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(54) Title: INFLUENZA A VIRUS VARIANTS

(57) Abrégé/Abstract:
The present invention relates to influenza A virus variants, particularly variants that are resistant to a polymerase inhibitors. Also provided are methods and compositions related to the influenza A virus variants. Further provided are methods of isolating, identifying, and characterizing multiple viral variants from a patient.

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(54) **Title**: INFLUENZA A VIRUS VARIANTS

(57) **Abstract**: The present invention relates to influenza A virus variants, particularly variants that are resistant to a polymerase inhibitors. Also provided are methods and compositions related to the influenza A virus variants. Further provided are methods of isolating, identifying, and characterizing multiple viral variants from a patient.

# INFLUENZA A VIRUS VARIANTS

## CROSS REFERENCE TO RELATED APPLICATION

**[0001]** This PCT application claims the benefit of U.S. provisional application Serial No. 62/058,945, filed on October 2, 2014. This document is hereby incorporated by reference in its entirety.

## SEQUENCE LISTING

**[0002]** This application incorporates by reference in its entirety the Sequence Listing entitled "355617_ST25.txt" (8.06 kilobytes), which was created on October 2, 2014 and filed electronically herewith.

## BACKGROUND OF THE INVENTION

**[0003]** Influenza spreads around the world in seasonal epidemics, resulting in the deaths of hundreds of thousands annually - millions in pandemic years. For example, three influenza pandemics occurred in the 20th century and killed tens of millions of people, with each of these pandemics being caused by the appearance of a new strain of the virus in humans. Often, these new strains result from the spread of an existing influenza virus to humans from other animal species.

**[0004]** Influenza is primarily transmitted from person to person via large virus-laden droplets that are generated when infected persons cough or sneeze; these large droplets can then settle on the mucosal surfaces of the upper respiratory tracts of susceptible individuals who are near (e.g. within about 6 feet) infected persons. Transmission might also occur through direct contact or indirect contact with respiratory secretions, such as touching surfaces contaminated with influenza virus and then touching the eyes, nose or mouth. Adults might be able to spread influenza to others from 1 day before getting symptoms to approximately 5 days after symptoms start. Young children and persons with weakened immune systems might be infectious for 10 or more days after onset of symptoms.

**[0005]** Influenza viruses are RNA viruses of the family Orthomyxoviridae, which comprises five genera: Influenza virus A, Influenza virus B, Influenza virus C, Isavirus and Thogoto virus.

**[0006]** The Influenza virus A genus has one species, influenza A virus. Wild aquatic birds are the natural hosts for a large variety of influenza A. Occasionally, viruses are transmitted to other species and may then cause devastating outbreaks in domestic poultry or give rise to human influenza pandemics. The type A viruses are the most virulent human pathogens among the three influenza types and cause the most severe disease. The influenza A virus

can be subdivided into different serotypes based on the antibody response to these viruses. The serotypes that have been confirmed in humans, ordered by the number of known human pandemic deaths, are: H1N1 (which caused Spanish influenza in 1918), H2N2 (which caused Asian Influenza in 1957), H3N2 (which caused Hong Kong Flu in 1968), H5N1 (a pandemic threat in the 2007–08 influenza season), H7N7 (which has unusual zoonotic potential), H1N2 (endemic in humans and pigs), H9N2, H7N2, H7N3 and H10N7.

**[0007]** The Influenza virus B genus has one species, influenza B virus. Influenza B almost exclusively infects humans and is less common than influenza A. The only other animal known to be susceptible to influenza B infection is the seal. This type of influenza mutates at a rate 2–3 times slower than type A and consequently is less genetically diverse, with only one influenza B serotype. As a result of this lack of antigenic diversity, a degree of immunity to influenza B is usually acquired at an early age. However, influenza B mutates enough that lasting immunity is not possible. This reduced rate of antigenic change, combined with its limited host range (inhibiting cross species antigenic shift), ensures that pandemics of influenza B do not occur.

**[0008]** The Influenza virus C genus has one species, influenza C virus, which infects humans and pigs and can cause severe illness and local epidemics. However, influenza C is less common than the other types and usually seems to cause mild disease in children.

**[0009]** Influenza A, B and C viruses are very similar in structure. The virus particle is 80–120 nanometers in diameter and usually roughly spherical, although filamentous forms can occur. Unusually for a virus, its genome is not a single piece of nucleic acid; instead, it contains seven or eight pieces of segmented negative-sense RNA. The Influenza A genome encodes 11 proteins: hemagglutinin (HA), neuraminidase (NA), nucleoprotein (NP), M1, M2, NS1, NS2(NEP), PA, PB1, PB1-F2 and PB2.

**[0010]** HA and NA are large glycoproteins on the outside of the viral particles. HA is a lectin that mediates binding of the virus to target cells and entry of the viral genome into the target cell, while NA is involved in the release of progeny virus from infected cells, by cleaving sugars that bind the mature viral particles. Thus, these proteins have been targets for antiviral drugs. Furthermore, they are antigens to which antibodies can be raised. Influenza A viruses are classified into subtypes based on antibody responses to HA and NA, forming the basis of the H and N distinctions (vide supra) in, for example, H5N1.

**[0011]** Influenza produces direct costs due to lost productivity and associated medical treatment, as well as indirect costs of preventative measures. In the United States, influenza is responsible for a total cost of over $10 billion per year, while it has been estimated that a

future pandemic could cause hundreds of billions of dollars in direct and indirect costs. Preventative costs are also high. Governments worldwide have spent billions of U.S. dollars preparing and planning for a potential H5N1 avian influenza pandemic, with costs associated with purchasing drugs and vaccines as well as developing disaster drills and strategies for improved border controls.

**[0012]** Current treatment options for influenza include vaccination, and chemotherapy or chemoprophylaxis with anti-viral medications. Vaccination against influenza with an influenza vaccine is often recommended for high-risk groups, such as children and the elderly, or in people that have asthma, diabetes, or heart disease. However, it is possible to get vaccinated and still get influenza. The vaccine is reformulated each season for a few specific influenza strains but cannot possibly include all the strains actively infecting people in the world for that season. It takes about six months for the manufacturers to formulate and produce the millions of doses required to deal with the seasonal epidemics; occasionally, a new or overlooked strain becomes prominent during that time and infects people although they have been vaccinated (as by the H3N2 Fujian flu in the 2003–2004 influenza season). It is also possible to get infected just before vaccination and get sick with the very strain that the vaccine is supposed to prevent, as the vaccine takes about two weeks to become effective.

**[0013]** Further, the effectiveness of these influenza vaccines is variable. Due to the high mutation rate of the virus, a particular influenza vaccine usually confers protection for no more than a few years. A vaccine formulated for one year may be ineffective in the following year, since the influenza virus changes rapidly over time, and different strains become dominant.

**[0014]** Also, because of the absence of RNA proofreading enzymes, the RNA-dependent RNA polymerase of influenza vRNA makes a single nucleotide insertion error roughly every 10 thousand nucleotides, which is the approximate length of the influenza vRNA. Hence, nearly every newly-manufactured influenza virus is a mutant—antigenic drift. The separation of the genome into eight separate segments of vRNA allows mixing or reassortment of vRNAs if more than one viral line has infected a single cell. The resulting rapid change in viral genetics produces antigenic shifts and allows the virus to infect new host species and quickly overcome protective immunity.

**[0015]** Antiviral drugs can also be used to treat influenza, with neuraminidase inhibitors being particularly effective, but viruses can develop resistance to the standard antiviral drugs.

**[0016]** Thus, there is still a need for drugs for treating influenza infections, such as for drugs with expanded treatment window, and/or reduced sensitivity to viral titer.

**[0017]** Accordingly, there exists a need in identifying mutated influenza A viruses or other viruses that exhibit resistance to drugs or other therapies and in developing new viral therapeutics effective against these mutated viruses.

## SUMMARY OF THE INVENTION

**[0018]** Accordingly, the present invention provides influenza A virus variants, and related methods and compositions. In particular, influenza A virus variants and variant influenza A virus polymerases that have reduced sensitivity to one or more polymerase inhibitors are provided.

**[0019]** In one aspect, this invention provides an isolated influenza A virus polynucleotide, a biologically active analog thereof, or a biologically active fragment thereof, comprising a mutation in the gene encoding the influenza A virus polymerase wherein said mutation results in at least one amino acid substitution corresponding to amino acid residues selected from the group consisting of amino acid 306, 323, 324, 325, 357, 376, 404, 406, 431, and 510 of a wild-type influenza A virus.

**[0020]** In certain embodiments, the isolated influenza A virus polynucleotide comprises a nucleotide corresponding to amino acid 306 of the wild-type influenza A virus polynucleotide that does not encode Q. In certain embodiments, the nucleotide encodes H.

**[0021]** In certain embodiments, the isolated influenza A virus polynucleotide comprises a nucleotide corresponding to amino acid 323 of the wild-type influenza A virus polynucleotide that does not encode F. In certain embodiments, the nucleotide encodes L.

**[0022]** In certain embodiments, the isolated influenza A virus polynucleotide comprises a nucleotide corresponding to amino acid 324 of the wild-type influenza A virus polynucleotide that does not encode S. In certain embodiments, the nucleotide encodes I, N or R.

**[0023]** In certain embodiments, the isolated influenza A virus polynucleotide comprises a nucleotide corresponding to amino acid 325 of the wild-type influenza A virus polynucleotide that does not encode S. In certain embodiments, the nucleotide encodes V.

**[0024]** In certain embodiments, the isolated influenza A virus polynucleotide comprises a nucleotide corresponding to amino acid 357 of the wild-type influenza A virus polynucleotide that does not encode H. In certain embodiments, the nucleotide encodes Q.

**[0025]** In certain embodiments, the isolated influenza A virus polynucleotide comprises a nucleotide corresponding to amino acid 376 of the wild-type influenza A virus polynucleotide that does not encode K. In certain embodiments, the nucleotide encodes Q or R.

**[0026]** In certain embodiments, the isolated influenza A virus polynucleotide comprises a nucleotide corresponding to amino acid 404 of the wild-type influenza A virus polynucleotide

that does not encode F. In certain embodiments, the nucleotide encodes Y.

**[0027]** In certain embodiments, the isolated influenza A virus polynucleotide comprises a nucleotide corresponding to amino acid 406 of the wild-type influenza A virus polynucleotide that does not encode F. In certain embodiments, the nucleotide encodes K.

**[0028]** In certain embodiments, the isolated influenza A virus polynucleotide comprises a nucleotide corresponding to amino acid 431 of the wild-type influenza A virus polynucleotide that does not encode M. In certain embodiments, the nucleotide encodes I.

**[0029]** In certain embodiments, the isolated influenza A virus polynucleotide comprises a nucleotide corresponding to amino acid 510 of the wild-type influenza A virus polynucleotide that does not encode N. In certain embodiments, the nucleotide encodes K or T.

**[0030]** In certain embodiments, the nucleotides that correspond to any 2 amino acids selected from the group consisting of amino acids 306, 323, 324, 325, 357, 376, 404, 406, 431, and 510 are mutated such that the nucleotides encode an amino acid different from the amino acid encoded by the corresponding wild-type influenza A virus polynucleotide. In certain embodiments, the nucleotides that correspond to any 3 amino acids selected from the group consisting of amino acids 306, 323, 324, 325, 357, 376, 404, 406, 431, and 510 are mutated such that the nucleotides encode an amino acid different from the amino acid encoded by the corresponding wild-type influenza A virus polynucleotide.

**[0031]** In further embodiments, this invention provides methods and compositions involving an influenza A virus of the invention. For example, an expression system comprising the influenza A virus is provided, and such expression system may include a vector that comprises the influenza A virus operably linked to a promoter; also provided is a host cell transfected, transformed, or transduced with the vector. Alternatively, an expression system of the invention is based on an mRNA display technology, e.g., the RNA-protein fusion technology as described in U.S. Patent No. 6,258,558 or the in vitro "virus" technology as described in U.S. Patent No. 6,361,943.

**[0032]** In another aspect, this invention provides An isolated influenza A virus variant comprising a polynucleotide encoding an influenza A virus polymerase, wherein at least one amino acid at at least one position selected from the group consisting of 306, 323, 324, 325, 357, 376, 404, 406, 431, and 510 is mutated such that it encodes an amino acid different from the corresponding amino acid of the wild-type influenza A virus polynucleotide. Further embodiments of the invention provide methods and compositions involving the influenza A virus variants. For example, a method is provided to identify a compound that can inhibit replication of an influenza A virus variant of the invention; a cell is provided that is infected

by an influenza A virus variant of the invention.

**[0033]** In another aspect, this invention provides an isolated influenza A virus polymerase comprising an amino acid sequence in which the amino acid at at least one position selected from the group consisting of 306, 323, 324, 325, 357, 376, 404, 406, 431, and 510 is different from the amino acid at the corresponding position of the wild-type influenza A virus polymerase. In some embodiments, the influenza A virus polymerase comprises a biologically active analog of an influenza A virus polymerase. In some embodiments, the influenza A virus polymerase comprises a biologically active fragment of an influenza A virus polymerase.

**[0034]** In a further aspect, this invention provides an anti-influenza A virus polymerase antibody that recognizes an influenza A virus polymerase comprising an amino acid sequence in which the amino acid at at least one position selected from the group consisting of 306, 323, 324, 325, 357, 376, 404, 406, 431, and 510 is different from the amino acid at the corresponding position of the wild-type influenza A virus polymerase. Further embodiments of the invention provide methods and compositions involving an anti-influenza A virus polymerase antibody of the invention. For example, a diagnostic kit comprising an antibody of the invention, and a pharmaceutical composition comprising an antibody of the invention and a pharmaceutically acceptable carrier are provided.

**[0035]** In another aspect, this invention provides a nucleotide probe or primer capable of hybridizing under stringent conditions to a nucleic acid sequence of an influenza A virus polynucleotide of the invention. Further embodiments of the invention provide methods and compositions involving the probe or primer. For example, a diagnostic or detection kit comprising a probe or primer of the invention is provided, and the kit is useful in, e.g., determining whether an influenza A virus variant or an influenza A virus polymerase of the invention is present in a sample.

**[0036]** In a further aspect, this invention provides a method for evaluating drug resistance or sensitivity to a polymerase inhibitor of an influenza A virus infection in a patient comprising: a) collecting a biological sample from the influenza A virus infected patient; and b) evaluating whether the sample comprises a nucleic acid encoding an influenza A virus polymerase that comprises an amino acid sequence in which the amino acid at at least one position selected from the group consisting of 306, 323, 324, 325, 357, 376, 404, 406, 431, and 510 is different from the amino acid at each corresponding position of the wild-type influenza A virus polymerase.

**[0037]** Also provided is a method for guiding a treatment for an influenza A virus infection

in a patient comprising: a) evaluating drug resistance or sensitivity to a polymerase inhibitor of the patient according the method of claim 23; and b) optimizing the treatment regimen for the patient based on the drug resistance or sensitivity evaluated in a). For example, if drug resistance is predicted or detected (e.g., reduced sensitivity to a polymerase inhibitor), one or more other compounds or agents may be included in the patient's treatment plan or therapeutic regimen. The method may comprise any combination of determining the sequence (e.g., genotyping) of an influenza A virus polymerase in the patient, determining the sensitivity to a polymerase inhibitor (e.g., phenotyping), or determining the viral fitness level of the patient's influenza A virus. The phenotyping may be carried out in a cell-free system (e.g., in vitro protease assays) as well as a cell-based system (e.g., replicon assays or viral infection or replication assays).

