**Antiviral Agents**

**Translation of Mycobacterium Survival Strategy to Develop a Lipopeptide based Fusion Inhibitor**

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**Abstract:** The entry of enveloped virus requires the fusion of viral and host cell membranes. An effective fusion inhibitor aiming at impeding such membrane fusion may emerge as a broad-spectrum antiviral agent against a wide range of viral infections. Mycobacterium survives inside the phagosome by inhibiting phagosome–lysosome fusion with the help of a coat protein coronin 1. Structural analysis of coronin 1 and other WD40-repeat protein suggest that the trp-asg (WD) sequence is placed at distorted β-meander motif (more exposed) in coronin 1. The unique structural feature of coronin 1 was explored to identify a simple lipo-peptide sequence (myr-WD), which effectively inhibits membrane fusion by modulating the interfacial order, water penetration, and surface potential. The mycobacterium inspired lipo-dipeptide was successfully tested to combat type 1 influenza virus (H1N1) and murine coronavirus infections as a potential broad-spectrum antiviral agent.

**Introduction**

Emerging infectious diseases remain a significant risk to public health and have a severe impact on the global economy. A large number of these diseases are zoonotic, and the incidence of such zoonoses increases when human and environmental factors are compromised owing to the unintended overlap of previously distinct ecological domains. In one such recent protrude, a novel coronavirus (SARS-CoV-2) strain has emerged, infected circa 56 million people globally, and The World Health Organization declared the outbreak of a pandemic. This pandemic has posed severe threats to global public health and socio-economic stabilities and warrants the need for effective measures to control the infection. The infection rate in terms of both mortality and morbidity is fast escalating with no vaccine currently available to tackle the virus infection. On the other hand, seasonal influenza epidemics also cause around 3–5 million illness cases and up to 650,000 deaths globally even after 100 years of the first H1N1 pandemic.[2,3] Strategies to prevent and treat these viral infections have been limited to developing a vaccine and with a relatively small arsenal of antiviral drugs. Given the emergence of a large number of emerging as well as reemerging viral pathogens, which are often lethal to humans, the traditional one bug–one drug approach is unlikely to become an effective modality to treat such pathogens.[4] Moreover, various subtypes of influenza virus such as Type A(H1N1) pdm2009, A(H3N2), A(H7N9), and A(H5N1) exhibited resistance to many approved antiviral drugs, such as oseltamivir and zanamivir.[5–9] Thus, the development of broad-spectrum antivirals is urgently needed to control pandemic viral infections.[4,10]

The broad-spectrum antivirals target some standard, but essential features of the viral life cycle and are beneficial over the strategies that are conventionally focused on individual viruses or specific target protein. Notably, many viral pathogens such as smallpox virus, flu virus, HIV, flavivirus (dengue, ebola, zika), coronavirus, and arbovirus, are membrane-enveloped viruses.[11] To replicate inside the host cells, these viruses mainly rely on a typical biological process, namely membrane fusion. The fusion between virus and host cellular or subcellular membranes is a molecular choreography between viral fusion proteins and host cell components.[12,13] The underlying biophysical and biochemical features of the membrane fusion show noticeable similarities among the wide range of enveloped viruses. Thus, blocking of the classical feature of membrane fusion has appeared to be a new paradigm for the development of broad-spectrum antivirals.[4]

Membrane fusion inhibitors have been developed for a long and enfuvirtide (T20) is used in combination therapy for HIV-1 infection.[14] T20 based inhibitors were modified further, and lipopeptides (such as LP40) was shown to exhibit a more potent effect.[14] Additionally, several small molecule-based fusion inhibitors have been reported as potent antiviral agent against severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2),[15] influenza,[16,17] and respiratory syncytial virus.[18] However, these molecules inhibit the fusion upon interacting with a specific viral fusion protein and, in most cases, lack potential broad-spectrum antiviral nature. Therefore, targeting the membrane properties may be useful to develop potential broad-spectrum antivirals.

