Introduction:

It seems simple to think that viruses infect organisms by crossing the lipid bilayer. However, there are a lot more going on while these viruses try to cross our cells through the lipid bilayer. Lipids in our cells can undergo several types of modifications, which may permit these viruses to enter our cells; thereby, infecting us.

Viruses can have an envelope, which is an outer membrane of the virus and encloses the protein coat where viruses store their DNA or RNA (Cohen, 2016). There are also viruses that do not have envelopes or outer membranes. Non-enveloped viruses lack the lipid bilayer membrane or outer membrane (Dormitzer et al., 2004). Some of enveloped viruses can enter the host cell plasma membrane directly or through endocytosis, and then fuse their envelope with an endosome (Cohen, 2016). An example of enveloped viruses that directly fuse with plasma membrane are retroviruses such as, HIV (Feneant et al. 2015). HIV-1 Gag polyprotein has myristoylated matrix domain, which targets the host plasma membrane for entry (Barros et al, 2016). Another example of envelope virus that uses endocytosis is Dengue virus (DEN), which uses different environmental factors to trigger virus-endosome fusion and delivers its viral genome into cytosol (Zaitseva et al., 2010). Dengue virus uses bis(monoacylglycerol)phosphate, which is an anionic lipid specific to late endosome and required as co-factor for the endosomal acidification-dependent fusion (Zaitseva et al., 2010).

Non-enveloped viruses may contain capsid proteins or "penetration proteins" that mediate membrane penetration, where it can be primed (often by proteolytic cleavage) and induce conformational change to allow membrane entry (Chandran et al, 2002). Membrane penetration of non-enveloped viruses such as, reovirus can induce membrane disruptions by ISVPs (infectious subvirion particles) (Chandran et al., 2002). ISVPs are intermediates produced by virions, which initiates disassembly (Chandran et al., 2002). It was also mentioned that virions do not induce membrane disruption, but ISVPs do. Another example of non-enveloped virus is poliovirus, Chandran et al. (2002) incubated poliovirus virions with receptor and showed that it externalized the N-myristoylated VP4 peptide, and the amphipathic VP1 sequences were exposed, which can insert into liposomes. Interestingly, Bubeck, D. et al (2005) showed the three-dimensional structure of the poliovirus-receptor-liposome complex using cryo-electron microscopy.

In addition, lipids can also act as a signal for viral replication. Hepatitis C virus (HCV) and enteroviruses (such as, poliovirus, EV71) recruit phosphatidylinositol (PI)-4 kinases and generates PI4P, which is essential for viral replication (Chukkapalli et al, 2012). PI-4 kinase alpha (PI4KA) is a cofactor for HCV RNA replication, which phosphorylates phosphatidylinositol (PI) at the 4^{th} position of inositol head group to allow recruitment of proteins containing lipid-binding motifs (Berger et al, 2011). HCV is an enveloped virus within the family *Flaviviridae*, which has structural and non-structural proteins (Reiss et al, 2011). The structural proteins are core, E1, and E2, and the non-structural proteins are required for replication such as, NS5A, which is

responsible for recruiting PI4KA by binding to it (Reiss et al, 2011). It was found that the core of HCV is palmitoylated, which is important to enhance hydrophobicity, membrane affinity, and protein trafficking (Majeau et al, 2009). The enteroviruses (such as, poliovirus) rely on phosphatidyl 4-kinase III Beta (PI4KB) for viral RNA replication. (Xiao, X. et al., 2017). Xiao, X. et al (2017) showed that the 3A protein of enterovirus 71 interacts with the ACBD3 host factor and recruits PI4KB, which produces PI4P. In this paper, the role of lipids in viral infection through viral membrane fusion and penetration will be discussed. In addition, some of the mechanisms involved in enveloped and non-enveloped viruses and their importance for developing new therapeutic drugs for certain illnesses will be examined. Lastly, the importance of lipid signalling in viral infection will be discussed.

