result, additional screens with lowered dosages

of MSH are required to analyse the strength of inhibition by MSH while lowering its toxicity levels on *Vero E6* cells to minimise cell death.

A

Virus : MERS-CoV/KOR/KNIH/002_02_2015, 5 MOI

Cell : Vero cells

Test Compound : MSH 25 μ M

References : Chloroquine (CQ) 100 μ M, Lopinavir (LPV) 25 μ M, CE-5 50 μ M

Negative control compound : 7,8 DHF 25 μ M

B

MERS-CoV Spike/Nucleus

Figure 3.14: Time of addition assay of MSH with live MERS-CoV on *Vero E6* cells.

(A) Schematic layout of the time of addition assay where the live MERS-CoV was inoculated at 0 hour and the final harvesting time point was set at 7 hours post infection (hpi). (B) Fluorescence microscopy results of the time of addition assay showing visible toxicity of MSH as compared to the other commonly used compounds for MERS infections such as CQ, LPV and CE-5. The negative control used was 7,8-DHF, a natural flavone which does not exert any effect on this infection pathway.

3.2.6.2 Inhibitory studies on MSH using live MERS-CoV

Further experiments to study the inhibitory effect of MSH to the binding between MERS-CoV and hDPP4 was performed on *Vero E6* cells using live MERS-CoV. Preliminary toxicity screening of MSH on *Vero E6* cells were found to be low, with a TD_{50} around 20 μ M. However, initial experiments with MSH and live MERS-CoV revealed increased toxicity where the addition of MSH at a concentration of above 7 μ M led to significant cell death, causing inconsistencies in the results (Figure 3.15A). Therefore, we decided to lower MSH concentrations for subsequent experiments.

4 conditions of drug addition and viral inoculation were performed: Pre-incubation, Co-treatment, Pre-treatment and Post-treatment. Pre-incubation refers to the incubation of MSH with MERS-CoV for 1 hour before addition to the *Vero E6* cells. Co-treatment is the addition of MSH at the same time as MERS-CoV to the cells. Pre-treatment is to incubate MSH with the *Vero E6* cells for 1 hour before inoculation of MERS-CoV. Lastly, Post-treatment is the infection of MERS-CoV in *Vero E6* cells for 1 hour before the addition of MSH.

This assay revealed the strong potency of MSH on MERS infection in the pre-incubation, co-treatment as well as post-treatment conditions, where the average IC_{50} was approximated to be 3 μ M. Upon lowering of the dosage of MSH used in our studies, the IC_{50} of MSH could be estimated more accurately with the reduced cell death.

A

B

C

D

E

Figure 3.15: Quantification of the inhibitory effect of MSH on MERS-CoV infection in *Vero E6* cells.

(A) Summary of dose dependent titration of MSH in *Vero E6* cells using live MERS-CoV. Dose response curves (DRC) of MSH in comparison with CQ and LPV in 4 different conditions: (B) Pre-incubation, (C) Co-treatment, (D) Pre-treatment and (E) Post-treatment. Points and lines in blue represent percentage of inhibition of MERS-CoV infection and those in red refer to percentage of cell viability.

3.3 Approach 2: New fragment library screening

A library of chemical compounds was provided by Prof. Tan Choon Hong from NTU-School of Physical and Mathematical Sciences/Division of Chemistry and Biological Chemistry (NTU-SPMS/CBC). Similar to the binding assays conducted in the screening of *in-silico* hits, intrinsic fluorescence quenching assay was used for binding studies with our recombinant MERS-CoV RBD sample.

3.3.1 Identification of intrinsically fluorescent compounds

Negative control runs consisting of the compounds in our FPLC buffer (25 mM Tris-HCl pH 8.0 and 30 mM NaCl), were carried out to test if there were any compounds emitting intrinsic fluorescent signals which might affect the overall screening results. From these negative control runs, those compounds: Plate 1 – C5 and D4; Plate 2 – A8 and B10, which were found to have intrinsic fluorescence were removed from the downstream screening process (Figure 3.16).