Lipids such as phosphatidylethanolamine (PE), diacylglycerol (DAG), and oleic acid induce negative curvature at the outer leaflet of the membrane and promote fusion.[19] Conversely, enrichment of the outer leaflet with lipids such as lysophosphatidylcholine and lysophosphoglycans, favor pos-
itive curvature and thus, prevent membrane fusion ability of enveloped viruses.\textsuperscript{[19–21]} Unfortunately, use of these fusion inhibitors (lysolipids) as antivirals are often associated with disruption of host cell membranes, cytotoxic effects, and they are rapidly metabolizable. Recently, the mechanism of actions of lysolipid helped to develop rigid amphipathic fusion inhibitors (RAFIs).\textsuperscript{[22]} RAFIs was designed on the basic principle based on their ability to promote positive curvature and, thus, inhibit fusion of several enveloped viruses even at nanomolar concentration. However, the exact mechanism of RAFI action is unclear, and the possibility of a geometric curvature-oriented mechanism and photosensitization induced generation of singlet oxygen was proposed.\textsuperscript{[23]} More importantly, no clinical or in vivo efficacy data is available with RAFIs to date.\textsuperscript{[24]} Therefore, the need for a cost-effective broad-spectrum fusion inhibitor to tackle the viral infection in the face of an outbreak (such as Covid-19) is very critical. To this end, we have explored the mechanism of mycobacterium evasion of phagosome-lysocome fusion and tested the antiviral potential of the designed lipo-peptides.

Intracellular survival of Mycobacterium tuberculosis in the phagosomes by avoiding phagosome-lysosome fusion and degradation remains an exciting area of research.\textsuperscript{[25,26]} Ferrari et al. reported that Coronin 1 protein recruited at the phagosomal membrane plays a crucial role in the survival of mycobacteria inside the macrophage.\textsuperscript{[27,28]} Although several other biochemical pathways were proposed regarding the mycobacterial evasion of phagosome-lysoosome fusion,\textsuperscript{[29]} the role of coronin 1 to inhibit fusion cannot be undermined.\textsuperscript{[27,29,30]} We hypothesized that structural analysis of coronin 1 coat protein would pave the way to the development of a novel fusion inhibitor. Analysis of the 3D structure suggests that coronin 1 has multiple tryptophan-aspartic acid domains (WD40).\textsuperscript{[31]} WD40 proteins are one of the largest class of proteins and are involved in various cellular processes.\textsuperscript{[32–34]} These proteins have similar architecture (domain) but exhibit diverse functionalities. Therefore, mechanistic understanding of such selective functionality of the WD40 proteins needs further investigation. Interestingly, it was found that in coronin 1 the tryptophan-aspartic acid (WD) moiety is exposed outside the distorted β-meander motif (Figure 1). Therefore, in the present study, we have synthesized a series of WD-based peptides. We found that lipo-dipeptide (myr-WD) acts as a potent fusion inhibitor, which can tackle H1N1 and murine coronavirus infections.

