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Strategies for Antiviral Screening Targeting Early Steps of Virus Infection*

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Abstract: Viral infection begins with the entry of the virus into the host target cell and initiates replication. For this reason, the virus entry machinery is an excellent target for antiviral therapeutics. In general, a virus life cycle includes several major steps: cell-surface attachment, entry, replication, assembly, and egress, while some viruses involve another stage called latency. The early steps of the virus life cycle include virus attachment, receptor binding, and entry. These steps involve the initial interactions between a virus and the host cell and thus are major determinants of the tropism of the virus infection, the nature of the virus replication, and the diseases resulting from the infection. Owing to the pathological importance of these early steps in the progress of viral infectious diseases, the development of inhibitors against these steps has been the focus of the pharmaceutical industry. In this review, Herpes Simplex Virus (HSV), Hepatitis C Virus (HCV), and Human Enterovirus 71 (EV71) were used as representatives of enveloped DNA, enveloped RNA, and non-enveloped viruses, respectively. The current mechanistic understanding of their attachment and entry, and the strategies for antagonist screenings are summarized herein.

Key words: Virus Infection; Antiviral therapeutics; Virus life cycle; Inhibitor screening

GENERAL THEME OF VIRUS ENTRY

Enveloped viruses enter cells by fusing with plasma or endocytic membranes (Fig 1.) [37]. Membrane fusion is a complex process initiated by specific interactions between host-cell-surface receptors and viral envelope glycoproteins[12, 23]. Virus entry consists of three basic steps: recognition of cellular receptors by a viral glycoprotein, triggering of fusion, and fusion execution. These steps are carried out and regulated by viral glycoproteins in concert with their cognate receptors.

The surface of non-enveloped viruses is covered with viral capsid proteins [52]. The capsid proteins of EV71 and picornaviruses in general are encoded by the P1 region of the genome. The capsid particles comprise of 60 copies of four P1-encoded poly-peptides (VP1-VP4). The first three viral proteins

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(VP1-VP3) reside on the outer surface of the virus, while the shorter VP4 is located on the inner surface of the capsids [63, 76]. The capsid proteins mediate the initiation of infection by binding to a receptor on the host membrane [54, 81, 82]. Although non-enveloped viruses do not require membrane fusion for entry into cells, a membrane-binding motif is still present in capsid surface proteins [15, 24], which is responsible for activating the host cell membrane to allow the entry of the capsid.

The current understanding on the key viral and cellular components involved in the early steps of HSV, HCV, and EV71 infections is summarized in Table 1.

HSV Entry

HSV infection is one of the most common communicable diseases in humans, causing recurrent cold sores, keratoconjunctivitis, genital herpes, and even life-threatening herpes encephalitis [67]. HSV undergoes primary replication following entry into a host

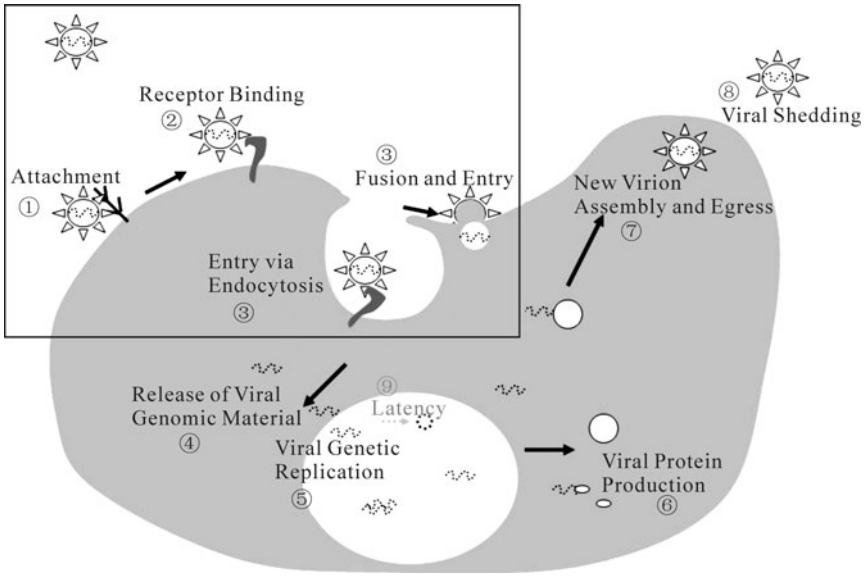

Fig. 1. Life-cycle of a generalized virus infection. Early steps of virus infection include attachment, receptor binding and entry (via fusion with cytoplasmic membrane directly, or via endocytosis).