A

B

Figure 3.16: Detection of intrinsically fluorescent compounds from the fragment library.

A total of approximately 200 compounds was used for this screening, of which the compounds were split into 2 sets of 96-well plates. Each plate was further split into 2 sets of plates for screening in duplicates. The results of (A) Plate 1, first 100 compounds and (B) Plate 2, next 100 compounds were summarised into 4 separate charts each. From Plate 1, compounds C5 and D4 were observed to have intrinsic fluorescent qualities and were omitted from subsequent screenings with the recombinant MERS-CoV RBD protein samples. Similarly, from Plate 2, compounds A8 and B10 were removed from downstream screenings.

3.3.2 Compound screening with recombinant MERS-CoV RBD sample

Preliminary compound screening was set at a protein-to-compound ratio of 1:5 and duplicates were performed to ensure replicability. From this screen, 4 compounds: E3, E4, E9 and E10 was found with the highest quenching effect as compared to the negative control (Figure 3.17A, C). As a result, a dose dependent titration was conducted on these 4 compounds ranging from a protein-to-compound ratio of 1:5 to 1:40 to study if the initial observations were accurate.

However, dose dependent titration experiments revealed inconsistencies with the initial findings, with only E10 having a slight quenching effect, though it was statistically insignificant (Figure 3.17D, E). Consistent with our quenching titration results, co-crystallisation and soaking screens conducted concurrently with the quenching assays also showed the lack of ligand density (Appendix IV). Therefore, the compound screening from this library was not successful in revealing any potentially significant candidates which are able to interact with the recombinant MERS-CoV RBD protein sample. Further screening of more compounds is required for the identification of potential hits.

B

C

Figure 3.17: Screening of ligands from the compound fragment library

Summary charts of the screening process for (A) Plate 1: first 100 compounds and (B) Plate 2: next 100 compounds. (C) Potential hits: E3, E4, E9 and E10 with their respective individual quenching curves and structures from initial screening at 1:5 ratio. (D) Summary of dose-dependent titration for potential hits from 1:5 to 1:40 protein-to-compound ratios. (E) Individual curves for the dose-dependent titration for the top 4 potential hits.

3.4 Approach 3: Designing of peptide mimetics targeting MERS-CoV RBD

Another approach to the discovery of therapeutics against MERS-CoV was the study of mimetic peptides. As the high-resolution crystal structure of MERS-CoV RBD-hDPP4 was previously published in 2013, it is possible to analyse this interface and design a short mimetic peptide to target and block this binding region to inhibit RBD-DPP4 complex formation.

3.4.1 Design of DPP4 mimetic peptide: DP12m

From our preliminary analysis of the MERS-CoV RBD-hDPP4 crystal structure (PDB: 4L72), a short helical region on the DPP4 protein lies at the interface region (Figure 3.18A). Further probing on HotRegion, a database containing cooperative hotspots in protein-protein interactions, revealed this helical region was a potential hotspot (Figure 3.18B) (Cukuroglu, Gursoy, & Keskin, 2011). Therefore, a short peptide from Ile-287 to His-298 containing this short helical region was procured and used for binding studies with our recombinant RBD protein. This DPP4 mimetic peptide was labelled DP12m peptide (DPP4-12mer peptide) and used in this study.

3.4.2 Circular Dichroism (CD) Spectroscopy analysis of peptide helicity

From our analysis of the RBD-DPP4 interface, this DP12m peptide is predominantly helical in structure. This helical property was important as it fits in the small binding pocket of MERS-CoV RBD. Hence, CD Spectroscopy was performed to check its estimated helical content. From the preliminary results of the CD analysis via the Bestsel software (Micsonai et al., 2018), the peptide was found to be approximately 10% helical and mainly unordered (Figure 3.18C, D). This suggested that the peptide was not in the native helical conformation, which might affect its binding with MERS-CoV RBD.