Results:

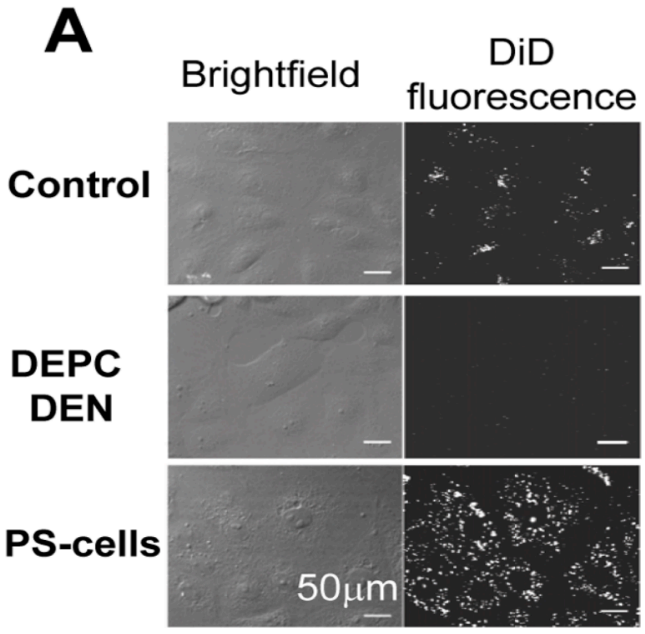

Figure 1: Anionic lipids promotes intracellular fusion and viral infection in DEN. After allowing internalization of virus for PS-treated cells (PS-cells), untreated cells (control), and untreated cells with DEPC-inactivated DEN (DEPC DEN), an increase in cell fluorescence was observed for PS-cells upon fusion of DiD-labeled DEN within the endocytic pathway of MA104 cells. Image from: Zaitseva et al. (2010).

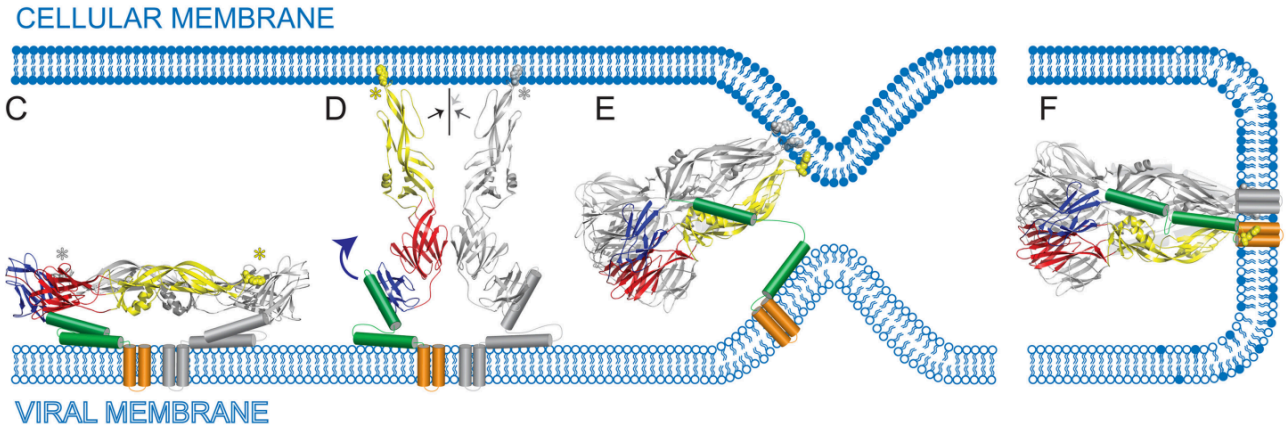

Figure 2: Schematic Diagram of Fusion Process in Dengue Virus. Top lipid bilayer shows the host cell membrane and the bottom shows the virus membrane with its anchored Glycoprotein E (E protein). **(C)** Pre-fusion state, where the dimer lies flat on the viral membrane. The fusion loops (asterisks) are buried in the dimer. Two of the amphipatic helices (green) lie against the membrane. **(D)** At low pH the dimer dissociates, and the monomers project outward. The fusion loops are shown buried in the target cell membrane. The arrows show how the angle between domain I (red) and II (yellow) changes, which allows the fusion loops of domain II (3 copies) to come together. Domain III (blue) folds back and rebinds with domain I, which stabilizes the trimer. **(E)** The stem extends along the seam between two domains II, but the distal stem and membrane anchor are not yet in contact with fusion loop. **(F)** Post-fusion, where 3 stems cross the entire length of domain II, which brings transmembrane anchor and fusion loop together. Both membranes start to merge and forms a pore. Image from: Klein et al. (2013).

