Antiviral drug discovery by targeting the envelope glycoproteins of influenza A virus

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ABSTRACT

Influenza A viruses are enveloped RNA viruses and composed of eight single-stranded, negative-sensed RNA segments. Among eight single strand components of influenza A virion hemagglutinin and neuraminidase, two surface glycoproteins, are interesting targets for antiviral drug discovery. Scientists are trying to discover newer antiviral compounds for the prevention of future pandemic and resistant strains of influenza A viruses targeting the surface glycoproteins. These viruses cause respiratory diseases in humans and animals with a high morbidity and mortality. In this review, various methods are described for screening of antiviral compounds of influenza A viruses by targeting the surface glycoproteins hemagglutinin and neuraminidase.

Key words: Antiviral, hemagglutinin, neuraminidase, fusion, hemagglutination, sialidase

INTRODUCTION

Influenza virus infection, commonly known as the flu, is an infectious disease of birds and mammals caused by RNA viruses. The most common symptoms are chills, fever, sore throat, muscle pain, headache (often severe), coughing, weakness, fatigue and general discomfort. The influenza virus causes yearly epidemics of respiratory infections in millions of human beings and the threat of a pandemic caused by new strains of this virus stimulates investigation of its pathogenic properties.[1]

The influenza virus belongs to the orthomyxoviridae family of enveloped viruses.[2] Influenza virions are made up of a ribonucleoprotein (RNP) core composed of eight RNA-protein complexes[3] associated with viral polymerase.[4] The virions consists of a lipid envelope containing two types of spike glycoproteins, hemagglutinin (HA)[5,6] and neuraminidase (NA).[7]

HA and NA are important for viral entry into the host cell and progeny virion release from the host cell. The matrix protein M1 forms a layer associated with the inside of the membrane and connects RNPs to the envelope.[8,9] The infection cycle of influenza A viruses is shown in Fig. 2. Influenza A viruses bind with the cell surface receptors of the host cell through its HA and enter into the cytoplasm through endocytosis process. Then viruses undergo low pH induced fusion process and uncoat into the nucleus. There viruses perform RNA replication. The progeny viral particles reassembled in ribosomes and golgi bodies and form a bud in the host cell membrane.

By action of NA protein sialidase enzymes cleave the bud and progeny viruses are released into the environment.

Figure 1: Infection cycle of influenza A virus
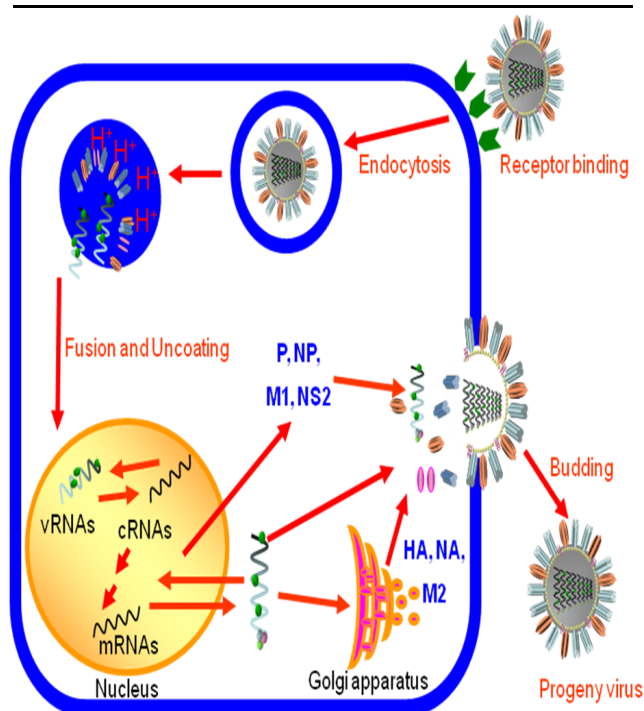
Vaccinations can be used as prophylactics for controlling influenza infections. Besides, other prophylactic and therapeutic antiviral drugs are needed as well especially during an outbreak and for people with weaker immune systems, such as children, elderly, or individuals undergoing other medical treatments.[10,11] Due to the effect of antigenic drift and antigenic shift, influenza virus readily develops resistance to conventional therapeutic agents. For example, high percentages of circulating influenza strains have developed resistance to fusion inhibitors amantadine and rimantadine.[12] Viruses that are resistant to the neuraminidase inhibitor Oseltamivir have also been reported since 2005.[13] More surprisingly, the new 2009 H1N1 virus, also called S-OIV (Swine-Originated Influenza Virus), was susceptible to Oseltamivir initially,[14] but developed Oseltamivir-resistant variants in 4 months.[15] Therefore, developing a new antiviral against seasonal and pandemic strain of influenza virus is a challenge for scientists.