**[0038]** In another aspect, this invention provides a method for identifying a candidate compound for treating an influenza A virus infection in a patient comprising: a) providing a sample infected with the influenza A virus variant; and b) assaying the ability of the candidate compound in inhibiting an activity of the influenza A virus variant in the sample. The sample may be obtained from a patient's cells or plasma. The sample infected with an influenza A virus variant may also be cultured cells. The activity of the influenza A virus variant may be determined by its ability to infect, replicate, and/or become released.

**[0039]** Alternatively, such a method may comprise providing a replicon RNA comprising an influenza A virus polynucleotide of the invention and determining whether the candidate compound inhibits replication of the replicon RNA in a suitable assay.

**[0040]** Another alternative method may comprise providing an isolated influenza A virus polymerase of invention and a polymerase substrate, and determining whether the influenza A virus polymerase activity is reduced in the presence of a candidate compound; the influenza A virus polymerase and/or the polymerase substrate may be in a cell-based system, for example expressed in cultured cells, or the influenza A virus polymerase and/or the polymerase substrate may be in a cell-free system, for example a reaction mixture including an influenza A virus polymerase and a peptide substrate.

**[0041]** A further alternative method for evaluating a candidate compound for treating an influenza A virus infection in a patient may include introducing a vector comprising an influenza A virus polynucleotide of the invention and an indicator gene encoding an indicator into a host cell and measuring the indicator in the presence of the candidate compound and in the absence of the candidate compound.

**[0042]** Further provided is a method for identifying a compound effective in reducing an

influenza A virus polymerase activity. The method may comprise obtaining a three dimensional model of an influenza A virus polymerase of the invention and designing or selecting a compound. The method may further comprise evaluating, in silico, in vitro, and/or in vivo, the ability of the compound to bind to or interact with the polymerase. The method may also involve determining whether the designed or selected compound can inhibit the activity of an influenza A virus polymerase, in particular, a variant influenza A virus polymerase with reduced sensitivity to a polymerase inhibitor, in a cell-free or cell-based assay. The method may further or alternatively include assaying the ability of a designed or selected compound to inhibit influenza A virus replication in a cell or sample. The influenza A virus replication can be determined by measuring the replication of an influenza A virus variant of the invention or an influenza A virus replicon of the invention.

**[0043]** A further aspect of this invention provides a method for treating an influenza A virus infection in a patient. The method may comprise administering to the patient a pharmaceutically or therapeutically effective amount of a compound identified by a method of the invention alone or in combination with another anti-viral agent.

**[0044]** Another aspect of the invention relates to computer tools, which provides a machine-readable data storage medium comprising a data storage material encoded with machine-readable data, wherein the machine-readable data comprise index values for at least two features associated with an influenza A virus variant or biological sample.

**[0045]** The features are selected from: a) the ability to exhibit resistance for reduced sensitivity to a polymerase inhibitor; b) an influenza A virus polymerase comprising an amino acid sequence in which the amino acid at at least one position selected from the group consisting of: 306, 323, 324, 325, 357, 376, 404, 406, 431, and 510 of a wild-type influenza A virus is different from the amino acid at the corresponding position of the wild-type influenza A virus polymerase; c) morbidity or recovery potential of a patient; and d) altered replication capacity (increased or decreased) of the influenza A virus variant.

**[0046]** A further aspect of the invention provides a method of obtaining a profile of influenza A virus variants in an influenza A virus-infected patient. The method may comprise obtaining a sample (e.g., a plasma sample) from the patient and genotyping and/or phenotyping an influenza A virus polymerase from at least 2, 20, 50, 100, 200, 500 or more influenza A virus virions from the sample. For example, such genotyping may include determining the nucleotide sequence of an influenza A virus polymerase from at least 2, 20, 50, 100, 200, 500 or more influenza A virus virions from the plasma sample.

**[0047]** In certain embodiments, the patient subjected to such profiling may have been

treated or be selected to be treated with a polymerase inhibitor.

**[0048]** In certain embodiments, plasma samples are obtained from the patient subjected to such profiling at two or more different time points.

## DETAILED DESCRIPTION OF THE INVENTION

**[0049]** The present invention relates to influenza A virus variants. In particular, influenza A virus variants that exhibit resistance to a polymerase inhibitor are provided. Also provided are methods and compositions related to the influenza A virus variants. The methods and compositions are useful in identifying viral variants, including variants of an influenza A virus and other viruses, evaluating and identifying anti-viral compounds, and developing and optimizing therapeutics against viral infections.

**[0050]** ***Influenza A virus Variants and Related Polynucleotides and Polymerases***

**[0051]** The present invention provides influenza A virus variants. In particular embodiments, an influenza A virus variant includes a polynucleotide sequence that encodes an influenza A virus polymerase with reduced sensitivity to a polymerase inhibitor (also termed "a variant influenza A virus polymerase"), such as Compound 1. As used herein, a wild-type influenza A virus refers to an influenza A virus comprising a polynucleotide (also termed "a wild-type polynucleotide") that encodes an influenza A virus polymerase with normal or desirable sensitivity to a polymerase inhibitor, and in particular embodiments, the polymerase inhibitor is Compound 1. Similarly, a wild-type influenza A virus polymerase refers to an influenza A virus polymerase with normal or desirable sensitivity to a polymerase inhibitor, and in particular embodiments, the polymerase inhibitor is Compound 1.

**[0052]** In some embodiments, the wild-type influenza A virus comprises the amino acid sequence of SEQ ID NO: 1. In some embodiments, the wild-type influenza A virus comprises an amino acid sequence having at least 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 97 or 99 % homology to SEQ ID NO: 1. Additional influenza A virus strains are known in the art and can be found in the GenBank sequence database provided by the National Center for Biotechnology Information (NCBI). Specific examples include influenza A/Wisconsin/67/2005: PB2 759 aa protein-- AHG97066.1, AHG97054.1, AHG97042.1, AHG97006.1, etc.; A/Puerto Rico/8/34: PB2 759 aa protein--AAM75155.1; A/California/07/2009: PB2 759 aa protein--AFM72841.1, ACQ63273.1, ACP44175.1, ACP41956.1; A/Hamburg/NY1580/2009(H1N1) PB2 domain gene--GU480807.1; and A/turkey/Ontario/FAV110/2009(H1N1) PB2 domain gene--HM370957.1, HM370972.1.

**[0053]** The terms "influenza virus mediated condition", "influenza infection", "Influenza", or "flu" as used herein, are used interchangeable to mean the disease caused by an infection

with an influenza virus.

**[0054]** Influenza is an infectious disease that affects birds and mammals caused by influenza viruses. Influenza viruses are RNA viruses of the family Orthomyxoviridae, which comprises five genera: Influenzavirus A, Influenzavirus B, Influenzavirus C, Isavirus and Thogotovirus. Influenzavirus A genus has one species, influenza A virus which can be subdivided into different serotypes based on the antibody response to these viruses: H1N1, H2N2, H3N2, H5N1, H7N7, H1N2, H9N2, H7N2, H7N3 and H10N7. Influenzavirus B genus has one species, influenza B virus. Influenza B almost exclusively infects humans and is less common than influenza A. Influenzavirus C genus has one species, Influenzavirus C virus, which infects humans and pigs and can cause severe illness and local epidemics. However, Influenzavirus C is less common than the other types and usually seems to cause mild disease in children.

**[0055]** In some embodiments of the invention, influenza or influenza viruses are associated with Influenzavirus A or B. In some embodiments of the invention, influenza or influenza viruses are associated with Influenzavirus A. In some specific embodiments of the invention, Influenzavirus A is H1N1, H2N2, H3N2 or H5N1.

**[0056]** In humans, common symptoms of influenza are chills, fever, pharyngitis, muscle pains, severe headache, coughing, weakness, and general discomfort. In more serious cases, influenza causes pneumonia, which can be fatal, particularly in young children and the elderly. Although it is often confused with the common cold, influenza is a much more severe disease and is caused by a different type of virus. Influenza can produce nausea and vomiting, especially in children, but these symptoms are more characteristic of the unrelated gastroenteritis, which is sometimes called "stomach flu" or "24-hour flu".

**[0057]** Symptoms of influenza can start quite suddenly one to two days after infection. Usually the first symptoms are chills or a chilly sensation, but fever is also common early in the infection, with body temperatures ranging from 38-39 °C (approximately 100-103 °F). Many people are so ill that they are confined to bed for several days, with aches and pains throughout their bodies, which are worse in their backs and legs. Symptoms of influenza may include: body aches, especially joints and throat, extreme coldness and fever, fatigue, Headache, irritated watering eyes, reddened eyes, skin (especially face), mouth, throat and nose, abdominal pain (in children with influenza B). Symptoms of influenza are non-specific, overlapping with many pathogens ("influenza-like illness). Usually, laboratory data is needed in order to confirm the diagnosis.

**[0058]** The terms, "disease", "disorder", and "condition" may be used interchangeably here

to refer to an influenza virus mediated medical or pathological condition.

**[0059]** As used herein, the terms "subject" and "patient" are used interchangeably. The terms "subject" and "patient" refer to an animal (e.g., a bird such as a chicken, quail or turkey, or a mammal), specifically a "mammal" including a non-primate (e.g., a cow, pig, horse, sheep, rabbit, guinea pig, rat, cat, dog, and mouse) and a primate (e.g., a monkey, chimpanzee and a human), and more specifically a human. In one embodiment, the subject is a non-human animal such as a farm animal (e.g., a horse, cow, pig or sheep), or a pet (e.g., a dog, cat, guinea pig or rabbit). In a preferred embodiment, the subject is a "human".

**[0060]** The term "biological sample", as used herein, includes, without limitation, cell cultures or extracts thereof; biopsied material obtained from a mammal or extracts thereof; blood, saliva, urine, feces, semen, tears, or other body fluids or extracts thereof.

**[0061]** As used herein, "multiplicity of infection" or "MOI" is the ratio of infectious agents (e.g. phage or virus) to infection targets (e.g. cell). For example, when referring to a group of cells inoculated with infectious virus particles, the multiplicity of infection or MOI is the ratio defined by the number of infectious virus particles deposited in a well divided by the number of target cells present in that well.

**[0062]** As used herein the term "inhibition of the replication of influenza viruses" includes both the reduction in the amount of virus replication (e.g. the reduction by at least 10 %) and the complete arrest of virus replication (i.e., 100 % reduction in the amount of virus replication). In some embodiments, the replication of influenza viruses are inhibited by at least 50 %, at least 65 %, at least 75 %, at least 85 %, at least 90 %, or at least 95 %.

**[0063]** Influenza virus replication can be measured by any suitable method known in the art. For example, influenza viral titre in a biological sample (e.g. an infected cell culture) or in humans (e.g. lung viral titre in a patient) can be measured. More specifically, for cell based assays, in each case cells are cultured in vitro, virus is added to the culture in the presence or absence of a test agent, and after a suitable length of time a virus-dependent endpoint is evaluated. For typical assays, the Madin-Darby canine kidney cells (MDCK) and the standard tissue culture adapted influenza strain, A/Puerto Rico/8/34 can be used. A first type of cell assay that can be used in the invention depends on death of the infected target cells, a process called cytopathic effect (CPE), where virus infection causes exhaustion of the cell resources and eventual lysis of the cell. In the first type of cell assay, a low fraction of cells in the wells of a microtiter plate are infected (typically 1/10 to 1/1000), the virus is allowed to go through several rounds of replication over 48-72 hours, then the amount of cell death is measured using a decrease in cellular ATP content compared to uninfected controls. A

second type of cell assay that can be employed in the invention depends on the multiplication of virus-specific RNA molecules in the infected cells, with RNA levels being directly measured using the branched-chain DNA hybridization method (bDNA). In the second type of cell assay, a low number of cells are initially infected in wells of a microtiter plate, the virus is allowed to replicate in the infected cells and spread to additional rounds of cells, then the cells are lysed and viral RNA content is measured. This assay is stopped early, usually after 18-36 hours, while all the target cells are still viable. Viral RNA is quantitated by hybridization to specific oligonucleotide probes fixed to wells of an assay plate, then amplification of the signal by hybridization with additional probes linked to a reporter enzyme.

**[0064]** As used herein a "viral titer" (or titre) is a measure of virus concentration. Titer testing can employ serial dilution to obtain approximate quantitative information from an analytical procedure that inherently only evaluates as positive or negative. The titer corresponds to the highest dilution factor that still yields a positive reading; for example, positive readings in the first 8 serial twofold dilutions translate into a titer of 1:256. A specific example is viral titer. To determine the titer, several dilutions will be prepared, such as 10-1, 10-2, 10-3,...,10-8. The lowest concentration of virus that still infects cells is the viral titer.

**[0065]** As used herein, the terms "treat", "treatment", and "treating" refer to both therapeutic and prophylactic treatments. For example, therapeutic treatments includes the reduction or amelioration of the progression, severity and/or duration of influenza viruses mediated conditions, or the amelioration of one or more symptoms (specifically, one or more discernible symptoms) of influenza viruses mediated conditions, resulting from the administration of one or more therapies (e.g., one or more therapeutic agents such as a compound or composition of the invention). In specific embodiments, the therapeutic treatment includes the amelioration of at least one measurable physical parameter of an influenza virus mediated condition. In other embodiments the therapeutic treatment includes the inhibition of the progression of an influenza virus mediated condition, either physically by, e.g., stabilization of a discernible symptom, physiologically by, e.g., stabilization of a physical parameter, or both. In other embodiments the therapeutic treatment includes the reduction or stabilization of influenza viruses mediated infections. Antiviral drugs can be used in the community setting to treat people who already have influenza to reduce the severity of symptoms and reduce the number of days that they are sick.

**[0066]** The term "chemotherapy" refers to the use of medications, e.g. small molecule drugs

(rather than "vaccines") for treating a disorder or disease.

**[0067]** The terms "prophylaxis" or "prophylactic use" and "prophylactic treatment" as used herein, refer to any medical or public health procedure whose purpose is to prevent, rather than treat or cure a disease. As used herein, the terms "prevent", "prevention", and "preventing" refer to the reduction in the risk of acquiring or developing a given condition, or the reduction or inhibition of the recurrence or said condition in a subject who is not ill, but who has been or may be near a person with the disease. The term "chemoprophylaxis" refers to the use of medications, e.g. small molecule drugs (rather than "vaccines") for the prevention of a disorder or disease.

**[0068]** As used herein, prophylactic use includes the use in situations in which an outbreak has been detected, to prevent contagion or spread of the infection in places where a lot of people that are at high risk of serious influenza complications live in close contact with each other (e.g. in a hospital ward, daycare center, prison, nursing home, etc.). It also includes the use among populations who require protection from the influenza but who either do not get protection after vaccination (e.g. due to weak immune system), or when the vaccine is unavailable to them, or when they cannot get the vaccine because of side effects. It also includes use during the two weeks following vaccination, since during that time the vaccine is still ineffective. Prophylactic use may also include treating a person who is not ill with the influenza or not considered at high risk for complications, in order to reduce the chances of getting infected with the influenza and passing it on to a high-risk person in close contact with him (for instance, healthcare workers, nursing home workers, etc.).

**[0069]** According to the US CDC, an influenza "outbreak" is defined as a sudden increase of acute febrile respiratory illness (AFRI) occurring within a 48 to 72 hour period, in a group of people who are in close proximity to each other (e.g. in the same area of an assisted living facility, in the same household, etc.) over the normal background rate or when any subject in the population being analyzed tests positive for influenza. One case of confirmed influenza by any testing method is considered an outbreak.

**[0070]** A "cluster" is defined as a group of three or more cases of AFRI occurring within a 48 to 72 hour period, in a group of people who are in close proximity to each other (e.g. in the same area of an assisted living facility, in the same household, etc.).