Table 1. Current molecules involved in the early steps of HSV, HCV and EV71 infection

	Attachment		Receptor Binding		Entry
	Viral Component	Cellular Component	Viral Component	Cellular Component	Viral Component
HSV	gC, gB	HS	gD, gB	Nectin1, Nectin2, HVEM, 3O-S PILRalpha	gB, gH/gL
HCV	E1, E2	HS, LDL	E2	CD81, SRBI Claudin-1, Occludin	E2
EV71	VP1	SA	VP1	SCARB2; PSGL-1	VP1, VP4 (?)

HS: Heparin Sulfate. SA: Sialic Acid. SCARB2: Scavenger receptor class B member 2. PSGL-1: P-selectin glycoprotein ligand-1.

cell through the skin or mucosae. The virus then gains access to the distal axon terminals of the sensory neurons and ganglia, where latent infection occurs[2]. The initial replication phase, when the IE genes of the HSV are expressed in the absence of viral protein synthesis, is critical. IE proteins are required for subsequent viral protein expression and successful completion of the virus life cycle [44]. The current clinical management of HSV-related diseases uses chemotherapeutic agents mainly targeting viral DNA polymerase, such as acyclovir (ACV) [10, 21, 26, 50, 65], which are limited by the emergence of drug-resistant viruses or their side effects [18, 48]. An estimated five percent of the isolates from immunocompromised patients with HSV lesions have evidence of resistance [10, 21, 26, 50, 65].

HSV is considered as the paradigm of herpes viruses with respect to virus entry into the cell. The HSV entry process is by far the most complicated because it requires a number of viral glycoproteins functioning in concert to complete the virus life cycle [17, 33, 64, 68]. An HSV first attaches itself to a cell membrane by interacting with gC and gB to heparan sulphate (HS) non-specifically. Since the viruses recovered at this point remain infective, dramatic conformational changes resulting from such interactions is unlikely [8, 39, 55, 62, 73]. The second step of HSV entry requires binding between the gD and HSV entry receptors. Several HSV receptors have been identified, including nectin 1 and 2, herpes virus entry mediator (HVEM), as well as specific o-sulphates (3O-S) moieties in heparin sulfate (HS), which is a glycosaminoglycan [27, 33, 68]. The alternative usage and global expression of these receptors probably account for the entry of HSV into a wide range of cell types. The third step of HSV entry is the fusion of the virion envelope with the plasma membrane of the target cell. gB and gH-gL are required and constitute the conserved fusion machinery across the herpes virus family [14, 17, 27, 33, 62, 64, 68]. Although its structure is still unknown, molecular and biochemical analysis of gH suggests a class I fusion protein. On the other hand, the crystal structure of gB exhibits a remarkable similarity to that of vesicula stomatitis G protein and to viral fusion glycoprotein in general [3, 32, 79]. The sequential interactions of gD, gB, and gH-gL were recently demonstrated [3]. The results of this study clearly showed that gD-receptor interaction induced conformational changes of gB, which in turn promoted gB-(gH-gL) interaction, followed by membrane fusion [3]. The manner by which gB and gH-gL cooperate to obtain fusion and the reason for the requirement of two fusion executors and not one are required in the herpes virus family remains unclear. The fact that entry by fusion at the plasma membrane, fusion in the endocytic vesicle, or cell-cell fusion between infected and uninfected cells requires all four glycoproteins (gD, gB, gH, and gL) is interesting. However, mechanism differences and the requirement of different cellular components of these three membrane fusion processes are yet to be clarified.