3.4.3 Binding studies of DP12m peptide with recombinant MERS-CoV RBD

Similar to previous approaches, intrinsic fluorescence quenching experiments were used to study the potential binding of MERS-CoV RBD with the DP12m peptide. Dose-dependent titration was carried out from a low protein-to-peptide ratios of 1:5 to high ratios of 1:40. However, results showed weak interactions between RBD and DP12m peptide which were considered statistically insignificant (Figure 3.18E, F). Repeated runs were conducted but similar results were obtained. Further studies with this mimetic peptide would first require the stability of the peptide's secondary structure.

A

B

Interface Name	Residue Number	Residue Type	Chain	Relative Complex ASA	Relative Monomer ASA	Pair Potential	Hotspot Status	Hotregion Status	Complex ASA	Monomer ASA
4L72AB	267	LYS	A	12.06	50.3	10.81	NH		24.22	101.01
4L72AB	269	PHE	A	3.86	15.41	26.4	H	-	7.69	30.74
4L72AB	286	GLN	A	10.4	40.71	20.18	H	-	18.56	72.67
4L72AB	288	THR	A	14.51	54.43	10.19	NH		20.21	75.8
4L72AB	289	ALA	A	5.81	10.56	17.39	NH		6.27	11.4
4L72AB	291	ALA	A	0	71.41	19.62	H	1	0	77.09
4L72AB	292	SER	A	3.03	26.66	22.38	H	1	3.53	31.06
4L72AB	294	LEU	A	1.88	58.41	33.21	H	1	3.36	104.34
4L72AB	295	ILE	A	15.2	79.89	20.21	H	1	26.61	139.91
4L72AB	298	HIS	A	7.44	19.96	25.03	H	2	13.61	36.5
4L72AB	317	ARG	A	16.51	26.63	24.45	H	2	39.42	63.58
4L72AB	322	TYR	A	3.47	13.29	23.16	H	2	7.38	28.27
4L72AB	334	SER	A	20.87	59.5	10.43	NH		24.31	69.32
4L72AB	336	ARG	A	14.42	55.95	24.58	H	-	34.44	133.58
4L72AB	341	VAL	A	32.2	71.63	7.82	NH		48.76	108.47
4L72AB	344	GLN	A	16.11	27.32	22.83	H	-	28.75	48.77
4L72AB	346	ILE	A	28.97	62.03	24.95	NH		50.74	108.62
4L72AB	392	LYS	A	69.8	87.5	4.99	NH		140.17	175.71
4L72AB	455	ASP	B	6.42	31.03	16.98	NH		9.01	43.57
4L72AB	463	PRO	B	6.66	25.53	11.06	NH		9.07	34.76
4L72AB	499	TYR	B	5.17	16.07	32.09	H	-	10.99	34.19
4L72AB	501	ASN	B	10.82	36.45	12.57	NH		15.58	52.46

C

D

Secondary Structure	Estimated content (%)
Helix	10.0
Antiparallel beta sheets	23.9
Parallel beta sheets	0.0
Turns	9.4
Others	56.8

DP12m peptide – dissolved in DMSO, 200μM concentration

E

F

Figure 3.18: DPP4 mimetic peptide studies with recombinant MERS-CoV RBD

(A) Design of DPP4 mimetic peptide: DP12m, showing the region of the helical peptide on the MERS-CoV RBD-hDPP4 complex highlighted in red. Obtained from PDB: 4L72. (B) Hotspot prediction via HotRegion software showing Ala-291, Ser-292, Leu-294, Ile-295 and His-298 as residues residing in a potential hotspot for RBD-DPP4 binding interface. (C) CD Spectroscopy conducted on the DP12m peptide to study its secondary structure. Results revealed a lack of helicity as compared to the estimated values. (D) Summary chart of predicted secondary structure of DP12m peptide. (E) Intrinsic fluorescence quenching curves of dose-dependent titration from 1:5 to 1:40. (F) Summary chart of the quenching results showed statistically insignificant quenching of fluorescence signals upon addition of DP12m peptide.