**[0071]** As used herein, the "index case", "primary case", or "patient zero" is the initial patient in the population sample of an epidemiological investigation. When used in general to refer to such patients in epidemiological investigations, the term is not capitalized. When the term is used to refer to a specific person in place of that person's name within a report on

a specific investigation, the term is capitalized as Patient Zero. Often scientists search for the index case to determine how the disease spread and what reservoir holds the disease in between outbreaks. Note that the index case is the first patient that indicates the existence of an outbreak. Earlier cases may be found and are labeled primary, secondary, tertiary, etc.

**[0072]** In one embodiment, the methods of the invention are a preventative or "pre-emptive" measure to a patient, specifically a human, having a predisposition to complications resulting from infection by an influenza virus. The term "pre-emptive" as used herein as for example in pre-emptive use, "pre-emptively", etc., is the prophylactic use in situations in which an "index case" or an "outbreak" has been confirmed, in order to prevent the spread of infection in the rest of the community or population group.

**[0073]** In another embodiment, the methods of the invention are applied as a "pre-emptive" measure to members of a community or population group, specifically humans, in order to prevent the spread of infection.

**[0074]** As used herein, an "effective amount" refers to an amount sufficient to elicit the desired biological response. In the present invention the desired biological response is to inhibit the replication of influenza virus, to reduce the amount of influenza viruses or to reduce or ameliorate the severity, duration, progression, or onset of an influenza virus infection, prevent the advancement of an influenza viruses infection, prevent the recurrence, development, onset or progression of a symptom associated with an influenza virus infection, or enhance or improve the prophylactic or therapeutic effect(s) of another therapy used against influenza infections. The precise amount of compound administered to a subject will depend on the mode of administration, the type and severity of the infection and on the characteristics of the subject, such as general health, age, sex, body weight and tolerance to drugs. The skilled artisan will be able to determine appropriate dosages depending on these and other factors. When co-administered with other antiviral agents, e.g., when co-administered with an anti-influenza medication, an "effective amount" of the second agent will depend on the type of drug used. Suitable dosages are known for approved agents and can be adjusted by the skilled artisan according to the condition of the subject, the type of condition(s) being treated and the amount of a compound described herein being used. In cases where no amount is expressly noted, an effective amount should be assumed. For example, compounds described herein can be administered to a subject in a dosage range from between approximately 0.01 to 100 mg/kg body weight/day for therapeutic or prophylactic treatment.

**[0075]** Generally, dosage regimens can be selected in accordance with a variety of factors

including the disorder being treated and the severity of the disorder; the activity of the specific compound employed; the specific composition employed; the age, body weight, general health, sex and diet of the patient; the time of administration, route of administration, and rate of excretion of the specific compound employed; the renal and hepatic function of the subject; and the particular compound or salt thereof employed, the duration of the treatment; drugs used in combination or coincidental with the specific compound employed, and like factors well known in the medical arts. The skilled artisan can readily determine and prescribe the effective amount of the compounds described herein required to treat, to prevent, inhibit (fully or partially) or arrest the progress of the disease.

**[0076]** Dosages of the compounds described herein can range from between about 0.01 to about 100 mg/kg body weight/day, about 0.01 to about 50 mg/kg body weight/day, about 0.1 to about 50 mg/kg body weight/day, or about 1 to about 25 mg/kg body weight/day. It is understood that the total amount per day can be administered in a single dose or can be administered in multiple dosing, such as twice a day (e.g., every 12 hours), three times a day (e.g., every 8 hours), or four times a day (e.g., every 6 hours).

**[0077]** For therapeutic treatment, the compounds described herein can be administered to a patient within, for example, 48 hours (or within 40 hours, or less than 2 days, or less than 1.5 days, or within 24 hours) of onset of symptoms (e.g., nasal congestion, sore throat, cough, aches, fatigue, headaches, and chills/sweats). The therapeutic treatment can last for any suitable duration, for example, for 5 days, 7 days, 10 days, 14 days, etc. For prophylactic treatment during a community outbreak, the compounds described herein can be administered to a patient within, for example, 2 days of onset of symptoms in the index case, and can be continued for any suitable duration, for example, for 7 days, 10 days, 14 days, 20 days, 28 days, 35 days, 42 days, etc.

**[0078]** Various types of administration methods can be employed in the invention, and are described in detail below under the section entitled "Administration Methods".

**[0079]** The present invention also provides isolated influenza A virus variants, isolated variant influenza A virus polymerases, and isolated polynucleotide that encodes a variant influenza A virus polymerase. The term "isolated" generally means separated and/or recovered from a component of natural environment of a subject virus, protease, or polynucleotide.

**[0080]** In certain embodiments, a variant influenza A virus polymerase may be a variant influenza A virus polymerase that comprises an amino acid sequence in which the amino acid(s) at one or more positions from positions 306, 323, 324, 325, 357, 376, 404, 406, 431,

and 510 of a wild-type influenza A virus polymerase is (are) different from the amino acid at each corresponding position of the wild-type influenza A virus polymerase.

**[0081]** Expression systems are provided, for example, to make the variant influenza A virus polymerases of the invention. An expression system may include an expression vector that comprises an influenza A virus polynucleotide of the invention. Suitable prokaryotic or eukaryotic vectors (e.g., expression vectors) comprising an influenza A virus polynucleotide (or "nucleic acid", used interchangeably herein) of the invention can be introduced into a suitable host cell by an appropriate method (e.g., transformation, transfection, electroporation, infection), such that the polynucleotide is operably linked to one or more expression control elements (e.g., in the vector or integrated into the host cell genome). For production, host cells can be maintained under conditions suitable for expression (e.g., in the presence of inducer, suitable media supplemented with appropriate salts, growth factors, antibiotic, nutritional supplements, etc.), whereby the encoded polypeptide is produced. If desired, the encoded protein can be recovered and/or isolated (e.g., from the host cells or medium). It will be appreciated that the method of production encompasses expression in a host cell of a transgenic animal (see e.g., WO 92103918). An expression system may be based on a cell-free system such as the RNA-protein fusion technology described in U.S. Patent No. 6,258,558 or the in vitro "virus" described in U.S. Patent No. 6,361,943. Ribosome display methods can also be used, such as the method described in U.S. Patent No. 5,843,701.

**[0082]** Various assays are provided, for example, assays suitable for phenotyping influenza A viruses. The assays may be directed to measuring a viral activity (e.g., infection, replication, and/or release of viral particles) or an enzymatic activity (e.g. polymerase activity). Viral activity assays may employ cells or samples infected with a virus or viral variant of which the activity is to be measured. The cells or samples may be obtained from a patient such as a human patient. Alternatively, the cells or samples may be cultured and infected with a virus or viral variant in vitro. Viral activity assays may employ a replicon-based system.

**[0083]** Enzymatic activity can be determined in cell-free or cell-based systems which generally include the enzyme of interest or a biologically active fragment or analog thereof and a substrate for the enzyme of interest.

**[0084]** In certain embodiments, the identified compound is formulated into a composition comprising the compound and a pharmaceutically acceptable carrier, adjuvant or vehicle. Preferably the composition contains the compound in an amount effective to reduce the

activity of an influenza A virus polymerase. Even more preferably the composition is formulated for administration to a patient. The compositions also may comprise an additional agent selected from an immunomodulatory agent; an anti-viral agent; a second inhibitor of influenza A virus polymerase; an inhibitor of another target in the influenza A virus life cycle; or combinations thereof. The various compositions are described in greater details below.

**[0085]** In another aspect, the present invention provides antibodies that are specific to an influenza A virus polymerase, in particular, an influenza A virus polymerase with one or more amino acids altered as compared to a wild type influenza A virus polymerase. The term "antibody" is used in the broadest sense and specifically covers, without limitation, intact monoclonal antibodies, polyclonal antibodies, chimeric antibodies, multispecific antibodies (e.g., bispecific antibodies) formed from at least two intact antibodies, and antibody fragments, so long as they exhibit the desired biological activity. The term "immunoglobulin" includes a variety of structurally related proteins that are not necessarily antibodies.

**[0086]** "Antibody fragments" comprise a portion of an intact antibody, preferably the antigen-binding or variable region of the intact antibody. Examples of antibody fragments include Fab, Fab', F(ab')2, and Fv fragments; diabodies; linear antibodies; single-chain antibody molecules; and multispecific antibodies formed from antibody fragments. "Single-chain Fv" or "scFv" antibody fragments comprise the VH and VL domains of an antibody, wherein these domains are present in a single polypeptide chain. Preferably, the Fv polypeptide further comprises a polypeptide linker between the VH and VL domains that enables the scFv to form the desired structure for antigen binding.

**[0087]** The term "diabodies" refers to small antibody fragments with two antigen binding sites, which fragments comprise a heavy-chain variable domain (VH) connected to a light-chain variable domain (VL) in the same polypeptide chain (VHVL). By using a linker that is too short to allow pairing between the two domains on the same chain, the domains are forced to pair with the complementary domains of another chain and create two antigen-binding sites.

**[0088]** An antibody against a variant influenza A virus polymerase may be developed from a known antibody against an influenza A virus protein, for example through molecular evolution. Amino acid sequence variants are prepared by introducing appropriate nucleotide changes into the DNA of a known antibody, or by peptide synthesis. Such variants include, for example, deletions from, and/or insertions into and/or substitutions of, residues within the

amino acid sequences of the known antibody. Any combination of deletion, insertion, and substitution is made to arrive at the final construct, provided that the final construct possesses the desired characteristics. The amino acid changes also may alter post-translational processes of the antibody, such as changing the number or position of glycosylation sites.

**[0089]** An antibody of the invention may have diagnostic as well as therapeutic applications. In certain embodiments, an antibody of the invention is labeled. The various antibodies of the present disclosure can be used to detect or measure the expression of a variant influenza A virus polymerase, and therefore, they are also useful in applications such as cell sorting and imaging (e.g., flow cytometry, and fluorescence activated cell sorting), for diagnostic or research purposes. As used herein, the terms "label" or "labeled" refers to incorporation of another molecule in the antibody. In one embodiment, the label is a detectable marker, e.g., incorporation of a radiolabeled amino acid or attachment to a polypeptide of biotinyl moieties that can be detected by marked avidin (e.g., streptavidin containing a fluorescent marker or enzymatic activity that can be detected by optical or colorimetric methods). In another embodiment, the label or marker can be therapeutic, e.g., a drug conjugate or toxin. Various methods of labeling polypeptides and glycoproteins are known in the art and may be used. Examples of labels for polypeptides include, but are not limited to, the following: radioisotopes or radionuclides (e.g., $^{3}H$, $^{14}C$, $^{15}N$, $^{35}S$, $^{90}Y$, $^{99}Tc$, $^{111}In$, $^{125}1$, $^{131}I$), fluorescent labels (e.g., FITC, rhodamine, lanthanide phosphors), enzymatic labels (e.g., horseradish peroxidase, beta-galactosidase, luciferase, alkaline phosphatase), chemiluminescent markers, biotinyl groups, predetermined polypeptide epitopes recognized by a secondary reporter (e.g., leucine zipper pair sequences, binding sites for secondary antibodies, metal binding domains, epitope tags), magnetic agents, such as gadolinium chelates, toxins such as pertussis toxin, taxol, cytochalasin B, gramicidin D, ethidium bromide, emetine, mitomycin, etoposide, tenoposide, vincristine, vinblastine, colchicin, doxorubicin, daunorubicin, dihydroxy anthracin dione, mitoxantrone, mithramycin, actinomycin D, 1-dehydrotestosterone, glucocorticoids, procaine, tetracaine, lidocaine, propranolol, and puromycin and analogs or homologs thereof. In some embodiments, labels are attached by spacer arms of various lengths to reduce potential steric hindrance.

**[0090]** In certain aspects, kits for use in detecting the presence of an influenza A virus, a variant influenza A virus polynucleotide, or a variant influenza A virus polymerase in a biological sample can also be prepared. Such kits may include an antibody that recognizes a variant influenza A virus polymerase of the invention, as well as one or more ancillary reagents suitable for detecting the presence of a complex between the antibody and the

variant polymerase or a portion thereof. Alternatively, such kits may include a probe or primer of the invention, such a probe or primer can hybridize with a variant influenza A virus polynucleotide of the invention under stringent conditions. A probe or primer of the invention may be suitable for PCR or RT-PCR that can be employed to detect a subject of interest. Alternatively, such kits may be based on PCR or non-PCR based influenza A virus diagnostic kits available commercially.

**[0091]** Another aspect of the invention provides pharmaceutical compositions or formulations including a compound of the invention, for example, a secondary compound that is identified as being able to rescue the activity of a polymerase inhibitor, or a compound that is identified as effective against an influenza A virus variant (e.g., capable of reducing replication of the viral variant) and/or a variant influenza A virus polymerase (e.g., capable of reducing the enzymatic activity of the variant polymerase).

**[0092]** Another aspect of the invention provides uses of a compound of the invention in the manufacture of a medicament, such as a medicament for treating an influenza A virus infection in a patient.

**[0093]** Another aspect of the invention provides methods for treating an influenza A virus infection in a patient. Such methods generally comprise administering to the patient a pharmaceutically or therapeutically effective amount of a compound of the invention alone or in combination (sequentially or contemporaneously) with another anti-viral agent. "Effective amount" of a compound or agent generally refers to those amounts effective to reproducibly reduce influenza A virus polymerase expression or activity, influenza A virus production, replication, or virulence, influenza A virus infection, or produce an amelioration or alleviation of one or more of the symptoms of influenza A virus infection in comparison to the levels of these parameters in the absence of such a compound or agent.

**[0094]** In another aspect, the methods and compositions of this invention include a polymerase inhibitor and another anti-viral agent, preferably an anti-influenza A virus agent. Combination therapy targeting influenza A virus is also described in WO 2010/148197.

**[0095]** As used herein, the terms "in combination" or "co-administration" can be used interchangeably to refer to the use of more than one therapy (e.g., one or more prophylactic and/or therapeutic agents). The use of the terms does not restrict the order in which therapies (e.g., prophylactic and/or therapeutic agents) are administered to a subject.

**[0096]** Coadministration encompasses administration of the first and second amounts of the compounds of the coadministration in an essentially simultaneous manner, such as in a single pharmaceutical composition, for example, capsule or tablet having a fixed ratio of first and

second amounts, or in multiple, separate capsules or tablets for each. In addition, such coadministration also encompasses use of each compound in a sequential manner in either order.

**[0097]** As used herein, the term "synergistic" refers to a combination of a compound of the invention and another therapy (e.g., a prophylactic or therapeutic agent), which is more effective than the additive effects of the therapies. A synergistic effect of a combination of therapies (e.g., a combination of prophylactic or therapeutic agents) can permit the use of lower dosages of one or more of the therapies and/or less frequent administration of said therapies to a subject. The ability to utilize lower dosages of a therapy (e.g., a prophylactic or therapeutic agent) and/or to administer said therapy less frequently can reduce the toxicity associated with the administration of said therapy to a subject without reducing the efficacy of said therapy in the prevention, management or treatment of a disorder. In addition, a synergistic effect can result in improved efficacy of agents in the prevention, management or treatment of a disorder. Finally, a synergistic effect of a combination of therapies (e.g., a combination of prophylactic or therapeutic agents) may avoid or reduce adverse or unwanted side effects associated with the use of either therapy alone.

**[0098]** The presence of a synergistic effect can be determined using suitable methods for assessing drug interaction. Suitable methods include, for example, the Sigmoid-Emax equation (Holford, N.H.G. and Scheiner, L.B., Clin. Pharmacokinet. 6: 429-453 (1981)), the equation of Loewe additivity (Loewe, S. and Muischnek, H., Arch. Exp. Pathol Pharmacol. 114: 313-326 (1926)) and the median-effect equation (Chou, T.C. and Talalay, P., Adv. Enzyme Regul. 22: 27-55 (1984)). Each equation referred to above can be applied with experimental data to generate a corresponding graph to aid in assessing the effects of the drug combination. The corresponding graphs associated with the equations referred to above are the concentration-effect curve, isobologram curve and combination index curve, respectively.