HCV Entry

The World Health Organization (WHO) estimated that at least 170 million people are currently infected with HCV, with three to four million new cases each year [40]. This number is predicted to continue to increase. At the present, however, there is still no prophylactic vaccine against HCV, and the only approved treatments for infection are pegylated interferon and ribavirin. This clearly illustrates the need

for new antiviral therapies.

HCV enters cells through clathrin-mediated endocytosis [6, 34]. Productive infection requires a low pH compartment and depends on the presence of cholesterol in the target cell membrane [13]. Initial association of HCV with the host cell surface involves nonspecific attachment to the subdomains of the cell surface [22]; one of the identified attachment factors is HS [5, 30, 51, 83]. HCV infection is inhibited in the presence of heparin, as well as by treatment of cells with heparinase I or III prior to infection[30]. However, whether HS associates with HCV particles or lipoproteins (e.g., low-density lipoproteins [LDL] and very low-density lipoproteins [VLDL]) commonly found in association with the virus is unclear [34]. The LDL receptor is implicated as an attachment factor, and adsorption of HCV particles can be inhibited by antibodies specifically for either purified VLDL or its receptor [1, 84]. C-type lectins, including DC-SIGN and LSIGN, are also implicated in HCV attachment [25, 46, 47, 75]. However, the role of each of these attachment factors in HCV entry remains unclear because they have not been shown to be required for productive infection. Moreover, the role for DC/SIGN and L/SIGN in productive entry is uncertain because they are not expressed on hepatocytes.

HCV E1 and E2 glycoproteins are responsible for interaction with cellular receptors. An increasing number of cellular factors have been implicated in HCV entry, including CD81, SR-BI, CLDN1, and highly sulfated glycosoaminoglycans (GAGs) [22, 34, 84]. Since certain cell lines remain non-permissive for HCV entry despite the expression of all of these molecules, this finding suggests that either there are still unidentified additional entry factor(s), or there are special mechanisms missing downstream from the receptor level of the cell lines [13, 61].

CD81 has four transmembrane domains as well as a small and large extracellular loop (LEL). LEL interacts with E2[85, 86] while SR-BI also binds to HCV E2[20]. However, contrary to CD81, SR-BI was highly expressed on hepatocytes to selectively uptake cholesterol and cholesterol esters from high-density lipoprotein (HDL) particles, thus enhancing HCV infectivity [66]. HCV infection is inhibited by CD81 and SR-BI antibodies[85, 86], specific RNAi, and protein fragments[78]. The ligand of SR-BI, oxidized LDL, also inhibited HCV entry. CLDN1 is a recently identified HCV entry receptor. It is a tight junction tetraspan membrane protein highly expressed in the liver. HCV entry requires residues within extracellular loop 1. HCVpp infection of 293T cells expressing CLDN1 remains CD81 dependent, further supporting the model that no one factor is sufficient for HCV entry [60].

EV71 Entry

Enterovirus 71 (EV71) is a small non-enveloped virus which has a icosahedral capsid that encloses a single-stranded (+)RNA genome. Its infection causes hand, foot, and mouth disease (HFMD) and herpangina [45, 76]. It is also associated with severe neurological diseases, such as brain stem encephalitis and poliomyelitis-like paralysis, mainly in infants and young children [56]. Although the mechanistic understanding of EV71 entry is still very limited, its cell entry involves virus surface attachment, receptor binding, uptake through endocytic pathways followed by release of the genome from the capsid, and delivery of the genome across the endosome membrane into the cytoplasm [54, 81]. There is evidence

supporting the notion that cell surface sialic acid is important for initial viral attachment [82]. The entry of EV71 is similar to that of enveloped viruses. It is initiated from the binding between the virus and the receptor. At physiological temperatures, the formation of the initial complex induces conformational changes in the virus to form a tight-binding complex. Subsequent structural changes in the virus cause the viral capsid surface VP1 to move away from the five-fold axes, resulting in more extensive contacts with the receptor.