**Results and Discussion**

The N-terminus of coronin 1, a WD40-repeat domain, have β-propeller architecture typically comprising of seven blades.\textsuperscript{[32,39]} With an overall doughnut shape, the WD40 domain is among the most interacting domains in eukaryotic genomes.\textsuperscript{[32–34]} The analysis of the structure of murine coronin 1 suggests that the trp-asp dipeptide sequence of the protein was placed mostly at the distorted β-meander motif.\textsuperscript{[33]} There are four trp-asp (WD)/WE (trp-glu) sequences in murine coronin 1, and all of them (sequence: 35–36; 109–110; 159–160 and 250–251) were exposed outside and not buried (Figure 1; Supporting Information, Figure S2). Interestingly, 75% of them were placed at distorted β-meander motif, where the third anti-parallel β-strand H-bonded poorly with second β-strand and the residues (blue: trp, red: asp/glu) at the edge were more exposed (Figure 1; Supporting Information, Figure S1). The question that arises is whether the preference of trp-asp sequence at distorted β-meander motif is unique to coronin 1 or a common feature in other WD40-repeat domain proteins? To investigate, crystal structures of eight WD40-repeat proteins were analyzed (Figure 1; Supporting Information, Table S1). It was found that in most cases, the WD sequence was placed at a perfect β-meander motif, and WD is less exposed (Figure 1). It is worthwhile to mention that none of the eight proteins possess any fusion modulatory role (Supporting Information, Table S1). It appears that the coronin 1 structure is unique, and the more exposed trp-asp might have a role in fusion inhibition (Figure 1). Further, we have built the 3D model of human coronin 1 using I-TASSER server\textsuperscript{[35]} and noticed that 75% of the WD/WE were placed at exposed distorted β-meander motif (Supporting Information, Figure S3). Apart from coronin 1, we looked into other WD40-repeat protein having membrane fusion modulatory role. The protein, tomosyn with N-terminal WD40 repeat, was reported to impair the membrane bending activity of synaptotagmin-1 to inhibit SNARE complex-mediated membrane fusion.\textsuperscript{[36]} The model of tomosyn was built in I-TASSER and it was found that 50% of trp-asp of tomosyn reside at distorted β-meander motif (Supporting Information, Figure S10). The structural analysis of eleven WD40-repeat proteins points out that the trp-asp is more exposed in fusion inhibitory coronin 1 and mostly less exposed in other proteins (Figure 1; Supporting Information, Table S1). These findings led us to explore the effect of trp-asp (WD) and similar small dipeptides towards fusion inhibition. Therefore, inspired by the coronin 1 structure, we have synthesized a dipeptide containing L-tryptophan and L-aspartic acid, which can act as a potent fusion inhibitor.
aspartic acid (WD) and investigated its role in membrane fusion.

Approximately 50 nm vesicles composed with DOPC/DOPPE/Chol (55:25:20) and DOPC/DOPPE/Chol/NBD-PE/Rh-PE (55:22:20:1.5:1.5) were incubated at pH 5.5, and the fusion was triggered by 6% (w/v) polyethylene glycol (PEG).\(^ {37} \) It was noticed that 6% (w/v) PEG has no influence on the tryptophan fluorescence intensity of the WD, suggesting that PEG does not alter the microenvironment of WD. The control vesicles exhibit \( \approx 25\% \) fusion (FRET assay), and the addition of WD in membrane does not change the extent of fusion (Figure 2). This suggests that WD peptide may not be a close mimic to coronin 1 as it cannot inhibit fusion. We recall that coronin 1 is a coat protein, anchored at the surface, and the WD region is exposed outside the membrane. We hypothesize that to exert the biological activity, the synthetic mimic (dipeptide) must be anchored/bound to the surface. The binding affinity of the WD peptide was evaluated by monitoring the change in the intensity of tryptophan fluorescence with an increasing concentration of membrane. It was found that the WD peptide does not bind to the membrane and, therefore, does not influence the fusion. To accomplish a surface anchoring, we introduced lipidation at the N-terminus of the WD peptide. N-acyl tryptophan aspartic acid (N-acylWD) was synthesized by acylating the N-terminus of tryptophan with fatty acyl chains \( (n = 12–16, \text{ even}) \). The binding affinity of lauroyl-WD (lau-WD), myristoyl-WD (myr-WD), and palmitoyl-WD (pal-WD) to the membrane were determined by fluorescence spectroscopy\(^ {38} \) and listed in the Supporting Information, Table S2. It was found that all the lipo-peptides anchor to the membrane, and the equilibrium dissociation constant \( (K_d) \) decreases with the increase of acyl chain-length (Supporting Information, Table S2). After confirming the membrane anchoring ability of lipo-peptides, we examined the effect of membrane fusion. Interestingly, the lipo-peptides were also capable of inhibiting the fusion. The myr-WD and pal-WD more effective than lau-WD, and the inhibition nicely correlates with the membrane-binding (Figure 2B; Supporting Information, Table S2). Since the hydrophobic anchor plays a significant role in inhibiting fusion, we asked what is the role of cholesterol anchor compared to fatty acyl anchor. The chol-WD was synthesized, and the \( K_d \) was measured. We noticed that the chol-WD bound membrane weakly compared to myr-WD and could not effectively inhibit the fusion (Figure 2B). At a Chol-WD to lipid ratio of 1:100, the addition of peptide will increase the cholesterol content by a negligible amount of about 1 mol% in the membrane. It may be noted that a WD-containing 23 amino acid peptide (TG-23) exhibited significantly less inhibition of fusion when 20 mol% cholesterol was incorporated in the membrane.\(^ {39} \) We conclude that the hydrophobic anchor is important, and myristoyl chain is the smallest anchor to exert reasonable inhibition of fusion. Therefore, further experiments were performed with myristoyl chains. Now, the roles of tryptophan and aspartic acid were investigated. myr-WD di-methylenelester (myr-WDMe, devoid of two -COOH), myristoyl tryptophan (myr-W, no aspartic acid), myristoyl aspartic acid (myr-D, no tryptophan) were synthesized and their role in fusion was investigated (Figure 2A,C). The myr-WDMe could not block the fusion, suggesting critical role of a terminal acid moiety (Figure 2C).