**[0099]** Specific examples that can be co-administered with a compound described herein include neuraminidase inhibitors, such as oseltamivir (Tamiflu®) and Zanamivir (Rlenza®), viral ion channel (M2 protein) blockers, such as amantadine (Symmetrel®) and rimantadine (Flumadine®), and antiviral drugs described in WO 2003/015798, including T-705 under development by Toyama Chemical of Japan. (See also Ruruta et al., Antiviral Research, 82: 95-102 (2009), "T-705 (flavipiravir) and related compounds: Novel broad-spectrum inhibitors of RNA viral infections"). In some embodiments, the compounds described herein can be co-administered with a traditional influenza vaccine.

**[0100]** Nothing herein limits the methods or combinations of this invention to any specific

dosage forms or regime. Thus, each component of a combination according to this invention may be administered separately, together, sequentially or simultaneously, or in any combination thereof.

**[0101]** Formulations, doses, and routes of administration for the foregoing molecules are well-known in the art. Alternatively, once a compound that exhibits influenza A virus antiviral activity, particularly antiviral activity against a drug-resistant strain of influenza A virus, has been identified, a pharmaceutically effective amount of that compound can be determined using techniques that are well-known to the skilled artisan. Thus, the appropriate formulations, dose(s) range, and dosing regimens, of such a compound can be easily determined by routine methods.

**[0102]** The compositions related to combination therapies of the present invention can be provided to a cell or cells, or to a human patient, either in separate pharmaceutically acceptable formulations administered simultaneously or sequentially, formulations containing more than one therapeutic agent, or by an assortment of single agent and multiple agent formulations. Regardless of the route of administration, these drug combinations form an anti-influenza A virus effective amount of components of the pharmaceutically acceptable formulations.

**[0103]** In the event of enhanced influenza A virus antiviral effectiveness of the present influenza A virus polymerase inhibitors in the presence of immunomodulators and immunostimulants, reduced amounts of these influenza A virus polymerase inhibitors can be employed in the methods and compositions contemplated herein. Such reduced amounts can be determined by routine monitoring of influenza A virus titers in infected patients undergoing therapy. This can be carried out by, for example, monitoring influenza A virus RNA in patients' serum by slot-blot, dot-blot, or RT-PCR techniques, or by measurement of influenza A virus surface or other antigens. Patients can be similarly monitored during combination therapy employing the influenza A virus polymerase inhibitors disclosed herein and other compounds having anti- influenza A virus activity to determine the lowest effective doses of each when used in combination.

**[0104]** Upon improvement of a patient's condition, a maintenance dose of a compound, composition or combination of this invention may be administered, if necessary. Subsequently, the dosage or frequency of administration, or both, may be reduced, as a function of the symptoms, to a level at which the improved condition is retained when the symptoms have been alleviated to the desired level, treatment should cease. Patients may, however, require intermittent treatment on a long-term basis upon any recurrence of disease

symptoms.

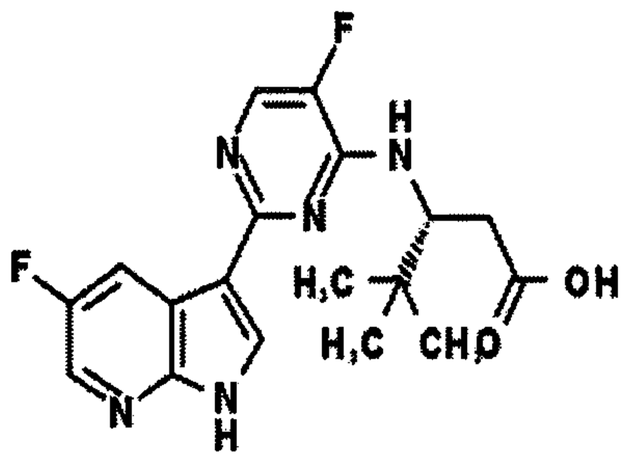
**[0105]** A specific dosage and treatment regimen for any particular patient will depend upon a variety of factors, including the activity of the specific compound employed, the age, body weight, general health, sex, diet, time of administration, rate of excretion, drug combination, and the judgment of the treating physician and the severity of the particular disease being treated. The amount of active ingredients will also depend upon the particular described compound and the presence or absence and the nature of the additional anti-viral agent in the composition.

**[0106]** According to another embodiment, the invention provides a method for treating a patient infected with or preventing infection by a virus characterized by a virally encoded influenza polymerase that is necessary for the life cycle of the virus by administering to said patient a pharmaceutically acceptable composition of this invention. Preferably, the methods of this invention are used to treat a patient suffering from an influenza A virus infection. Such treatment may completely eradicate the viral infection or reduce the severity thereof.

**[0107]** In another embodiment, the compounds of this invention may be used as laboratory tools to aid in the isolation of a virally encoded influenza A virus polymerase. This method comprises the steps of providing a compound of this invention attached to a solid support; contacting said solid support with a sample containing an influenza A virus polymerase under conditions that cause said polymerase to bind to said solid support; and eluting said influenza A virus polymerase from said solid support. Preferably, the viral polymerase isolated by this method is PB2 polymerase. More particularly, it is a mutant PB2 polymerase that is resistant to treatment by polymerase inhibitors. Exemplary such polymerase includes those described herein as having mutant (i.e., non-wild-type) residues at positions 306, 323, 324, 325, 357, 376, 404, 406, 431, and/or 510.

**[0108]** ***Compounds of the Invention***

**[0109]** In some embodiments, the influenza A virus polymerase inhibitor is Compound 1, or a pharmaceutically acceptable salt thereof for any of the uses specified above.



Compound 1

**[0110]** In yet another embodiment, the compounds described herein or pharmaceutically acceptable salts thereof can be used to reduce viral titre in a biological sample (e.g. an infected cell culture) or in humans (e.g. lung viral titre in a patient).

**[0111]** ***Pharmaceutically Acceptable Salts, Solvates, Chlatrates, Prodrugs and Other Derivatives***

**[0112]** The compounds described herein can exist in free form, or, where appropriate, as salts. Those salts that are pharmaceutically acceptable are of particular interest since they are useful in administering the compounds described below for medical purposes. Salts that are not pharmaceutically acceptable are useful in manufacturing processes, for isolation and purification purposes, and in some instances, for use in separating stereoisomeric forms of the compounds of the invention or intermediates thereof.

**[0113]** As used herein, the term "pharmaceutically acceptable salt" refers to salts of a compound which are, within the scope of sound medical judgment, suitable for use in contact with the tissues of humans and lower animals without undue side effects, such as, toxicity, irritation, allergic response and the like, and are commensurate with a reasonable benefit/risk ratio.

**[0114]** Pharmaceutically acceptable salts are well known in the art. For example, S. M. Berge et al., describe pharmaceutically acceptable salts in detail in J. Pharmaceutical Sciences, 1977, 66, 1-19, incorporated herein by reference. Pharmaceutically acceptable salts of the compounds described herein include those derived from suitable inorganic and organic acids and bases. These salts can be prepared in situ during the final isolation and purification of the compounds.

**[0115]** Where the compound described herein contains a basic group, or a sufficiently basic bioisostere, acid addition salts can be prepared by 1) reacting the purified compound in its

free-base form with a suitable organic or inorganic acid and 2) isolating the salt thus formed. In practice, acid addition salts might be a more convenient form for use and use of the salt amounts to use of the free basic form.

**[0116]** Examples of pharmaceutically acceptable, non-toxic acid addition salts are salts of an amino group formed with inorganic acids such as hydrochloric acid, hydrobromic acid, phosphoric acid, sulfuric acid and perchloric acid or with organic acids such as acetic acid, oxalic acid, maleic acid, tartaric acid, citric acid, succinic acid or malonic acid or by using other methods used in the art such as ion exchange. Other pharmaceutically acceptable salts include adipate, alginate, ascorbate, aspartate, benzenesulfonate, benzoate, bisulfate, borate, butyrate, camphorate, camphorsulfonate, citrate, cyclopentanepropionate, digluconate, dodecylsulfate, ethanesulfonate, formate, fumarate, glucoheptonate, glycerophosphate, glycolate, gluconate, glycolate, hemisulfate, heptanoate, hexanoate, hydrochloride, hydrobromide, hydroiodide, 2-hydroxy-ethanesulfonate, lactobionate, lactate, laurate, lauryl sulfate, malate, maleate, malonate, methanesulfonate, 2-naphthalenesulfonate, nicotinate, nitrate, oleate, oxalate, palmitate, palmoate, pectinate, persulfate, 3-phenylpropionate, phosphate, picrate, pivalate, propionate, salicylate, stearate, succinate, sulfate, tartrate, thiocyanate, p-toluenesulfonate, undecanoate, valerate salts, and the like.

**[0117]** Where the compound described herein contains a carboxy group or a sufficiently acidic bioisostere, base addition salts can be prepared by 1) reacting the purified compound in its acid form with a suitable organic or inorganic base and 2) isolating the salt thus formed. In practice, use of the base addition salt might be more convenient and use of the salt form inherently amounts to use of the free acid form. Salts derived from appropriate bases include alkali metal (e.g., sodium, lithium, and potassium), alkaline earth metal (e.g., magnesium and calcium), ammonium and N+(C1-4alkyl)4 salts. This invention also envisions the quaternization of any basic nitrogen-containing groups of the compounds disclosed herein. Water or oil-soluble or dispersible products may be obtained by such quaternization.

**[0118]** Basic addition salts include pharmaceutically acceptable metal and amine salts. Suitable metal salts include the sodium, potassium, calcium, barium, zinc, magnesium, and aluminum. The sodium and potassium salts are usually preferred. Further pharmaceutically acceptable salts include, when appropriate, nontoxic ammonium, quaternary ammonium, and amine cations formed using counterions such as halide, hydroxide, carboxylate, sulfate, phosphate, nitrate, lower alkyl sulfonate and aryl sulfonate. Suitable inorganic base addition salts are prepared from metal bases which include sodium hydride, sodium hydroxide, potassium hydroxide, calcium hydroxide, aluminum hydroxide, lithium hydroxide,

magnesium hydroxide, zinc hydroxide and the like. Suitable amine base addition salts are prepared from amines which are frequently used in medicinal chemistry because of their low toxicity and acceptability for medical use. Ammonia, ethylenediamine, N-methyl-glucamine, lysine, arginine, ornithine, choline, N, N'-dibenzylethylenediamine, chloroprocaine, diethanolamine, procaine, N-benzylphenethylamine, diethylamine, piperazine, tris(hydroxymethyl)-aminomethane, tetramethylammonium hydroxide, triethylamine, dibenzylamine, ephenamine, dehydroabietylamine, N-ethylpiperidine, benzylamine, tetramethylammonium, tetraethylammonium, methylamine, dimethylamine, trimethylamine, ethylamine, basic amino acids, dicyclohexylamine and the like.

**[0119]** Other acids and bases, while not in themselves pharmaceutically acceptable, may be employed in the preparation of salts useful as intermediates in obtaining the compounds described herein and their pharmaceutically acceptable acid or base addition salts.

**[0120]** It should be understood that this invention includes mixtures/combinations of different pharmaceutically acceptable salts and also mixtures/combinations of compounds in free form and pharmaceutically acceptable salts.

**[0121]** In addition to the compounds described herein, pharmaceutically acceptable solvates (e.g., hydrates) and clathrates of these compounds may also be employed in compositions to treat or prevent the herein identified disorders.

**[0122]** As used herein, the term "pharmaceutically acceptable solvate," is a solvate formed from the association of one or more pharmaceutically acceptable solvent molecules to one of the compounds described herein. The term solvate includes hydrates (e.g., hemihydrate, monohydrate, dihydrate, trihydrate, tetrahydrate, and the like).

**[0123]** As used herein, the term "hydrate" means a compound described herein or a salt thereof that further includes a stoichiometric or non-stoichiometric amount of water bound by non-covalent intermolecular forces.

**[0124]** As used herein, the term "clathrate" means a compound described herein or a salt thereof in the form of a crystal lattice that contains spaces (e.g., channels) that have a guest molecule (e.g., a solvent or water) trapped within.

**[0125]** In addition to the compounds described herein, pharmaceutically acceptable derivatives or prodrugs of these compounds may also be employed in compositions to treat or prevent the herein identified disorders.

**[0126]** A "pharmaceutically acceptable derivative or prodrug" includes any pharmaceutically acceptable ester, salt of an ester or other derivative or salt thereof of a compound described herein which, upon administration to a recipient, is capable of

providing, either directly or indirectly, a compound described herein or an inhibitorily active metabolite or residue thereof. Particularly favored derivatives or prodrugs are those that increase the bioavailability of the compounds when such compounds are administered to a patient (e.g., by allowing an orally administered compound to be more readily absorbed into the blood) or which enhance delivery of the parent compound to a biological compartment (e.g., the brain or lymphatic system) relative to the parent species.

**[0127]** As used herein and unless otherwise indicated, the term "prodrug" means a derivative of a compound that can hydrolyze, oxidize, or otherwise react under biological conditions (in vitro or in vivo) to provide a compound described herein. Prodrugs may become active upon such reaction under biological conditions, or they may have activity in their unreacted forms. Examples of prodrugs contemplated in this invention include, but are not limited to, analogs or derivatives of compounds of the invention that comprise biohydrolyzable moieties such as biohydrolyzable amides, biohydrolyzable esters, biohydrolyzable carbamates, biohydrolyzable carbonates, biohydrolyzable ureides, and biohydrolyzable phosphate analogues. Other examples of prodrugs include derivatives of compounds described herein that comprise -NO, -$NO^2$, -ONO, or -$ONO^2$ moieties. Prodrugs can typically be prepared using well-known methods, such as those described by Burger's Medicinal Chemistry and Drug Discovery (1995) 172-178, 949-982 (Manfred E. Wolff ed., 5th ed).

**[0128]** A "pharmaceutically acceptable derivative" is an adduct or derivative which, upon administration to a patient in need, is capable of providing, directly or indirectly, a compound as otherwise described herein, or a metabolite or residue thereof. Examples of pharmaceutically acceptable derivatives include, but are not limited to, esters and salts of such esters.

**[0129]** Pharmaceutically acceptable prodrugs of the compounds described herein include, without limitation, esters, amino acid esters, phosphate esters, metal salts and sulfonate esters.

**[0130]** ***Pharmaceutical Compositions***

**[0131]** The compounds described herein can be formulated into pharmaceutical compositions that further comprise a pharmaceutically acceptable carrier, diluent, adjuvant or vehicle. In one embodiment, the present invention relates to a pharmaceutical composition comprising a compound of the invention described above, and a pharmaceutically acceptable carrier, diluent, adjuvant or vehicle. In one embodiment, the present invention is a pharmaceutical composition comprising an effective amount of a compound of the present

invention or a pharmaceutically acceptable salt thereof and a pharmaceutically acceptable carrier, diluent, adjuvant or vehicle. Pharmaceutically acceptable carriers include, for example, pharmaceutical diluents, excipients or carriers suitably selected with respect to the intended form of administration, and consistent with conventional pharmaceutical practices.

**[0132]** An "effective amount" includes a "therapeutically effective amount" and a "prophylactically effective amount". The term "therapeutically effective amount" refers to an amount effective in treating and/or ameliorating an influenza virus infection in a patient infected with influenza. The term "prophylactically effective amount" refers to an amount effective in preventing and/or substantially lessening the chances or the size of influenza virus infection outbreak. Specific examples of effective amounts are described above in the section entitled Uses of Disclosed Compounds.