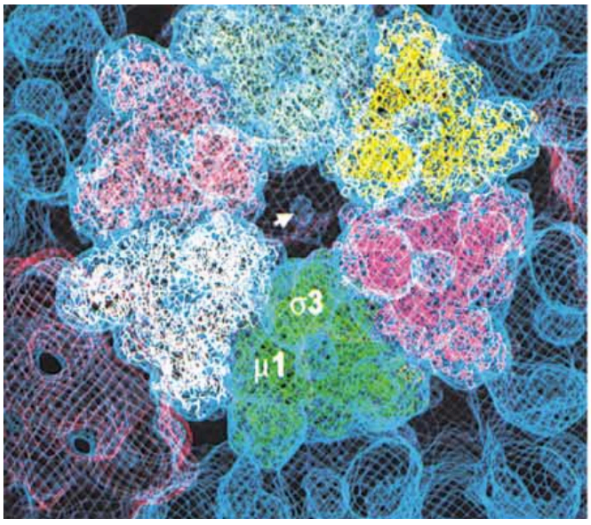

Figure 3: Reovirus membrane-penetration (μ1) complex with protector protein structure (σ3) view from outside the particle. The arrow shows an isolated blob in the center of six μ1 trimers represent the probable clustering of C-terminal arms from the six μ1 neighbouring subunits. Image from: Liemann et al. (2002).

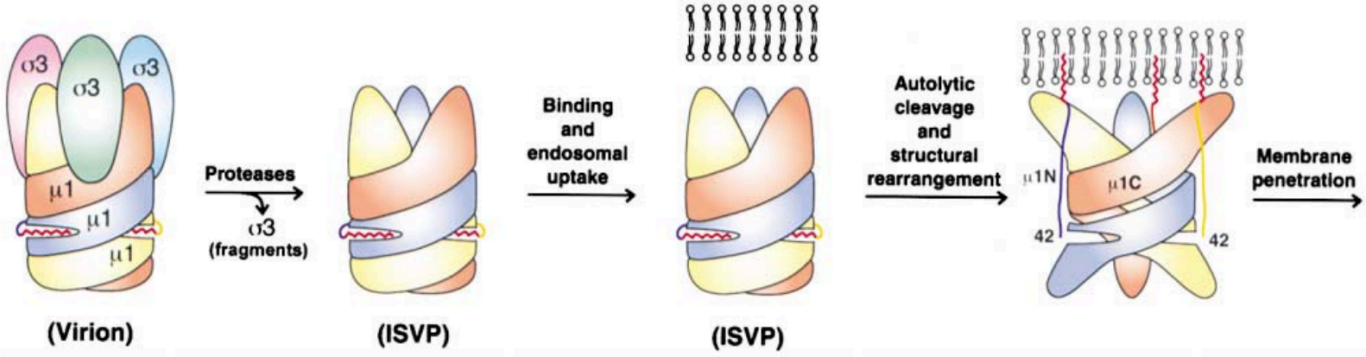

Figure 4: Virion to ISVP transformation in Reovirus. Presence of proteases remove σ3 and exposes μ1 for conformational change in endosome. It was suggested that the folding of μ1C is due to the uncoiling of the trimer; however, the actual rearrangement may be more extensive. μ1N release allows the myristoyl group to insert in the membrane. They reported that autolytic cleavage, where μ1N and μ1C separate, is not yet known. Image from: Liemann et al. (2002).