Different types of screening methods have been discovered to discover various types of antiviral drug compounds for preventing viral infection and replication against influenza virus. However, very few have been recognized as successful medicines for influenza treatment due to their toxicities and non selective activities with virus-host targets. Since viral glycoproteins HA and NA are present only on the viral surface envelope and not in the host cellular construction therefore, HA and NA may be considered as selective and safe targets for antiviral drug discovery.

Many lead compounds targeting HA and NA proteins have been identified as promising anti-influenza drugs. Oseltamivir has been used as a widely used medicine for influenza treatment. However, effectiveness of neuraminidase inhibitors like oseltamivir also compromised due to the emergence of drug-resistant viruses that escape interaction of their inhibitors with the active site of viral neuraminidase.[14,15] Therefore, antiviral drugs that can inhibit both currently circulating human influenza and avian influenza viruses are urgently needed for future prevention of pandemic influenza.

Here, we will discuss about different types of screening approaches generally used in antiviral drug screening by targeting glycoproteins HA and NA of influenza A virus.

METHOD FOR DATA COLLECTION

For this review article, resources which were relevant to the drug discovery on influenza virus were searched in the months January to April, 2013 from online papers (pubmed) using the key words influenza, antiviral, influenza pandemic, hemagglutination, and neuraminidase drug targets. About 70 articles from pubmed were retrieved (including abstracts) and 47 out of them were used for preparation of the review.

All the data gathered was then used to draft a comprehensive review article about the classical and novel screening methods of antiviral drug discovery by targeting the envelope glycoproteins of influenza A virus, by the authors, with an aim to cover all its important aspects.

REVIEW

Discovery of antiviral drugs by targeting hemagglutinin of influenza virus

Hemagglutination inhibition assay

Influenza virus particles have an envelope protein called the hemagglutinin, or HA, which binds to sialic acid receptors on cells. The virus also binds to erythrocytes (red blood cells), causing the formation of a lattice. This property is called hemagglutination, and is the basis of a rapid assay to determine levels of influenza virus present in a sample. To conduct the assay, two-fold serial dilutions of a virus are prepared, mixed with a specific amount of red blood cells, and added to the wells of a plastic tray. The red blood cells that are not bound by influenza virus sink to the bottom of a well and form a button. The red blood cells that are attached to virus particles form a lattice that coats the well. The assay can be performed within 30 minutes, and is therefore a quick indicator of the relative quantities of virus particles.[16,17]

In order to perform hemagglutination inhibition assay (HAI) of an inhibitor or drug, generally 25ml of virus suspension (4 HA unit titer) is incubated for 1 h at 4 °C with 25ml of viral inhibitors serially diluted two-fold with PBS (pH 7.2, 131mM NaCl, 14mM Na_2HPO_4, 1.5mM KH2PO4, and 2.7mM KCl) in 96-well microtiter plastic plates. After adding 50 mL of 0.5% (v/v) guinea pig erythrocytes to each well, the plates are kept for 8 h at 4°C. The maximum dilution of the agents of the inhibitor showed complete inhibition of hemagglutination was defined as the HAI titer.[18]

HAI titer of the inhibitor is measured as the maximum dilution of the inhibitor required for the complete hemagglutination of the blood. Therefore, hemagglutinin inhibitors may be considered as a potential target for influenza prevention. Small ligand molecules can also inhibit the low pH-induced conformational change of hemagglutinin, thereby blocking viral entry into host cells.[19] If the inhibitor has the specificity to bind with the viral glycoprotein residue specific for binding with red blood cells, the HAI titer can be determine clearly.