**[0133]** A pharmaceutically acceptable carrier may contain inert ingredients which do not unduly inhibit the biological activity of the compounds. The pharmaceutically acceptable carriers should be biocompatible, e.g., non-toxic, non-inflammatory, non-immunogenic or devoid of other undesired reactions or side-effects upon the administration to a subject. Standard pharmaceutical formulation techniques can be employed.

**[0134]** The pharmaceutically acceptable carrier, adjuvant, or vehicle, as used herein, includes any and all solvents, diluents, or other liquid vehicle, dispersion or suspension aids, surface active agents, isotonic agents, thickening or emulsifying agents, preservatives, solid binders, lubricants and the like, as suited to the particular dosage form desired. Remington's Pharmaceutical Sciences, Sixteenth Edition, E. W. Martin (Mack Publishing Co., Easton, Pa., 1980) discloses various carriers used in formulating pharmaceutically acceptable compositions and known techniques for the preparation thereof. Except insofar as any conventional carrier medium is incompatible with the compounds described herein, such as by producing any undesirable biological effect or otherwise interacting in a deleterious manner with any other component(s) of the pharmaceutically acceptable composition, its use is contemplated to be within the scope of this invention. As used herein, the phrase "side effects" encompasses unwanted and adverse effects of a therapy (e.g., a prophylactic or therapeutic agent). Side effects are always unwanted, but unwanted effects are not necessarily adverse. An adverse effect from a therapy (e.g., prophylactic or therapeutic agent) might be harmful or uncomfortable or risky. Side effects include, but are not limited to fever, chills, lethargy, gastrointestinal toxicities (including gastric and intestinal ulcerations and erosions), nausea, vomiting, neurotoxicities, nephrotoxicities, renal toxicities (including such conditions as papillary necrosis and chronic interstitial nephritis), hepatic toxicities

(including elevated serum liver enzyme levels), myelotoxicities (including leukopenia, myelosuppression, thrombocytopenia and anemia), dry mouth, metallic taste, prolongation of gestation, weakness, somnolence, pain (including muscle pain, bone pain and headache), hair loss, asthenia, dizziness, extra-pyramidal symptoms, akathisia, cardiovascular disturbances and sexual dysfunction.

**[0135]** Some examples of materials which can serve as pharmaceutically acceptable carriers include, but are not limited to, ion exchangers, alumina, aluminum stearate, lecithin, serum proteins (such as human serum albumin), buffer substances (such as tween 80, phosphates, glycine, sorbic acid, or potassium sorbate), partial glyceride mixtures of saturated vegetable fatty acids, water, salts or electrolytes (such as protamine sulfate, disodium hydrogen phosphate, potassium hydrogen phosphate, sodium chloride, or zinc salts), colloidal silica, magnesium trisilicate, polyvinyl pyrrolidone, polyacrylates, waxes, polyethylene-polyoxypropylene-block polymers, methylcellulose, hydroxypropyl methylcellulose, wool fat, sugars such as lactose, glucose and sucrose; starches such as corn starch and potato starch; cellulose and its derivatives such as sodium carboxymethyl cellulose, ethyl cellulose and cellulose acetate; powdered tragacanth; malt; gelatin; talc; excipients such as cocoa butter and suppository waxes; oils such as peanut oil, cottonseed oil; safflower oil; sesame oil; olive oil; corn oil and soybean oil; glycols; such a propylene glycol or polyethylene glycol; esters such as ethyl oleate and ethyl laurate; agar; buffering agents such as magnesium hydroxide and aluminum hydroxide; alginic acid; pyrogen-free water; isotonic saline; Ringer's solution; ethyl alcohol, and phosphate buffer solutions, as well as other non-toxic compatible lubricants such as sodium lauryl sulfate and magnesium stearate, as well as coloring agents, releasing agents, coating agents, sweetening, flavoring and perfuming agents, preservatives and antioxidants can also be present in the composition, according to the judgment of the formulator.

**[0136]** ***Administration Methods***

**[0137]** The compounds and pharmaceutically acceptable compositions described above can be administered to humans and other animals orally, rectally, parenterally, intracisternally, intravaginally, intraperitoneally, topically (as by powders, ointments, or drops), bucally, as an oral or nasal spray, or the like, depending on the severity of the infection being treated.

**[0138]** Liquid dosage forms for oral administration include, but are not limited to, pharmaceutically acceptable emulsions, microemulsions, solutions, suspensions, syrups and elixirs. In addition to the active compounds, the liquid dosage forms may contain inert diluents commonly used in the art such as, for example, water or other solvents, solubilizing

agents and emulsifiers such as ethyl alcohol, isopropyl alcohol, ethyl carbonate, ethyl acetate, benzyl alcohol, benzyl benzoate, propylene glycol, 1,3-butylene glycol, dimethylformamide, oils (in particular, cottonseed, groundnut, corn, germ, olive, castor, and sesame oils), glycerol, tetrahydrofurfuryl alcohol, polyethylene glycols and fatty acid esters of sorbitan, and mixtures thereof. Besides inert diluents, the oral compositions can also include adjuvants such as wetting agents, emulsifying and suspending agents, sweetening, flavoring, and perfuming agents.

**[0139]** Injectable preparations, for example, sterile injectable aqueous or oleaginous suspensions may be formulated according to the known art using suitable dispersing or wetting agents and suspending agents. The sterile injectable preparation may also be a sterile injectable solution, suspension or emulsion in a nontoxic parenterally acceptable diluent or solvent, for example, as a solution in 1,3-butanediol. Among the acceptable vehicles and solvents that may be employed are water, Ringer's solution, U.S.P. and isotonic sodium chloride solution. In addition, sterile, fixed oils are conventionally employed as a solvent or suspending medium. For this purpose any bland fixed oil can be employed including synthetic mono- or diglycerides. In addition, fatty acids such as oleic acid are used in the preparation of injectables.

**[0140]** The injectable formulations can be sterilized, for example, by filtration through a bacterial-retaining filter, or by incorporating sterilizing agents in the form of sterile solid compositions which can be dissolved or dispersed in sterile water or other sterile injectable medium prior to use.

**[0141]** In order to prolong the effect of a compound described herein, it is often desirable to slow the absorption of the compound from subcutaneous or intramuscular injection. This may be accomplished by the use of a liquid suspension of crystalline or amorphous material with poor water solubility. The rate of absorption of the compound then depends upon its rate of dissolution that, in turn, may depend upon crystal size and crystalline form. Alternatively, delayed absorption of a parenterally administered compound form is accomplished by dissolving or suspending the compound in an oil vehicle. Injectable depot forms are made by forming microencapsule matrices of the compound in biodegradable polymers such as polylactide-polyglycolide. Depending upon the ratio of compound to polymer and the nature of the particular polymer employed, the rate of compound release can be controlled. Examples of other biodegradable polymers include poly(orthoesters) and poly(anhydrides). Depot injectable formulations are also prepared by entrapping the compound in liposomes or microemulsions that are compatible with body tissues.

**[0142]** Compositions for rectal or vaginal administration are specifically suppositories which can be prepared by mixing the compounds described herein with suitable non-irritating excipients or carriers such as cocoa butter, polyethylene glycol or a suppository wax which are solid at ambient temperature but liquid at body temperature and therefore melt in the rectum or vaginal cavity and release the active compound.

**[0143]** Solid dosage forms for oral administration include capsules, tablets, pills, powders, and granules. In such solid dosage forms, the active compound is mixed with at least one inert, pharmaceutically acceptable excipient or carrier such as sodium citrate or dicalcium phosphate and/or a) fillers or extenders such as starches, lactose, sucrose, glucose, mannitol, and silicic acid; b) binders such as, for example, carboxymethylcellulose, alginates, gelatin, polyvinylpyrrolidinone, sucrose, and acacia; c) humectants such as glycerol; d) disintegrating agents such as agar--agar, calcium carbonate, potato or tapioca starch, alginic acid, certain silicates, and sodium carbonate; e) solution retarding agents such as paraffin; f) absorption accelerators such as quaternary ammonium compounds; g) wetting agents such as, for example, cetyl alcohol and glycerol monostearate; h) absorbents such as kaolin and bentonite clay; and i) lubricants such as talc, calcium stearate, magnesium stearate, solid polyethylene glycols, sodium lauryl sulfate, and mixtures thereof. In the case of capsules, tablets and pills, the dosage form may also comprise buffering agents.

**[0144]** Solid compositions of a similar type may also be employed as fillers in soft and hard-filled gelatin capsules using such excipients as lactose or milk sugar as well as high molecular weight polyethylene glycols and the like. The solid dosage forms of tablets, dragees, capsules, pills, and granules can be prepared with coatings and shells such as enteric coatings and other coatings well known in the pharmaceutical formulating art. They may optionally contain opacifying agents and can also be of a composition that they release the active ingredient(s) only, or preferentially, in a certain part of the intestinal tract, optionally, in a delayed manner. Examples of embedding compositions that can be used include polymeric substances and waxes. Solid compositions of a similar type may also be employed as fillers in soft and hard-filled gelatin capsules using such excipients as lactose or milk sugar as well as high molecular weight polyethylene glycols and the like.

**[0145]** The active compounds can also be in microencapsulated form with one or more excipients as noted above. The solid dosage forms of tablets, dragees, capsules, pills, and granules can be prepared with coatings and shells such as enteric coatings, release controlling coatings and other coatings well known in the pharmaceutical formulating art. In such solid dosage forms the active compound may be admixed with at least one inert diluent such as

sucrose, lactose or starch. Such dosage forms may also comprise, as is normal practice, additional substances other than inert diluents, e.g., tableting lubricants and other tableting aids such a magnesium stearate and microcrystalline cellulose. In the case of capsules, tablets and pills, the dosage forms may also comprise buffering agents. They may optionally contain opacifying agents and can also be of a composition that they release the active ingredient(s) only, or preferentially, in a certain part of the intestinal tract, optionally, in a delayed manner. Examples of embedding compositions that can be used include polymeric substances and waxes.

**[0146]** Dosage forms for topical or transdermal administration of a compound described herein include ointments, pastes, creams, lotions, gels, powders, solutions, sprays, inhalants or patches. The active component is admixed under sterile conditions with a pharmaceutically acceptable carrier and any needed preservatives or buffers as may be required. Ophthalmic formulation, eardrops, and eye drops are also contemplated as being within the scope of this invention. Additionally, the present invention contemplates the use of transdermal patches, which have the added advantage of providing controlled delivery of a compound to the body. Such dosage forms can be made by dissolving or dispensing the compound in the proper medium. Absorption enhancers can also be used to increase the flux of the compound across the skin. The rate can be controlled by either providing a rate controlling membrane or by dispersing the compound in a polymer matrix or gel.

**[0147]** The compositions described herein may be administered orally, parenterally, by inhalation spray, topically, rectally, nasally, buccally, vaginally or via an implanted reservoir. The term "parenteral" as used herein includes, but is not limited to, subcutaneous, intravenous, intramuscular, intra-articular, intra-synovial, intrasternal, intrathecal, intrahepatic, intralesional and intracranial injection or infusion techniques. Specifically, the compositions are administered orally, intraperitoneally or intravenously.

**[0148]** Sterile injectable forms of the compositions described herein may be aqueous or oleaginous suspension. These suspensions may be formulated according to techniques known in the art using suitable dispersing or wetting agents and suspending agents. The sterile injectable preparation may also be a sterile injectable solution or suspension in a non-toxic parenterally-acceptable diluent or solvent, for example as a solution in 1,3-butanediol. Among the acceptable vehicles and solvents that may be employed are water, Ringer's solution and isotonic sodium chloride solution. In addition, sterile, fixed oils are conventionally employed as a solvent or suspending medium. For this purpose, any bland fixed oil may be employed including synthetic mono- or di-glycerides. Fatty acids, such as

oleic acid and its glyceride derivatives are useful in the preparation of injectables, as are natural pharmaceutically-acceptable oils, such as olive oil or castor oil, especially in their polyoxyethylated versions. These oil solutions or suspensions may also contain a long-chain alcohol diluent or dispersant, such as carboxymethyl cellulose or similar dispersing agents which are commonly used in the formulation of pharmaceutically acceptable dosage forms including emulsions and suspensions. Other commonly used surfactants, such as Tweens, Spans and other emulsifying agents or bioavailability enhancers which are commonly used in the manufacture of pharmaceutically acceptable solid, liquid, or other dosage forms may also be used for the purposes of formulation.

**[0149]** The pharmaceutical compositions described herein may be orally administered in any orally acceptable dosage form including, but not limited to, capsules, tablets, aqueous suspensions or solutions. In the case of tablets for oral use, carriers commonly used include, but are not limited to, lactose and corn starch. Lubricating agents, such as magnesium stearate, are also typically added. For oral administration in a capsule form, useful diluents include lactose and dried cornstarch. When aqueous suspensions are required for oral use, the active ingredient is combined with emulsifying and suspending agents. If desired, certain sweetening, flavoring or coloring agents may also be added.

**[0150]** Alternatively, the pharmaceutical compositions described herein may be administered in the form of suppositories for rectal administration. These can be prepared by mixing the agent with a suitable non-irritating excipient which is solid at room temperature but liquid at rectal temperature and therefore will melt in the rectum to release the drug. Such materials include, but are not limited to, cocoa butter, beeswax and polyethylene glycols.

**[0151]** The pharmaceutical compositions described herein may also be administered topically, especially when the target of treatment includes areas or organs readily accessible by topical application, including diseases of the eye, the skin, or the lower intestinal tract. Suitable topical formulations are readily prepared for each of these areas or organs.

**[0152]** Topical application for the lower intestinal tract can be effected in a rectal suppository formulation (see above) or in a suitable enema formulation. Topically-transdermal patches may also be used.

**[0153]** For topical applications, the pharmaceutical compositions may be formulated in a suitable ointment containing the active component suspended or dissolved in one or more carriers. Carriers for topical administration of the compounds of this invention include, but are not limited to, mineral oil, liquid petrolatum, white petrolatum, propylene glycol, polyoxyethylene, polyoxypropylene compound, emulsifying wax and water. Alternatively,

the pharmaceutical compositions can be formulated in a suitable lotion or cream containing the active components suspended or dissolved in one or more pharmaceutically acceptable carriers. Suitable carriers include, but are not limited to, mineral oil, sorbitan monostearate, polysorbate 60, cetyl esters wax, cetearyl alcohol, 2 octyldodecanol, benzyl alcohol and water.

**[0154]** For ophthalmic use, the pharmaceutical compositions may be formulated as micronized suspensions in isotonic, pH adjusted sterile saline, or, specifically, as solutions in isotonic, pH adjusted sterile saline, either with or without a preservative such as benzylalkonium chloride. Alternatively, for ophthalmic uses, the pharmaceutical compositions may be formulated in an ointment such as petrolatum.

**[0155]** The pharmaceutical compositions may also be administered by nasal aerosol or inhalation. Such compositions are prepared according to techniques well-known in the art of pharmaceutical formulation and may be prepared as solutions in saline, employing benzyl alcohol or other suitable preservatives, absorption promoters to enhance bioavailability, fluorocarbons, and/or other conventional solubilizing or dispersing agents.

**[0156]** The compounds for use in the methods of the invention can be formulated in unit dosage form. The term "unit dosage form" refers to physically discrete units suitable as unitary dosage for subjects undergoing treatment, with each unit containing a predetermined quantity of active material calculated to produce the desired therapeutic effect, optionally in association with a suitable pharmaceutical carrier. The unit dosage form can be for a single daily dose or one of multiple daily doses (e.g., about 1 to 4 or more times per day). When multiple daily doses are used, the unit dosage form can be the same or different for each dose.