3.4.4 Stapled DP12m peptide studies

The molecular basis of stapling is the generation of a cross-link between 2 previously identified sites on the peptide. The stapling process generally requires the addition of olefin-bearing non-natural amino acids at the appropriate sites for cross-linking. Then, these peptides are placed in a synthesizer to catalyse the ring-closing olefin metathesis process to generate the hydrocarbon cross-link (Y.-W. Kim, Grossmann, & Verdine, 2011).

With the assistance of Dr. Lee Su Seong from A*star, a short DPP4 mimetic peptide: DP12m was stapled. The stapling of this short helical peptide was challenging due to its length and the short helical region which it contained. Therefore, upon the stapling of this peptide, its helicity was tested using secondary structure prediction via CD spectroscopy before any binding studies were carried out (Figure 3.19A). However, our results analysed using the Bestsel software revealed that the stapled peptide was still lacking in helicity where the overall helical content was predicted to be approximately 8% when the theoretical

helicity should be close to 50% (Figure 3.19B). Despite the stapling process, the folding of the DP12m peptide to its native helical structure could not be achieved. Nonetheless, a dose-dependent fluorescence quenching titration from a protein-to-peptide ratio of 1:5 to 1:40 was performed to check if the stapled peptide was able to interact with our recombinant MERS-CoV RBD sample. However, our results did not show any significant quenching of fluorescence emission as expected (Figure 3.19C, D). Hence, further optimisations such as the lengthening of the peptide are required for this mimetic peptide-based inhibitor design.

Figure 3.19: Stapled DP12m peptide secondary structure prediction and dose-dependent fluorescence quenching titrations

(A) Secondary structure prediction of the stapled DP12m peptide via CD spectroscopy. (B) Analysis of CD data via Bestsel software, revealing a low helical content in the stapled peptide sample. (C) Dose-dependent intrinsic fluorescence (Trp) based quenching experiments to

examine the presence or absence of any interactions between MERS-CoV RBD and stapled DP12m peptide. (D) Summary Trp quenching chart showing the overall quenching effect upon addition of stapled DP12m peptide.

4. Discussion

From previous structure-guided *in silico* screens on FDA-approved drugs by Dr. Harikishore and Dr. Sreekanth, MSH was predicted to fit and fill the MERS-CoV RBD pocket well (Figure 3.8). Biophysical assays revealed significant interaction between RBD and MSH in a dose-dependent manner, estimating the K_D value to be $62.99 \pm 7.62 \mu\text{M}$. Subsequent cell-based assays using MERS Spike PV suggested potential inhibitory effect of MSH on viral entry and live MERS-CoV experiments confirmed these findings and analysis on this inhibition led to a calculated IC_{50} of $3 \mu\text{M}$, which is significantly different from the K_D value. However, as binding affinity and inhibition efficiency are measurements of 2 distinct characteristics of the compound, a high binding affinity might not necessarily result in a strong inhibition and vice versa. Therefore, the K_D and IC_{50} values might not directly correlate with each other but are still considered to be significant.

The K_D value of RBD-MSH interaction falls in the micromolar range, suggesting a relatively weak affinity (Salahudeen & Nishtala, 2017). However, this might be due to *in-vitro* stability of the truncated RBD construct from the full length Spike glycoprotein (Billington et al., 2007; Figueira-Mansur, Aguilera, Stoque, Ventura, & Mohana-Borges, 2019). Live MERS-CoV experiments revealed a stronger inhibitory effect with MSH, which may be caused by the fully assembled virions carrying the stable full-length Spike protein. As the primary objective is to study and develop inhibitors specific to this RBD region on the Spike glycoprotein, the use of the truncated RBD protein was preferred over the full-length spike.