However, if the inhibitor has nonspecific binding affinity with the surface glycoprotein, it shows weaker or absence of HAI activity. In such cases, the inhibitors show viral entry inhibition activity in cells and inhibit viral binding activity with the sialic acid receptors in solid phase viral binding assay.[18] Besides, glycan dependent immunogenicity of recombinant soluble trimeric HA showed that HA proteins carrying terminal mannose moieties induce significantly lower hemagglutination inhibition antibody titers than HA proteins carrying complex glycans or single N-acetylglucosamine side chains.[20]

Fusion and hemifusion inhibition assay:

HA protein controls two critical aspects of entry including virus binding and membrane fusion. In order for HA to carry out these functions, it must first undergo a priming step, proteolytic cleavage, which renders it fusion competent. Membrane fusion commences from inside the endosome after a drop in luminal pH and an ensuing conformational change in HA that leads to the hemifusion of the outer membrane leaflets of the virus and endosome, the formation of a stalk between them, followed by pore formation.

Thus, the fusion machinery is an excellent target for antiviral compounds, especially those that target the conserved stem region of the protein.[21]

Hemolysis inhibition assay

In order to perform hemolysis inhibition assay, fresh chicken or guinea pig red blood cells (RBC) are washed twice with PBS and resuspended to make a 2% (v/v) suspension in phosphate buffer solution (PBS) that should be stored at 4°C until use. 100 ml of compound diluted in PBS is mixed with an equal volume of virus in a 96-well plate. Usually the whole viral particle is used in hemolysis assay.

After incubating virus-compound mixture at room temperature for 30 minutes, 200 ml of 2% chicken erythrocytes pre-warmed at 37°C is added. The mixture is incubated at 37°C for another 30 minutes. To initiate hemolysis, 100 ml of sodium acetate (0.5 M, pH 5.2) should be added and mixed well with erythrocyte suspension. The mixture is incubated at 37°C for 30 minutes for HA acidification and hemolysis.

To separate non-lysed erythrocytes, plates are centrifuged at the end of incubation at 1,200 rpm for 6-10 minutes. 300 ml of supernatant is transferred to another flat bottom 96-well plate. OD_{540} is read on a microtiter plate reader. Percentage of protection is calculated as [1- (mean of OD_{540}compound - mean of OD_{540}PBS)/(mean of OD_{540}solvent 2 mean of OD_{540}PBS)]X100%. IC_{50} is defined as the compound concentration that generates 50% of the maximal protection.

Special attention should be paid when a compound is colorful or is hemolytic. Suitable controls should be set up to adjust absorption backgrounds since both of those features of a compound could result in a false negative outcome in hemolysis inhibition assay.[22] This

is a rapid method of identifying the fusion inhibitor targeting influenza virus. Low pH induced influenza virus fusion to the RBC is an effective in vitro method to determine the fusion inhibitor of influenza virus. However, in some cases the method cannot explain the fusion between virus to host cell and cell to cell fusion activity. We can solve such types of limitations using the in vitro fusion assay.

Virus-cell fusion inhibition assay

In order to screen the fusion inhibitor, in vitro fusion assays for studying virus fusion to membranes were reported as either membrane mixing between the virus and the cell membrane or content release of the virus; but not both at the same time.[23,24] In brief, viral vesicles with encapsulated dye that upon fusion with either intact virions or reconstituted viral envelopes release their contents and give rise to a change in fluorescent signal in presence or in absence of inhibitor.

Two common approaches are the release of a calcium indicator dye that fluoresces when exposed to the surrounding buffer or changes in fluorescence due to dequenching of internal dye or fluorescence energy transfer (FRET). In membrane mixing assays, virus fusion is typically reported by changes in fluorescence resulting from fluorescence dequenching within the membrane upon fusion, or FRET between fluorophore pairs residing in the membrane.[24,25]

In the most direct measurement of membrane mixing and associated kinetics, intact viral membranes are first labeled with fluorophores until the fluorescence signal is quenched. Then, the labeled virus is mixed in a cuvette with unlabeled host cell mimics containing the viral receptor, such as liposomes or ghost cells, and a baseline florescence signal is obtained in a fluorimeter.

To initiate fusion, a small amount of acid is added to the cuvette while the sample is rapidly mixed. Inhibitor can be incubated in this stage to investigate the effect of inhibitor on fusion activities. The temporal change in the fluorescence signal is collected as the viruses fuse with the host membranes and the fluorophores originally in the viral membranes become diluted and dequench.

Alternatively, a FRET method can be used to avoid labeling the virus itself by creating liposomes containing both fluorophores of a FRET pair that separate when virus fuses to vesicle. From the change in the fluorescence signal in either approach, some information about the kinetics of virus fusion can be obtained. These approaches characterize the overall rate from the binding to the hemifusion step, determined over an ensemble population of virions within the cuvette. Many studies of virus fusion to date have been conducted using this type of assay[24, 25] and a great deal of what is known about virus fusion has been learned using this ensemble approach.