Two receptors of EV71 have recently been identified. These are the scavenger receptor class B member 2 (SCARB2), a membrane protein previously implicated in the endocytosis of high-density lipoprotein and the internalization of pathogenic bacteria, which is a functional receptor for EV71[81]. Human P-selectin glycoprotein ligand-1 (PSGL-1), a mucin-like protein involved in the tethering and rolling of leukocytes on vascular endothelium, is used by several EV71 isolates to infect lymphocyte cell lines [54]. When expressed in mouse cells, PSGL-1 and SCARB2 display sufficiency in terms of virus attachment and entry [54, 81]. Several EV71 isolates infect lymphocytes independent of PSGL-1, while SCARB2-specific antibodies could not block infection completely on some cell lines, suggesting that additional receptor(s) have yet to be identified. Furthermore, since EV71-receptor interactions do not cause viral instability or uncoating, these interactions probably result in the aggregation of other receptors or act as a trigger to subsequent endocytosis [58].

Despite the major differences between enveloped and non-enveloped viruses, common themes have emerged in the membrane penetration processes of non-enveloped viruses, including the presence of small, membrane active peptides in one or more of the capsid proteins [4, 11]. These amphipathic or hydrophobic regions, while analogous to the enveloped virus fusion peptides, cause membrane disruption rather than promote membrane fusion. While host cell entry of non-enveloped viruses remains a diverse and largely unresolved area, current data indicate that the membrane active peptides of the non-enveloped virus are comparable to those of fusion peptides in enveloped viruses [4, 11, 15, 24].

STRATEGIES FOR ENTRY INHIBITOR SCREENING

In the past years, assays specifically designed for the screening of inhibitors against the early steps of virus infection have been developed. These assays are based on the understanding of virus entry mechanisms, some of which are capable of robotic high-throughput-screening (HTS). With a more detailed understanding of virus entry mechanisms, more advanced assays will certainly be developed in the near future.

Recombinant Reporter Virus System

For HSV, a recombinant HSV-1, HSV-1/Blue, which contains an HSV-1 ICP4 promoter directing a lacZ gene inserted into the HSV-1 TK gene, was generated (Fig. 2) [19]. The ICP4 promoter is an HSV immediate-early promoter and could drive efficient LacZ expression within five hours after HSV-1/Blue infection [29]. Therefore, the inhibition activities displayed are most likely due to the inhibition of the early steps of virus infection. In addition to HSV-1/Blue, there are a number of different versions of recombinant HSVs generated [70].

For HCV, reporter viruses have become available

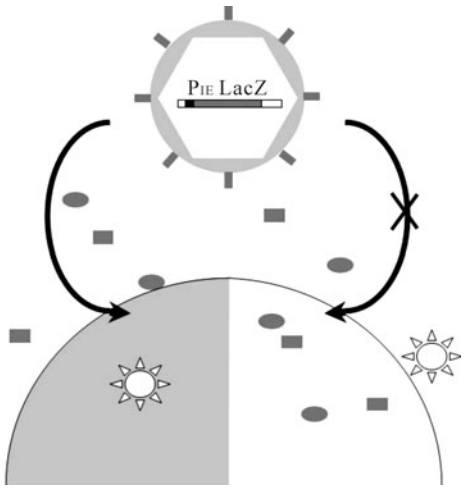

Fig. 2. Recombinant reporter virus system. A recombinant reporter virus with a reporter protein (such as LacZ, or EGFP, or Luc, or SEAP) under a viral immediately promoter is used in this system. The level of virus entry is measured as the level of reporter protein expression.

only recently when the JFH-1-based HCV infectious clone was constructed. Recombinant HCV-carrying reporter genes in their genomes, typically encoding luciferase or green fluorescent protein (GFP), are used for the measurement of HCV RNA replication. These reporter viruses were either bicistronic or moncistronic, as illustrated in Fig. 3. A chimeric bicistronic JFH1 virus that carries the luciferase reporter gene in the viral cDNA sequence was developed to characterize the early steps of HCV entry [38, 59]. Analogous chimeric monocistronic reporter virus systems were used to demonstrate time- and temperature-dependent activations of HCV for low-pH-triggered entry[74], as well as mutations in the HSV NS proteins important for efficient HCV replication[31].