MSH was found to exhibit increased toxicity during the live MERS-CoV studies compared to the PV entry inhibition assays. Preliminary cell viability screens of MSH on *Vero E6* cells identified the highest non-toxic concentration to be $20.086 \pm 0.001 \mu\text{M}$, consistent with previous literature (Igde & Yazici, 2012). However, when coupled with MERS-CoV infection, the overall toxicity was enhanced such that even at $7 \mu\text{M}$, significant cell death ($> 50\%$) was recorded. A possible explanation for this phenomenon might be the synergistic effect of viral infection weakening the cells and the intrinsic toxicity of MSH. As its overall cytotoxicity was found to be high when coupled with MERS-CoV infections, it might lead to difficulties in quantification of viral inhibition. However, it is still not impossible to perform such measurements as shown in the case of Lopinavir, which showed high cytotoxicity in infection assays with an IC_{50} of $8 \mu\text{M}$ (Chan et al., 2015), similar to our calculations of $14 \mu\text{M}$.

Additionally, MSH was shown to be highly sensitive to the body condition and dosage administered. According to the FDA approval information, the main route of administration is oral. MSH has a bioavailability of 64%. This dosage administered for asthmatic patients was considered safe. Under poor conditions and low dosages, MSH could exhibit beneficial responses such as neuro- and cardio-protective effects (Hoxha, Lewis-Mikhael, & Bueno-Cavanillas, 2018). Conversely, in good body conditions and high dosages, MSH disrupts cell proliferation, inducing negative effects (Eriksson et al., 2018). Hence, the optimisation of MSH concentration to balance out its cytotoxicity is crucial in our cell-based live MERS-CoV experiments.

The calculated IC_{50} of MSH was $3 \mu\text{M}$, which is less potent compared to other drugs with IC_{50} in the nanomolar to picomolar ranges. Yet, MSH was highly

efficient in inhibiting viral entry as shown in the live MERS-CoV assays (Figure 3.15). Furthermore, previously reported compounds such as NUD-1 for Influenza replication via NP-NP (nucleoprotein-nucleoprotein) interactions was reported to have an IC_{50} of $1.8 \pm 0.2 \mu M$, but demonstrated potent suppression of viral replication (Makau et al., 2017). Therefore, our IC_{50} value is considered acceptable and significant.

From the live MERS-CoV infection assays, similar IC_{50} values were generated from the pre-incubation, co-treatment and post-infection conditions, which suggests that the MSH interaction with MERS-CoV RBD is unaffected by incubation time and thus is characterised by rapid equilibrium (Swinney et al., 2016). Consistent with our experimental data obtained from *in-silico* and *in-vitro* assays, the live MERS-CoV infection assays supports that MSH is a viral entry inhibitor (Liu et al., 2011).

5. Conclusion

In this thesis, recombinant MERS-CoV RBD and hDPP4 constructs were cloned, expressed using a baculoviral system and purified for binding and inhibition studies. Cell-based PV studies were also performed on *Vero E6* cells for viral entry inhibition assays. Various approaches to drug discovery were conducted: 1) Drug repurposing via structure-guided screening of FDA-approved drugs, 2) New fragment library screening, and 3) Designing of peptide mimetics targeting MERS-CoV RBD. Of which, the fragment library and peptide mimetics studies still require further optimisation and screenings for potential lead identification.

For the drug repurposing approach, binding studies on the purified recombinant MERS-CoV RBD revealed interaction with MSH, a previously FDA-approved leukotriene receptor antagonist used for the treatment of asthma and seasonal allergies. Fluorescence quenching studies revealed a micromolar range dissociation constant. Analysis via size exclusion chromatography showed slight shifts in fractionation peaks upon MSH incubation with the recombinant protein samples, suggesting possible inhibitory effect.