However, there are several limitations that have restricted the amount of information that can be collected from these assays. First, virus fusion is stochastic and thus only averaged kinetic information is obtained from these assays, which can obscure intermediate steps.[26] Second, since individual events cannot be observed in this assay, viral binding and fusion events cannot be distinguished visually making it difficult to study either processes individually. Third, the binding sites of the chromophore and the inhibitor may compete with each other that may reduce the signal. To circumvent this limitation, these assays can be conducted at cold temperatures to bind viruses first and then with acidic buffer to trigger decoupling of binding and fusion processes kinetically from each other.[27] An instantaneous pH change from neutral to acidic is ideal to trigger virus fusion at the same time point at a uniform pH value.

Asynchronous triggering of events masks the magnitude of the pH dependence of fusion,[28] which may be an important criterion when assessing infectivity. Therefore, due to the finite volume of the cuvette, rapid mixing of contents is required to quickly distribute the acid throughout the cuvette, but this rapid mixing leads to shearing, which can interrupt virus binding and does not mimic the quiescent environment inside an endosome. A third important consideration is that the curvature of the two opposing membranes is opposite to that inside the endosome. It is unclear if this non-native geometry could result in membrane binding energies that alter the kinetics or pathway of fusion of the membranes.

Finally, monitoring pore-opening kinetics is difficult to conduct simultaneously with membrane hemifusion in this ensemble approach. Many of these drawbacks can be overcome using single virion imaging of virus fusion to cell membrane techniques.

Single virion imaging of virus fusion inhibition to cell membrane

Using single virion imaging, reconstituted viral envelopes (virosomes), or HA-expressing cells interacting with other cells or cell membrane mimics, several fusion studies of intact virus to erythrocytes or individual human erythrocytes to fibroblasts expressing the influenza virus hemagglutinin were reported.[29-31] Such an experiment can be used to investigate the effect of an inhibitor on fusion process. These assays employed a flow chamber mounted to a microscope stage in presence or in absence of an inhibitor. Fusion is triggered by rapid acidification of the flow chamber and fusion monitored by a fluorescence increase due to redistribution of fluorescent dyes between either membrane or cytoplasmic

compartments of fusing cells. Significant heterogeneity in lag times for events was reported, which could be in part due to asynchronous initiation of fusion, a point we will return to later. Another group also used a video epifluorescence microscope setup to study individual virions fusing to a planar black lipophilic membrane (BLM) formed across the orifice of a teflon support positioned within the field of view of a microscope.[32,33]

In the execution of this assay, fluorescently-labeled, quenched viruses were loaded into a micropipette tip which was positioned in one side of the Teflon chamber already at the desired fusion pH. To coordinate the triggering of fusion, virus solution (at neutral pH inside the micropipette) is gently expelled near the acidified BLM surface. Varions on coming in contact with the BLM, are either immediately bound to it or fall out of the field of view quickly. Bound viruses could then undergo fusion with the BLM. A video camera recorded the fluorescent images of individual fusion events, detected as single dequenching events. These images were later processed to obtain hemifusion kinetic parameters. It has been reported that individual virion fusion technique showed that receptor binding alters fusion kinetics. In the presence of receptor, the kinetics followed Markovian behavior characteristic of Poisson process described by a rate constant defining the jump between distinct states.

Fusion triggered in the absence of receptor followed non-Markovian behavior with no characteristic rate parameter. This clear distinction in fusion kinetics was made possible by (1) decoupling of binding and fusion processes; (2) temporal synchronization of fusion initiation; and (3) statistical analysis of the individual fusion events. It was known from previous ensemble studies that gangliosides increase fusion rate.[34] Therefore, imaging assays that combined lipid mixing, contents mixing, and electrical conductance measurements in one assay provided important experimental details on the intermediate steps leading to pore formation and that it might proceed initially by formation of series of small pores.[35] Therefore, if the target inhibitor is lipid in nature such experiment may affect the result of fusion inhibition. Then, supporting experiments may suggest their binding affinity with HA.