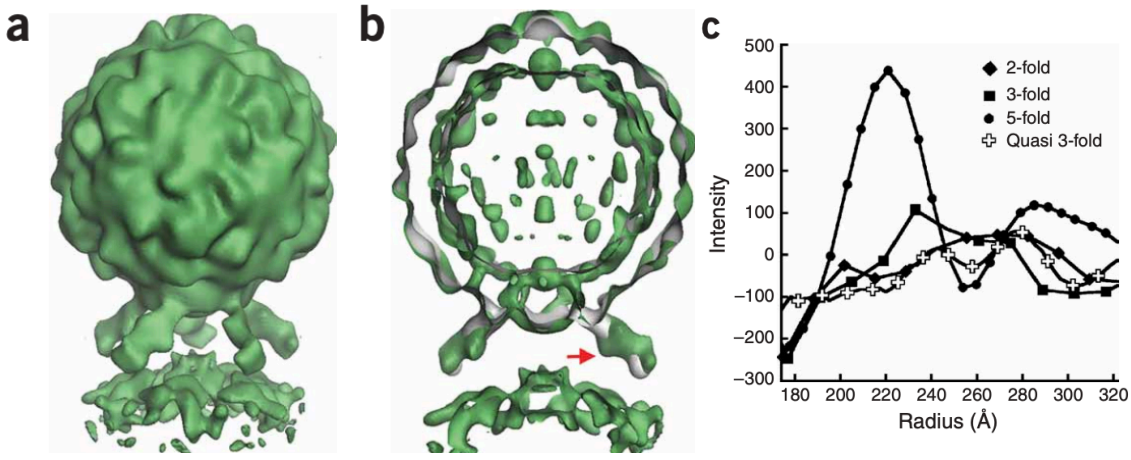

Figure 5: Poliovirus-receptor-liposome complex structure. (a) five-fold symmetric three-dimensional reconstruction of poliovirus bound to receptor-decorated liposomes. Surface rendering of complex (green) shows that the icosahedral features of the virus capsid are maintained, and density for receptors and membrane are well defined. **(b)** cut-away view shows the three-dimensional reconstruction of the complex (green) superimposes on electron density (gray), which corresponds to virus-receptor complex. The red arrow shows glycosylation site on domain II of poliovirus. **(c)** Density values sampled along various radial directions, measured from the center of virus. At 221 A, a peak value is significantly higher than other directions. Image from: Bubeck et al. (2005).

Discussion:

Enveloped viruses such as, dengue virus (DEN) enters mosquito and human cells through receptor-mediated endocytosis (Zaitseva, E. et al, 2010). DEN envelope fuses with endosomal membrane through its envelope glycoprotein E, which is structurally similar to E protein of other *flaviviruses* (Zaitseva, E. et al., 2010). Glycoprotein E binds to a receptor and undergoes conformational changes due to low pH (Stauffer, F. et al, 2007). Zaitseva, E. et al, (2010) found that anionic lipids (such as, bis(monoacylglycerol)phosphate) enriched in late endosomal membranes are required for low-pH dependent DEN fusion. DiD fluorescence and brightfield microscopy were used to show that anionic lipids promote DEN fusion and viral infection (figure 1). Zaitseva, E. (2010) used MA104 and BS-C-1 cells with pre-bound DEN labelled with DiD in a self-quenching concentration to study the fusion events during viral infection. It was found that there was no intracellular fusion when chloroquine was added to inhibit endosomal acidification or when DEN was DEPC-inactivated (Zaitseva et al, 2010). Zaitseva et al (2010) treated cells with anionic lipid (such as, PS) and found DEN fused in early endosomes that normally would not have anionic lipid. The increase in DiD fluorescence indicated that there was a high efficiency of DEN-endosome fusion (figure 1) (Zaitseva et al, 2010). Early endosomes have low pH but do not have anionic lipids, which ensures DEN fusion in late endosomes, where there are enriched anionic lipids present (Zaitseva et al, 2010). Thus, their experiment using DiD fluorescence showed that DEN fusion is dependent on anionic lipids, which are present in late endosomal membranes. Furthermore, Stauffer et al. (2007) found that the dengue virus fusion peptide (DEN Fpep) has high affinity for vesicles containing anionic lipids and has an electrostatic interaction using fluorescence quenching experiments. Interestingly, crystal structures of E protein showed the merging of viral and target cell membranes (figure 2). Klein et al. (2013) showed the arrangement of the three folded domains in dimeric prefusion conformation (Figure 2C) and low-pH induced post-fusion trimer (figure 2F). Stauffer et al. (2007) reported that conversion of dimers to trimers is a two-step process: (1) ectodomains dissociate reversibly, which is important to make the tip of domain-II (putative fusion peptide) accessible to the target membrane, and (2) Irreversible trimerization. The 'fusion loops' located at the tip of trimers allows them to insert into the host cell membrane, so that viral nucleocapsid is released into the host cytoplasm (Stauffer et al, 2007). Fusion loops are hydrophobic, which is buried at the dimer interface and cluster into a large hydrophobic surface at the end of post-fusion trimer (figure 2F) (Klein et al, 2013). Along with the clustered fusion loops, the N-terminal (proximal) stem stabilizes the trimer, while the central part has few interactions with the folded domains in E trimer (Klein et al, 2013). Also, it was suggested that E trimers induce curvature of the membrane, which helps promote membrane fusion (Modis et al, 2004).