Cell-based assays using MERS Spike PV and *Vero E6* cells demonstrated potent inhibition, supporting our previous findings. Further validation studies were performed with the assistance of Dr Kim SeungTaek's team in Institut Pasteur Korea using live MERS-CoV samples isolated from patients during the 2015 outbreak in South Korea. These results confirmed our *in-vitro* data and demonstrated significant inhibition of viral entry upon MSH addition at the pre-incubation, co-treatment and post-treatment conditions, producing a significant half-maximal inhibitory concentration (IC_{50}).

6. Future work

Further studies on MSH would be the primary focus in our future work. Confirmation of live MERS-CoV assay results could be conducted via *in vivo* studies on MERS-CoV transgenic mouse models (Coleman, Matthews, Goicochea, & Frieman, 2014) to study the potential therapeutic effects of MSH. However, to begin with the *in-vivo* studies, the Median Lethal Dose (MLD) of MSH has to be determined prior to the infection screens (Akhila, Shyamjith, & Alwar, 2007). After this, infection screens on the transgenic mice could then be performed by monitoring of survival and weight loss (JCU, 2018; Ray, Johnston, Verhulst, Trammell, & Toth, 2010).

To obtain structural information of MERS-CoV RBD-MSH interaction, further optimisation to the crystallisation conditions could be carried out. Another avenue that we could possibly explore is the use of NMR Spectroscopy. However, unlike X-ray crystallographic studies, NMR structural determination and analyses require stable isotope labelling of recombinant proteins (Muchmore, McIntosh, Russell, Anderson, & Dahlquist, 1989). Recent publications demonstrated the use of BioExpress® 2000 medium for stable isotope labelling of baculoviral expressed recombinant proteins which could possibly be adopted for structural studies (Saxena, Dutta, Klein-Seetharaman, & Schwalbe, 2012; Strauss, Fendrich, & Jahnke, 2019).

For the fragment library screening, additional screens are required for the identification of a good target for drug development. Therefore, more purified recombinant MERS-CoV RBD samples could be prepared for screening. Additionally, for the DPP4 mimetic peptide studies, previous stapling was shown to be successful, but the helicity of the stapled peptide was not enhanced.

Therefore, other methods to induce the helical conformation of the peptide have to be considered and tested with the MERS-CoV RBD sample.

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Appendix I – Anion Exchange Chromatography (ResourceQ column) for purification of impure RBD samples

Appendix II – Full report for Mass Spectrometry Protein Identification on (A) MERS-CoV RBD and (B) hDPP4 samples

A

Gel Idx/Pos	15/A15	Instr./Gel Origin	ak072/161123	Process Status	Analysis Succeeded				
Plate [#] Name	[1] mw1	Instrument Sample Name		Spectra	11				
Rank	Protein Name	Species	Accession No.	Protein MW	Protein Score	Protein Score C. I. %	Total Ion Score	Total Ion C. I. %	Confirmed
1	Dipeptidyl peptidase 4 OS=Homo sapiens GN=DPP4 PE=1 SV=2		sp P27487 DPP4_HUMAN	88906.7	302	100	114	100	.T.
Peptide Information									
Calc. Mass	Obsrv. Mass	± da	± ppm	Start Seq.	End Sequence Seq.	Ion Score	C. I. %	Modification	Rank Result Type
854.4665	854.4902	0.0237	28	590	596 IMHAINR				Mascot
856.4886	856.5063	0.0177	21	259	267 AGAVNPTVK				Mascot
888.4785	888.4938	0.0153	17	141	147 QLITEER				Mascot
892.5614	892.5779	0.0165	18	55	61 LKLYSLR				Mascot
915.541	915.5601	0.0191	21	311	317 ISLQWLR				Mascot
915.541	915.5601	0.0191	21	311	317 ISLQWLR				Mascot
918.4614	918.4822	0.0208	23	337	343 WNCLVAR			Carbamidomethyl (C)[3]	Mascot
959.5672	959.5808	0.0136	14	251	258 TVRVPYPK	14	0		Mascot
959.5672	959.5808	0.0136	14	251	258 TVRVPYPK				Mascot
966.4825	966.4985	0.016	17	616	623 MGFVDNKR				Mascot
966.4825	966.4985	0.016	17	616	623 MGFVDNKR				Mascot
982.4774	982.4994	0.022	22	616	623 MGFVDNKR			Oxidation (M)[1]	Mascot
1010.5676	1010.5854	0.0178	18	590	597 IMHAINRR				Mascot
1029.551	1029.5724	0.0214	21	649	658 CGIAPVSR			Carbamidomethyl (C)[1]	Mascot