Solid phase virus binding inhibition assay

Solid phase virus binding assay is an enzyme linked immunosorbant assay (ELISA). Receptor-binding properties of influenza viruses are usually characterized by the ability of viruses to interact with more or less defined sialic acid-containing carbohydrates, glycoproteins, or glycolipids assayed by haemagglutination (HA) or HA inhibition (HAI) tests. To overcome some limitations of these tests a solid-phase enzyme linked assay analogous to sandwich ELISA is developed.[36]

In order to investigate the binding inhibition activity of any inhibitor with HA, a microtiter plastic plate is generally used. The virus particles or HA protein in presence or in absence of inhibitors is adsorbed specifically to the well of plastic microtitre plates coated with fetuin. After washing with PBS, the plate is incubated with primary antibodies and then with horseradish peroxidase (HRP) labelled secondary antibodies by the solid phase-attached virions is measured.[37]

Besides such type of analysis, the binding of unlabelled compounds is measured by competition with the attachment of a standard fetuin-HRP conjugate. The binding of unlabelled compounds is measured by competition with the attachment of a standard fetuin-HRP conjugate. The assay is easy to perform, quantitative (allows the determination of affinity constants), and sensitive (even the weak binding of free N-acetylneuraminic acid).

Due to a higher stability of components of the present test system, as compared to red blood cells, the influence of pH, ionic strength, and other factors on virus-receptor interaction can also be investigated.[36] We can also investigate the binding affinity of an inhibitor on another surface glycoprotein neuramidase of influenza virus or hemagglutinin-neuraminida - se parainfluenza virus.[38,39]

Discovery of antiviral drugs by targeting neuraminidase of influenza virus

The principle underlying the functional methods relies on the enzymatic nature of the neuraminidase (NA), a viral surface glycoprotein and antigen. NA acts by cleaving the terminal sialic acid (molecular species are more than 50, and N-acetyl- and *N*-glycolyl-neura - minic acid are the predominant molecular species found in animals) from receptors, thus facilitating the release of progeny virions from infected cells and preventing self-aggregation.[40] Structurally, neuraminidase inhibitors (NAIs) mimic the natural substrate, neuraminic acid, and produce tight interactions, with conserved residues of the NA active site competing with neuraminic acid for binding.[41] Preincubation of virus with NAIs leads to the inhibition of enzyme activity, which is detected after the addition of enzyme substrate.

Most neuraminidase inhibition (NI) assays commonly used for virus surveillance utilize as substrates small synthetic conjugates that produce either a luminescent or a fluorescent signal upon cleavage by the NA enzyme. The chemiluminescent (CL) assay uses the 1,2-dioxetane derivative of neuraminic acid substrate in the influenza neuraminidase inhibitor resistance detection (NA-Star) kit,[42] while the fluorescent (FL) assay employs 2′-O-(4-methylumbelliferyl)-N-acetylneuraminic acid substrate (MUNANA).[43] The colorimetric (CM) assay, which utilizes fetuin as the substrate of the NA, is typically used to determine the titer of anti-NA antibodies because small substrates do not effectively compete with antibodies.[44] The results of the NI assays are expressed as the 50% inhibitory concentration (IC_{50}), which represents the NAI concentration that inhibits 50% of the enzyme activity.[45]

DISCUSSION

Reasons for the development of new antiviral agents for the treatment of influenza are antiviral resistance; limited antiviral efficacy in severe cases of influenza, including highly pathogenic avian influenza A(H5N1) disease, lack of parenteral agents and preparation for future pandemics. Numerous efforts are under way to develop new antiviral agents or influenza treatment targeting viral nucleoproteins, polymerase and matrix proteins with an improved spectrum of activity or better pharmacologic profiles, relative to current treatments.

Understanding the relationship between antigenic structure and immune specificity, the receptor binding specificity in virus transmission, how the cleavage site controls pathogenicity, and how the fusion peptide causes membrane fusion for the entry of influenza virus into the host cell should provide information to find more effective ways to prevent and control influenza.[46] In order to develop a new anti-influenza drug, we should consider about its effectiveness against both influenza A and B viruses, its effectiveness against all reassortants of influenza A viruses, i.e., effectiveness against all hemagglutinin and neuraminidase subtypes of influenza A viruses, its effectiveness against all mutants with amino acid substitution of viral protein, its no production of mutants with drug resistance, and it is safe for patients.[47]

Therefore, development of novel methods for screening prospective antiviral compounds will be beneficial for future antiviral drug discoveries.

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