For EV71, reporter viruses were constructed using viral cDNA clones. However, there have been no other reports on its application in entry inhibitor screenings [81].

There are several limitations associated with the use of screening systems with recombinant reporter viruses. For example, recombinant reporter HCV viruses are often attenuated and encounter difficulties in their passage. In addition, the activities against the primary viral isolates of the candidate inhibitors cannot be elucidated using these recombinant viruses.

Virus Infection Reporter Cell System

Another reporter system, which is complementary to the recombinant reporter virus system, is the reporter cell system for virus infection. This system either expresses a reporter protein (GFP or luciferase) under a virus infection-activated promoter or a virus infection-activated reporter protein.

For HSV, several reporter cells have been reported, including Vero-ICP10-SEAP [77] and BHKICP6LacZ-5[69]. Our laboratory recently generated an HSV reporter cell line stably transfected with an HSV-2 ICP10 promoter-directed luciferase (Luc) expression cassette (Fig. 4). The ICP10 promoter is generally defined as an early promoter, but it can also be regulated as an immediate-early promoter [80]. This

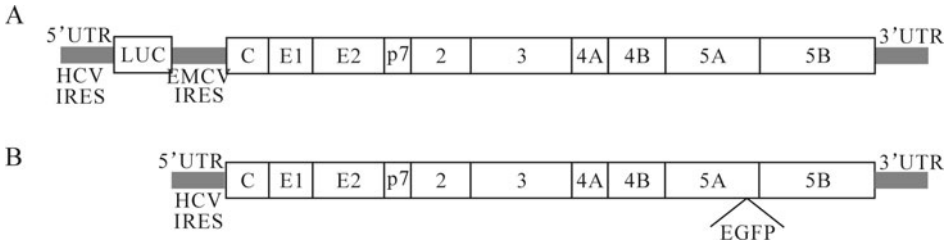

Fig. 3. HCV reporter viruses. A: A bicistronic reporter construct. B:A monocistronic reporter construct.

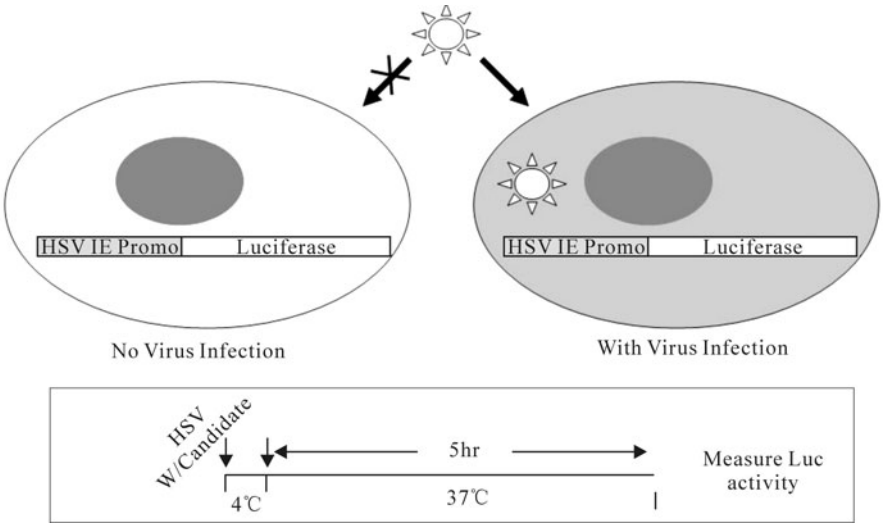

Fig. 4. Virus entry assay based on reporter cells. A cell line with a reporter protein (such as LacZ, or EGFP, or Luc, or SEAP) under a viral immediately promoter is used in this system. The level of virus entry is measured as the level of reporter protein expression 5 hours post infection.

cell line could efficiently and rapidly report HSV infection.