Now the next question that arises is what the role of tryptophan is. Both myr-D and myr-W inhibited the fusion, and the extent of inhibition is slightly more with myr-W (Supporting Information, Figure S23). These suggest that although the myr-D have two carboxylic acids, the myr-W with one carboxylic acid is more potent, inferring a vital role of tryptophan. However, both the peptides were less effective than myr-WD, suggesting that the WD sequence is unique for significant fusion inhibition. Myr-FD and myr-GD peptides were synthesized to evaluate the role of aromatic amino acids. Myr-FD inhibits more than myr-GD, suggesting that the aromatic amino acid may be a more potent inhibitor (Figure 2D). However, comparing all the peptides, it was observed myr-WD is the most effective inhibitor than other peptides. We conclude that the myristoyl chain, tryptophan, and aspartic acid worked in tandem to exhibit significant fusion inhibition.

We recall that the WD sequence of coronin 1 is placed at the edge of the \( \beta \)-sheet, and our results suggest that the specific sequence also acts as the best inhibitor of fusion. The question is whether the myr-WD peptide works in tandem, and what is the effective lipid: peptide ratio for fusion? Concentration-dependent fusion kinetics were performed, and it was found that the myr-WD peptide inhibits fusion in a concentration-dependent manner (Figure 3A). The initial rate and the extent of fusion were shown in the Supporting Information, Figure S24, and it was observed myr-WD effectively inhibits fusion at a peptide to lipid ratio of 1:100. The size of the vesicles at various lipid: peptide ratio was determined to rule out the effect of curvature in the fusion. The dynamic light scattering data suggest that the size of vesicles does not change significantly by the addition of the peptide at a 1:50 ratio.

![Figure 2](image-url)
Since the ordering of the membrane interface was found to inhibit fusion in vitro effectively, we expected that it could tackle viral infection by inhibiting membrane fusion. The life-cycle of the majority of the enveloped viruses mostly dependent on membrane fusion. To address the broader goal of our present study, we tested the efficacy of mycobacterium inspired lipopeptides in H1N1 influenza virus and murine coronavirus infection. Briefly, the antiviral activity of myr-WD peptide was tested in MDCK (Madin–Darby canine kidney) cells against influenza type A/PR/8/34 (H1N1) virus challenge by standard plaque assay as well as MTT based cell cytotoxicity assay (Figure 4). It was observed that 200 μM myr-WD exhibited low cytotoxicity against MDCK cell (CC₅₀) (Figure S26). The infection of MDCK cells (0.05 × 10⁵ cells per well) was initiated with 100 MOI of virus for 2 h at 37°C under 5% CO₂ pressure in a humidified chamber.