**[0157]** ***Exemplification***

**[0158]** The disclosure now being generally described, it will be more readily understood by reference to the following examples, which are included merely for purposes of illustration of certain aspects and embodiments of the present disclosure, and are not intended to limit the disclosure.

**[0159]** EXAMPLES

**[0160]** **Example 1: Identification of Influenza A Virus Variants**

**[0161]** In vitro selection experiments were performed on 3 influenza viruses: A/Puerto Rico/8/34, a widely characterized H1N1 laboratory strain, A/California/07/2009, a contemporary pandemic swine origin H1N1 strain, and A/Wisconsin/67/2005, a H3N2 strain selected for use in Phase 2A clinical trials. (Table 1.) Antiviral activity of Compound 1 against wild-type and variant influenza isolates was measured using MDCK cells in a three

day cytopathic effect (CPE)-based assay. Variants with reduced sensitivity to Compound 1 were selected in vitro by infecting replicate wells containing MDCK cells with influenza virus at an MOI of 1.0, and monitoring the development of CPE as a surrogate for viral replication. Selection experiments were performed in 384-well plates using 32 replicate wells at each of eight concentrations of Compound 1, ranging from 1x to 128x the $EC_{50}$. Virus was sampled from the supernatant and passaged to new cells. Supernatants from wells showing virus growth in the presence of inhibitor were used to grow small virus stocks, which were characterized for Compound 1 sensitivity and replication capacity. For viruses with reduced sensitivity to Compound 1, viral RNA was extracted, reverse transcribed, and PCR-amplified, followed by Sanger-based population sequencing of the PA, PB1 and PB2 coding regions. Confirmation of the effect of the primary amino acid changes on virus sensitivity to Compound 1 was performed using the reverse genetics system, and natural frequency of the identified variants was analyzed.

**[0162]** Variants with reduced sensitivity to Compound 1 were isolated by repeated passage of A/Puerto Rico/8/34, A/California/07/2009, or A/Wisconsin/67/2005 influenza virus strains in the presence of a range of concentrations of inhibitor. With each selection, variants that conferred resistance to Compound 1 were identified in the cap-binding pocket of the PB2 protein. Isolation of resistance variants in the cap binding pocket supports the mechanism of action of Compound 1 as an inhibitor of the essential 'cap-snatching' activity of the influenza polymerase complex. Variants at PB2 amino acid positions 306, 323, 324, 325, 357, 376, 404, 406, 431, and 510 conferred 3.5 to >767 fold-resistance, but were not commonly observed in recent or historical human influenza sequences (frequency below 0.01 %).

**[0163]** Variants with reduced susceptibility to Compound 1 showed specific changes in the influenza virus PB2 gene segment but not in PA or PB1. Variants at PB2 amino acid positions Q306, F323, S324, F325, H357, K376, F404, Q406, M431, and N510 showed greater than 10-fold shifts in sensitivity to Compound 1. Reverse genetics-generated viruses containing these PB2 alterations confirmed the reduced sensitivity to Compound 1.

**[0164]** Most of the selected variants with reduced susceptibility to Compound 1 (at amino acid positions F323, S324, F325, H357, K376, F404, Q406, and M431) are located within the PB2 cap-binding region. The others, at Q306 and N510, are located in PB2 regions for which structural information is not available.

**[0165]** The amino acid changes associated with reduced susceptibility to Compound 1 are rare in naturally occurring influenza strains. The primary Compound 1-selected variants

were not observed in a survey dataset of approximately 9000 PB2 sequences from naturally occurring human isolates.

**[0166] Table 1 Table of Viruses Used for Selection of Variants with Reduced Sensitivity to Compound 1**

| Virus Name | Subtype | Source |
|---|---|---|
| A/Puerto Rico/8/34 | A/H1N1 | ATCC, VR-1469 |
| A/California/07/2009 | A/H1N1 | IRR, FR-201 |
| A/Wisconsin/67/2005 | A/H3N2 | IRR, FR-397 |
| ATCC: American Type Culture Collection; IRR: Influenza Resource Reagents | | |

**[0167] Example 2: Compounds, Growth Media and Media Supplements**

**[0168]** Compound 1 was synthesized at Vertex Pharmaceuticals Incorporated, dissolved in 100 % DMSO at a concentration of 10 mM and stored at -20 °C. DMEM (catalog number 11960), 200 mM L-glutamine (catalog number 25030-081), penicillin-streptomycin liquid (catalog number 15140-122) and HEPES buffer (catalog number 15630) were purchased from Invitrogen (Carlsbad, CA, USA). Fetal bovine serum (FBS; catalog numbers F4135 or 10091-148) was purchased from Sigma-Aldrich (St Louis, MO, USA), or Invitrogen, respectively. Dimethyl sulfoxide (DMSO; catalog number D2650) and Ex-CELL serum-free medium (catalog number M8303) were purchased from Sigma-Aldrich. CellTiterGlo® (catalog number G7573) was purchased from Promega (Madison, WI, USA). Tolylsulfonyl phenylalanyl chloromethyl ketone (TPCK)-treated trypsin (catalog number 22725) was purchased from USB Corporation (Affymetrix, Fremont, CA, USA).

**[0169] Example 3: Viral Stocks**

**[0170]** Influenza virus A/Puerto Rico/8/34 (American Type Culture Collection, VR-95, Manassas, VA, USA), A/California/07/2009 (Influenza Reagent Resources, FR-201, Manassas, VA, USA) and A/Wisconsin/67/2005 (IRR, FR-397) stocks were prepared by standard methods (World Health Organization). Briefly, MDCK cells (CCL-34, ATCC) were maintained in DMEM supplemented with 2 mM L glutamine, 1X non essential amino acids, 100 U/mL penicillin, 100 µg/mL streptomycin (complete DMEM; cDMEM) with 10 % FBS. Cells were infected at low multiplicity of infection (MOI) in cDMEM with 1 µg/mL TPCK-treated trypsin (viral growth medium; VGM) for approximately 48 h, after which the supernatant was harvested by centrifuged at 650 x g for 10 min with a Beckman GS-6R centrifuge. Virus stocks were frozen at –80 °C until used. To adapt A/Wisconsin/67/2005 to infection of MDCK cells, the virus was serially passaged in MDCK cells 10 times, plaque

purified and grown up as stated above. The $TCID_{50}$ (amount of infective agent that will produce pathological change in 50 % of cell cultures) infectious titer was determined by testing serial dilutions of the virus stock on MDCK cells in a 4-day cytopathic effect-based assay, with the results calculated by the Karber method.

**[0171] Table 2: Equipment.**

| Item | Model | Company | Location |
|---|---|---|---|
| Centrifuge | GS-6R | Beckman Coulter | Brea, CA, USA |
| Incubator | NU-8700 | Nuaire | Plymouth, MN, USA |
| Liquid Handler | Biomek FX | Beckman Coulter | Brea, CA, USA |
| Luminometer | EnVision 2103 Multilabel Plate Reader | PerkinElmer | Waltham, MA, USA |
| Spectrophotometer | NanoDrop, 8000 v.1.1 | Thermo Fisher Scientific | Waltham, MA, USA |

**[0172] Table 3: Software.**

| Purpose | Software | Company | Location |
|---|---|---|---|
| CPE Assay | Condoseo | Genedata | Basel, Switzerland |
| Data Storage and $TCID_{50}$ | Excel | Microsoft | Redmond, WA, USA |
| Reporting | Word | Microsoft | Redmond, WA, USA |
| Sequence Analysis | Mutation Surveyor v3.20 | SoftGenetics | State College, PA, USA |

**[0173] Example 4: In Vitro Selection of Influenza Variants**

**[0174]** A Biomek FX liquid handler (Beckman Coulter, Brea, CA, USA) was used to plate MDCK cells ($4x10^5$ cells/mL) into black, clear bottom, 384-well plates at a density of $2x10^4$ cells per well in 50 µL of viral growth media (VGM: Dulbecco's modified eagle medium (DMEM) supplemented with penicillin/streptomycin, L-glutamine, HEPES and 1 µg/mL TPCK-treated trypsin). Cells were incubated for 5 h at 37 °C, 5 % $CO_2$, with humidity to allow cells to adhere and form a monolayer. Using a Biomek FX, 40 µL of media was removed, while 25 µL of VGM containing diluted drug (final DMSO concentration of 0.5 % DMSO) and 15 µL of virus at an MOI of 1 (20,000 $TCID_{50}$/well) was added, for a total of

50 µL. Drug was added to 32 replicate wells at each of eight concentrations of Compound 1, ranging from 1x to 128x the $EC_{50}$. Internal controls consisted of 64 wells containing cells only and 64 wells with virus infected cells in the absence of compound. Plates were incubated at 37 °C, 5 % $CO_2$, with saturating humidity, for 72 h. After incubation, 20 µL of supernatant was harvested and diluted in 150 µL of cDMEM. CellTiter-Glo was added to the plate containing 30 µL of media and cells using a Biomek FX and incubated at room temperature for 10 min. Luminescence, which is a measure of cell viability via total ATP in cells, was measured using an EnVision plate reader (PerkinElmer, Waltham, MA, USA) to monitor the effect of virus grown in the presence of compound. Diluted supernatant virus was passaged to new wells. Two additional virus passages were performed. After a total of three passages, wells showing virus growth in the presence of inhibitor were expanded to small virus stocks, and these stocks were characterized for Compound 1 sensitivity in a 3 day MDCK cell protection assay. For stocks showing reduced sensitivity to Compound 1 the PB1, PB2, and PA coding sequences were sequenced using RNA isolation, reverse transcription PCR, PCR amplification and Sanger-based population sequencing. Amino acid changes from the parent strain were identified and cloned into the A/Puerto Rico/8/34 influenza background using the 12-plamid reverse genetics system. The phenotype of the reverse genetics influenza strains was again characterized by the 3 day MDCK cell protection, inhibitor sensitivity assay. The natural frequency of the identified variants was analyzed by searching public databases of influenza sequences.

**[0175]** To allow productive infections in a canine kidney cell line (i.e., MDCK), A/Wisconsin/67/2005 was serially passaged though MDCK cells 10 times and plaque purified to produce A/Wisconsin/67/2005-p5 (herein referred to as A/Wisconsin/67/2005).

**[0176]** Selection of influenza virus variants with reduced susceptibility to Compound 1 was performed at compound concentrations ranging from the $EC_{50}$ to 128x-$EC_{50}$. Following three passages of virus at the same compound concentration per well, enriched viral populations were harvested from the supernatant. Sixteen potential Compound 1-resistant variant containing wells (i.e., those with a greater than 50 % decrease in ATP levels compared to uninfected wells) and 16 control wells for each of the 3 viruses, were selected for confirmatory phenotypic analysis and polymerase gene sequencing (Table 4 through Table 9).

**[0177]** Thirty-one isolates showed greater than a 10-fold reduction in sensitivity to Compound 1, and in 29 of these isolates PB2 variants were observed; Q306H, F323L, S324I/N/R, F325V, H357Q, K376Q/R, F404Y, Q406K, M431I, and N510K/T. The most

commonly isolated variants were K376R (n=6), S324I (n=4), M431I (n=4), and N510K (n=3), however, due to this low sample size no obvious correlation between Compound 1 selective pressure (i.e., fold EC50 selection) and variant position or identity can be made. The biological isolates had a 10- to 1720-fold shift in Compound 1 $EC_{50}$. For the remaining 2 isolates, with $EC_{50}$ shifts of 286 and 500-fold, no polymerase variation was identified using Sanger sequencing methods in the PB2, PB1 or PA regions. One potential explanation is the presence of a resistant variant below the threshold for sequence identification. No primary influenza A virus variants were found in PB1 or PA genes.

**[0178]** To identify potential Compound 1 resistant variants for testing in the reverse genetics-based system, several criteria were used. First, the virus needed an $EC_{50}$ shift of greater than 10-fold in Compound 1 sensitivity, which is outside the assay-to-assay variation of 3- to 5-fold. Second, to remove common polymorphisms not related to Compound 1, the variant was excluded if observed in DMSO passaged virus.

**[0179]** Characterization of the individual potential Compound 1-resistant variants using reverse genetics methods is underway. To date these viruses have conferred Compound 1 $EC_{50}$ shifts ranging from 3.5- to >769-fold (Table 10). This indicates that single amino acid changes in PB2 can result in reduced sensitivity to Compound 1. Since the reverse genetics system used in these studies was generated from A/Puerto Rico/8/34, which contains sequences conferring M2-inhibitor resistance and NAI sensitivity, cross-resistance studies were not performed.

**[0180] Table 4: A/Puerto Rico/8/34 Variants Selected with Compound 1.**

| | | | PB2 | | | | | | | | | PB1 | | PA |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Sample Number** | **$EC_{50}$ Shift** | **Variant Summary** | **F323** | **S324** | **H357** | **K376** | **F404** | **Q406** | **N510** | **I560** | **L571** | **F166** | **E656** | **D529** |
| 1 | >714 | **S324I,** PA D529N | | I | | | | | | | | | | N |
| 5 | >714 | Q406K, I560V, L571L/Q | | | | | | K | | V | L/Q | | | |
| 8 | >714 | **N510T,** I560V, PB1 F166L | | | | | | | T | V | | L | | |
| 10 | >714 | **S324I** | | I | | | | | | | | | | |
| 11 | >714 | **S324I** | | I | | | | | | | | | | |
| 2 | 471 | F404Y, PB1 F166F/L | | | | | Y | | | | | F/L | | |

| | | | PB2 | | | | | | | | | PB1 | | PA |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Sample Number** | **$EC_{50}$ Shift** | **Variant Summary** | **F323** | **S324** | **H357** | **K376** | **F404** | **Q406** | **N510** | **I560** | **L571** | **F166** | **E656** | **D529** |
| 9 | 374 | **K376R,** PB1 F166F/L, PB1 E656E/K | | | | R | | | | | | F/L | E/K | |
| 3 | 286 | | | | | | | | | | | | | |
| 12 | 245 | **K376R** | | | | R | | | | | | | | |
| 14 | 18 | K357Q | | | Q | | | | | | | | | |
| 16 | 12 | I560V | | | | | | | | V | | | | |
| 13 | 9 | **F323L,** I560V | L | | | | | | | V | | | | |
| 4 | 1 | PB1 F166L | | | | | | | | | | L | | |
| 15 | 1 | I560V, PB1 F166L | | | | | | | | V | | L | | |
| 6 | NA | Low Titer | | | | | | | | | | | | |
| 7 | NA | Low Titer | | | | | | | | | | | | |

**[0181] Table 5: A/California/07/2009 Variants Selected with Compound 1.**

| | | | PB2 | | | | | | PB1 | | | PA |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Sample Number** | **$EC_{50}$ Shift** | **Variant Summary** | **D253** | **S324** | **F325** | **K376** | **M431** | **N510** | **L10** | **A652** | **E18** | **A256** |
| 2 | 1720 | F325V | | | V | | | | | | | |
| 5 | 500 | | | | | | | | | | | |
| 1 | 417 | **N510K** | | | | | | K | | | | |
| 3 | 183 | **K376R**, PB1 A652V | | | | R | | | | V | | |
| 4 | 176 | F325V | | | V | | | | | | | |
| 7 | 130 | **S324N** | | N | | | | | | | | |
| 8 | 95 | **K376R** | | | | R | | | | | | |
| 11 | 74 | **N510N/K** | | | | | | N/K | | | | |
| 6 | 31 | **M431I** | | | | | I | | | | | |
| 10 | 10 | **M431I**, PB1 L10V, PAE18G | | | | | I | | V | | G | |
| 9 | 2 | D253N | N | | | | | | | | | |
| 16 | 2 | | | | | | | | | | | |
| 12 | 1 | | | | | | | | | | | |
| 13 | 1 | PA E18G | | | | | | | | | G | Q |
| 14 | 1 | PA E18G, PA A256Q | | | | | | | | | G | |

| | | | PB2 | | | | | | PB1 | | | PA |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Sample Number** | **$EC_{50}$ Shift** | **Variant Summary** | **D253** | **S324** | **F325** | **K376** | **M431** | **N510** | **L10** | **A652** | **E18** | **A256** |
| 15 | 1 | PA E18G | | | | | | | | | G | |