B

Gel Idx/Pos		106/E5	Instr./Gel Origin		ak072/190107		Process Status		Analysis Succeeded		
Plate [#] Name		[1] mw1	Instrument Sample Name				Spectra		2		
Rank	Protein Name		Species		Accession No.	Protein MW	Protein Score	Protein Score C. I. %	Total Ion Score	Total Ion C. I. %	Confirmed
1	Spike glycoprotein OS=Middle East respiratory syndrome-related coronavirus (isolate United Kingdom/				sp K9N5Q8 SPIKE_CVEMC	151710.3	129	100	121	100	
Peptide Information											
	Calc. Mass	Obsrv. Mass	± da	± ppm	Start Seq.	End Seq.	Sequence	Ion Score	C. I. %	Modification	Rank Result Type
	1799.881	1799.8119	-0.0691	-38	454	470	SDLSVSSAGPISQFNYK	121	100		Mascot
	1799.881	1799.8119	-0.0691	-38	454	470	SDLSVSSAGPISQFNYK				Mascot

Appendix III – Thermal Shift Assay results

A

Negative controls – ligands only

B

Thermal Shift - Ligand screens

C

Thermal Shift Assay (RBD)

Appendix IV – Table of Co-crystallisation and soaking conditions (MERS-CoV RBD with MSH/E3/E4/E9/E10)

S/N	Crystal ID	Resolution (Å)	Conditions
1	8-RBD-MSH-1	2.40	Soaked in 3.75mM MSH for 7min
2	27-RBD-MSH-5	2.36	Overnight Soaking in 2.0mM MSH
3	28-RBD-MSH-6	2.70	Overnight Soaking in 2.0mM MSH
4	0-RBD-MSH-co	2.49	Co-crystallized with MSH
5	10-RBD-E4-1	2.26	Soaked in 2mM E4 for 1min
6	11-RBD-E4-2	2.00	Soaked in 2mM E4 for 3min
7	26-RBD-E4-4	2.45	Overnight soaking in 2mM E4
8	17-RBD-E10-2	2.55	Soaked in 2mM E10 for 10min
9	18-RBD-E10-3	2.33	Soaked in 2mM E10 for 7min
10	19-RBD-E10-4	2.50	Soaked in 2mM E10 for 5min
11	13-RBD-E9-1	2.20	Soaked in 2mM E9 for 7min
12	15-RBD-E9-3	2.35	Soaked in 2mM E9 for 3min
13	16-RBD-E9-4	2.40	Soaked in 2mM E9 for 2min
14	20-RBD-cocktail-2	2.65	Co-crystallized
15	23-RBD-cocktail-4	2.19	Co-crystallized
16	25-RBD-cocktail-5	2.42	Overnight soaking in 2mM E3/E4/E9/E10

Co-crystallization was conducted in a 1:4 molar ratio

Soaking of RBD apo crystals with MSH at 2 mM – 5 mM was carried out for 2 minutes - 10 minutes and overnight in 2 mM

For the fragments, RBD crystals were soaked in 2 mM E3/E4/E9/E10 for 1 minute - 10 minutes and overnight.

The space group was similar to apo RBD in all (P2₁2₁2₁; a=46.97Å, b=108.47Å, c=125.93Å; α= β=γ=90 °)

Only information on diffracted crystals alone are shown in the table