To investigate how the unique lipopeptide myr-WD inhibits fusion, extensive biophysical characterization with three distinct peptides (myr-WD, myr-WDMε and myr-GD) were performed. It was noticed earlier that myr-WD inhibited the fusion, myr-WDMε has no role in fusion, and myr-GD slightly inhibit the fusion (Figure 2). The membrane order at the interfacial region was measured at various peptide to lipid ratio by monitoring the steady-state fluorescence anisotropy of a fluorophore, TMA-DPH. The TMA-DPH anisotropy increased with the increase of myr-WD peptide in the membrane, myr-GD slightly increased the anisotropy, whereas myr-WDMε in the membrane did not change the anisotropy (Figure 3 B). The increase in TMA-DPH anisotropy by the most effective fusion inhibitory peptide (myr-WD) indicates that the peptide effectively orders the membrane interface. Since the ordering of the membrane interface inhibits the lipid protrusion required for hemifusion, we suggest that myr-WD inhibit the fusion by ordering the membrane interface. Next, the water penetration to the bilayer was determined at various peptide to lipid ratio by measuring the lifetime of TMA-DPH. The average lifetime increased with the addition of two fusion inhibitory peptide, myr-GD and myr-WD in the membrane, whereas the average lifetime did not increase with myr-WDMε (Figure 3 C). The increase in average lifetime suggests that water penetration into the membrane decreases with fusion inhibitory peptides. Membrane fusion is generally believed to proceed via a lipid-water mixture to form a hemifusion intermediate. Therefore, the reduction of water penetration in the membrane by the myr-WD peptide likely interfere with the lipid protrusion required for hemifusion. Next, the zeta potential was measured as the surface potential was shown earlier to modulate the fusion. It was observed that while myr-WDMε does not change the zeta potential with increasing the peptide concentration, myr-WD decreases the zeta potential. The other peptide, myr-GD also reduces the zeta potential but not to the same extent as myr-WD. It is interesting to note that although both myr-GD and myr-WD have two-COOH groups with identical charged headgroup at the interface, the extent of reduction of zeta potential with myr-WD is almost twice as compared to myr-GD (Figure 3 D). The more negative zeta potential of myr-WD may be responsible for the inhibition as the negative surface potential was reported to inhibit the fusion. All these experiments suggest that myr-WD is a unique peptide sequence, and it effectively inhibit the fusion by the increase in the interfacial order and decrease in water penetration, zeta potential. It may be suggested that the unique nature of the WD sequence was chosen in coronin 1 to inhibit phagosome-lysosome fusion.

Figure 3. Mechanistic studies on the inhibition of fusion. A) myr-WD peptide inhibits the fusion in a concentration-dependent manner, B) change in the fluorescence anisotropy of TMA-DPH in DOPC/DOPE/Chol (55:25:20) membranes with increasing concentration of myr-WD peptide (black, η), myr-GD peptide (red, γ) and myr-WDMε peptide (blue, μ), C) change in the average fluorescence lifetime of TMA-DPH, D) change in the zeta potential with increasing concentration of the above peptides. The lines were drawn to guide the eye.

Figure 4. myr-WD protects the influenza-A virus infection. A) myr-WD reduces viral plaque formation in influenza type A/PR/8/34 (H1N1) virus-infected MDCK cells in a dose-dependent manner. B) myr-WD provides protection against influenza viral infection in H1N1-virus-infected MDCK cells (MOI = 100) evaluated by MTT assay and C) cell cytopathic effect and image of the cells viewed under an inverted light microscope (Nikon, Japan) at 20× magnification.
A significant reduction in viral plaque formation was observed with the addition of myr-WD peptide (Figure 4A). MTT based cell survivability assay also supports that the designed lipo-peptide protects the cells from influenza challenge at low concentration (ca. 3 μM) (Figure 4B). The corresponding cell cytopathic effect (CPE) characterized by rounding up and detachment of cells was observed by an inverted light microscope. The reduction of CPE in myr-WD treated cells suggested that the fusion inhibitory myr-WD provided protection against influenza viral infection. The cells treated with the peptide were observed to retain their cellular morphology compared to cells that did not receive myr-WD treatment (Figure 4C).