Many versions of reporter cell systems have been developed for HCV. Most of these systems take advantage of viral protease activity. The NS3/4A protease of different HCV genotypes recognizes the conserved NS4A/4B sequence DEMEEC-S/AXXX. Lee *et al*. constructed a fusion protein composed of EGFP-SEAP linked by an octapeptide spacer, and the HCV NS4A/4B cleavage site [42, 43]. This fusion protein could act as a substrate for the NS3/4A serine protease. The role of EGFP is to retain the entire fusion protein within the cell. Upon HCV infection, NS3/4A separates SEAP from EGFP-SEAP, which is further secreted into the extracellular culture medium. Recently, Iro *et al*. took this construct and generated a stable Huh7 cell line (Fig. 5) [36]. The reporter cell line enabled rapid and sensitive quantification of HCV infection and quantifies virus entry efficiency.

For EV71, similar to that for HCV, the current reporter cell systems are also built upon viral protease activity. EV71 contains $2A^{pro}$ and $3C^{pro}$ two proteases. Using fluorescence resonance emission transfer (FRET) technology, an assay measuring EV71 $2A^{pro}$ was designed[28]. In this system, GFP and RFP were

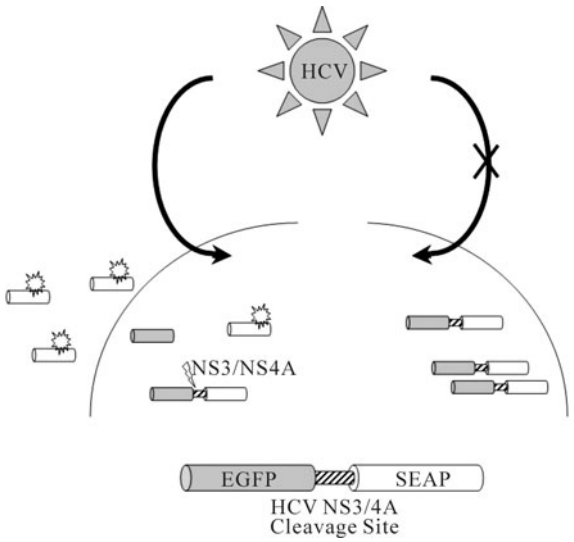

Fig. 5. Reporter cell based HCV infection assay. A EGFP-SEAP fusion protein linked with a HCV NS3/4A protease cleavage sequence is expressed in the reporter cell. Upon HCV infection and viral protein expression, EGFP and SEAP is separated via NS3/4A cleavage. SEAP is secreted to the culture media. The level of HCV infection is measured as the level of SEAP activity.

connected with the $2A^{pro}$ cleavage motif as a FRET pair. Upon protease cleavage in the context of virus infection, the separation of the tandem fluorophore substrate was monitored as a FRET disruption in real time by fluorescent microscopy and in a quantitative fashion by fluorometry. This type of system should also be adaptable to $3C^{pro}$.

In addition to its use as a cell-based entry inhibitor screening system, the reporter virus and reporter cell system could also be utilized to determine the detailed working mechanisms of the candidate compounds. Using the HSV reporter systems as an example and as illustrated in Fig. 6, when the virus is allowed to mix with the cells for attachment before a candidate agent is added (Fig. 6A), the assay helps to determine whether attachment is required. If an antiviral effect is observed only in this case, a post-attachment target for the candidate agent is indicated. Similarly, when the candidate agent is mixed with cells before testing virus is added (Fig. 6B), the assay helps illustrate if the agent functions before virus attachment to the cells. If the agent functions, this means there is a cellular pre-attachment target. On the other hand, when the candidate agent is mixed with the testing virus before its addition to cells for infection (Fig. 6C), the assay shows whether the exposure of the virus to the agent inactivates the virus before infection. If the virus is inactivated, the candidate agent obviously has a viral pre-attachment target. Similarly, when the Vero cells in Fig. 6 are replaced with Vero-ICP10-Luc, HSV-1/Blue could be replaced with any laboratory (or primary isolated) strains of HSV, while the inhibition efficacy could be quantified with the measurement of luciferase activity.