Cell entry of non-enveloped viruses requires membrane penetration without membrane fusion (Liemann et al, 2002). Non-enveloped viruses usually have capsids, which helps them

penetrate the target membranes (Chandran et al, 2002). For example, non-enveloped reoviruses are activated by proteolytic cleavages (Liemann et al, 2002), where σ3 is removed from a virion and primes the particle for membrane penetration by exposing μ1 to interact with target membrane (Chandran et al, 2002). When virions are exposed to proteases, "infectious subviral particles" (ISVPs) forms, which lacks a protector protein (σ3) (see figure 4) (Liemann et al, 2002). The μ1 undergoes an autolytic cleavage into a small N-terminal fragments (μ1N) and large C-terminal fragments (μ1C), which depends on σ3 on the N-terminal, μ1 myristoyl group, μ1 residues (Asn-Pro) at the cleavage site (Liemann et al, 2002). Once the μ1N gets released after autolytic cleavage, the myristoyl group gets inserted into the target membrane; then, membrane penetration occurs (figure 4) (Liemann et al, 2002). To prove the release of myristoylated μ1N in ISVPs, the structure of the membrane penetration protein (μ1) in complex with protein protector (σ3) in reovirus was revealed using a 2.8 -Å- resolution X-ray crystallography fitted onto the cryo-EM image of intact virion (figure 3) (Liemann et al, 2002; Tsai, B., 2007). In addition, the structure of non-enveloped poliovirus-receptor-liposome complex was reconstructed using cryo-electron microscopy at 32 Å resolution (figure 5) (Bubeck et al, 2005). It was demonstrated that the five receptor molecules bind to the virus and orient it with a five-fold axis toward the membrane (Bubeck et al, 2005). The viral five-fold axis is involved in externalized polypeptide interaction with membranes, and a probable RNA translocation site (Bubeck et al, 2005). It was also stated that the density level for the receptors strongly suggest that all the five receptor sites are fully occupied (figure 5C) (Bubeck et al, 2005). To prove the validity of methods used in this experiment, the density quality for the virus and receptor, the icosahedral appearance features in the capsid, and the consistency of density for the receptor were compared with previously reported reconstruction of soluble binary complex (Bubeck et al, 2005). Furthermore, the binding of virus to receptors create bilayer perturbations, which may function as membrane penetration and cell entry (Bubeck et al, 2005). Bubeck et al. (2005) proposed 3 explanations for these perturbations in the bilayer: (1) The virus-receptor complex formation could cause the outer leaflet of a bilayer to be distorted. (2) The formation of the virus-receptor complex can stimulate selective partitioning of lipids. In their experiment, they used the electron-dense Ni^{2+}-chelating NTA lipids to anchor the receptor. Bubeck et al. (2005) stated that either (1) or (2) may prime the membrane for penetration of viral peptides. (3) Receptor binding at room temperature facilitates "breathing", which was observed for free virus at 37 ^{o}C resulting in a brief global or local exposure of viral peptides; thereby, receptors associate with the membrane. Bubeck et al. (2005) concluded that NTA-liposome interaction with His-tagged receptor combined with new structural methods done in their experiment could be applied to study other virus-receptor complexes.

We have discussed how non-enveloped and enveloped viruses can penetrate or fuse to their target membranes. Next, we will discuss how viruses can initiate lipid signalling. HCV and enteroviruses (such as, poliovirus) infections can stimulate the release of PI4P by recruiting PI4-