The antiviral effect of myr-WD and myr-GD was further explored in L2 rat epithelial cell line using RSA59, an isogenic recombinant strain of mouse hepatitis virus, belongs to the β-coronavirus group (a prototype m-CoV: murine coronavirus). The CC₅₀ values for myr-WD and myr-GD were determined using MTT assay (Supporting Information, Figure S27). Myr-WD, myr-GD were not toxic to the cells even at high concentrations. For consequent experiments, approximate 1/10th of the CC₅₀ concentration, that is, 50 μM was used for myr-WD and myr-GD. Plaque assay in L2 cells cotreated with virus and inhibitor peptide suggests the potent antiviral effect of myr-WD (Figure 5A). RSA59 also expresses enhanced green fluorescent protein (EGFP) that allows visualization of viral spread and fusogenesis in vitro. The antiviral effect of myr-GD and myr-WD on the fusogenicity of RSA59 was assessed by analyzing the syncytial formation in L2 cells on cotreatment with the virus (at 0.5 MOI) and the inhibitor. Myr-WD and myr-GD both significantly reduced the syncytia formation; however, myr-WD was more effective (Figure 5B).

The global concerns for infectious diseases of viral origin remain as a serious risk throughout the history of human civilization. Therefore, developing a broad-spectrum antiviral is essential to tackle the impending risk associated with present or future pandemic viral infections. The broad-spectrum antivirals are expected to be superior to the traditional one bug—one drug approach as the drug development process always has some lag period. To develop broad-spectrum antivirals, we searched for a membrane-active compound to inhibit the fusion. Drugs targeting the fusion machinery likely be specific to a particular virus (such as T20 for HIV, IPB02 for SARS-CoV-2, cyclic peptide P4 for influenza, etc.) and may not be useful to target a variety of deadly viruses. Instead, the membrane-active compounds that passively interfere with the membrane fusion likely be a new class of molecules to inhibit fusion and may be used as a broad-spectrum antiviral agent. Inspired from coronin 1, several WD lipopeptides were synthesized, and their fusion inhibition potentials were evaluated. Myr-WD inhibits the fusion by increasing the interfacial order, decrease water penetration, surface potential. Interestingly, myr-WD also demonstrated that myr-WD increases interfacial order and decreases water penetration, surface potential. Interestingly, myr-WD also effectively in tackling the H1N1 and murine coronavirus infection at relatively low concentrations.[46] The membrane-active nature of the peptide likely reflects the micromolecular concentration required to combat H1N1 and murine coronavirus infection. We suggest that the mycobacterium inspired approach is new and will pave the way in the development of potential broad-spectrum antiviral to tackle several deadly viruses such as influenza, corona, and ebola.

**Conclusion**

The emergence of highly pathogenic viruses questions the unparalleled superiority of vaccines as the process of vaccine development is associated with a substantial amount of lag period with relatively high cost. To this end, a fusion inhibitor may emerge as an effective broad-spectrum antiviral as membrane fusion is important to the life cycle of many enveloped viruses. Here we utilized the survival strategy of mycobacterium inside the phagosome to develop a new class of fusion inhibitors. Mycobacterium coats the phagosome with coronin 1 to inhibit fusion with the lysosome. Structural analysis of coronin 1 and other WD40-repeat proteins suggests that the microenvironment of the trp-as (WD) sequence in fusion inhibitory protein is unique. This has inspired us to explore the role of several lipo-dipeptides and finally identify an effective small-molecule fusion inhibitor, myr-WD, that recapitulates the likely feature of coronin 1. We demonstrated that myr-WD increases interfacial order and decreases water penetration, surface potential to inhibit membrane fusion. The mycobacterium inspired simple lipo-dipeptide protects cells from prototypes type A influenza virus (H1N1) and murine coronavirus challenge as a potential broad-spectrum antiviral agent.

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Conflict of interest

The authors declare no conflict of interest.

Keywords: broad-spectrum antivirals · enveloped virus · lipo-peptide inhibitor · membrane fusion · water penetration

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