Pseudotyped Virus System

Pseudotyped viruses offer unique advantages for the screening of virus entry inhibitors. In addition to their safety and ease of experimental manipulation, they represent the surrogate approach for viruses that are difficult to culture, such as HCV.

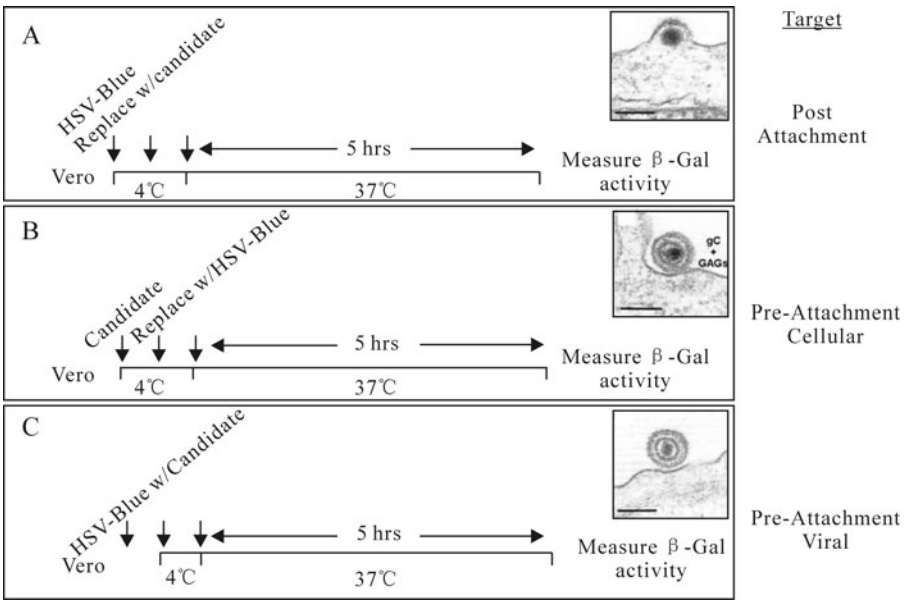

Fig. 6. Determination of potential mechanisms of HSV entry inhibitors. Vero cell monolayer in 96-well plates are treated with HSV-1/Blue and a candidate entry inhibitor as shown in panels A, B, and C. If the maximum antiviral effect appears in A, the candidate agent has a post-attachment target, if in B, a cellular pre-attachment target, and if in C, a viral pre-attachment target.

The retrovirus-based pseudovirus system is most commonly used for HCV. Pseudotyped viral particle (HCVpp) is considered as the most biologically relevant reporter system for the study of HCV attachment, receptor binding, and entry [9, 41, 57]. HCVpp consists of HCV enveloping the glycoproteins assembled onto the retroviral core particles carrying a reporter gene, such as luciferase or green fluorescence protein (Fig. 7). Numerous studies have indicated that HCVpp closely mimics the entry and serological properties of native HCV infection, such as the tropism for primary human hepatocytes and hepatocyte cell lines, pH dependence of the infection process, and neutralization by patient sera as well as monoclonal antibodies (mAbs) specific for E2[35]. The involvement of currently discovered receptors in HCV entry was also confirmed in this surrogate system [16, 53]. The use of HCVpp harboring a luciferase reporter permits easy detection of productive viral entry through very sensitive luciferase assay.

In addition to the retrovirus-based pseudotyping system, the vesicular stomatitis virus (VSV) has also been used to generate the HCV-enveloped pseudotyped virus. In this system, a VSV glycoprotein gene deleted virus (VSV-△G) is used to infect an HCV E1E2 expressing cell to generate the pseudotyped virus [7, 71]. This virus has been shown to be capable of infecting primary human hepatocytes.