**[0182] Table 6: A/Wisconsin/67/2005 Variants Selected with Compound 1.**

| | | | PB2 | | | | | PA |
|---|---|---|---|---|---|---|---|---|
| **Sample Number** | **$EC_{50}$ Shift** | **Variant Summary** | **Q306** | **S324** | **K376** | **M431** | **N510** | **K213** |
| 1 | >909 | **S324R** | | R | | | | |
| 5 | >909 | **S324I** | | I | | | | |
| 12 | >909 | **N510K**, PA K213K/R | | | | | K | K/R |
| 7 | 450 | **N510T** | | | | | T | |
| 4 | 309 | **K376Q** | | | Q | | | |
| 3 | 245 | **K376R** | | | R | | | |
| 11 | 209 | **Q306H** | H | | | | | |
| 2 | 209 | **K376R** | | | R | | | |
| 10 | 66 | **M431I** | | | | I | | |
| 9 | 65 | **M431I** | | | | I | | |
| 16 | 2 | | | | | | | |
| 13 | 1 | | | | | | | |
| 14 | 1 | | | | | | | |
| 15 | 1 | | | | | | | |
| 6 | NA | Low Titer | | | | | | |
| 8 | NA | Low Titer | | | | | | |

**[0183] Table 7: A/Puerto Rico/8/34 Control Variants Selected Without Compound 1.**

| | | | PB2 | | PB1 | PA | | |
|---|---|---|---|---|---|---|---|---|
| **Sample Number** | **$EC_{50}$ Shift** | **Variant Summary** | **M243** | **I560** | **F166** | **V90** | **D529** | **N675** |
| 17 | 1 | PB1 F166L | | | L | | | |
| 18 | 1 | I560V | | V | | | | |
| 19 | 1 | I560I/V | | I/V | | | | |
| 20 | 1 | PB1 F166F/L, PA N675N/T | | | F/L | | | N/T |
| 21 | 1 | M243K, PA V90I | K | | | I | | |
| 22 | 1 | | | | | | | |

| | | | PB2 | | PB1 | PA | | |
|---|---|---|---|---|---|---|---|---|
| **Sample Number** | **$EC_{50}$ Shift** | **Variant Summary** | **M243** | **I560** | **F166** | **V90** | **D529** | **N675** |
| 26 | 1 | M243K, PA D529N | K | | | | N | |
| 27 | 1 | PB1 F166L | | | L | | | |
| 29 | 1 | I560V, PB1 F166L | | V | L | | | |
| 23 | NA | | | | | | | |
| 24 | NA | | | | | | | |
| 25 | NA | | | | | | | |
| 28 | NA | | | | | | | |
| 30 | NA | | | | | | | |
| 31 | NA | | | | | | | |
| 32 | NA | | | | | | | |
| NA: titers were not high enough for phenotypic evaluation so $EC_{50}$ and sequence were not determined. | | | | | | | | |

**[0184] Table 8: A/California/07/2009 Control Variants Selected Without Compound 1**

| | | | PB2 | PB1 | PA |
|---|---|---|---|---|---|
| **Sample Number** | **$EC_{50}$ Shift** | **Variant Summary** | **K33** | **M111** | **E18** |
| 17 | 1 | PA E18E/G | | | E/G |
| 18 | 2 | K33K/R, PA E18E/G | K/R | | E/G |
| 19 | 1 | PA E18E/G | | | E/G |
| 20 | 0.4 | PA E18E/G | | | E/G |
| 21 | 2 | PA E18G | | | G |
| 22 | 1 | PA E18E/G | | | E/G |
| 23 | 1 | | | | |
| 24 | 0.3 | PA E18E/G | | | E/G |
| 25 | 1 | PA E18E/G | | | E/G |
| 26 | 1 | | | | |
| 27 | 1 | PA E18E/G | | | E/G |
| 28 | 1 | PB1 M111I, PA E18E/G | | I | E/G |
| 29 | 1 | PA E18E/G | | | E/G |
| 30 | 1 | | | | |
| 31 | 1 | | | | |
| 32 | 1 | PA E18E/G | | | E/G |

**[0185] Table 9: A/Wisconsin/67/2005 Control Variants Selected Without Compound 1.**

| | | | PB2 | | PB1 |
|---|---|---|---|---|---|
| **Sample Number** | **$EC_{50}$ Shift** | **Variant Summary** | **G222** | **I411** | **M290** |
| 31 | 2 | | | | |
| 32 | 2 | | | | |
| 17 | 1 | | | | |
| 18 | 1 | | | | |
| 19 | 1 | | | | |
| 20 | 1 | I411I/V | | I/V | |
| 22 | 1 | | | | |
| 23 | 1 | I411I/V | | I/V | |
| 24 | 1 | | | | |
| 25 | 1 | | ND | | |
| 26 | 1 | | | | |
| 27 | 1 | | | | |
| 28 | 1 | | | | |
| 29 | 1 | | | | |
| 30 | 1 | | | | |
| 21 | 0.4 | G222G/S, PA M290M/I | G/S | | M/I |
| ND: Unable to obtain sequence data for this sample. | | | | | |

**[0186] Table 10: Summary of PB2 Variants Selected by Compound 1 *In Vitro***

| | **Incidence of PB2 Substitution During In Vitro Selection** | | | **Sensitivity to Compound 1[a]** | | | |
|---|---|---|---|---|---|---|---|
| **Reverse Genetics Test Strain** | **A/PR/8/34 (H1N1)** | **A/Cali/07/2009 (pH1N1)** | **A/Wisc/67/2005 (H3N2)** | **$EC_{50}$ (SD) µM** | **Fold $EC_{50}$ Shift** | **Percent of Peak In Vitro Titer[c]** | **Prevalence in Human Dataset** |
| PR8- Wild Type | | | | 0.0013 (0.011) | 1.0 | NA | NA |
| PR8-PB2-Q306H | | | 1 | ND[b] | ND[b] | 6% | 0/8919 |

| | Incidence of PB2 Substitution During In Vitro Selection | | | Sensitivity to Compound 1[a] | | | |
|---|---|---|---|---|---|---|---|
| Reverse Genetics Test Strain | A/PR/8/34 (H1N1) | A/Cali/07/2009 (pH1N1) | A/Wisc/67/2005 (H3N2) | $EC_{50}$ (SD) µM | Fold $EC_{50}$ Shift | Percent of Peak In Vitro Titer[c] | Prevalence in Human Dataset |
| PR8-PB2-F323L | 1 | | | 0.11 (0.085) | 8.5 | ND[b] | 3/8942 |
| PR8-PB2-S324I | 3 | | 1 | ND[b] | ND[b] | 6% | 0/8942 |
| PR8-PB2-S324N | | 1 | | 0.40 (0.29) | 31 | 18% | 1/8942 |
| PR8-PB2-S324R | | | 1 | 0.43 (0.44) | 33 | 18% | 0/8942 |
| PR8-PB2-F325V | | 2 | | 0.045 (0.022) | 3.5 | ND[b] | 0/8942 |
| PR8-PB2-H357Q | 1 | | | 0.20 (0.12) | 15 | ND[b] | 0/8906 |
| PR8-PB2-K376Q | | | 1 | 5.3 (3.1) | 408 | ND[d] | 0/8910 |
| PR8-PB2-K376R | 2 | 2 | 2 | 3.0 (1.7) | 231 | 10% | 0/8910 |
| PR8-PB2-F404Y | 1 | | | ND[b] | ND[b] | 56% | 3/8912 |
| PR8-PB2-F406K | 1 | | | ND[b] | ND[b] | ND[b] | 8912 |
| PR8-PB2-M431I | | 2 | 2 | 1.7 (0.82) | 131 | 8% | 0/8914 |
| PR8-PB2-N510K | | 2 | 1 | ND[b] | ND[b] | 1% | 0/8926 |
| PR8-PB2-N510T | 1 | | 1 | ND[b] | ND[b] | 32% | 1/8926 |

$EC_{50}$: effective concentration at which ATP is half the maximum in the CPE-based assay; SD: standard deviation.

[a] Compound 1 $EC_{50}$ values for reverse genetics-generated viruses in the 3 day MDCK cell CPE-based assay. Values shown are the averages of at least three independent experiments.

[b] Values are listed as ND (not determined) because at least one experimental value was determined to be outside the range of compound concentration tested making determination of an average not possible.

| | Incidence of PB2 Substitution During In Vitro Selection | | | Sensitivity to Compound 1[a] | | | |
|---|---|---|---|---|---|---|---|
| Reverse Genetics Test Strain | A/PR/8/34 (H1N1) | A/Cali/07/2009 (pH1N1) | A/Wisc/67/2005 (H3N2) | $EC_{50}$ (SD) µM | Fold $EC_{50}$ Shift | Percent of Peak In Vitro Titer[c] | Prevalence in Human Dataset |
| [c] MDCK cells were infected with the indicated virus at an MOI = 0.01 and supernatants were titered over 62 hours. Percent of peak titer was determined as a percentage of variant mean antilog titer divided by wild-type mean antilog titer at 48 hours post infection. Virus replication capacity was assayed in one experiment with triplicate repeats.<br>[d] ND Not determined because titer was below the limit of detection at 48 hours. | | | | | | | |

**[0187] Example 5: Characterization of Sensitivity of Influenza Variants to Compound 1**

**[0188]** Compound antiviral activity was evaluated by its ability to prevent MDCK cell death as a consequence of influenza virus infection, as measured by cellular ATP levels using CellTiter Glo. Briefly, a Biomek FX liquid handler was used to plate MDCK cells ($4x10^5$ cells/mL) into black, clear bottom, 384-well plates at a density of $2x10^4$ cells per well in 50 µL VGM. Cells were incubated for 5 h at 37 °C, 5 % $CO_2$, with saturated humidity to allow cells to adhere and form a monolayer. Using a Biomek FX, 40 µL of media was removed, 25 µL of VGM containing diluted drug (final DMSO concentration of 0.5 % DMSO), and 15 µL of virus at a concentration of 100 $TCID_{50}$/well was added. Internal controls consisted of wells containing cells only and cells infected with virus in the absence of compound. Plates were incubated at 37 °C, 5 % $CO_2$, and saturating humidity for 72 h. After incubation, 20 µL of CellTiter-Glo was added to each well using a Biomek FX and incubated at room temperature for 10 min. Luminescence was measured using an EnVision plate reader (PerkinElmer). $EC_{50}$ (compound concentration at which CPE is half that of control) values were calculated by fitting the compound dose versus response data using a 4-parameter curve fitting method of Levenburg Marquardt algorithm (Condoseo software; Genedata Basel, Switzerland).

**[0189] Example 6: Determination of Viral Replication Competency**

**[0190]** Viral replication competency was assayed for reverse genetics variants via 62-hour growth curves in infected MDCK cells. MDCK cells were plated in 96-well plates at a

density of $4x10^4$ cells per well and infected with virus at an MOI=0.01 in the absence of compound. At various time points plates were harvested and supernatant was assayed for virus titer. Virus titers were plotted over time and peak titer for wild-type virus was determined to occur at 48 hours post infection.

**[0191] Example 7: Amplification and Sequencing of the Influenza A Polymerase Complex from Viral Stocks or Infected MDCK cells**

**[0192]** Sequence analysis of influenza A virus utilized reverse-transcriptase polymerase chain reaction (RT-PCR) amplification of the approximately 3 kilobase RNA fragments of the PB2, PB1, and PA coding regions. Viral RNA was extracted from 100 µL of viral stock or from $2x10^6$ infected MDCK cells in 300 µL of lysis buffer under denaturing conditions. Viral RNA was isolated by standard commercial silica-gel membrane using either the RNeasy Plus Mini method (catalog number 74134, Qiagen, Valencia, CA, USA) or the QIAamp Virus RNA Mini method (catalog number 52904, Qiagen). A complementary DNA (cDNA) fragment was synthesized from viral RNA in a 50 µL reaction containing 2.5 µM of a Universal 12 primer (AGCRAAAGCAGG) (SEQ ID NO: 2), 400 U of Superscript™ III Reverse Transcriptase (catalog number 18080-044, Invitrogen, Carlsbad, CA), 40 U of RNase OUT (catalog number 10777-019, Invitrogen), PC2 reaction buffer (50 mM Tris-HCl pH 9.1, 16 mM ammonium sulfate, 3.5 mM magnesium chloride, and 150 µg/mL bovine serum albumin) (catalog number 1001, AB Peptides, St. Louis, MO, USA), 500 µM dNTPs (catalog number 639125, Clontech, Mountain View, CA, USA), and 5 mM dithiolthreitol (catalog number 18080-044, Invitrogen), with a denaturation step (65 °C for 5 min) followed by ramping extension temperatures (25 °C for 10 min, 42 °C for 10 min, 50 °C for 20 min, 55 °C for 10 min, and 70 °C for 15 min) in the RT reaction. To amplify influenza A virus polymerase subunit-encoding fragments from the synthesized cDNA pool, 5 µL of the completed RT reaction was combined with PC2 reaction buffer, 200 µM dNTPs (catalog number 639125, Clontech), 1.5 M betaine (catalog number, B0300, Sigma Aldrich,), 3.2 U Klentaq DNA polymerase (catalog number 1001, AB Peptides), 1.6 U Pfu DNA polymerase (catalog number 600160, Stratagene, La Jolla, CA, USA), and 400 µM each primer for a final reaction volume of 50 µL (for PA segment: Forward: 5'-CGTCTCNGGGAGCGAAAGCAGGTACTGATCCAAAAT (SEQ ID NO: 3) and Reverse: 5'-CGTCTCNTATTAGTAGAAACAAGGTACTTTTTTGGA (SEQ ID NO: 4), for PB1 segment: Forward: 5'- CGTCTCNGGGAGCGAAAGCAGGCAAACCATTTGAA (SEQ ID NO: 5) and Reverse: 5'- CGTCTCNTATTAGTAGGAACAAGGCATTTTTTCATG (SEQ ID NO: 6), for PB2 segment: Forward: 5'-

CGTCTCNGGGAGCGAAAGCAGGTCAATTATATTCAA (SEQ ID NO: 7) and Reverse: 5'- CGTCTCNTATTAGTAGAAACAAGGTCGTTTTTAAAC (SEQ ID NO: 8)). The reaction was incubated at 94 °C for 2 min, followed by 40 cycles at 94 °C for 15 s, 68 °C -0.4 °C/cycle ('touchdown' PCR) for 20 s, and 68 °C for 3.5 min, followed by incubation at 68 °C for 7 min. The PCR product was purified using the QIAquick 96 PCR Purification kit (catalog number 28181, Qiagen) and an aliquot was analyzed by 1 % agaraose gel electrophoresis for quality. The purity and quantity of the purified PCR products were evaluated using spectrophotometry (NanoDrop, 8000 v.1.1; Thermo Fisher Scientific). Purified DNA was sequenced in-house or sent to Beckman-Coulter (Agencourt® Biosciences; Danvers, MA, USA) for sequencing of the PB2, PB1 and PA segments.