kinases. HCV activates PI4KA (PI4-kinase alpha), while enteroviruses activate PI4KB (PI4-kinase beta). The PI4KA localizes in the endoplasmic reticulum, whereas the PI4KB localizes at the Golgi compartment (Reiss et al, 2011). Both of the kinases produce PI4P, but they have different subcellular localizations and regulation (Reiss et al, 2011). Reiss et al (2011) investigated the PI4P levels in hepatocytes of HCV patients using immunohistochemistry and found that staining for NS5A, core, and PI4P showed that there was always an increased in PI4P levels in HCV-positive areas, which matched their in vitro data indicating that HCV infection results in elevated PI4P levels. Also, HCV infection can change the metabolism of PI4P, which activates catalytic activity of PI4KA and reduce PI4P distribution between different membrane compartments leading to decreased PI4P pool in plasma membrane (Bianco et al, 2012). Furthermore, NS5A expression leads to stimulation and altered staining pattern of PI4P, which is comparable to the observed pattern seen in infected cells suggesting that NS5A is required for recruitment of PI4KA and stimulation of PI4P (Reiss et al, 2011). To show the role of NS5A in recruitment of PI4KA, Reiss et al, (2011) co-expressed individual HCV proteins with HA-tagged PI4KA in Huh7-Lunet/T7 cells and coimmunoprecipitation experiments were done with monospecific antisera. It was suggested that the recruitment of PI4KA to the HCV membrane through NS5A prevents interaction of PI4KA with its cellular protein partners; thereby, decreasing PI4P concentration in plasma membrane (Bianco et al, 2012). In another study, it was found that the beta isoform of PI4K is not targeted by HCV to increase PI4P production (Berger et al, 2011). However, in another study, it showed that enteroviruses rely on phosphatidylinositol 4-kinase IIIβ (PI4KB) for RNA replication (Xiao et al, 2017). The researchers found that 3A protein of enterovirus 71 (EV71), a non-enveloped virus, requires the host factor, ACBD3, for recruitment of PI4KB to RNA replication sites (Xiao et al, 2017). This is analogous to HCV's recruitment of PI4KA, where it requires the NS5A (non-structural protein of HCV). It was also found that EV71 infection in cells lead to increased PI4KB and PI4P in the replication sites (Xiao et al, 2017).

PI4P is involved in signal transduction and vesicle transport to the plasma membrane (Xiao et al, 2017). So, what is the importance of PI4P in plasma membrane? PI4P in plasma membrane can support ion channel functions and can contribute to the anchoring of lipid-modified proteins containing polybasic domains (Balla, 2013). There are seven phosphoinositides (PI) named according to their position on inositol ring where phosphorylation occurs generated by a set of kinases residing in different subcellular compartments (Reiss et al, 2011). These kinases residing in different subcellular compartments give a molecular signature to a membrane where a PI is located (Reiss et al, 2011). However, it is important to note that while PIs can be a characteristic of specific intracellular membranes, it might be misleading to assign their functions to those membranes because these PIs may cycle between various compartments (Balla, 2013). As shown earlier with HCV and enteroviruses, PI4KA and PI4KB stimulate PI4P production in both the ER or Golgi compartment. The

difference is that PI4KA localizes in ER, while PI4KB localizes in Golgi compartment. We have seen that in HCV and enteroviruses infection an increase of PI4P pools were found in infected cells. Balla (2013) pointed out that the use of antibody tools can be misleading when making conclusion about the lack of lipids in internal compartments and its presence. It was mentioned that it is possible that one can detect a larger phosphorylated “precursor” pool, and dephosphorylated with short-lived intermediate, where the short-lived lipid is the important regulator (Balla, 2013). Thus, one should be careful of making strong conclusions just from inositol lipids location or the lack of it, and also one should consider the enzymes localization both for the production and elimination (ie. Kinases and phosphatases) (Balla, 2013).

By knowing how non-enveloped and enveloped viruses interact and affect lipid metabolism in host cell membrane, we could try to design drugs that specifically target these host cell membrane-virus interactions. It was reported that the DHA-derived protectin D1 isomer obstructs enveloped influenza virus replication in vitro by inhibiting the nuclear export of viral mRNA (Watanabe and Kawaoka, 2015). Interestingly, the IV administration of protectin D1 protected mice from influenza virus, which suggest that it has potential therapeutic treatment and prevention of influenza (Watanabe and Kawaoka, 2015). As shown earlier, enveloped viruses use membrane fusion to enter host cell membrane. St. Vincent et al. (2010) exploited the mechanism of viral fusion with the host cell membrane and demonstrated that by using RAFIs (Rigid Amphipathic Fusion Inhibitors), a synthetic rigid amphiphiles with similar shape as phospholipids, can inhibit infectivity of several enveloped viruses by targeting virion envelope lipids, such as HCV and HSV 1 and 2 with no cytotoxic or cytostatic effects. RAFIs inhibit the increased negative curvature required in the initial stages of viral fusion (St. Vincent et al, 2010).

In conclusion, understanding the mechanisms on how enveloped and non-enveloped viruses interact with the host cell membrane, and the role of lipid metabolism in viral infection could advance drug design and develop novel vaccines against certain viruses that may cause diseases in organisms.

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