Virus Free Cell-Cell Fusion System

Some enveloped viruses, such as HSV and HIV, express glycoproteins onto the plasma membrane of infected cells, which can in turn induce cell-cell fusion using similar mechanisms as in the virus-cell fusion process[49, 72]. The cellular expression of these viral enveloped glycoproteins has allowed for the measurement of membrane fusion events using cell-cell fusion or syncytia formation. This system is a powerful tool that helps the important attachments and fusion proteins and that identify entry inhibitors. The method has been enhanced by the addition of a reporter-gene system to the cell-cell fusion assay (Fig. 8). This improvement provided a high-throughput and quantitative aspect to the assay, which can serve as a surrogate for virus entry and is therefore ideally suited for the screening of viral membrane fusion inhibitors.

CONCLUSION

In addition to the high mutation rate of the viral genome, cellular toxicities and the evasion of host defense systems are the key challenges caused by virus infection and replication. They also highlight the importance of novel inhibitors in antiviral therapy. Regardless of whether viruses are enveloped or non-enveloped, the inhibitors targeting the early stages of the virus life cycle interfere with the establishment of efficient infection and prevent the initiation of replication.

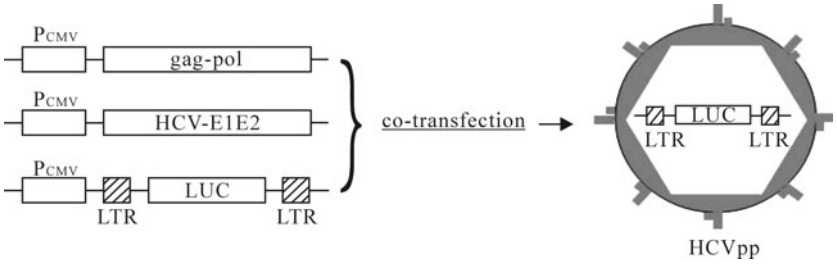

Fig. 7. Retrovirus pseudotyped with HCV E1E2.

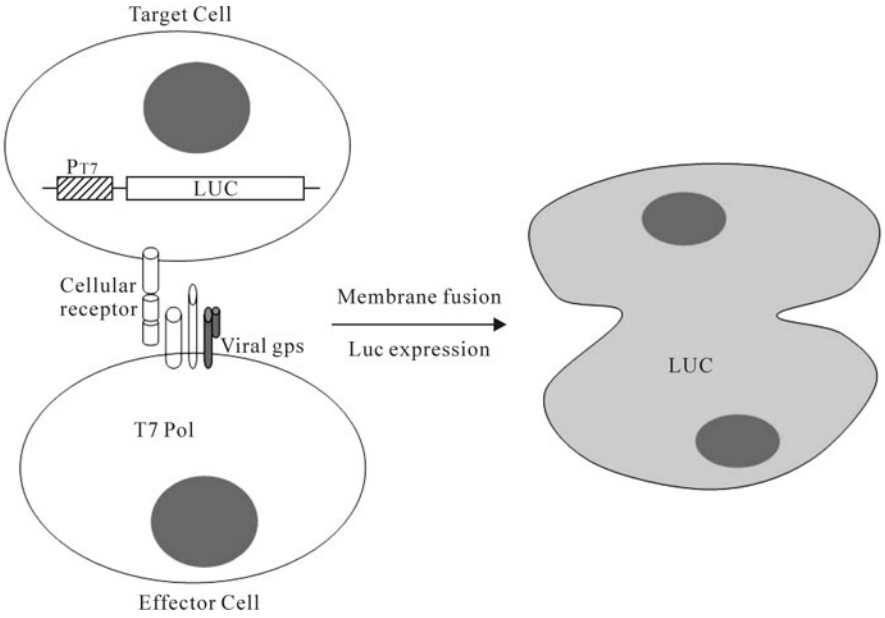

Fig. 8. Virus free fusion assay. Target cell: expresses virus receptor protein on its cellular membrane, and a reporter protein under T7 promoter. Effector cell: expresses viral entry glycoproteins and T7 polymerase. When target cell is cocultured with effector cell, membrane fusion is induced. T7 polymerase in effector cell will express the reporter protein expression. Level of membrane fusion is measured as the level of reporter protein expression.

Experiences from several well-studied viruses demonstrate that the availability of assay systems allowing specific quantification of virus attachment and fusion is paramount to the development of such inhibitors.

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