**[0193]** Sequence traces were aligned and interpreted using Mutation Surveyor software (SoftGenetics, State College, PA). Amino acid substitutions were detected by comparing sequences to the corresponding virus used for resistance selection (i.e., A/Puerto Rico/8/34, A/California/07/2009 or A/Wisconsin/67/2005).

**[0194] Example 8: Construction of Plasmids for Reverse Genetics**

**[0195]** Plasmid constructs encoding viral genome segments were created. Briefly, influenza A/Puerto Rico/8/34 virus RNA isolation and cDNA synthesis were. cDNA was amplified using oligos containing terminal *Bsm*B1 restriction endonuclease sites, and the amplified product was cloned into the pCR-XL-TOPO shuttle vector using a TOPO® XL PCR Cloning Kit (catalog number K4700 10, Invitrogen). The cDNA-containing TOPO vectors were digested with BsmB1 and inserts ligated into the RNA polymerase I-driven expression vector pHH21. Plasmids were sequenced to confirm proper integration of the cDNA fragment and the absence of unwanted mutations.

**[0196]** Amino acid changes from the parent strain, identified from resistance selection experiments, were cloned into the corresponding pHH21-A/Puerto Rico/8/34 plasmid using the Quick Change™ II XL Site-Directed Mutagenesis Kit (catalog number 200518, Agilent Technologies, Inc., Wilmington, DE, USA) following the manufacturer's protocol. Briefly, 10 ng of wild-type plasmid and 150 ng of each forward and reverse mutation-containing primer were combined with 5 µL 10x reaction buffer, 1 µL dNTP mix, 3 µL QuikSolution and 2.5 U of PfuUltra HF DNA polymerase in PCR grade water to 50 µL, and mutations were introduced by PCR. Thermal cycling was performed with an initial denaturation at 95 ˚C for 1 min, 18 cycles of 95 ˚C for 50 s, 60 ˚C for 50 s, and 68 ˚C for 5 min and a final extension for 7 min at 68 ˚C. Residual wild-type plasmid was digested with *Dpn* I restriction enzyme, and mutation-containing plasmid was transformed into bacteria and plasmid was isolated

using a QIAGEN Plasmid Midi Kit (catalog number 12143, Qiagen). Proper incorporation of point mutations into the plasmid was confirmed by sequencing.

**[0197]** Alternatively, mutations were introduced by splice overlap extension PCR, whereby internal primers containing the desired amino acid substitution were used to generate intermediate segments with overlapping 3'ends and flanking primers that included an appropriate restriction sites. First round PCR amplification reactions, was performed with 10 ng of template plasmid DNA, internal forward or reverse mutation-containing primers, and a High Fidelity PCR Master mix (catalog number 12140314001, Roche, Basel, Switzerland) according to the protocols provided. Thermal cycling temperatures and times were 94 ˚C for 4 min, 40 cycles of 94 ˚C for 45 s, 55 ˚C for 30 s, 72 ˚C for 2 min, and a final extension at 72 ˚C for 10 min. Products were gel purified by electrophoresis on a 1.5 % agarose gel. Five ng of each first round PCR product was used as template for a second PCR reaction using the flanking restriction site-containing primers. PCR round two products were again gel purified, and pHH21 plasmids were digested with appropriate restriction enzymes, ligated, and 5 μL of product was used for bacterial transformation. Proper incorporation of point mutations was confirmed by sequencing.

**[0198] Example 9: Generation of Recombinant Viruses**

**[0199]** To investigate mutations, recombinant viruses were generated by transient transfection of 293T cells (CRL-11268, ATCC) with a plasmid mixture containing 1.25 μg of the pHH21 mutation-containing plasmid, each of the seven remaining pHH21 wild-type plasmids, and the four RNP complex expression plasmids. Transient transfections were performed with TransIT-LT1 transfection reagent (catalog number MIR 2300, MirusBio, Madison, WI, USA). Briefly, a 1:2 mixture (w/v) of the plasmid mixture and transfection reagent was incubated in 1.5 mL of OPTI-MEM I media (catalog number 11058, Invitrogen) at room temperature for 15 min. The transfection mixture was then added to T-75 flasks containing 293T cells at 50 % confluency maintained in 15 mL OPTI-MEM I. Cells were incubated at 37 °C in 5 % $CO_2$ for 48 h. Following incubation, the supernatant was harvested and centrifuged at 650 x g for 10 min to remove cell debris and virus was further expanded in MDCK cells infected at low MOI after which viral stocks were harvested and titered.

## OTHER EMBODIMENTS

**[0200]** It is to be understood that while the invention has been described in conjunction with the detailed description thereof, the foregoing description is intended to illustrate and not limit the scope of the invention, which is defined by the scope of the appended claims. Other aspects, advantages and modifications are within the scope of the following claims.

WHAT IS CLAIMED IS:

1. An isolated influenza A virus polynucleotide, a biologically active analog thereof, or a biologically active fragment thereof, comprising a mutation in the gene encoding the influenza A virus polymerase wherein said mutation results in at least one amino acid substitution corresponding to amino acid residues selected from the group consisting of amino acid 306, 323, 324, 325, 357, 376, 404, 406, 431, and 510 of a wild-type influenza A virus.

2. The isolated influenza A virus polynucleotide of claim 1, wherein the nucleotide corresponding to amino acid 306 of the wild-type influenza A virus polynucleotide does not encode Q.

3. The isolated influenza A virus polynucleotide of claim 1, wherein the nucleotide corresponding to amino acid 306 of the wild-type influenza A virus polynucleotide encodes H.

4. The isolated influenza A virus polynucleotide of claim 1, wherein the nucleotides corresponding to amino acid 323 of the wild-type influenza A virus polynucleotide does not encode F.

5. The isolated influenza A virus polynucleotide of claim 1, wherein the nucleotide corresponding to amino acid 323 of the wild-type influenza A virus polynucleotide encodes L.

6. The isolated influenza A virus polynucleotide of claim 1, wherein the nucleotides corresponding to amino acid 324 of the wild-type influenza A virus polynucleotide does not encode S.

7. The isolated influenza A virus polynucleotide of claim 1, wherein the nucleotide corresponding to amino acid 324 of the wild-type influenza A virus polynucleotide encodes I.

8. The isolated influenza A virus polynucleotide of claim 1, wherein the nucleotide corresponding to amino acid 324 of the wild-type influenza A virus polynucleotide encodes N.

9. The isolated influenza A virus polynucleotide of claim 1, wherein the nucleotide corresponding to amino acid 324 of the wild-type influenza A virus polynucleotide encodes R.

10. The isolated influenza A virus polynucleotide of claim 1, wherein the nucleotides corresponding to amino acid 325 of the wild-type influenza A virus polynucleotide does not encode S.

11. The isolated influenza A virus polynucleotide of claim 1, wherein the nucleotide corresponding to amino acid 325 of the wild-type influenza A virus polynucleotide encodes V.

12. The isolated influenza A virus polynucleotide of claim 1, wherein the nucleotides corresponding to amino acid 357 of the wild-type influenza A virus polynucleotide does not encode H.

13. The isolated influenza A virus polynucleotide of claim 1, wherein the nucleotide corresponding to amino acid 357 of the wild-type influenza A virus polynucleotide encodes Q.

14. The isolated influenza A virus polynucleotide of claim 1, wherein the nucleotides corresponding to amino acid 376 of the wild-type influenza A virus polynucleotide does not encode K.

15. The isolated influenza A virus polynucleotide of claim 1, wherein the nucleotide corresponding to amino acid 376 of the wild-type influenza A virus polynucleotide encodes R.

16. The isolated influenza A virus polynucleotide of claim 1, wherein the nucleotide corresponding to amino acid 376 of the wild-type influenza A virus polynucleotide encodes Q.

17. The isolated influenza A virus polynucleotide of claim 1, wherein the nucleotides corresponding to amino acid 404 of the wild-type influenza A virus polynucleotide does not encode F.

18. The isolated influenza A virus polynucleotide of claim 1, wherein the nucleotide corresponding to amino acid 404 of the wild-type influenza A virus polynucleotide encodes Y.

19. The isolated influenza A virus polynucleotide of claim 1, wherein the nucleotides corresponding to amino acid 406 of the wild-type influenza A virus polynucleotide does not encode F.

20. The isolated influenza A virus polynucleotide of claim 1, wherein the nucleotide corresponding to amino acid 406 of the wild-type influenza A virus polynucleotide encodes K.

21. The isolated influenza A virus polynucleotide of claim 1, wherein the nucleotides corresponding to amino acid 431 of the wild-type influenza A virus polynucleotide does not encode M.

22. The isolated influenza A virus polynucleotide of claim 1, wherein the nucleotide corresponding to amino acid 431 of the wild-type influenza A virus polynucleotide encodes I.

23. The isolated influenza A virus polynucleotide of claim 1, wherein the nucleotides corresponding to amino acid 510 of the wild-type influenza A virus polynucleotide does not encode N.

24. The isolated influenza A virus polynucleotide of claim 1, wherein the nucleotide corresponding to amino acid 510 of the wild-type influenza A virus polynucleotide encodes T.

25. The isolated influenza A virus polynucleotide of claim 1, wherein the nucleotide corresponding to amino acid 510 of the wild-type influenza A virus polynucleotide encodes K.

26. The isolated influenza A virus polynucleotide of claim 1, wherein the nucleotides that correspond to any 2 amino acids selected from the group consisting of amino acids 306, 323, 324, 325, 357, 376, 404, 406, 431, and 510 are mutated such that the nucleotides encode an

amino acid different from the amino acid encoded by the corresponding wild-type influenza A virus polynucleotide.

27. The isolated influenza A virus polynucleotide of claim 1, wherein the nucleotides that correspond to any 3 amino acids selected from the group consisting of amino acids 306, 323, 324, 325, 357, 376, 404, 406, 431, and 510 are mutated such that the nucleotides encode an amino acid different from the amino acid encoded by the corresponding wild-type influenza A virus polynucleotide.

28. An isolated influenza A virus polymerase comprising an amino acid sequence in which the amino acid at at least one position selected from the group consisting of 306, 323, 324, 325, 357, 376, 404, 406, 431, and 510 is different from the amino acid at the corresponding position of the wild-type influenza A virus polymerase.

29. The influenza A virus polymerase of claim 28 comprising a biologically active analog of an influenza A virus polymerase.

30. The influenza A virus polymerase of claim 28 comprising a biologically active fragment of an influenza A virus polymerase.

31. An anti-influenza A virus polymerase antibody that recognizes an influenza A virus polymerase comprising an amino acid sequence in which the amino acid at at least one position selected from the group consisting of 306, 323, 324, 325, 357, 376, 404, 406, 431, and 510 is different from the amino acid at the corresponding position of the wild-type influenza A virus polymerase.

32. A nucleotide probe or primer capable of hybridizing under stringent conditions to a nucleic acid sequence of the influenza A virus polynucleotide of claim 1.

33. An expression system comprising the influenza A virus polynucleotide of claim 1.

34. The expression system of claim 17 comprising a vector, wherein the vector comprises the influenza A virus polynucleotide of claim 1 operably linked to a promoter.

35. A host cell transfected, transformed, or transduced with the vector of claim 18.

36. The expression system of claim 33 that is an mRNA display system.

37. An isolated influenza A virus variant comprising a polynucleotide encoding an influenza A virus polymerase, wherein at least one amino acid at at least one position selected from the group consisting of 306, 323, 324, 325, 357, 376, 404, 406, 431, and 510 is mutated such that it encodes an amino acid different from the corresponding amino acid of the wildtype influenza A virus polynucleotide.

38. A method for evaluating drug resistance or sensitivity to a polymerase inhibitor of an influenza A virus infection in a patient comprising:

a) collecting a biological sample from the influenza A virus infected patient; and

b) evaluating whether the sample comprises a nucleic acid encoding an influenza A virus polymerase that comprises an amino acid sequence in which the amino acid at at least one position selected from the group consisting of 306, 323, 324, 325, 357, 376, 404, 406, 431, and 510 is different from the amino acid at each corresponding position of the wild-type influenza A virus polymerase.

39. A method for guiding a treatment for an influenza A virus infection in a patient comprising:

a) evaluating drug resistance or sensitivity to a polymerase inhibitor of the patient according the method of claim 38; and

b) optimizing the treatment regimen for the patient based on the drug resistance or sensitivity evaluated in a).

40. A method for identifying a candidate compound for treating an influenza A virus infection in a patient comprising:

a) providing a sample infected with the influenza A virus variant of claim 37; and

b) assaying the ability of the candidate compound in inhibiting an activity of the influenza A virus variant in the sample.

41. The method of claim 40, wherein the activity of the influenza A virus variant is replication.

42. A method for identifying a candidate compound for treating or preventing an influenza A virus infection in a patient comprising:

a) providing a replicon RNA comprising the polynucleotide of claim 1; and

b) determining whether the candidate compound inhibits replication of the replicon RNA of a).

43. A method for identifying a candidate compound for treating an influenza A virus infection in a patient comprising:

a) providing an isolated influenza A virus polymerase of claim 28 and a polymerase substrate, wherein the polymerase and the substrate are in a cell-based system or in a cell-free system;

b) contacting the influenza A virus polymerase with the candidate compound in the presence of the substrate; and

c) determining whether the influenza A virus polymerase activity is reduced.

44. A method for evaluating a candidate compound for treating an influenza A virus infection in a patient comprising:

a) introducing a vector comprising a polynucleotide according to claim 1 and an indicator gene encoding an indicator into a host cell;

b) culturing the host cell; and

c) measuring the indicator in the presence of the candidate compound and in the absence of the candidate compound.

45. A method of treating an influenza A virus infection in a patient, comprising administering to the patient a pharmaceutically effective amount of the compound identified according to any of claims 40-43.

46. A machine-readable data storage medium comprising a data storage material encoded with machine-readable data, wherein the machine-readable data comprise index values for at least two features associated with an influenza A virus variant or biological sample; wherein the features are selected from the group consisting of:

a) the ability to exhibit resistance for reduced sensitivity to a polymerase inhibitor;

b) an influenza A virus polymerase comprising an amino acid sequence in which the amino acid at at least one position selected from the group consisting of 306, 323, 324, 325, 357, 376, 404, 406, 431, and 510 is different from the amino acid at the corresponding position of the wild-type influenza A virus polymerase;

c) morbidity or recovery potential of a patient; and

d) altered replication capacity (increased or decreased) of the influenza A virus variant.

47. A method of obtaining a profile of influenza A virus variants in an influenza A virus-infected patient comprising:

a) obtaining a plasma sample from the patient; and

b) determining the nucleotide sequence of an influenza A virus polymerase from at least 2 influenza A virus virions from the plasma sample.

48. The method of claim 47, wherein at least 20 influenza A virus virions were identified.

49. The method of claim 47, wherein at least 50 influenza A virus virions were identified.

50. The method of claim 47, wherein at least 100 influenza A virus virions were identified.

51. The method of claim 47, wherein at least 200 influenza A virus virions were identified.

52. The method of claim 47, wherein at least 500 influenza A virus virions were identified.

53. The method of claim 47, wherein the nucleotide sequence of an influenza A virus polymerase comprises a sequence of the polynucleotide of claim 1.

54. The method of claim 47, wherein the patient has been treated with a polymerase inhibitor.

55. The method of claim 47, wherein at least 2 plasma samples are obtained from the patient at at least two different time points.

56. A method for detecting the presence of an influenza A virus variant in a biological sample comprising detecting the presence of a polynucleotide according to claim 1in the biological sample.

57. A diagnostic kit comprising the antibody of claim 31.

58. A diagnostic kit comprising the nucleotide probe or primer of claim 32.